FRONT COVER

A “filleted” Drosophila embryo stained with an antibody that recognizes motor axons and a subset of central nervous system axons. The dark structure in the center of the photograph is the ventral nerve cord, and the lines radiating out from it are motor nerves that innervate body wall muscles. Anterior is down. Note that the axonal pattern observed in each abdominal (posterior) segment is almost identical between segments. For further information, see http://www.caltech.edu/~zinn/.

BACK COVER

Surface representation of the three dimensional structure of hemolin, an immune response protein that is involved in the anti-bacterial response in insects. Hemolin is a member of the immunoglobulin gene superfamily containing four Ig-like domains that fold into a shape resembling a closed horseshoe. Blue areas represent regions of the protein having an overall positive charge which may be involved in binding to the negatively charged lipopolysaccharide component of bacterial cell walls. (See Abstract 255 from the Bjorkman lab.)
A Report for the Year 1996-1997
On the Research and Other Activities
of the
Division of Biology
at the
California Institute of Technology
Pasadena, California
Mary Jeffries, Annual Report Coordinator
Thanks to all who contributed to the preparation of the material for this year's Report. May the process continue to evolve.

Research Reports
Much of the research work summarized here (covering the period June 1996 to May 1997) has not yet been reported in print. In many cases the work simply is not complete. For this reason the Biology Division Annual Report is not intended as a publication and should not be cited as such. Individual projects should be referred to only if specific permission to do so is obtained from the investigator responsible for the material. References are made here to published papers bearing on the projects reported. Publications by members of the Division, covering the period from about July, 1996 to August, 1997, are listed separately at the end of the research reports of each group.
# Table of Contents

**Introduction**......................................................................................................................... 3

**Staff of Instruction and Research**.......................................................................................... 9

**Administrative Staff**.............................................................................................................. 15

## Research Reports

### Cellular Biology and Biophysics

**Charles Brokaw**

Summary........................................................................................................................................... 19

1. Cytosolic motility activating factors of *Ciona* spermatozoa...................................................... 19
2. Transient disruptions of axonemal structure and microtubule sliding during bend propagation by *Ciona* sperm flagella......................................................... 19
3. Mechanical components of motor enzyme function........................................................................ 20
4. Reactivation at low [ATP] distinguishes among classes of paralyzed flagella mutants................. 20
5. A motility function for the parailgeellar rod of Leishmania parasites revealed by PFR-2 gene knockouts................................................................................... 20

**Raymond J. Deshaies**

Summary........................................................................................................................................... 21

6. Analysis of Cdc2p ubiquitination by the Anaphase Promoting Complex in yeast......................... 21
7. Regulated ubiquitination of the bHLH transcription factor Gcn4 in vitro...................................... 21
8. Destruction of the cyclin/CDK inhibitor Sic1 at the G1/S transition.................................................. 22
9. A functional analysis of human CUL1 and its potential role in ubiquitin-mediated proteolysis...... 22
10. CKSI is required for G1 cyclin/CDK activity in budding yeast....................................................... 23
11. Genetic analysis of the CDC15 pathway in the exit from mitosis.................................................. 23
12. Defining the requirements for cell cycle-specific proteolysis of substrate proteins....................... 23

**William G. Duaphy**

Summary........................................................................................................................................... 24

13. Molecular characterization of Msy1, a membrane-associated histidine kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15.............................................. 24
14. Cell cycle regulation of a Xenopus Wee1-like kinase....................................................................... 25
15. Search for proteins involved in coordination of S-phase with mitosis.......................................... 25
16. Identification of Xenopus Pix1 as a protein kinase that phosphorylates and activates the Cdc2 protein................................................................................................. 26
17. Role of protein phosphatases in cell cycle regulation...................................................................... 26
18. Identification of novel cell cycle control proteins............................................................................ 27
19. Segmentation and characterization of Xe-9, the Xenopus homolog of Sucl/CKI............................ 27
20. The Xenopus Origin Recognition Complex...................................................................................... 27
21. Characterization of Xenopus Cdc6 during the initiation of DNA replication in cell free extracts.... 28

**Jean-Francois Revel**

Summary........................................................................................................................................... 28

22. Examination of AFM tips by TEM.............................................................................................. 28
23. Avoiding crystals in freezing samples............................................................................................ 29
24. Neural tube/epidermal interactions are required for normal trigeminal placode formation.......... 30
25. Molecular differences between cranial and trunk neural crest/neural tube................................. 30
26. Roles of cadherins in neural crest migration and differentiation.................................................. 30
27. Functional role of the α subunit of integrin in neural crest migration .......................................... 31

### Developmental Biology and Genetics

**Marianne Bronner-Fraser**

Summary........................................................................................................................................... 33

28. Origins of the avian neural crest.................................................................................................. 33
29. Early- and late-crust cranial neural crest populations are not lineage-restricted in vivo.............. 34
30. Isolation and characterization of Npl, a novel gene expressed in neural crest.............................. 34
31. Segmental patterning of trunk neural crest migration.................................................................... 34
32. Tissue interactions in inner ear formation....................................................................................... 35
33. Coordinate actions of BMP, Wnt, Shh, and nodgin mediate patterning of the dorsal somite........ 35
34. Neural tube/epidermal interactions are required for normal trigeminal placode formation.......... 35
35. Molecular differences between cranial and trunk neural crest/neural tube................................. 36
36. Roles of cadherins in neural crest migration and differentiation.................................................. 36
37. Functional role of the α subunit of integrin in neural crest migration .......................................... 36

**Eric H. Davidson**

Summary........................................................................................................................................... 37

38. Transcriptional regulation of the Cysta actin gene in sea urchin embryos and larvae.................. 37
39. Functional intermodular interrelation and the delicate Cis-regulatory elements within the module A of Endo16 promoter............................................................... 37
40. Purification and cloning of SpMyb, a spatial repressor of Cysta transcription in sea urchin embryos........................................................................................................... 38
41. Isolation of genes encoding spicule matrix proteins in sea urchin *Strongylocentrotus purpuratus*... 38
42. Isolation of transcription factors from early stage sea urchin embryos and sea urchin immune effector cells......................................................................................... 39
43. Identification of a battery of downstream target genes of a transcription factor which specifies the spatial expression of genes into skeletogenic mesenchyme cells in *Strongylocentrotus purpuratus*. ............................................. 39
44. Construction of an arrayed cDNA library and expressed sequences tags of early cleavage-stage sea urchin embryo................................................................. 40
45. Analysis of transcriptional regulation of the sea urchin *Brachyura* (T ortholog SpT).................. 41
46. The genetic introduction of a specific single-chain antibody inhibits a transcriptional repressor in vivo........................................................................................................... 41
47. The genetic introduction of a specific single-chain antibody inhibits a transcriptional repressor in vivo........................................................................................................... 42
48. The genetic introduction of a specific single-chain antibody inhibits a transcriptional repressor in vivo........................................................................................................... 42
49. Isolation of transcription factors from early stage sea urchin embryos and sea urchin immune effector cells......................................................................................... 42
50. An enhancer trap strategy............................................................................................................. 42
51. Contributions to endoderm differ in oral versus aboral Vecl-derived clones.................................. 43
52. Sea urchin population genetics...................................................................................................... 43
53. Using gene expression patterns to test hypotheses concerning the evolution of deuterostomes.... 43
54. Mobile elements inserted in the distant past now have important functions............................... 45
Alexander Varshovsky

Summary

182. A mouse amidase specific for N-terminal asparagine: the gene, the enzyme, and their function in the N-end rule pathway

183. Construction and analysis of mouse mutants that lack the Ntan1-encoded, asparagine-specific N-terminal amidase (NtN-amidase)

184. Molecular and physiological evidence for the recombinant N-term-amidase

185. N-terminal sequences that confer UBR1-dependent metabolic instability in S. cerevisiae

186. Cdc44p interacts with U6p3p, a WD-repeat protein required for ubiquitin-mediated proteolysis in S. cerevisiae

187. Targeting complex of the N-end rule pathway

188. Biochemical and genetic dissection of N-recognin

189. The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor

190. The N-end rule pathway is modulated by a post-transcriptional mechanism

191. Detecting and measuring cotranslational proteolysis in vivo: the double-ubiquitin technique

192. Using split-ubiquitin sensor to detect transient in vivo interactions between a signal sequence and components of the translocation apparatus

193. Cleavage-regulated conditional toxins

194. Codominance-mediated conditional toxins

195. Using differential hybridization techniques to identify new functions of the N-end rule pathway

196. The N-end rule pathway in Xenopus egg extracts

197. Analysis of mitochondrial function of the Ntalp Nt-amidase in S. cerevisiae

Publications

Barbara J. Wold

Summary

198. Different MRF4 knockout alleles differentially disrupt Mgf55 expression: cis-regulatory interactions at the MRF4/Mgf55 locus

199. Targeted disruption of mouse M Guthrie gene

200. Isolation and culture of mouse skeletal muscle fibers and their associated satellite cells

201. Expression and function of skeletal myoblasts

202. Analysis of regulatory gene expression in single skeletal muscle satellite cells by multiplex RT-PCR

203. Optimizing and applying jellyfish green fluorescent protein (GFP) as a vital marker for transgenic mice

204. Multiple mechanisms of action for the bHLH regulator twist in myogenesis

205. Searching for BRCA1 function

206. Mouse embryonic expression of p27, a cyclin dependent kinase inhibitor

207. P16INK4a/p19ARF in mouse development: Expression and gene disruption

Publications

Molecular Biology and Biochemistry

John N. Abelson

Summary

209. Probing the active site of the spliceosome

210. Investigating the roles of specific sequences of U6snRNA

211. U2 RNA interactions in the spliceosome: The crosslinking approach

212. Probing 5' splice site interactions using a yeast trans-splicing system

213. PRP22p, a DEAD-box splicing protein, catalyzes RNA duplex unwinding

214. Dominant negative mutants in the search for PRP5 function

215. Affinity purification of yeast splicing complexes

216. Structural studies of DEAD box proteins

217. Initial studies of the coupling of transcription and splicing in yeast

218. The yeast RNA splicing endonuclease

219. Identification of the protein sequence from the yeast spliceosome

220. Diversity in serine-ylthetase function

Publications

Giuseppe Attardi

Summary

221. Very rare interorganellar complementation of mitochondrial frameshift and tRNA mutations in cultured human cells

222. Pathogenetic mechanisms of the mitochondrial RNA splicing precursor 74455 mutation and coexisting Complex I subunit mtDNA mutations associated with non-syndromic deafness

223. The putative nuclear gene(s) to the biochemical defects underlying the non-syndromic deafness associated with the mitochondrial 12s rRNA 1555 mutation

224. Cell loss in nervous system of MERRF patient does not correlate with proportion of tRNA<sup>3'.</sup> mutation

225. In vitro control of respiration by cytochrome c oxidase in wild-type and mitochondrial DNA mutation-carrying human cells

226. Role of post-transcriptional modifications in the structure and function of wild-type and MERRF A8344G mutation-carrying human mitochondrial tRNA<sup>3'</sup>

227. The pathogenesis of the contribution of mitochondrial DNA mutations to the aging process

228. Structural analysis of mitochondrial DNA in transmitochondrial cell lines derived from fibroblasts of human individuals covering a broad range of ages

229. Further characterization of the recombinant mitochondrial transcription termination factor (mTERF) and analysis of the in vitro expression of the native protein

230. Large-scale purification of the human mitochondrial transcription termination factor (mTERF)

231. Molecular cloning of the cDNA and gene of the mouse mitochondrial transcription termination factor (mTERF)

232. Functional analysis of in vitro and in vivo footprinting of Hela cell mitochondrial RNA DNA

233. Pathogenetic mechanism of the MELAS mutation

234. An ND5 frameshift mutation leads to a failure of assembly of mtDNA-encoded subunits of NADH dehydrogenase and a non functional enzyme

235. Isolation and characterization of a mouse NDS mutant cell line

236. Alkali expression of mitochondrial genes in human cells

Publications
385. Mutations affecting expression of the transmembrane glycoprotein Odr-10 "olfactory" cell marker in olfactory bulbs and cerebral cortex of the C. Elegans mutant odr-10

365. Expression of LIF in normal and Alzheimer's disease brain

366. The role of cytokines in long-term potentiation

367. Adenovirus-mediated expression of neurotrophic cytokines in brain

380. LIF is an autocrine/paracrine factor for sympathetic neurons

369. Bone morphogenetic proteins modulate STAT signaling by neurotrophic cytokines

370. Cardiotrophin-1 induces the same neuropeptides in sympathetic neurons as do neurotrophic cytokines

371. Analysis of neuronal and glial phenotypes in brains of mice deficient in LIF

372. Association of the tetraspan protein CD9 with integrins on the surface of S-16 Schwann cells

373. Perturbation of the tetraspan membrane protein CD9 promotes neurite formation in a partially ε71 integrin-dependent manner

368. LIF is an autocrine/paracrine factor for sympathetic neurons

374. Nitric oxide synthase subcellular localization and long-term potentiation

375. Temporal analysis of mossy fiber sprouting in hippocampal slices using two-photon laser scanning microscopy

376. Different role for TrkB signaling in long-term potentiation in the adult rat hippocampus

377. Localization of neurotrophins BDNF and NT-3 and their receptor tyrosine kinases TrkB and TrkC in the rat hippocampus

378. Involvement of cadherin molecules in synaptic plasticity in the adult hippocampus

379. High-frequency stimulation in stratum lacunosum-moleculare gates Schaffer collateral-evoked spiking in hippocampal CA1 pyramidal cells

381. Competition and cooperation among receptor tyrosine phosphatases control motoneuron growth cone guidance in Drosophila

382. Genetic analysis of the functions of DPTP1D and DPTP1D9 in Drosophila embryonic CNS development

383. The transmembrane glycoprotein gp150 is a substrate for the receptor tyrosine phosphatase DPTP1D in Drosophila cells

384. Isolation of gp150 mutations by T-element mutagenesis

385. Analysis of neuronal expression of the protein DPTP1D in the mouse embryonic midline

386. Characterization of dimer formation among fly CNS RPTPs

387. PKA in the development of the embryonic Drosophila midline

388. Regulated neurite tension as a mechanism for determination of neuronal arbor geometries in vivo

389. Development of the grasshopper neuromuscular system

390. The C. elegans 7-transmembrane protein Odr-10 functions as an odorant receptor in mammalian cells

391. Functional expression of the heteromeric "olfactory" cyclic nucleotide-gated channel in the hippocampus: a potential effector of synaptic plasticity in brain neurons

392. Nurturing and survival in primates: Caretakers live longer

393. Cytochrome oxidase blobs in primary visual cortex of Eulemur fulvus and Microcebus murinus

394. The structure of the visual pulvinar in nocturnal and diurnal primates

395. Topographical localization of iron in brains of the aged fat-tailed dwarf lemur (Cheirogaleus medius) and grey lesser mouse lemur (Microcebus murinus)

396. A quantitative trait loci (QTL) analysis of the forebrain, hindbrain, olfactory bulbs, and cerebellum in the mouse

397. Where in the what pathway: Object distance modulates responses in areas V1 and V4

398. Dynamic size illusions and what they tell us about the physiology of size contrast and size constancy

399. A quantitative trait loci (QTL) analysis of the forebrain, hindbrain, olfactory bulbs, and cerebellum in the mouse

400. Primate area MT activities reflect the perception of depth-from-motion

401. Frames of reference in primate parietal cortex: Separation between 7A and LIP; physiology and simulation

402. Auditory responses in LIP I: Training effects

403. Auditory responses in LIP II: Behavioral gating

404. Self-motion path perception during head and body rotation

405. Perception and neural representation of heading during gaze-rotation

406. Comparison of eye pursuit effects on expanding and rotating motion patterns

407. Lesion of a macaque parietal area produces visual extinction in an egocentric framework

408. A change in motor intention, without a spatial shift in attention, activates posterior parietal cortex in monkey

409. Time course of the eye position signal in posterior parietal cortex

410. Extra cellular recording from adjacent neurons: I. A maximum-likelihood solution to the spike separation problem

411. Extra cellular recording from adjacent neurons: II. Correlation in macaque parietal cortex

Publications
Introduction
The Division

We are saddened to note the death in 1996 of Professor James F. Bonner. Bonner first came to the Caltech Biology Division (as a visiting junior from the University of Utah) in 1929. He returned to Caltech as a graduate student, earning his Ph.D. in 1934. After a one-year postdoctoral period in Leiden and Zürich, he remained here as a faculty member until his death. He was a pioneer in the early work on the chemical nature of plant hormones, did the classical light-break experiments that revealed a crucial aspect of plant responses to photoperiod, and his laboratory performed some of the first modern analyses of plant proteins. These including the discovery of ribulose bisphosphate carboxylase, the photosynthetic enzyme said to be the most abundant protein on Earth, and the discovery that pea and cow histones are very similar in amino acid sequence. Later in his long career he became a pioneer in determining the structure and function of chromatin. Bonner was well-known as the author of several textbooks and monographs, including what was long the standard textbook of plant biochemistry, which went through three editions (1950, 1965, 1976). His own summary of his work was published in the Annual Review of Plant Physiology and Plant Molecular Biology, (45), 1-23, 1994.

A second sad event: Professor Derek Fender died in May, 1997. Derek took B.Sc. degrees in Physics at the University of Reading in England in 1939 and 1947. In the interval, he served as Senior Lecturer in Physics at The Royal Military College of Science; during and after World War II he had become expert on radar control and the technology of airborne bomb sights, and through that experience became interested in the remarkable attributes of the human eye and visual system. He came to Caltech in 1961 as a senior Research Fellow in Engineering, associated primarily with Professor Gilbert McCann in the lively program studying, among other things, the fly’s eye and behavior. He was appointed Associate Professor of Biology and Electrical Engineering in 1962, and in 1966 Professor of Biology and Applied Science. An unusually clever experimenter, his work was rather frequently featured in Engineering & Science, for example his demonstration of the scanning patterns of the eyes of human subjects as they viewed a photograph, the contributions of the very fine movements of the eye to visual acuity, and localization of brain activity in response to visual signals as recorded through electrodes applied externally to the scalp. He achieved broad recognition for diverse studies and contributions over many years, but as late as the 1995-96 American Men and Women of Science he listed his main research area as “interaction between the scanning motions of the human eye and the pattern recognition processes of which it is capable.” Meanwhile, he taught effectively and was for years the Caltech’s faculty’s one-person committee on the Independent Studies program.

On a happier note, the Division of Biology will be joined next year by two new faculty members. Dr. José Alberola-Ila will join us from the University of Washington, Seattle as Assistant Professor of Biology; his scientific work is on the function of the immune system. Dr. David Baltimore, who will become the new President of the Institute in the next academic year, will also be appointed Professor of Biology. He expects to maintain a research group in the Biology Division, also studying immune function. Detailed descriptions of these new appointments will appear in next year’s Annual Report.
Honors and Awards

Ferguson Prize

The Lawrence L. and Audrey W. Ferguson Prize, an award of $2,000 for the outstanding Biology Ph.D. thesis, goes this year to Hyejin Kang (Erin Schuman Laboratory) for her work on neurotrophic factors and synaptic plasticity in the rat hippocampus.

Professorial

Professor Giuseppe Attardi has been appointed Associate Member of the European Molecular Biology Organization.

Associate Professor Pamela Bjorkman was awarded the 1996 Investigator Award of the American Association of Immunologists, and the 1996 Outstanding Mentor Award from the Graduate Study Council here at Caltech. In 1997 she was elected to membership in the American Academy of Arts and Sciences, and won the James R. Klinenberg Science Award of the Arthritis Foundation.

Assistant Professor Raymond Deshaies was granted both a Burroughs-Wellcome New Investigator Award in the Basic Pharmacological Sciences, and a Beckman Young Investigator Award.

Professor Christof Koch won an Alexander von Humboldt Research award from the German Government.

Professor Elliot Meyerowitz shared the "Science pour l'Art" Science Prize of LVMH Moët Hennessy-Louis Vuitton, awarded in Paris in 1996, and was awarded the 1996 Genetics Society of America Medal. He gave the Bodenstein Lecture at the University of Virginia on March 23, 1997; and the A.E. Waller Memorial Lectures at Ohio State University on May 22 and 23, 1997.

Professor Ray Owen was honored with a symposium at the University of Wisconsin, in recognition of his lifelong contributions to immunology, and of the 50th anniversary of his discovery of immune tolerance. The proceedings of the symposium have been published as a special issue of the Journal Human Immunology 52(2), February 1997.

Professor Paul Patterson was granted an Ulf Von Euler Lectureship at the Karolinska Institutet, Stockholm.

Assistant Professor Erin Schuman was made an Assistant Investigator of the Howard Hughes Medical Institute in May, 1997.

The next two pages represent a photo collage of the 1st Biology Reunion held on May 16, 1997. (Photos by Maria Murphy.)
Reminiscing our Caltech years

Professor "Alumni"

Neighbors reunited

Oh boy- here comes that photographer again

See, the data show that *Drosophila* have six wings

Who said I can't be first author
This way to the food

We’re all smiles

Here’s to you

It’s about the future of biology

Social Hour

Smile, say “Hippocampus”

Future Caltech Alumni 2020
Staff of Instruction and Research
Division of Biology
Melvin I. Simon, Chairman
Norman Davidson, Executive Officer
Elliot Meyerowitz, Executive Officer
Paul H. Patterson, Executive Officer

Professors Emeriti
Norman H. Horowitz, Ph.D.
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Edward B. Lewis, Ph.D.
Thomas Hunt Morgan Professor of Biology (active)
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Ray D. Owen, Ph.D., Sc.D.
Biology

Professors
Christof Koch, Ph.D.
Biology and Engineering and Applied Science; Computation and Neural Systems
Masakazu Konishi, Ph.D.
Ring Professor of Behavioral Biology
Henry A. Lester, Ph.D.
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Alexander Varshavsky, Ph.D.
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Barbara J. Wold, Ph.D.
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James G. Boswell Professor of Neuroscience (active)

James F. Bonner, Ph.D.
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Norman Davidson, Ph.D.
Norman Chandler Professor Emeritus of Chemical Biology

Derek H. Fender, Ph.D.
Biology and Applied Science

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w}ave propagation, to vary the bending pattern to meet the needs of the cell, and to turn motility on and off when appropriate.

Our experimental study of flagellar motility exploits the ATP-reactivated movement of demembranated flagella, typically from sea urchins or tunicates that are easily obtained and maintained at the Kerckhoff Marine Laboratory. This in vitro assay system retains all of the motility capabilities and significant regulatory capabilities of intact spermatozoa. In particular, the regulatory system involved in activation of motility of spermatozoa of the tunicate, *Ciona*, can be triggered in demembranated spermatozoa by the addition of cAMP. The complex kinase activities involved in the initiation of motility are thus readily accessible for measurement and manipulation in this in vitro system.

Significant effort has been devoted to development of computerized methods for analysis of flagellar movement and for simulation of flagellar movement and the function of motor enzyme molecules. These have all been converted to Macintosh applications, which are accessible at http://www.cco.caltech.edu/~brokawc/software.html

1. Cytosolic motility activating factors of *Ciona* spermatozoa
   Prem S. Chaudhry

   Triton-demembranated spermatozoa of the tunicate, *Ciona*, become fully motile upon incubation with cAMP and ATP. Demembranated sperm can be separated into sperm pellet and triton supernatant fractions, both of which contain endogenous cAMP-dependent protein kinase (PKA) activity. Incubation of sperm pellet fraction with either cAMP or a large excess of exogenous PKA (catalytic subunit) does not activate motility unless some of the triton supernatant is added during the incubation. Gel permeation chromatography of Triton supernatant showed that the motility activating component emerged from the column as single peak around molecular weight 100 kDa. This active fraction loses its sperm motility activating potential upon storage at 4° C, but activity can be restored by addition of the casein kinase II (CKII) fraction isolated by phosphocellulose chromatography. The combination of this CKII fraction and PKA only partially activates motility, in the absence of other supernatant components. These results indicate that activation of motility requires exposure of the sperm flagellar axoneme to PKA, CKII, and another, relatively stable, component of the Triton supernatant. Examination of sperm pellet proteins labelled with [\(^{32}p\)] ATP during activation has shown that full activation of motility requires a rapid phosphorylation of a 25 kDa axonemal protein by PKA and a slower phosphorylation of 33 kDa and 36 kDa axonemal proteins that is dependent upon both CKII and the additional supernatant component.

Reference

2. Transient disruptions of axonemal structure and microtubule sliding during bend propagation by *Ciona* sperm flagella
Charles J. Brokaw

Demembranated sperm flagella of *Ciona* were reactivated at increased salt concentrations (0.45 to 0.5 M K acetate). In addition to a decrease in amplitude of propagated bends, some flagella switch between "stable" and "transient" bending cycles. In the transient bending cycles, there is increased inter-microtubule sliding, in the direction that forms a new principal bend at the base of the flagellum, during the first half of a bending cycle. The magnitude of this increased sliding may be as much as 1 radian, or 0.06 µm between adjacent doublet microtubules. Most transient bending patterns also show a characteristic disruption of axonemal structure, involving separation between strands of microtubule doublets over a distance of up to 5 µm, occurring within a principal bend, typically about 16 µm from the base of the flagellum. The disruptions usually disappear after the principal bend propagates beyond the region of the disruption. Formation of these disruptions requires additional sliding, in the direction that would form a principal bend at the base of the flagellum, of up to about 0.3 µm. Formation of these disruptions may be explained by weakening of structural interactions by increased salt concentration and transverse forces, proportional to curvature and transmitted force, that will tend to separate doublets in a bend. These observations indicate that an actively beating flagellum possesses active sliding capability that is activated but not expressed during normal bend initiation and propagation. The initiation and propagation of flagellar bends may not be explicable solely in terms of local activation and inactivation of dynein-driven sliding.

3. Mechanical components of motor enzyme function
Charles J. Brokaw

Motor enzymes use energy from ATP dephosphorylation to generate movement by a mechanical cycle, moving and pushing in one direction while strongly attached to their cytoskeletal substrate, and recovering by moving relative to their substrate to reach a new attachment site. Mainstream models assert that movement while attached to the
substrate results from preexisting strain in the attached motor. The additional ideas underlying these models can be described in terms of three distinct components for strain amplification: a rotating lever arm, multiple attached states, and an elastic compliance within the motor. These components determine the how energy is recovered during the mechanical cycle and stored in a strained motor. They may coexist in a real motor; the challenge is to discover the relative contributions of each component. Since these components can generate similar relationships between strain energy and strain, standard measurements of motor function, such as relationships between force, ATP usage, and steady-state velocity, do not discriminate easily between these components. However, important information is provided by observations that suggest weak coupling between chemical and mechanical cycles, observations of apparent negative force and movement events in single motor experiments, and the discovery that two motors that move in opposite directions along a microtubule have very similar structures. In models incorporating changes in conformation between attached states, these observations are only explained easily if the conformational changes are tightly coupled to changes in the strength of motor-substrate binding.

4. Reactivation at low [ATP] distinguishes among classes of paralyzed flagella mutants

Erica Frey¹⁹, Charles J. Brokaw, Charlotte K. Omoto¹

Paralyzed flagella (pf) mutants of Chlamydomonas have been distinguished by the inability of the intact cells to move. Demembranated flagella from these mutants are also immotile when reactivated under standard conditions, with millimolar ATP concentrations. Three of these pf mutants were previously found to be motile when reactivated under three alternate reactivation conditions: low ATP concentration (<= 50 mM); 0.1 mM ATP combined with >0.5 mM ADP; or 0.1 mM ATP combined with non-reactivating ATP analogs anthraniloyl ATP or methylanthraniloyl ATP. We have now surveyed all pf mutants in the Chlamydomonas Culture Collection and discovered that a great majority of these mutants can move under these alternate nucleotide conditions. Only pf22 and pf23, mutants missing multiple subsets of dynein arms did not reactivate under those conditions. This suggests that the paralysis observed in most pf mutants is the result of inhibition by physiological [ATP]. Except for pf12, which has an abnormally symmetric bending pattern, all other pf mutants exhibits asymmetric bending pattern similar to wild-type. Previously, motility that was restored by the presence of suppressor mutations was found to lack the normal asymmetry of wild type flagella or the suppressor by itself. The waveform of pf mutants at alternate reactivation conditions in

the absence of suppressor shows that pf mutants with radial spoke or central pair defects are capable of asymmetric bending similar to wild type. A complete radial spoke/central pair complex is not essential for the production of asymmetric bending patterns.

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5. A motility function for the paraflagellar rod of Leishmania parasites revealed by PFR-2 gene knockouts

Jonathan H. LeBowitz¹, Charles J. Brokaw

The paraflagellar rod (PFR) is a structure found in the flagella of kinetoplastid protozoa that appears to be a latticework of protein fibers running parallel to the axoneme. The major protein constituents of the PFR are recognized to be two related proteins (PFR-1 and PFR-2). As an approach to understanding the structure and function of the PFR in Leishmania, L. mexicana lines were generated that are null mutants of PFR-2. These cells grow and divide essentially normally in culture and express the PFR-1 protein, but lack most of the PFR structure. The PFR-2 null mutant cells have greatly reduced forward swimming velocity, which results from a significantly reduced wavelength for bending waves propagated from tip to base in these flagella, and from reduced stability of the bending wave pattern. These studies are indicating a functional role for the PFR in the motility of Leishmania parasites.

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Summary: Accurate control over the growth and division of cells is crucial for the development of complex, multicellular organisms. Consequently, aberrations in cell cycle control can have profound consequences; the inability to restrain cell division, for example, contributes to the genesis of cancer. We have been studying cell cycle control in the baker's yeast Saccharomyces cerevisiae, because this organism offers many advantages. Yeast provides a unique opportunity to apply both genetic and biochemical approaches to resolve a multicomponent system. A large catalog of yeast genes required for cell cycle progression and regulation has been assembled from genetic studies. Many of the cell cycle genes from yeast have homologs that perform identical tasks in human cells. This striking conservation of function allows for comparative studies of the cell division cycle in yeast and mammalian cells.

The key challenge in the study of cell cycle regulation is to understand how cells transit from one phase of the cell cycle to another. Cell cycle transitions are dramatic events in the life of a cell; at the transition from G2 phase to mitosis, for example, the cytoplasm and nucleus are extensively remodeled to promote the coordinated execution of chromosome segregation. Recent results suggest that cell cycle transitions also serve as regulatory watersheds for 'checkpoint' signaling pathways that maintain the temporal organization of cell cycle events and ensure the fidelity of chromosome replication and segregation. Thus, a molecular description of the mechanism and regulation of cell cycle transitions will greatly illuminate our understanding of how cells grow and divide. It is currently thought that transitions from one phase of the cell cycle to another are coupled to fluctuations in the activity of a family of cyclin-dependent protein kinases (CDKs). These kinases represent a special family of kinases that are activated by regulatory proteins known as cyclins. Cyclins bind to the catalytic kinase subunit and trigger a battery of post-translational modifications that culminate in the activation of the kinase. Eventually, the kinase activity is extinguished by proteolysis of the stimulatory cyclin subunit.

We are currently focusing our effort on two cell cycle transitions: the entry into S phase and the exit from mitosis. Surprisingly, both of these transitions are triggered by the ubiquitin-mediated destruction of key cell cycle regulatory proteins. Ubiquitin is a small protein that can be ligated posttranslationally to substrate proteins. Upon attachment of ubiquitin, the substrate protein is rapidly destroyed by a multisubunit protease complex known as the proteasome. The key step in this process is the ligation of ubiquitin to target proteins, which is a highly specific and tightly regulated event.

The transition from G1 to S phase is driven by the destruction of an inhibitor (Sic1p) that restrains the activity of a cyclin/CDK complex that triggers DNA replication. We have recently shown that the ubiquitination of Sic1p can be achieved with a defined set of proteins in vitro (see Figure). Like the G1/S transition, the exit from mitosis is also driven by selective ubiquitination. In this instance, however, the ubiquitination is carried out by a distinct multisubunit 'machine' known as the Anaphase-Promoting Complex/cyclosome, and the targets that are degraded are the cyclin that maintains the activity of the mitosis-specific CDK, and proteins that regulate sister chromatid cohesion. We are combining the power of genetics, molecular biology, and biochemistry to probe how the ubiquitination pathways that trigger entry into S phase and exit from mitosis work, and to address how their activity is regulated as cells proceed through the division cycle.

Figure 1: A molecular model for the G1/S transition in budding yeast
6. Analysis of Clb2p ubiquitination by the Anaphase Promoting Complex in yeast
Julie E. Archer
The correct timing and coordination of stages of the cell cycle are essential to ensure accurate division. A key oscillation in the cell cycle is provided by the sequential presence and action of various cyclins. The abundance of cyclins is controlled primarily by proteolysis, and cyclin degradation depends on the ubiquitin system. Recently, a convergence of experimental approaches has led to the identification of the activities required for the ubiquitination of cyclin B. These approaches include work on previously isolated CDC genes with mutant arrest points at G2/M, a genetic screen for mutant yeast strains unable to degrade Clb2p, and biochemical purification of cyclin-selective ubiquitin ligase activity from clam and Xenopus oocyte extracts. Several labs have identified a large 20S "Anaphase Promoting Complex (APC)" which is altered during mitosis to become active for cyclin B ubiquitination and contains at least eight subunits (including Cdc23p).

We are interested in understanding the mechanisms that affect the activity of the APC and coordinate the morphological events of late mitosis. We can purify APC using several steps of affinity chromatography from a Saccharomyces cerevisiae strain containing multiply tagged Cdc23p. We are now beginning to characterize the requirements for APC activation by assaying the ubiquitination of an MBP-Clb2p substrate in crude yeast extract.

7. Regulated ubiquitination of the bHLH transcription factor Gcn4p in vitro
Yong Chi
Selective protein degradation contributes to the regulation of diverse cellular processes. Protein degradation plays a key role in tethering the concentration of a protein in vivo to the rate of its synthesis. The ubiquitin system is the major cytoplasmic pathway for selective protein degradation in eukaryotes. Gcn4p, a transcription factor involved in the regulation of general amino acid biosynthesis, has been shown to be very unstable and rapidly degraded via the ubiquitin pathway. The instability of Gcn4p presumably allows for rapid modulation of Gcn4p-dependent transcription. The destruction of Gcn4p in vivo is dependent on the ubiquitin-conjugating enzyme Cdc34p (Kornitzer et al., EMBO, 13, 6021-6030, 1994). To investigate the mechanism and regulation of Gcn4p turnover, we have been studying Gcn4p ubiquitination in vitro using fractionated yeast extracts supplemented with recombinant proteins. Our results to date indicate that Gcn4p ubiquitination in vitro requires Cdc34p and Cdc4p (see also Abstract 8 by Correll & Feldman). Fractionation of crude yeast extracts revealed two fractions essential for Gcn4p ubiquitination; one fraction contained the ubiquitination enzymes, and the other contained an unidentified kinase activity that phosphorylated Gcn4p in vitro. We are currently purifying this kinase. The purification and identification of the Gcn4p kinase will facilitate the reconstitution of Gcn4p ubiquitination in vitro using purified protein components and will allow us to investigate the regulatory mechanisms that control Gcn4p stability in vivo.

8. Destruction of the cyclin/CDK inhibitor Sic1 at the G1/S transition
Craig Correll, Renny Feldman
In Saccharomyces cerevisiae, the transition from G1 to S phase requires the activity of CDC34, CDC4, CDC53, SKP1 and at least one member of a family of G1 cyclins (CLNs) complexed with the cyclin-dependent kinase (CDK) Cdc28. In vivo, these genes conspire to eliminate the CDK inhibitor Sic1, which normally acts during G1 phase by binding S-phase cyclin/CDK complexes whose kinase activity is required for the initiation of DNA synthesis. Whereas the cloning and sequence analysis of CDC34 identified it as an E2 ubiquitin conjugating enzyme, the sequences of CDC4, CDC53, and SKP1 provide no clue as to their function. To investigate the molecular basis by which Sic1 is targeted for destruction, we have devised an in vitro system which efficiently reconstitutes the ubiquitination of Sic1. Highly purified preparations of recombinant Cdc4, Cdc34, Cdc53, Skp1, G1 cyclin/Cdc28 and Cdc34 are sufficient to support the ubiquitination of Sic1. If any single component is omitted, no ubiquiti-nation is observed; thus, these proteins are both necessary and sufficient for the in vitro ubiquitination of Sic1.

We have now begun to dissect the functions of each of these individual proteins. The specific phosphorylation of Sic1 by the G1 cyclin/CDK complex precedes and is required for its subsequent ubiquitination. Phosphorylated Sic1 is recognized and specifically bound by Cdc4, which through its direct association with Cdc53 and Skp1, brings the ubiquitination machinery to bear on Sic1. Mutation of four out of the nine CDK consensus sites within Sic1 renders the protein incompetent for ubiquitination in vitro and significantly stabilizes the protein in vivo (See Verma & Deshaies, 1997). Furthermore, the mutant Sic1, though still phosphorylated by G1 cyclin/Cdc28, is no longer recognized and bound by Cdc4. We propose that Cdc4 is a receptor that targets phosphorylated substrates for ubiquitin-mediated degradation. Furthermore, given the identification of CDC4, CDC53, SKP1, and CDC34 homologues in higher eukaryotes, this may be a general mechanism by which phosphorylated proteins are targeted for destruction.
9. A functional analysis of human CUL1 and its potential role in ubiquitin-mediated proteolysis

*Svetlana Lyapina*

In budding yeast, ubiquitin-dependent degradation of cell cycle regulatory proteins plays a key role in orchestrating cell cycle progression during Gl phase. For example, G1 cyclins, the G1 cyclin/Cdk inhibitor Far1p, and the S phase cyclin/Cdk inhibitor Sic1p are degraded via a ubiquitin-dependent proteolysis pathway that is comprised of the Cdc4p, Cdc34p, Cdc53p, and Skp1p proteins (see Abstracts 12 by Verma, and 8 by Correll & Feldman). The importance of this pathway in cell cycle progression is demonstrated by the phenotypes of cdc34, cdc53 or cdc4 temperature sensitive mutants, which arrest at the G1/S transition at the restrictive temperature.

We wish to address whether a homologous pathway of regulated protein destruction also plays an important role in controlling progression through Gl phase in human cells. There are unstable cell cycle regulators in mammalian cells whose abundance might be controlled via a similar pathway, including cyclins D and E, p53, E2F, β-catenin, and the Cdk inhibitor p27Kip1. A human homologue of CDC34 has been reported, and p27Kip1 is ubiquitinated in an *in vitro* reaction that is dependent on human CDC34. In addition, recent results suggest that C. elegans genes related to CDC4 and CDC53 play important roles in regulating cell division during nematode development. Six human homologues of CDC53 have been identified. We are studying the role of HsCUL1, the gene with the greatest homology to yeast CDC53. We have shown that expression of human CUL1 in yeast can complement the cdc53 temperature sensitive phenotype. We have used a two-hybrid approach to identify components of the CUL1 pathway in human cells. Through this approach we isolated HsSKP1, which is homologous to a protein shown to be important in this pathway in yeast. Coimmunoprecipitation studies demonstrated that CUL1 can also assemble into a complex with cyclin A/CDK2/SP2/SPK1. This result suggests that the CUL1/SP2/SPK1 complex might be functionally homologous to the yeast Cdc53p/Cdc4p/Skp1p complex and may act to target proteins for ubiquitination in human cells. We are currently trying to identify substrates for this degradation system.

10. CKS1 is required for G1 cyclin/CDK activity in budding yeast

*Greg Reynard*

p13*<sup>del</sup>/CKS5 proteins have been implicated in the regulation of cyclin/cyclin-dependent kinase (CDK) activity. However, the mechanism by which p13*<sup>del</sup>/CKS5 regulates the function of cyclin/CDK complexes has remained elusive. We have observed that CKS1 is required for the formation of active complexes between budding yeast G1 cyclin (CLN2) and CDK (CDC28) subunits. CLN2 and CDC28 subunits assembled in baculovirus-infected insect cells fail to exhibit protein kinase activity towards multiple substrates in the absence of CKS1. In contrast, CDC28 complexed to the B-type cyclins CLB1, CLB4, or CLB5 is active as a protein kinase regardless of whether CKS1 is present. Mutant cks1-38 cells lack detectable CLN2/CDC28 protein kinase activity, and fail to sustain the CDC28-dependent accumulation of CLN2 during Gl phase. CKS1 can stabilize CLN2/CDC28 complexes and can activate intact complexes, suggesting that it plays multiple roles in the biogenesis of active CLN2/CDC28 protein kinase. The ability of CKS1 to promote CLN2/CDC28 protein kinase activity provides a simple molecular rationale for the essential role of CKS1 in the G1/S transition. Ongoing work seeks to further elucidate the specific mechanism of this interaction.

11. Genetic analysis of the CDC15 pathway in the exit from mitosis

*Wenying Shou*

Cell cycle progression is driven by oscillations in activities of a family of kinases called cyclin-dependent kinases (Cdks). In *S. cerevisiae*, exit from mitosis is accompanied by degradation of B-type cyclins such as Clb2p as well as putative sister chromatin cohesion molecules. These proteolytic events require the activity of a multi-protein assembly called the Anaphase Promoting Complex, which is active from the beginning of anaphase until late G1 of the following cycle. In a number of temperature-sensitive mutants, including dbf2, cdc5, cdc14, cdc15, and tem1, the degradation of Clb2p is initiated but not completed, whereas degradation of putative sister-chromatin-adhesion molecules proceeds normally. Consequently, when shifted to restrictive conditions, these mutants are able to segregate chromosomes, but arrest with microtubule spindles characteristic of late anaphase and elevated levels of Cdk-associated kinase activity. To understand the regulation of this process, we have screened for mutants that interact with these known anaphase-defective mutants. Several mutants that suppress cdc15 or tem1 loss of function mutations have been isolated, and their molecular characterization is underway.

12. Defining the requirements for cell cycle-specific proteolysis of substrate proteins

*Rafi Verma*

Entry into S phase in budding yeast requires the activities of CDC34, CDC4, CDC53, SKP1, and the G1 cyclins CLN1-3. Genetic experiments suggest that these proteins collaborate to bring about the degradation of the cyclin-dependent kinase (Cdk) inhibitor Sic1p, which otherwise inhibits a cyclin/Cdk complex that is required for DNA replication. To investigate in more detail the relationship between Sic1p phosphorylation, Sic1p ubiquitination, and cell cycle progression, we sought to determine the sites at which G1 cyclin/Cdk complexes phosphorylate Sic1p. A
purified Maltose Binding Protein-Sic1 (MBP-Sic1p) chimera was phosphorylated with purified Cln2/Cdc28 protein kinase complexes. Phosphorylated MBP-Sic1p was digested with trypsin, tryptic peptides were fractionated by HPLC, and phosphopeptides were identified by electrospray mass spectrometry (ES-MS) in the negative ion mode (The ES-MS work was performed in collaboration with R. Annan, M. Huddleston, and S. Carr at SmithKline Beecham). ES-MS of the phosphopeptides in the positive ion mode was then employed to deduce the identity of the phosphorylated peptides and the sites of modification. This analysis revealed that Cln2/Cdc28 phosphorylates all eight S/T P dipeptides within Sic1p. In addition, 3-4 nonconsensus serines and threonines are modified. A subset of these phosphorylations also occur in vivo.

To test the significance of the phosphorylations observed in vitro and in vivo, we have mutated seven of the nine phosphorylation sites individually and in various combinations, and analyzed the Cdc34-dependent ubiquitination of each mutant in vitro. The properties of these mutants indicate that no single phosphorylation site is either necessary or sufficient to sustain ubiquitination of Sic1p. A triply-mutated Sic1p that fails to be ubiquitinated in vitro is stable in vivo and blocks DNA synthesis upon expression from the regulated GAL promoter. We propose that G1 cyclin/CDK activity triggers entry into S phase by phosphorylating Sic1p, which marks Sic1p for ubiquitin-mediated destruction via the Cdc34p pathway. These data provide the clearest example to date for how a cyclin-dependent kinase triggers a transition from one phase of the cell cycle to another.

Future studies will be focused on the mechanism by which ubiquitated phosphoSic1p is targeted to the proteasome.

**Publications**


**Associate Professor:** William G. Dunphy

**Senior Research Associate:** Akiko Kumagai

**Senior Research Fellows:** Phillip B. Carpenter, Thomas R. Coleman, Paul R. Mueller, Debabrata Patra

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**Summary:** In eukaryotic cells, the cyclin-dependent kinases (CDks) control the progression of the cell cycle by regulating the accurate replication of the genome during S-phase and the faithful segregation of the chromosomes at mitosis (M-phase). The entry into these phases of the cell cycle is controlled by CDks called S-phase promoting factor (SPF) and M-phase promoting factor (MPF). The action of these CDks must be controlled both temporally and spatially in a very stringent manner. This strict regulation is imparted by a number of checkpoint mechanisms. For example, cells containing unreplicated DNA cannot enter mitosis due to the mobilization of the replication checkpoint. The Dunphy laboratory is engaged in the elucidation of the molecular mechanisms underlying the regulation of SPF and MPF during the cell cycle. Most of these experiments are conducted with *Xenopus* egg extracts, a system in which the entire cell cycle can be reconstituted in vitro.

The first member of the cyclin-dependent protein kinase family described is M-phase promoting factor (MPF), which contains the Cdc2 protein kinase and a regulatory subunit known as cyclin B. Since the identification of the molecular components of MPF, there has been rapid and extensive progress in unraveling the biochemistry of mitotic initiation. It is
now well established that MPF acts by phosphorylating a myriad of structural and regulatory proteins that are involved directly in mitotic processes such as nuclear membrane disintegration, chromosome condensation, and mitotic spindle assembly. An ongoing challenge to the cell cycle field is the elucidation of how these phosphorylation reactions regulate the structural and functional properties of the various targets of MPF.

We have been most interested in how the cyclin-dependent protein kinases are regulated during the cell cycle. The principal focus of our laboratory has been on the regulatory mechanisms that govern the activation of MPF at the G2/M transition. Some immediate and long-term issues that we are tackling include:

1) What controls the timing of MPF activation so that it occurs at a defined interval following the completion of DNA replication?

2) How do various checkpoint or feedback controls influence the Cdc2/cyclin B complex?

3) What are the molecular differences between the simple biphasic cell cycle found in early embryonic cells and the more complex cell cycles that arise later in development?

More recently, we have been able to study at the molecular level some of the key events leading to the initiation of DNA replication at the G1/S transition. These events involve a cooperative interaction between the Origin Recognition Complex (ORC), the Cdc6 protein, and members of the Mcm family. These studies may ultimately help us understand how S-phase and M-phase are integrated with one another.

In principle, the regulation of cyclin-dependent kinases such as MPF could occur at any of several levels, including synthesis of the cyclin protein, association between the Cdc2 and cyclin proteins, or posttranslational modification of the Cdc2/cyclin complex. Posttranslational regulation of the Cdc2/cyclin complex is particularly important, even in early embryonic cells which manifest the simplest cell cycle programs. In recent years, many of the elaborate details of this Cdc2 modification process have been defined. For example, the binding of cyclin results in three phosphorylations of Cdc2: one at threonine 161 that is required for Cdc2 activity, and two dominantly inhibitory phosphorylations at threonine 14 and tyrosine 15. A variety of genetic and biochemical experiments have established that the inhibitory tyrosine phosphorylation of Cdc2 is an especially important mechanism of cell cycle regulation. A thorough understanding of the kinase/phosphatase network that controls the phosphotyrosine content of Cdc2 will provide a firm foundation for understanding other facets of mitotic regulation.

Our laboratory has made substantial contributions to understanding the molecular mechanisms controlling the activation of the Cdc2 protein. For our studies, we utilize cell-free extracts from Xenopus eggs. Due to pioneering work in a number of the laboratories, it is now possible to re-create essentially all of the events of the cell cycle in these extracts. Consequently, it is feasible to study the molecular mechanisms of Cdc2 regulation in intricate detail with this experimental system. To facilitate these studies, we make extensive use of recombinant DNA technology to overproduce cell cycle proteins in either bacteria or baculovirus-infected insect cells. Moreover, in conjunction with our biochemical studies, we are taking advantage of the fission yeast system to exploit genetic approaches to identify novel Xenopus regulators of the cell cycle.

13. Molecular characterization of Myt1, a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15

Sophie X. Wang, Paul R. Mueller

Entry into mitosis is controlled by the Cdc2 protein kinase which is in turn controlled by its association with cyclin B and by its phosphorylation state. Phosphorylation at two key residues of Cdc2, Thr14 and Tyr15, inhibit its activity. A variety of genetic and biochemical studies have indicated that Wee1 is the kinase that phosphorylates Cdc2 on Tyr15. In human and Xenopus, Wee1 is a soluble enzyme that phosphorylates Cdc2 on Tyr15, but not Thr14. Recently, Thr14-specific kinase activity has been detected in the membrane fraction of Xenopus egg extracts, but the molecular identity of the enzyme had not been established.

We have isolated a cDNA encoding Xenopus Myt1, a novel member of the Wee1 family that phosphorylates Cdc2 efficiently on both Thr14 and Tyr15. Phosphorylation of Cdc2 by this dual-specificity kinase dramatically reduces Cdc2 activity. Fractionation of Xenopus egg extracts showed that Myt1 is a membrane-associated protein, probably via its putative transmembrane segment. Immunodepletion studies indicate that Myt1 is the predominant, if not the sole, Cdc2-specific inhibitory kinase in Xenopus egg membranes. Moreover, since all of the Thr14-directed activity resides in the membrane fraction, Myt1 is apparently the major Thr14-specific kinase in Xenopus eggs. Finally, Myt1 activity is highly regulated during the cell cycle, suggesting that this novel relative of Wee1 plays an important role in mitotic control.

14. Cell cycle regulation of a Xenopus Wee1-like kinase

Paul R. Mueller, Sophie X. Wang, Peter Yakowec

Using a PCR-based strategy, we have isolated a gene encoding a Wee1-like kinase from Xenopus eggs. The recombinant Xenopus Wee1 protein efficiently phosphorylates Cdc2 exclusively on Tyr15 in a cyclin-dependent manner. The addition of exogenous Wee1 protein to Xenopus cell cycle extracts results in a dose-dependent delay of mitotic initiation that is accompanied by enhanced tyrosine phosphorylation of Cdc2. The activity of the Wee1 protein is highly regulated during the cell cycle: the interphase,
underphosphorylated form of Wee1 (68 kD) phosphorylates Cdc2 very efficiently, whereas the mitotic, hyperphosphorylated version (75 kD) is weakly active as a Cdc2-specific tyrosine kinase. The down-modulation of Wee1 at mitosis is directly attributable to phosphorylation, since dephosphorylation with protein phosphatase 2A (PP2A) restores its kinase activity. During interphase, the activity of this Wee1 homolog does not vary in response to the presence of unreplicated DNA. The mitosis-specific phos-phorylation of Wee1 is due to at least two distinct kinases: the Cdc2 protein and another activity (kinase X) that may correspond to an MPM-2 epitope (ME) kinase. These studies indicate that the down-regulation of Wee1-like kinase activity at mitosis is a multi-step process that occurs after other biochemical reactions have signaled the successful completion of S-phase.

15. Search for proteins involved in coordination of S-phase with mitosis

Zijian Guo

In eukaryotic cells, biochemical pathways have evolved to ensure that initiation of a late cell cycle event is dependent upon an early event. Particularly, entry into mitosis normally depends on successful completion of DNA replication. Perturbation of DNA synthesis by DNA replication inhibitors or DNA damage arrests the cell cycle at a point termed the mitotic entry checkpoint. Mutants that disrupt the checkpoint control have been isolated from various organisms, suggesting that checkpoint control is due to an active mechanism rather than an intrinsic feature of mitosis itself. By using the Xenopus egg extract system, it has been shown that ongoing chromosome replication can induce the mitotic checkpoint, while inhibition of DNA replication by DNA polymerase inhibitors enhances this effect. Furthermore, DNA synthesis on artificial single-stranded DNA templates in S-phase extract is sufficient to delay mitosis, indicating that the DNA replication itself generates a signal triggering the arrest at S/M transition. Based on these observations, it is generally believed that the S-M checkpoint mechanism can sense a particular structure of DNA replication, e. g., protein complex assembled at the replication fork or the single-stranded DNA, and transmit a signal to the downstream cell cycle machinery. I am currently trying to employ a few different approaches, including homology-based PCR and biochemical purification, to isolate the key players in the checkpoint pathway, and to characterize their function in Xenopus egg extracts.

16. Identification of Xenopus Plx1 as a protein kinase that phosphorylates and activates the Cdc25 protein

Akiko Kumagai

The Cdc25 protein is a dual-specificity phosphatase that dephosphorylates both threonine-14 and tyrosine-15 on Cdc2 and activates the kinase activity of Cdc2 at the onset of mitosis. The Cdc25 protein has a highly conserved C-terminal catalytic domain and less conserved N-terminal regulatory domain rich in Ser-Pro and Thr-Pro motifs. The activity of the Cdc25 protein is regulated by phosphorylation. During mitosis, the N-terminal regulatory domain becomes highly phosphorylated on serine and threonine residues and the catalytic activity increases 5-10 fold. Many studies have indicated that both the Cdc2/cyclin B complex as well as an additional unidentified kinase phosphorylate Cdc25 at mitosis.

We set out to purify the unknown kinase responsible for activating Cdc25 from Xenopus extracts. This attempt was aided greatly by our finding that a major Cdc25-specific kinase distinct from Cdc2/cyclin B associates with the N-terminal domain of Cdc25. Using a Xenopus Cdc25 affinity column and several conventional chromatographic methods, we purified a Cdc25-specific kinase activity approximately 2500-fold. We isolated several micrograms of this protein (p67) and sequenced four of its tryptic peptides. PCR primers designed for two of these peptides were used to amplify a segment of the cDNA encoding p67. The PCR fragment was used to isolate a full-length 2.4 kb cDNA that contains all four tryptic peptide sequences. The amino acid sequence indicates that it is a typical Ser-Thr kinase with catalytic domain and less conserved N-terminal regulatory domain and more conserved C-terminal catalytic domain.

Accordingly, we have named it Plxl, for Polo-like kinase from Xenopus. Recombinant six-histidine-tagged Plxl was then produced in Sf9 insect cells and purified on nickel agarose. This kinase phosphorylates and activates Cdc25 in vitro. We are now studying the regulation of this enzyme, which may be a key regulator of mitosis in all eukaryotic cells.

17. Role of protein phosphatases in cell cycle regulation

Debabrata Patra

A second enzymatic activity that regulates the activity of the Cdc25 protein is an inhibitory phosphatase that functions during interphase to maintain Cdc25 in its weakly active, 70 kDa form. We have found that this Cdc25-specific phosphatase is highly sensitive to okadaic acid, a potent inhibitor of serine/threonine phosphatases. This finding is consistent with our observation that the most active form of the Cdc25 protein is phosphorylated on serine and threonine residues. Dose-response studies with okadaic acid suggest that this Cdc25-inhibitory phosphatase may be type 2A-like, but definitive evaluation of this possibility will require direct examination of the responsible enzyme. A number of studies have demonstrated that treatment of Xenopus extracts with okadaic acid can accelerate the entry into mitosis. The consensus derived from these studies is...
that okadaic acid blocks the action of a phosphatase(s) that serves a negative regulator of cell-cycle progression. Here we have directly established that treatment of Xenopus extracts with okadaic acid results in the generation of a more highly active version of the Cdc25 protein. This observation suggests that at least one target of this inhibitory phosphatase is the Cdc25 protein. Moreover, we have found that the activity of this Cdc25-inhibitory phosphatase is turned down at the onset of mitosis, thereby facilitating the activation of the Cdc25 protein. We are taking a number of molecular approaches to study this mitotic regulator.

18. Identification of novel cell cycle control proteins

Phillip B. Carpenter, Paul R. Mueller, Debabrata Patra

The eukaryotic cell cycle machinery is highly conserved from yeast to vertebrates, and many factors are homologous and interchangeable between species. This functional conservation allows the cloning of cDNAs from higher eukaryotes by complementing temperature-sensitive lethal yeast mutants. The basic premise is to cure a mutation or deficiency by expressing a foreign cDNA and then to rescue and characterize that cDNA. A key advantage of this approach is that it does not rely on sequence similarities to isolate a gene. Thus, the cDNAs isolated will include those homologous to the wildtype genes as well as novel genes that encode suppressors or bypass proteins. We are particularly interested in using interspecies complementation to isolate novel, non-homologous genes since these will provide new insights into the regulation of the cell cycle.

We have constructed several Xenopus cDNA expression libraries that can shuttle back and forth between expression in yeast and growth in bacteria. Xenopus oocytes were used as the source of RNA for the library construction because they contain large stores of mRNA that are used throughout maturation and early development. The libraries differ in their yeast promoters so that we can modulate the level of Xenopus cDNA expression. The ability to modulate expression level is important because in some cases overexpression of a product can bypass the requirement caused by a mutation, whereas in other cases the overexpression of a protein can be deleterious. To date, we have used these libraries to rescue several Schizosaccharomyces pombe mutants that are deficient in their cell cycle regulation. At the restrictive temperature, one mutant enters mitosis before DNA replication is completed, and the other cannot enter mitosis. Both of these are lethal phenotypes. Several hundred Xenopus cDNAs have been isolated and these are being grouped into several classes based on restriction mapping and sequence information. We plan to assess the biochemical function of these novel cell cycle factors in extracts from Xenopus eggs.

19. Biochemical characterization of Xe-p9, the Xenopus homolog of Suc1/Cks

Debabrata Patra

A Xenopus homolog of the Suc1/Cks1 family of proteins from a Xenopus oocyte cDNA library has been identified and cloned. This protein has been named as Xe-p9 (or p9; Mr = 9 kD). Using recombinant p9 protein and anti-p9 antibodies, we have investigated the role of p9 in controlling the mitotic Cdc2/cyclin B complex (MPF) in cell cycle extracts from Xenopus eggs. The following is a brief summary of this study: (A) Xe-p9 is present in Xenopus egg extracts where it exists in a complex with Cdc2 and cyclin B2, suggesting that it is a functional homolog of the Suc1/Cks proteins. (B) Addition of recombinant p9 to egg extracts in the form of a 6-histidine fusion protein (His6-p9) can delay the entry into mitosis. The addition of His6-p9 can induce a significant delay, whose duration is directly proportional to the amount of His6-p9 added. (C) The mechanism of delay by His6-p9 is dependent on Thr-14 and Tyr-15 residues of the Cdc2/cyclin B complex, residues that need to be dephosphorylated by Cdc25 phosphatase at the G2/M transition. This suggests a possible role of p9 in modulating the activation of MPF. (D) Complete immunodepletion of p9 from Xenopus interphase egg extracts prevents the entry into M-phase; moreover, the capacity to enter mitosis is fully restored by the addition of His6-p9. This suggests that p9 is essential for entry into mitosis. (E) Significantly, immunodepletion of p9 also abolishes the ability of Xenopus egg extracts to degrade mitotic cyclins. Consequently, the p9-depleted extracts are unable to enter interphase and the next round of the cell cycle. Collectively, these observations suggest that p9 is an important molecule that plays a dynamic role in regulating cell cycle transitions. p9 is needed not only for activation of MPF but also for its inactivation through proteolysis of the cyclin B protein. Significantly, this implies that p9, besides binding to Cdc2/cyclin B, may interact not only with positive regulators that play crucial roles in the activation of the Cdc2/cyclin B complex at the G2/M transition, but also with negative regulators of the cell cycle.

20. The Xenopus Origin Recognition Complex

Phillip B. Carpenter

The six-subunit origin recognition complex (ORC) is essential for the initiation of DNA replication at specific origins in the budding yeast Saccharomyces cerevisiae. One important issue is whether DNA replication in higher eukaryotes, in which the characteristics of replication origins are poorly defined, occurs by an ORC-dependent mechanism. We have previously demonstrated that the Xenopus laevis Xorc2 protein resides in a large molecular weight complex and is required for the initiation of DNA replication in embryonic extracts (Carpenter et al., 1996). We now show that anti-Xorc2...
antibodies are capable of immunoprecipitating components of the *Xenopus* Origin Recognition Complex (ORC). The composition of immunopurified *Xenopus* ORC is in good agreement with other published results (Rowles *et al.*, 1996). We demonstrate that some members of the Xenopus ORC are phosphorylated, possibly by cyclin-dependent kinases. These modifications appear to be cell cycle regulated. A more thorough description of the cell cycle control of the Xenopus ORC is being conducted.

References

21. Characterization of *Xenopus* Cdc6 during the initiation of DNA replication in cell free extracts

*Christina A. Waters, Thomas R. Coleman*

DNA replication is one of the most critical events in the process of cell division. In order to ensure the fidelity required for eucaryotic nuclear DNA replication, evolution has dictated a highly conserved process and the proteins involved are conserved across species. Many proteins involved in replication have been identified in yeast, but the exact functions of these proteins have remained elusive. The use of cell free extracts from *Xenopus* offers a more tractable way to determine the role of these important proteins.

Initially discovered in yeast, CDC6 was determined to be essential for DNA replication. Our lab previously identified the Xenopus homolog, Xcdc6, and found that it is not only essential for replication, but is required for initiation of DNA replication. Immunodepletion of Xcdc6 abolishes chromosomal DNA replication, but not elongation on single stranded templates which emphasizes its requirement for initiation. By indirect immunofluorescence, we have shown that Xcdc6 binds to chromatin at the beginning of interphase and disappears from DNA in post-replicative nuclei. An association of Xcdc6 with the nuclear envelope in postreplicative nuclei has been observed. It remains to be determined whether the interaction with the nuclear envelope has any regulatory function.

Subsequent studies of Xcdc6 are aimed to further characterize the events necessary for binding of Xcdc6 to chromatin prior replication and its release after initiation. It has previously been determined that the association of Xcdc6 to DNA requires the presence of Xorc2, a component of the origin recognition complex. It has also been shown that chromatin binding by other proteins involved in DNA replication is Xcdc6-dependent. For example, Xmcm3 cannot bind chromatin in the absence of Xcdc6. This suggests the sequential formation of a replication complex in which Xcdc6 plays an essential role. The events necessary for the binding of Xcdc6 to chromatin via the ORC complex and necessary events for its proper and timely release are currently under study.

22. Examination of AFM tips by TEM

*Jim DeRose, Jean-Paul Revel*

As described in our previous reports, we have devised a system which allows the repetitive examination of...
the same AFM tip, say before and after use in the TEM. Using this device (DeRose & Revel, 1997), we have found evidence for tip wear during scanning of surfaces. Even an approach which should yield minimal wear, such as tapping mode which is believed not to involve contact between sample and probe, is violent enough to cause alteration to the tip shape. One would imagine that it is likely also to cause serious damage to delicate samples.

We have also obtained direct evidence for "contamination" of the tip. By that we mean debris from the sample accumulates at or near the end of the tip, and changes its shape during scanning. This greatly complicates the job of deconvoluting the actual shape of the sample, from an image of the sample and of the tip itself.

23. The gold standard

Jim DeRose, Jean-Paul Revel

Gold beads, easily obtained in different sizes, have often been used in various types of microscopy, including AFM, as a standard of known shape. We have found that, contrary to commonly held views, gold particles deviate substantially from sphericity. This was found by direct examination in the EM and by measuring the ratio of the shadow length at known shadowing angles (height) and particle circumference. Less than 40% of the particles deviate within 5% error from sphericity when using this criterion. A number of the gold beads are found actually to be single quasi-triangular (rounded corners) plates and many others to be composed of several overlapping plates. We suggest that the apparently spherical particles may actually consist of overlapping plates which only mimic sphericity. Obviously, they would not be useful as a calibration standard at high resolution.

24. Avoiding crystals in freezing samples

Tatiana Myagkova, Garun Gupta, Jean-Paul Revel

We have reported a method to prepare mixtures of LN2 and N2 ice which are stable at atmospheric pressure. Using this method, we were able to freeze red cells, and after etching, image both the fractured membrane and its external surface. Using standard holders, we found however that the freezing was not uniform across the whole sample, which we attribute to the fact that the samples are too massive to freeze uniformly. We have been devising a sample holder of low mass which would allow freeze cleaving from water (buffer solution) without antifreeze to avoid ice crystal formation in the whole sample. The holders consist of a square sheet of thin copper foil which is nearly cut in two, except for a tab holding the two pieces together. The side away from the tab is rolled into a cylinder. The sample is placed into the cylinder by capillarity, frozen in the melting N2, and then transferred to a holder that fits on the stage of the Balzer's freeze etch plant. The Knife holder is fitted with a piece of metal shaped so as to cleanly break off the sample holder in two.
Developmental Biology and Genetics

Marianne Bronner-Fraser
Eric H. Davidson
Scott E. Fraser
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Summary: This laboratory is concerned with analyzing the cellular and molecular events underlying the formation, cell lineage decisions and migration of neural crest cells. The neural crest is a segregated population in the early ectoderm, arising from the ectoderm of vertebrate embryos, which gives rise to a diverse range of cell types. Following neurulation, neural crest cells emerge from the neural tube and undergo extensive movements along pathways that often are characteristic of their axial level of origin. After migration, these assume one of a number of possible fates, ranging from neurons and glia of the peripheral nervous system to pigment cells and cells of the facial skeleton. What dictates the cell lineage decisions of these cells into one of numerous possible derivatives and what controls the precise and stereotypic pattern of neural crest migration? These areas represent the focus of my research.

It has been classically assumed that the neural crest is a segregated population in the early ectoderm, lying between the neural plate and presumptive epidermis. However, our recent studies on avian embryos show that individual precursor cells within the "neural folds" can form neural tube (central nervous system), neural crest (peripheral nervous system) and epidermal derivatives. This led us to explore the interactions that impart the potential to form the neural crest. Interestingly, we found that neural crest cells are generated when epidermis and neural plate are juxtaposed—a classical type of embryonic induction. Our current goal is to characterize the inductive interactions that lead to formation of the neural crest and to examine how regional differences arise in the neural crest populations along the rostrocaudal axis.

With respect to neural crest migrations, we are examining the mechanisms controlling cell movement by manipulating the environment using reagents that block specific cell-cell and cell-matrix interactions as well as ectopic expression of molecules of interest by retrovirally-mediated gene transfer. We can directly observe the effects of disrupting cell interactions on neural crest cell guidance by preparing trunk explants of living embryos in which neural crest cells are labeled with a membrane intercalating dye. Importantly, we have found that inhibitory cues inherent to the somites and notochord prevent neural crest cells from entering these domains. In addition, cell-matrix interactions mediated by integrins and extracellular matrix molecules are required for normal neural crest cell migration. Our goal is to identify interactions that are important for cell migration that subsequently leads to segmentation of the peripheral nervous system.

These studies shed important light on the mechanisms of neural crest formation and migration. Because neural crest cells are involved in a variety of birth defects and cancers such as neurofibromatosis, melanoma, neuroblastoma, our results on the normal mechanisms of neural crest development provide important clues regarding the mistakes that may lead to abnormal development or loss of the differentiated state.

25. Origins of the avian neural crest
Mark Selleck, Marianne Bronner-Fraser

Numerous fate mapping experiments performed on the ectoderm have shown that prospective neural crest cells lie at the lateral margins of the neural plate, adjacent to presumptive epidermis. Because of this geometry, we have tested whether neural crest cells are generated as a result of interactions between these two flanking tissues. By performing in situ and in vitro tissue recombination experiments in the chick embryo, we have demonstrated that neural crest cells are indeed generated as a result of interactions between neural and non-neural ectoderm. To investigate the time at which neural crest cells become committed to their lineage, we have performed single cell lineage analysis on the ectoderm at various stages of neurulation. Our results indicate that the non-neural (epidermal) lineage segregates from the neural/neural crest lineages around the time of neural tube closure, and the neural and neural crest lineages separate later, around the time when neural crest migrate from the dorsal neural tube. Therefore, commitment to a neural crest lineage is a late event and may result from the concomitant epithelial-to-mesenchymal conversion.

What is the molecular basis of the neural/non-neural (epidermal) interaction? A number of different genes have been isolated whose transcripts are localized to dorsal neural tube and might therefore play a role in neural crest cell formation. We have investigated whether three such genes (Wnt-1, Wnt-3a, Slug) are regulated by neural/non-neural interactions, and whether their in vitro expression...
correlates with the development of neural crest cells. Neural plate/epidermal interactions can induce expression of these three genes, but Wnt-1 and Wnt-3a do not appear to be necessary for the expression of many neural crest-associated markers. Our present experiments are directed toward establishing the nature of the molecules mediating the neural/ non-neural interactions, and the repertoire and function of genes expressed as a consequence of this induction.

26. Early- and late-onset cranial neural crest populations are not lineage-restricted in vivo

Clare Baker, Marianne Bronner-Fraser

We have studied the long-term fate and potential of early-migrating and late-migrating mesencephalic neural crest cell populations in vivo by performing isochronic and heterochronic quail-to-chick grafts. Both early- and late-migrating populations form melanocytes, neurons, glia, cartilage and bone in a segmental manner throughout the somites, providing a more complete context in which to examine the cues that guide neural crest. Previous studies have shown that cues intrinsic to the somites are responsible for this segmental patterning of neural crest. Early- and late-migrating populations form melanocytes, neurons, glia, cartilage and bone in vivo. Early-migrating populations distribute both dorsally and ventrally during normal development, while the late-migrating population is confined dorsally and forms much less cartilage and bone. When the late-migrating population is substituted heterochronically for the early-migrating population, it contributes extensively to ventral derivatives such as jaw cartilage and bone. Conversely, when the early-migrating population is substituted heterochronically for the late-migrating population, it no longer contributes to the jaw skeleton and only forms dorsal derivatives. When the late-migrating population is grafted into a late-stage host with neural crest that had previously been ablated, it migrates ventrally into the jaws. Thus, the dorsal fate restriction of late-migrating mesencephalic neural crest in normal development is due to the presence of earlier-migrating neural crest cells, rather than to any change in the environment or to any intrinsic difference in migratory ability or potential between early- and late-migrating cells. These results highlight the plasticity of the neural crest and show that its fate is determined primarily by the environment.

27. Isolation and characterization of NP1, a novel gene expressed in neural crest

Meyer Barembaum, Marianne Bronner-Fraser

We have isolated a clone from a cDNA library made from neural crest cultures which encodes a putative secreted protein which we refer to as NP1. The RNA was examined by in situ hybridization. NP1 is expressed in the early neural plate and then becomes restricted to the dorsal neural tube. Later NP1 is expressed by the migrating neural crest cells and down regulated in the dorsal neural tube. Once the neural crest begins to be differentiated, NP1 is present primarily in various ganglia in the head. Since the trigeminal ganglia is derived from both neural crest and placodal cells we determine whether NP1 was expressed from cells of neural crest or placodal origin. By injecting the lipophillic dye Dil into the neural tube prior to neural crest migration, we can label the neural crest cells. In situ revealed that these labeled neural crest cells expressed NP1. Similarly, the ectodermally derived placodal cells could be labeled by adding Dil to the outside of the embryo after the neural tube had closed. The placodal cells began to express NP1 after they began to migrate out of the ectoderm. Thus both the neural crest and placodal component of the trigeminal ganglia express NP1. Further experiments are underway to try to determine the function of NP1. We are using retro virus vectors to ectopically express NP1 in chick embryos. We have also isolated several mouse genomic clones of NP1 which we are using to develop mice lacking functional NP1.

28. Segmental patterning of trunk neural crest migration

Catherine E. Krull, Rusty Lansford, Andres Collazo, Christophe Marcelle, Scott Fraser, Marianne Bronner-Fraser

In avian embryos, trunk neural crest cells migrate in a segmental manner through the somites, entering the rostral but not caudal sclerotome. Previous studies have shown that cues intrinsic to the somites are responsible for the segmental patterning of neural crest. Attractive/permissive molecules located rostrally, inhibitory/repellent cues positioned caudally or a combination of these two types of influences could establish this remarkable cellular pattern. Although several cell surface and extracellular matrix molecules demonstrate the correct spatiotemporal distribution to influence neural crest patterning, little is known the roles these molecules play in vivo. To this end, we developed methods to explant whole trunk regions of chick embryos, providing a more complete context in which to examine the cues that guide neural crest. These explants offer several unique features including accessibility to a wide variety of perturbing agents and the ability to visualize the subsequent effects of these substances on neural crest migration over time. Previous studies have suggested that integrins, receptors for extracellular matrix molecules, participate in the segmental patterning of neural crest migration. To examine whether β1 integrin was involved in this process, a function-blocking antibody against β1 integrin was added to trunk explants. Surprisingly, no effects on the segmental nature of neural crest migration was noted although effects were observed on the rates and organization of cell movement. Two glycoproteins that bind the lectin peanut agglutinin (PNA) are distributed in caudal sclerotome and are thought to influence the patterning of motor axons in the somites. We recently tested whether these molecules played a role in patterning trunk neural crest migration by adding the lectin PNA to whole trunk explants. In the presence of the lectin, neural crest entered both
the rostral and caudal sclerotome, suggesting the PNA-binding proteins normally influence the segmental patterning of trunk neural crest. Analysis of the trajectories taken by labeled neural crest cells in the treated trunk explants revealed that rostral cells moved in their typical linear fashion but caudal cells navigated in a disoriented manner. One possibility is that caudal cells experienced the confusing influence of an additional caudal cue. Alternatively, caudal cells may miss a rostral cue that is required for coordinated migration.

Recently, the Eph family of receptor tyrosine kinases (RTK) and their ligands has been identified. Previous studies have suggested that these molecules pattern the retinal axonal projections to the tectum. We analyzed the distribution of receptors and ligands during trunk neural crest migration. Interestingly, the RTK QEK10 (EphB3) is expressed by neural crest and rostral sclerotome cells while the ligand Elk-L (Ephrin B1) is distributed in caudal sclerotome. To test whether receptor/ligand interactions were involved in patterning trunk neural crest, we examined the behavior of isolated neural crest migrating on stripes composed of ligand vs fibronectin. Neural crest avoided ligand-containing stripes and preferred the fibronectin lanes. This avoidance behavior was abolished in the presence of soluble ligand; neural crest migrated equally well on all lanes. Excess ligand was also added to whole trunk explants to disrupt receptor/ligand interactions. Neural crest cells entered both rostral and caudal sclerotome domains in the presence of exogeneous ligand, suggesting the ligand acted as a competitive inhibitor of receptor function.

These results, in combination, suggest that multiple cues influence trunk neural crest migration. Some cues appear to be responsible for coordinated migratory behavior rostrally while others prohibit entry of neural crest into caudal somite. This type of analysis should help to unravel the mechanisms that pattern the embryonic nervous system.

29. **Tissue interactions in inner ear formation**
   
   Andrew Groves, Marianne Bronner-Fraser
   
   The inner ear develops from a simple patch of ectoderm next to the hindbrain, called the otic placode. The mechanisms by which this piece of ectoderm is singled out to give rise to the otic placode are not clear. We have shown that two genes, BMP-7 and Pax-2 are expressed by the developing otic placode, and furthermore, are expressed in a thin stripe of ectoderm immediately adjacent to the hindbrain before the placode becomes morphologically distinct. These two genes are thus excellent early markers of the presumptive placode. We have performed studies in culture to show that cranial ectoderm becomes specified to express these two markers only a few hours before their normal appearance, and that once initiated, expression can be maintained in the absence of other signals. We are now using in ovo surgery to determine which structures are necessary for the initial induction of the otic placode.

30. **Coordinate actions of BMP, Wnt, Shh, and noggin mediate patterning of the dorsal somite**
   
   Christophe Marcelle, Michael Stark, Marianne Bronner-Fraser
   
   We have examined the early events that lead to the formation and organization of muscles, with emphasis on the tissue and molecular interactions that underlie patterning of the somites, from which skeletal muscle arise. We demonstrated that patterning of the dorsal compartment of the somite (the dermomyotome) is dependent upon BMP and Wnt signaling in the dorsal neural tube. Using Wnt-11 as a molecular marker for medial dermomyotome, we find that BMP indirectly induces Wnt-11 by up-regulating Wnt-1 expression in the neural tube. Wnt-11 induction is antagonized by Sonic Hedgehog, secreted by the notochord and the floor plate. Together, our results implicate the coordinated actions of the dorsal neural tube (via BMP and Wnts) and the ventral neural tube/notochord (via Shh) in patterning the dorsal compartment of the somite.

   These results are important, because they shed light on some of the complex molecular interactions that lead to the formation of an organized, functional muscle. We are now trying to understand the specific roles of some of the molecules implicated in somite patterning; the function that Wnt-11 is playing during myotome formation and organization.

31. **Neural tube/epidermal interactions are required for normal trigeminal placode formation**
   
   Michael R. Stark, John Sechrist, Marianne Bronner-Fraser, Christophe Marcelle
   
   In vertebrates, most cranial sensory ganglia develop with a dual contribution from the neural crest and ectodermally-derived placodes. Although much is known about the neural crest contribution, relatively little is known about how placode cells form, invaginate and migrate to their targets. We have identified Pax-3 and FREK as markers of trigeminal placode cells from early induction through ganglion formation. To explore the mechanisms of early placode formation, we have challenged normal tissue interactions through embryonic manipulation. After neural crest and neural fold ablation, we found Pax-3 expression in the placodal ectoderm to be normal or slightly reduced, indicating that the neural crest is not required for placode formation. However, after physical separation of the neural tube from the epidermis with foil barriers, epidermal Pax-3 expression was absent, suggesting that an interaction between the neuroectoderm and epidermis is required for normal placode induction. To determine the nature of this interaction, polycarbonate barriers which selectively prohibit cell contact but allow molecular signalling were used to separate the neural tube from
the epidermis. These experiments demonstrate that formation of the trigeminal placode is mediated by a secreted signal emanating from the neural tube. Current efforts include identifying the molecules involved in placode specification.

32. Molecular differences between cranial and trunk neural crest/neural tube

Brad Martinsen, Marianne Bronner-Fraser

The avian neural crest is a useful model system in which to study cell fate specification because it gives rise to diverse cell types including autonomic neurons, sensory neurons, glia, pigment cells, endocrine cells, paracrine cells, cartilage, and bone. Different populations of neural crest cells exist along the rostrocaudal axis that form region-specific derivatives. For example, only cranial neural crest cells have the ability to develop into cartilage and bone. The factors that enable the neural crest to generate diversity among an initially homogeneous population remain to be determined. One possible mechanism leading to fate specification could be that subpopulations of neural crest cells are pre-programmed to form specific cell types before the onset of migration. In order to isolate molecules potentially involved in position-specific determination, chicken RNA isolated from cranial neural fold tissue (4-6 somite embryo) was compared to RNA from neural tube tissue (16-22 somite embryo) using Differential Display. This technique used anchored and arbitrary primers to generate cDNA fragments by reverse transcription followed by PCR. The cDNA fragments were then electrophoretically separated and subsequently compared. The differences in cDNA patterns indicated differences in the mRNA levels and composition. The resulting 9 polyacrylamide gels were analyzed and approximately 100 bands were identified to be only expressed in the cranial tissue and not in the trunk tissue. The differentially displayed cDNAs were then isolated, sequenced, and used as probes for whole mount in situ hybridization. One cDNA fragment, CNC, showed expression in a sub-population of migrating cranial neural crest cells, as well as, the lateral aspect of the somites. Another cDNA fragment, DNC, was expressed in the neural fold and migrating neural crest cells. A third cDNA fragment, TNT, only showed expression in the trunk neural tube. The results verified that the isolated cDNAs were truly differentially expressed.

33. Roles of cadherins in neural crest migration and differentiation

Ben A. Murray, Martin I. Garcia-Castro, Marianne Bronner-Fraser

Avian neural crest cells arise from the neural fold of the ectoderm. They constitute a transient population of migratory cells that give rise to diverse cell types after reaching specific locations. Their emigration from the dorsal part of the neural tube involves an epithelial to mesenchymal transformation, however, the molecular nature of this event is largely unknown. The relevance of the regulation of adhesion molecules on epithelial-mesenchymal transformations seems obvious and has been proposed many times. Adhesion molecules of the cadherin family have been suggested to play a role in neural crest cells migration. In mouse and chick embryos, premigratory neural crest cells down-regulate the expression of some cadherins prior or immediately after migration. Recent evidence has demonstrated that in both organisms, sub-populations of migratory neural crest cells express different cadherins. Furthermore, since migrating neural crest cells express catenins, the cytoplasmic partners-in-function of cadherins, it is believed that other cadherins, that remain to be identified, are active on migrating neural crest cells. The main interest of this project is to understand the role of cadherins on the appearance, migration and differentiation of the neural crest cells. We are studying the regulation of cadherin expression by combined immunohistochemical and in situ hybridization analysis to determine whether expression is controlled by message abundance, polypeptide turnover, or both. We also are using ectopic expression of wild type and dominant negative forms of cadherin to understand how and if, cadherin function affects the origin, migration, and differentiation of neural crest cells in various experimental systems.

34. Functional role of the α4 subunit of Integrin in neural crest migration

Sung-Hee Kil, Marianne Bronner-Fraser

Interactions between cells and the extracellular environment are crucial for normal development. The extracellular matrix (ECM) is an organized network composed of glycoproteins, proteoglycan, and glycosaminoglycans that are secreted locally by cells. Cells adhere to these ECM molecules primarily through cell-surface receptors known as integrins. Integrins are composed of an α and β subunit that heterodimerize non-covalently. There are 20 αβ heterodimers known to date. Presently, we have cloned a full length avian integrin α4 subunit cDNA. In situ hybridization or antibody staining with integrin α4 mRNA probe showed that it is expressed on both migrating cranial and trunk neural crest cells, in chick and mouse embryos, respectively. In a threedimensional explant system which allows visualization of neural crest migration, we find that an anti-integrin α4 antibody blocks migration of neural crest cells at both hindbrain and trunk levels in developing mouse embryos. These results suggest that integrin α4 subunit is a major extracellular matrix receptor on neural crest cells and plays an important role in their migration.

Publications


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**Summary:** The major focus of research in our laboratory is on the molecular mechanisms by which specification of embryonic blastomeres occur early in development. Most of our work is carried out on sea urchin embryos, which provide key experimental advantages. These include an easy gene transfer technology; virtually unlimited availability of embryonic material, necessary for isolation of rare molecules such as transcription factors; an optically clear, easily manipulated embryo that is remarkably able to withstand micromanipulation, blastomere recombination and disaggregation procedures; a very well understood, and relatively simple embryonic process; and in-house egg-to-egg culture of the species we work with, *Strongylocentrotus purpuratus* (in a special culture system we have developed at Caltech's Kerckhoff Marine Laboratory).

We pursue an integrated, "vertical" mode of experimental analysis, in that our experiments are directed at all levels of biological organization, from the cell interactions by which many of the blastomeres achieve their identity, to the transcription factor-DNA interactions that control spatial and temporal expression of specific genes in the embryo. Current projects include: i. Analysis of cis-regulatory systems of territorially expressed genes: We are using in vitro mutagenesis and reconstruction of regulatory systems with synthetic DNA fragments in order to unravel the cis-regulatory "information processing system" by which genes determine where they are in the embryo, and whether to be expressed. In these studies synthetically altered cis-regulatory DNA from a territorially expressed gene, associated with a reporter sequence, is injected into eggs and its time and place of expression are assayed by whole mount *in situ* hybridization. The complex functional organization of these regulatory elements is turning out to be extraordinarily interesting and illuminating. At present studies of this kind are focused on the sea urchin Brachyury gene; the *Endo 16* gene; the mesenchyme and gut-specific CyIIa actin gene. We are also exploring local mechanisms of spatial repression in upstream regions of the CyIIa actin gene. ii. Isolation, cloning, and functional analysis of transcription factors: We have developed technologies for purification of transcription factors from embryo nuclear extract, followed by microsequencing, and hence cloning.
Transcription factors that interact with target sites of known function can thus be characterized, and their embryonic provenance determined. Six different transcription factors are at present being studied in this way: two gut-specific factors, and a repressor that serves as a terminus of a signal pathway, identified in studies on the Endo16 gene, which is expressed in the embryonic gut; one that causes skeletogenic gene expression; and a repressor and an activator of the aboral ectoderm specific Cyllia gene. iii. Sea urchin homebox gene cluster, and other embryonically expressed homebox transcription factors: We have isolated the sea urchin HOM-C genes and shown that they reside in a single genomic cluster. Their genomic organization and pattern of expression during development are of particular comparative interest, since echinoderms are the surviving sister group of all other major deuterostome members of the Animal Kingdom, i.e., chordates and hemichordates. We have also found a number of divergent homeobox regulators, and are searching for the earliest expressed cohorts of these and certain other classes of embryonic transcription factors. iv. Modern experimental embryology: We are using molecular markers, microsurgery, and microinjected lineage tracers to identify interactions among cleavage stage blastomeres that are essential for embryonic specification, and to determine the lineage origins of virtually all of the cell types and structures of the embryo. In this past year we have focused on lineage and specification of the vegetal plate and embryonic gut; and on the perturbations resulting from application of the teratogen LiCl. v. Development of new technologies for sea urchin embryogenesis: Among these projects are development of gene trap vectors; identification of sea urchin germ line progenitor cells; methods of studying spatial gene expression in the imaginal rudiments of advanced larvae; methods for interference with specific transcription factor functions in vivo; development of a series of genotyped inbred lines of adult sea urchins, using simple sequence repeat polymorphisms as markers. vi. Exploration of gene networks: We are isolating batteries of genes required for skeletogenesis, and other batteries of genes that are controlled by specific sets of transcription factors. In addition we are utilizing new highly sensitive protein microsequencing chemistry utilizing a mass spectrometer detector to directly isolate the complete set of skeletogenic matrix proteins from 2D gels. In another project we are utilizing high density arrayed cDNA libraries to seek sets of genes that respond to given embryonic transcription factors, a direct approach to isolation of batteries of unknown genes downstream of specific trans-regulators. vii. Methods for specific interference with gene expression in living embryos: We have built and are testing several "magic bullets" based on recombinant monoclonal antibodies and knowledge of transcription factors that play specific regulatory roles in embryogenesis.

viii. Retrotransposons of sea urchin and other marine invertebrate genomes: We have discovered that these genomes harbor retrotransposon sequences, some active in transposition and self recognition, at least on a recent evolutionary time scale. Sequence and polymorphism information obtained with reference to such cloned mobile elements may reveal the extent of insertion and deletion processes.

Transcription Factor Resource Center

The Transcription Factor Resource Center is now in its third year in the Beckman Institute. Its mission is to extend and enrich technology for working with transcription factors, particularly on a very small scale, and to use the new technologies to solve some major outstanding problems. Transcription factors are the immediate molecular operators of gene regulatory systems. The patterns of gene expression, and thus all cellular functions, depend on the constitution of the sets of active transcription factors that the cell presents at any given stage. We have partially purified and cloned a relatively large number of transcription factors from sea urchin embryos, which for various biological and technical reasons are among the most accessible experimental systems for transcription factor biology. We shall continue to exploit the sea urchin system. If successful, our new technologies will greatly increase accessibility to molecular analyses of transcription factors in many other systems, where amounts of material are far more limiting.

Technologies on which the studies summarized in the following Abstracts are based include: (i) New protein microsequencing technologies, including innovations in both pre-sequencer handling of protein samples and a new sequencing chemistry, the output of which is analyzed by mass spectrometry; (ii) use of capillary electrophoresis for detection of DNA-protein interactions under physically controlled conditions in extracts from only a small number of embryos or cells; and (iii) high resolution, quantitative 2D gel electrophoresis. This report describes the initial accomplishments and ongoing activities of the Center. The first three reports (15-17) describe the use and/or development of electrophoresis-based instrumentation and chemistry to better purify and analyze (in one- and two-dimensional electrophoresis and microscaled DNA/protein binding assays) the transcription factors involved in regulating the spatial and temporal development of the sea urchin embryo.

The final two topics were both initiated last year; one is a collaboration with the Physics Division at Caltech to develop microchip-based devices for manipulating and analyzing transcription factors; the others involve the application of high resolution, two dimensional electrophoresis to identify and characterize proteins involved in the transmissible dementia of humans, termed Creutzfeldt-Jakob disease, and a protein that affects sheep prostaglandin D synthesis, that is altered in various diseases of the nervous system.
The Transcription Factor Resource Center is also a co-sponsor, with the Genomics Resource Center of the Beckman Institute, of the new Genomics Technology Facility. This facility will provide to the Caltech research community automated DNA sample preparation, a high throughput robotic arraying service for plasmid-based recombinant DNA libraries; and production from these of high density clone filters.  

35. Transcriptional regulation of the CyIIa actin gene in sea urchin embryos and larvae  
M. Ina Arnone  
During all of early sea urchin embryogenesis the mRNA transcribed from a cytoskeletal actin gene, CyIIa, is specifically expressed in cells that utilize contractile pseudopodia for morphogenetic purposes. Primary mesenchyme, secondary mesenchyme and endodermal cells all express CyIIa at different times during development. In the mature plutei, by 72 hours, CyIIa expression is restricted to endodermal regions; in particular, strong midgut and hindgut staining are observed.  

To understand the transcriptional regulation of this complex CyIIa expression pattern, a study of the cis-regulatory system of this gene has been undertaken. Previous gene-transfer experiments have identified a 4.3 kb upstream domain of CyIIa which is able to drive accurate expression of a reporter gene (the chloramphenicol acetyl transferase, CAT) in plutei. Progressive deletions from the 5' end of this region have been made in order to determine the minimal domain that is necessary and sufficient for correct spatial and temporal control of CyIIa gene expression. After injection of those constructs into fertilized eggs, the level of CAT activity was measured at different times of development and the localization of CAT message was determined by whole-mount in situ hybridization. This analysis showed that the 1.35 kb proximal domain of CyIIa promoter contains all of the necessary information to correctly drive expression in all stages, from blastulae to plutei. A 1.5 kb region, located at the 5' end of the original 4.3 kb domain, has been also found to modulate the amplitude of expression driven by the proximal domain. A detailed analysis of the proteins that bind to a smaller region within the proximal domain, an element of 900 bp which contains all the positively acting cis-regulatory sequences responsible for the spatial control of CyIIa expression, is currently in progress. Among these interactions, a particularly interesting one involves the binding of an AP-1-like factor responsible for the late pattern of expression of the endodermal specific gene Endo16. The role of this factor in regulating the expression of CyIIa in the gut of late embryos will be examined by constructing a mutated version of the proximal element in which the AP-1-like site had been disrupted and assaying the effect on spatial expression in vivo. For this type of analysis we use Green Fluorescent Protein (GFP) as a reporter gene.  

The gene coding for this intrinsically fluorescent protein has been successfully used in our laboratory as reporter gene in live sea urchin embryos and turned out to be particularly effective for those genes, like CyIIa, which display intermittent expression in different cell types. The high stability of GFP and high sensitivity of fluorescence techniques together with the high resolution of confocal microscopy allowed us to precisely determine the spatial expression patterns controlled by different elements (including the ones with lower transcriptional activity) of the CyIIa cis-regulatory system during embryogenesis and also during larval development. GFP expression driven by the CyIIa proximal element has been detected in fact in the gut of one and two week-old plutei, thus giving information on a previously unknown spatial domain of expression of the endogenous CyIIa gene at larval stages.  

36. Functional intermodular interrelation and the delicate Cis-regulatory elements within the module A of Endo16 promoter  
Chiou-Hwa Yuh  
The Endo16 gene encodes a polyfunctional secreted glycoprotein of the sea urchin S. purpuratus. Endo16 is an endoderm-specific gene with an upstream regulatory region that can be divided into seven modules (A-G) in addition to the basal promoter (Bp). Each module displays a specific regulatory subfunction when linked with the basal promoter and in some cases other modules (Yuh and Davidson, Dev. 122, 1996). Module A primarily establishes the transcription of Endo16 in the vegetal plate and adjacent areas. Modules E and F negatively regulate expression in ectodermal cells and module DC negatively regulates expression in primary mesenchyme cells. Module B is responsible for the late stomach expression. Module A is very important since it exhibits at least four functions including: (1) it has early vegetal plate input; (2) it transmits the negative modules' input to the basal promoter; (3) it cooperates with modules B and G, and (4) it transmits all upstream information to basal promoter.  

A key endoderm specific element in module A is homologous to the Otx binding site. To further explore the mode of function of other target sites in module A, we analyzed quantitative time course measurements of CAT enzyme output in embryos bearing expression constructs controlled by various Endo16 regulatory modules including several mutations in different elements of module A. Module A amplifies the output of the others (B and G) by four-fold when linked with the basal promoter of Endo16. Mutation of the core sequence for Otx binding site did not effect the synergistic amplification function, on the other hand, mutation of the either the CG1 or the CP sites effected the cooperation between A and B modules. The time course profiles of expression constructs which the CG1 and CP mutation on module A linked with module B are the same as module A only. The AP1 site in module A
is needed for the activity of the negative modules. That is mutation of the API site in module A together with negative module abolishes the negative function and LiCl effect. The CG2 site mutation decreases output of A module by ~1/2 or BA by ~1/2 (the input of which is 4XB). Therefore we think CG2 interacts directly with basal promoter probably through the two CG sites in basal the promoter.

37. Purification and cloning of SpMyb, a spatial repressor of CyIIIa transcription in sea urchin embryos

James A. Coffman, Carmen V. Kirchhamer, Michael G. Harrington

Previously we demonstrated that the P7II target site within the middle module of the CyIIIa regulatory domain is necessary and sufficient to prevent ectopic expression of CyIIIaCAT reporter constructs in the oral ectoderm and skeletogenic mesenchyme of the embryo (see 1996 Annual Report Abstract 26). Because constructs lacking both the P7II site and the positively-acting SpRunt-1 site (see 1996 Annual Report Abstract 25) are expressed in a spatially normal pattern, the P7II target site must be specifically required to prevent ectopic activation by SpRunt-1. The P7II target site contains a perfect Consensus sequence for the myb family of transcription factors, that is, TAACGG. Furthermore, a protein purified on a P7II oligonucleotide affinity column was immuno-precipitated using a polyclonal antibody specific to the highly conserved myb DNA-binding domain, and the same affinity-purified protein was shown to contain amino acid sequences identical to sequences found in the myb domain of phylogenetically diverse species. We therefore used homologous sequence information to obtain cDNA clones encoding SpMyb, the myb domain transcription factor expressed in embryos of *S. purpuratus*. In addition to the highly conserved, N-terminal myb DNA-binding domain, SpMyb contains conserved C-terminal regions that are also found in vertebrate myb proteins. These regions contain several potential sites for phosphorylation by proline-directed serine/threonine kinases, one of which has known regulatory significance in murine c-Myb. Together these findings suggest that SpMyb functions as an intramodular repressor of CyIIIa transcription, i.e. to specifically interfere with ectopic activation by SpRunt-1 in the oral ectoderm and skeletogenic mesenchyme.

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38. Isolation of genes encoding spicule matrix proteins in sea urchin *Strongylocentrotus purpuratus*

Gabriele Amore

In sea urchin embryos two triradiate spicules develop, forming a skeleton which continues to elaborate in the larva. These spicules are secreted by the primary mesenchyme cells (PMCs), a group of cells that arise from the micromeres.

Before the onset of gastrulation, PMCs detach from the vegetal plate, ingress into the blastocoel and, after migrating along the wall of the blastocoel, aggregate to form a ring around the base of the invaginating archenteron. Two major clusters of cells accumulate in the ventrolateral position and from these two sites skeleton formation is initiated. Cellular interaction between the ectoderm and the PMCs regulate the shape, the size and the timing of spicules formation in a species specific manner.

The spicules are made of minerals and proteins. The mineral is calcite (95% calcium carbonate, 5% magnesium carbonate). By means of acrylamide 2D gels, 48 different proteins have been identified. The proteins constitute a matrix into which are embedded microcrystals of calcite. The proteins are mostly acidic and N-linked glycosylated. They seem to regulate the growth of the spicules by interaction with specific faces of the calcite crystals.

In *S. purpuratus* cDNAs encoding two different spicules matrix proteins have been isolated: SM50 (which encodes a non N-linked glycosylated protein with an alkaline net charge and a molecular mass of 50 kD) and SM30 (which encodes a 30 kD N-linked glycosylated protein with an acidic net charge). However genomic DNA gel blots suggest that two or four genes encoding for SM30 exist in *S. purpuratus*.

The aim of this study is to identify more genes encoding spicules matrix protein in sea urchin *S. purpuratus*. To do this, peptide sequences of each single protein will be obtained and corresponding oligonucleotides will be synthesized in order to identify the encoding genes in arrayed libraries. The major difficulties of this approach consist in separating each single protein, because of their high tendency to aggregate. To solve this problem a combination of acrylamide electrophoresis and reverse phase HPLC is being used.

39. Isolation and characterization of sea urchin homeobox genes: Expression pattern and genomic organization

P. Martinez

We have been interested during the last few years in the expression pattern of different homeobox-containing genes during the embryogenesis of the sea urchin *Strongylocentrotus purpuratus*, including genes of the *Antennapedia*, *engrailed* and *paired* classes. With sequence information currently available for homeobox genes from different species and using PCR, we have isolated and characterized clones corresponding to twelve different genes expressed at the pluteus stage. Eight of these clones belong to the *Antennapedia* class (we call them: Hbox1, Hbox3, Hbox4, Hbox6, Hbox7, Hbox9, Hbox10 and Hbox11). Two others are Antp-related genes, one similar to the *Xenopus XlHbox8* gene (Hbox8) and the other to the vertebrate H6-homeobox gene family (SpHmx). The final SpEn and Spalx are
the sea urchin homologues of the *Drosophila engrailed* and *aristaless* genes.

With the sequence now available, and using a RACE-PCR protocol, we have isolated clones spanning the 3′ region of two of these genes (Hbox1 and SpHmx). We have used these fragments as probes to screen a cDNA library from gastrula embryos made in the phage vector ZAP. Several cDNAs have been isolated covering most of the transcriptional units of Hbox1 and SpHmx. Their complete coding sequences has been determined. The Hbox1 gene appears to be homologous to the members of the paralogue group number 8 in vertebrates. At the gastrula stage of development both genes are represented by a unique transcript of 6.5 kb for the RACE-PCR protocol, we have isolated clones spanning Hbox1 and 3.5 kb for SpHmx.

The spatial expression patterns of the SpHmx and Spalx genes have been characterized by whole-mount *in situ* hybridization. SpHmx expression is confined to a subpopulation of mesenchymal cells, the pigment cells. The prd-related gene, Spalx, is expressed during gastrulation in a striking pattern that marks a left-right asymmetry in the embryo. This pattern arises probably as a consequence of the asymmetrical segregation of a maternal message during the first two cleavages as revealed by whole mount *in situ* hybridization1. We are trying to knock out the expression of this gene using antisense-oligonucleotides technology.

We have also isolated genomic clones for the Hbox genes: Hbox3, Hbox6, Hbox9, Hbox10 and Hbox11. From all these genes we have subcloned different pieces outside the homeodomain that we are currently using as specific probes to map these genes in the *S. purpuratus* genome. At the same time, most of them have been used to obtain fosmid clones.

With the aid of the pulsed-gel electrophoresis we have been able to show that in sea urchins there is a unique cluster of Hbox genes containing the genes Hbox1,2,3,4,6,7,9,10 and 11, the size of which is about 400 kb. We are currently making a PAC library2 of the sea urchin genome to fine-map, also with the help of the fosmid clones, the position of every gene in the cluster. We have already made a two fold coverage library with an average size per clone of 80-120 kb. The screening of this library with specific probes will speed-up considerably the construction of the contig covering the region of the homeobox cluster.

1In collaboration with Dr. Leroy Hood’s lab at Center for Molecular Biotechnology, University of Washington

41. Construction of an arrayed cDNA library and expressed sequences tags of early cleavage-stage sea urchin embryo

*Youn-Ho Lee, Jimmy Moore, Roy J. Britten*

In the purple sea urchin, *Strongylocentrotus purpuratus*, the first phase of cell type specification of an embryo is completed at 5th or 6th cleavage (7 hours post-fertilization) with the establishment of five embryonic territories. These are the presumptive aboral and oral ectoderm, the vegetal plate, the skeletogenic mesenchyme and the small micromeres. Considering the time of this specification, it is of particular interest to examine the genes expressed during this cleavage stage. To do this, we first constructed a plasmid cDNA library from the 7-hour embryo. From this library, clones were picked and arrayed in 384-well plates using a robotic system. In total, approximately 50,000 clones were arrayed. Then, high density colony blots, each containing 18,400 clones, were made for screening. We randomly chose approximately 760 clones and obtained the expressed sequence tags (ESTs) in collaboration with Leroy Hood at the University of Washington at Seattle. The results of this sequencing and comparison with known sequences in Genbank are summarized in the following table.
Table 1. Preliminary EST Sequencing Results

<table>
<thead>
<tr>
<th>Total clones sequenced</th>
<th>758</th>
<th>100.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins identified by homology with data banks</td>
<td>179</td>
<td>24.0%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Unidentified sequences</th>
<th>417</th>
<th>55.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>rRNA</td>
<td>109</td>
<td>14.0%</td>
</tr>
<tr>
<td>Mitochondrial sequences</td>
<td>42</td>
<td>5.5%</td>
</tr>
<tr>
<td>Other non-translatable sequences</td>
<td>11</td>
<td>15.0%</td>
</tr>
</tbody>
</table>

Total identified protein sequences 179
Receptors and transporters 28
Signal transduction proteins and signaling ligands (growth factors) 16
Transcription factors 45
Metabolic and synthetic enzymes 11
RNA processing, splicing factors, polymerase, and RNA binding proteins 14
Histones and HMG proteins 10
Cytoskeletal proteins 19
Translational initiation factors and ribosomal proteins 7
Miscellaneous other categories 28

42. Analysis of transcriptional regulation of the sea urchin Brachyury (T) ortholog SpTa
Yoshito Harada, Armenia Gregorian, Andrew R. Cameron

The most prominent characteristic of the chordates is the notochord, the namesake of the phylum. An understanding of the evolution of the notochord is crucial for an understanding of the origin and evolution of the chordates in general. Chordates, along with hemichordates and echinoderms, constitute the superphyletic assemblage Deuterostomia, whose monophyly is well supported from both morphological and molecular analyses. Therefore, developmental studies on echinoderms may shed insight into the origin of the chordates because although they are closely related to chordates, they do not have a notochord or any obvious notochord homologue.

Harada et al. (1995, 1996) have investigated sea urchin homologues of chordate transcription factors, viz. Brachyury and HNF-3b, which are implicated in notochord formation. The expression patterns of these genes in sea urchins suggest that they might function in some evolutionary conserved ways with respect to expression pattern(s) in chordates, but also in some divergent ways as well. Now our major interest is how differences in the regulation of these genes, or in their target genes, may lead to the radically different body plans of echinoderms and chordates.

To gain insight into the comparative developmental analysis of the regulation mechanism(s) of Brachyury expression, we originally isolated the orthologue of Brachyury in the sea urchin Hemicentrotus pulcherrimus (called HpTa) and then isolated the orthologue of HpTa in Strongylocentrotus purpuratus (called SpTa). We determined the transcriptional start site of SpTa and cloned a fragment of genomic DNA 10 kb upstream. In order to understand which region of this fragment is sufficient for the correct spatial and temporal expression, CAT/GFP reporter constructs were examined. Analysis is currently in progress.

43. The genetic introduction of a specific single-chain antibody inhibits a transcriptional repressor in vivo
Leonard Bogard, Ina Arnone, Chieh Chang, Armenia Gregorians, Eric Davidson

We have developed the technology and a test system that has demonstrated our ability to specifically block the function(s) of a transcription factor in vivo. In our test case, a sea urchin transcription factor of known function (P3A2) is the target of inhibition by the genetic introduction of an engineered antibody. An expression construct was designed to express, translate, and transport a highly specific single chain antibody to the proper place and time to inhibit the P3A2 protein from carrying out its developmental program.

The specific single chain antibody was selected by its ability to tightly bind P3A2 and inhibit P3A2 from contacting its own specific DNA binding site. To direct the antibody to the nucleus, a nuclear localization sequence was added to the N-terminus. To monitor the antibody in vivo a FLAG epitope tag was cloned at the C-terminus. Our construct is modular and we can readily “swap” promoters enabling us to express the antibody anywhere and when in the embryo. We cloned the 3' end of the SV40 T gene to splice, stabilize, terminate and polyadenylate the transcripts of our antibody producing construct. In vitro gel shift assays have shown the ability of our single chain antibody to inhibit P3A2-DNA interactions. This antibody was chosen over others which caused a super-shift; i.e., they bound P3A2 but did not inhibit DNA binding. With this in vitro data and our single chain antibody producing clone in hand we then proceeded with our in vivo test system.

A wild-type CyIIIa regulatory construct expresses exclusively in the aboral ectoderm, however, a CyIIIa regulatory construct which lacks P3A2 binding sites shows ectopic expression in oral ectoderm. These experiments demonstrates clearly that P3A2 is needed to repress the expression of CyIIIa in oral ectoderm. Our test system is to mimic this effect by creating ectopic expression of CyIIIa at high levels in the oral ectoderm by co-injecting a wild-type CyIIIa reporter construct (i.e., with both P3A2 sites intact) along with the anti-P3A2 expression construct.

Our microinjection experiments demonstrated that high levels of ectopic expression of the wild-type reporter can be caused, but only in the presence of the specific antibody expression construct. These levels cannot be achieved with control constructs or with the wild-type CyIIIa reporter alone. Hence, our antibody is generated, folded, transported and functions to Technically Knock Out P3A2 at least in the oral ectoderm. Interestingly, we have also observed a consistent mutant phenotype with this construct which
we are in the process of further characterization. It is
our hope to extend this approach to other
developmental and clinical applications.

44. A GAL4-driven targeted system for the
overexpression and misexpression of transcription
factors in the developing sea urchin
Jonathan P. Rast, Eric H. Davidson

The sea urchin embryo has proven to be an efficient
model system in which to explore the control of gene
expression during early metazoan development. Given
a gene of interest, reporter construct analysis and
affinity chromatography can be used to isolate the
proteins which regulate its transcription. The ability
to isolate these transcription factors provides a means
to climb upstream in the transcriptional regulatory
network from any given expressed gene. While this
type of analysis has become almost routine in the sea
urchin system, no analogous method is available in any
developmental model system to move in the opposite
direction. That is, given a transcription factor, there
is no standard means to identify the genes that it
regulates. To this end, we have begun work on a gene
construct system by which the overexpression or
misexpression of any transcription factor can be
targeted to a particular region of the sea urchin embryo.
This system utilizes modifications of two constructs
which have been employed previously to target gene
expression during Drosophila development: (1) the
yeast GAL4 gene under the control of a defined promoter
that is active in the sea urchin embryo and (2) a second
construct containing the gene of interest under the
transcriptional control of 5 UAS-G (GAL4 target) sites.
We have shown that the GAL4 gene under the control
of the complete sea urchin Endo16 promoter is able to
drive the expression of a UAS-green fluorescent protein
(GFP) construct in an manner that is temporally and
spatially consistent with the expression of the
endogenous Endo16 gene. Similar results were obtained
with an H2B-GAL4/UAS-GFP system. Comparisons
of embryos injected with Endo16-GAL4/ UAS-CAT
constructs to those injected with an Endo16-CAT
construct demonstrate a 3-5-fold enhancement of CAT
activity in the GAL4 system. Cells overexpressing the
transcription factor of interest can be identified by the
coinjection of UAS-GFP. The mode of construct
incorporation into the DNA of injected embryos ensures
that those cells which express GFP also express the
UAS controlled transcription factor. Fluorescing cells
can then be sorted from dissociated embryos and their
mRNA content compared to GAL4/UAS-GFP injected
controls. We are currently working on cDNA
amplification techniques that will allow the direct
analysis of the transcription products from embryonic
cells which overexpress a transcription factor. This
analysis will be done in the context of the arrayed
cDNA library screening (currently being developed
by L. Bogarad) in which we can globally identify
and quantitatively characterize genes responding
both positively and negatively to the manipulated
transcription factor.

45. Isolation of transcription factors from early stage
sea urchin embryos and sea urchin immune effector
cells
Michael Rhoads, Jonathan P. Rast,
Michele K. Anderson, Eric H. Davidson

We have initiated a PCR-based approach to
isolate transcription factor (TF) genes expressed in the
eyear sea urchin embryo (15 hours post-fertilization).
At this stage of development, the primary territories
of the sea urchin larva have been established and
differential gene expression has been initiated. A
previous investigation enumerating early sea urchin
embryo nuclear extract protein species that have
characteristics of transcription factors (TFs) indicates
that less than 200 different proteins are represented.
PCR primer sets that target phylo-genetically
conserved motifs from a range of TF gene families are
being used to amplify expressed genes that
are candidate regulators of early embryonic
transcription. Template for amplification consists of
first strand cDNA generated from polyadenylated
RNA by reverse transcription from both poly-T and
random primers. The primers used for amplification
in this screen are degenerate and complement 4-6 amino
acid motifs which are conserved throughout the gene
family of interest and, when possible, over a
phylogenetic range that encompasses the echinoderms
(i.e., conserved in both protostome and deuterostome
representatives). These targeted TF families have
been demonstrated to play a role in vertebrate
hematopoiesis and have been successfully employed
in the isolation of a wide variety of TFs from mouse
thymocytes in a separate investigation. To begin to
understand the role of these genes in the generation of
the echinoid immune system, TF genes present in cDNA
derived from coelomocytes, the sea urchins immune
effector cells, are also being investigated. While a
PCR based screen of this type is unlikely to isolate
all TFs present, it has the potential to identify a
significant fraction of those operating in these systems
and will complement other methods (e.g., affinity
chromatography) being used to isolate the regulators
of sea urchin gene expression.

46. An enhancer trap strategy
R. Andrew Cameron, Len Bogarad,
Armenia Gregorians

The cis-regulatory regions of territorially
expressed genes in the sea urchin embryo constitute
information processing systems that determine how
genomes are expressed in time and space. Since the control
regions of conditionally specified genes are usually a
few kilobases in length and there are probably about
10,000 genes active in developing embryos, we estimate
that at least 1% of randomly-cloned fragments should
contain an enhancer expressed during early
development. We are developing a strategy that
employs chloramphenicol acetyl transferase (CAT) reporter constructs to find novel enhancers. Libraries of genomic DNA selected to a size of 3-6 kb are prepared in a plasmid vector containing a basal promoter that shows no spatial specificity. Pools of library clones are injected into sea urchin zygotes and assayed for reporter construct activity by enzyme assay. Pools with high activity are deconvolved and the individual active clones are analyzed by whole mount in situ hybridization in order to find those which are expressed in a spatially restricted manner. In parallel, we are testing the ability of different basal promoters to support expression by known cis-regulatory regions and by the cloned genomic fragments.

47. Contributions to endoderm differ in oral versus aboral Veg1-derived clones
Andrew Ransick

Cell lineage analyses have been employed to investigate the lineages contributing to the endoderm of the pluteus larva of the sea urchin Stronglylocentrotus purpuratus. This detailed analysis was prompted by the observation that Veg1 tier derived cells make a significant contribution to the endoderm in the sea urchin L. variegatus. This observation was confirmed in S. purpuratus using the application of ‘Di’ to single macromere granddaughter cells in the 6th cleavage embryo, (i.e., Veg1 or Veg2 tier cells). These studies not only confirmed that some progeny of each of the eight Veg1 tier cells contributes to the endoderm, but also showed that the entire hindgut and a portion of the stomach derives from the Veg1 tier cells. Additional new findings include: 1) that Veg1 derived cells begin invaginating beyond the blastopore region after the archenteron is in contact with the oral field, i.e., between 35 and 45 hours post-fertilization; previously it was thought that gastrulation was essentially complete when the archenteron contacted the oral field; and 2) in S. purpuratus the contributions of Veg1-derived cells to the endoderm differ significantly circumferentially around the oral-aboral axis, with oral clones contributing >50%, aboral clones contributing <50%, and lateral clones contributed approximately 50% of their cells to the endoderm.

These findings prompted a reinvestigation of the spatial domain of Endo16 transcription, a gene which is expressed in the vegetal plate and endoderm. A detailed analysis of the lineages expressing this gene was made using microinjection of lysinated-biotinylated dextran into macromere daughter and granddaughter cells, followed at the appropriate stage by an avidin/HRP reaction to detect the dextran and whole mount in situ hybridization to detect Endo16 mRNA. The results show that Endo16 expression is confined to a subset of Veg2 derived cells until the late mesenchyme blastula stage (~26 hours); however, by the initial phase of gastrulation (~30-32 hours) the Endo16 expression domain has expanded include 10-20% of the Veg1 derived progeny. Thereafter, the percentage of each Veg1 clone expressing Endo16 (from 10 to 70%) is directly related to the particular clone's contribution to the endoderm.

48. Sea urchin population genetics
R. Andrew Cameron, Jane Wyllie, Paul Kim

A variety of techniques that display a range of precision are employed to measure the genetic variation in populations of organisms. We have adapted a precise method utilizing microsatellites to map genomic variation in purple sea urchin genomes. We are characterizing genomic repeat sequences of GA which average about 15 repeats. Using the adjacent single copy sequences as primers in the polymerase chain reaction, we amplify these sequences from individual genomic DNA samples and measure the size of the DNA fragment. These can be treated as alleles in the calculation of genetic variation. As a first step we have employed eight different microsatellite loci to determine the genetic variation in our inbred line of sea urchins which is 6 generations deep. An analysis of the second and third generations of this line showed several interesting points: 1) There are no more than four alleles at each locus, as Mendelian ratios would predict for a line started from two individuals; 2) Several loci showed a loss of alleles going from the second to the third generation; 3) The degree of heterozygosity is quite variable for different loci, suggesting that the regions of the genome that bear the microsatellite loci are under very different degrees of selection. Analysis of individuals from the fourth and fifth generations are continuing.

To test the efficacy of these markers in the assessment of the genetic structure of natural populations we are collecting microsatellite sizes for two widely separated populations of purple sea urchins. Preliminary tests with just two markers yield calculations of genetic distance at levels similar to those between species of terrestrial animals. We conclude that these measurements are probably a reflection of the high intraspecific genomic variation as well as of the separation of the two test populations.

49. Using gene expression patterns to test hypotheses concerning the evolution of deuterostomes
Kevin J. Peterson, Yoshito Harada, Eric H. Davidson

Davidson et al. (1995) and Peterson et al. (1997) noted a very interesting taxonomic paradox within deuterostomes: echinoderms and enteropneust hemichordates share embryonic and larval, but not adult, characters; whereas enteropneusts and chordates share adult, but not larval, characters. This leads to the hypothesis that enteropneusts and chordates share a common adult body plan, one with a notochord, dorsal hollow nervous system, and gill slits, but that enteropneusts share only a larval body plan with echinoderms, the adult body plans of each evolving independently. A prediction of this hypothesis is that expression of a gene involved in a chordate structure
should be expressed in the putative adult hemichordate homologue, and this same gene will be expressed in similar structures in both echinoderm and enteropneust embryos/larvae. Numerous studies in all three chordate subphyla have identified several genes involved in the development of the notochord, including the transcription factors Brachyury, Forkhead, and Not. Studies in sea urchins, however, have shown that both Brachyury and Forkhead are expressed in the vegetal plate of sea urchins. We have now cloned the orthologue of Not in Strongylocentrotus purpuratus (SpNOT). This gene shares 75% amino acid identity in the homeobox with XNOT and 77% with CNOT2, and groups with the NOT family in a maximum parsimony analysis of homeobox genes. It also shares similarity with the vertebrate NOT genes in the short carboxy-terminal extension of the homeodomain. Northern analysis shows that NOT is present as a maternal message and expression is upregulated at 36 hours post-fertilization which corresponds to the early stages of gastrulation, and then is downregulated by 48 hours to egg levels. We are now examining the spatial expression patterns of SpNOT, and expect elevated expression to be associated with the vegetal plate.

We are also in the process of cloning the NOT orthologue in the enteropneust hemichordate Ptychodera flava. We will examine its expression pattern during both embryogenesis and metamorphosis. Expression in the vegetal plate during gastrulation, and then reexpression during metamorphosis in the stomochord, would confirm the evolutionary ideas discussed above, and provide the first experimental test of an evolutionary hypothesis regarding deuterostome evolution.

References

50. Mobile elements inserted in the distant past now have important functions
Roy. J. Britten

The project studying the long-term evolutionary effect of insertion of mobile sequence elements is continuing and the number of clear cases has grown. There are twenty-one examples from Drosophila, sea urchin, human and mouse genomes that meet the following criteria: (1) the element was inserted far in the past and thus the event is not a transient mutation; (2) the element is a member of a large group of similar sequences; (3) the inserted element now serves a useful function. One remarkable case of long-standing use of mobile elements is in the telomeres of Drosophila. The telomeres of most eukaryotes contain short simple repeats that are highly conserved. Drosophila does not have such sequences but has one or more LINE-like retrotransposons. Instead of elongation by telomerase, incomplete DNA replication at the termini is counterbalanced by transposition of these elements at high frequency specifically to the termini.

Among the 21 cases of now useful mobile element insertions are eight examples in which Alu repeats perform regulatory functions in the human genome. SINEs such as the Alu sequences may be important since they contain a variety of binding sites for control proteins or at least potential binding sites. The Alu that forms a hypersensitive site in an intron of the CD8 gene contains four binding sites that are important to its function as an enhancer. The sites apparently evolved to their functional form after insertion, possibly under the influence of positive selection. Many of the human Alu sequences contain effective binding sites for the retinoic acid receptor and the thyroid hormone receptor. In some cases these sites have been shown to be significant to the control of transcription. Taken together these 21 examples show that the insertion of sequence elements in the genome has been a significant source of regulatory variation in evolution.

51. Rapid exchange between ends of different yeast chromosomes
Roy J. Britten

In studying the complete DNA sequence of the Saccharomyces cerevisiae genome a reverse complementary relationship between individual repeats in the left and right subterminal regions of yeast chromosomes was observed. This led to an examination of the relationships of the complete terminal regions which uncovered large terminal regions of all chromosomes that were the reverse complement of the other ends of other chromosomes. For every one of the sixteen chromosomes a good (88-100%) reverse complementary copy of several kilobases of the left terminal region is present in another chromosome as the right terminal region. The length of the reverse complementary regions ranges from 3 to 19 kilobases. In all but one case the inverted sequence similarity extends to the exact end of one or the other chromosome. For any given chromosome the reverse complementary relationship between its two ends is short, imprecise or absent. There is no significant direct sequence similarity between the opposite ends of same or different chromosomes. It appears there has been an extensive history of sequence exchange or copying between the terminal regions of inverted pairs of distinct chromosomes. It is likely that the process has been regenerative since, once inverted sequence regions are present between opposite ends, the chromosomes may possibly be aligned as inverted pairs by the mechanisms that permit normal recombination to occur between matching pairs of diploid chromosomes.
52. Amino acid sequence determinations of sea urchin transcription factors at different stages of embryo development

Miki Yun, Jina Yun, Michael Harrington

The mechanism by which genes are temporally and spatially regulated with precision in an embryo directly involves the transcriptional proteins that control these genes. To understand how this is achieved requires detailed analysis of the structure and interactions of these proteins at different stages of development when their activities are known to vary. We have found that all proteins studied thus far (nine) have different isoforms. It seems likely that the underlying structural basis for these isoform variations is related to their known variable function in gene regulation during the early stages of development. It will be necessary to determine the structural basis for these isoform modifications. Thus far, one of the factors, SpP3A2, is shown to be phosphorylated.

We are using standard Edman degradation chemistry to obtain partial amino acid sequences when there is at least 3-5 picomoles of pure peptide available. When transcription factors are present in low abundance we have adopted a chemistry suitable for greater sensitivity, based on a modified phenyl isothiocyanate (PITC-311). This reagent has been shown to have kinetics that are comparable to standard PITC but is also efficiently ionized for mass spectrometry detection. The sensitivity of detection of the PITC-311 derivatized amino acids by mass spectrometry is in the attomolar range and its sensitivity in an automated sequencer mode has been demonstrated at 100-500 femtomoles. We are currently attempting to implement this chemistry with a micro-liquid chromatography interface directly from a commercial protein sequencer to an electrospray mass spectrometer. In order to achieve the necessary volume reduction from the sequencer, we are using a miniaturized conversion flask that will deliver one fifth the normal volume from the sequencer to an enrichment pre-column before the final micro-LC/mass spectrometry step. This approach will allow both sequence determination and analysis of post-translational modifications in the further structural characterization of these transcription factors form the different staged embryos.

53. Nano-fabricated devices for picoliter-scale analyses of embryonic gene regulation

Mi Chang1, Jeremy Mans1, Michael Roukes1, Michael Harrington

Many important analytical studies of biomolecules are not feasible because current technology is both too insensitive and at a scale too gross for experimentation with extremely small sample volumes. The overall objective of this work is to explore the potential of micro- and nanometer scale technology to overcome these limitations. Two underlying principles motivate this work: molecular detection capability can be greatly enhanced in micro/nanometer scale devices; small volumes, from micro- to picoliters or less, can be more appropriately handled in small devices.

To study TF/DNA binding from small regions within an embryo, or from individual cells, microdevices that provide the following functions will be sequentially constructed, evaluated and optimized in this research program: manipulation and transport of cells, small tissue fragments and solutions; lysis of cells/organelles to open their contents for further analysis; reagent mixing, to combine synthetic, fluorescent oligonucleotides and unlabelled, non specific DNA with cell extracts; electrical conductivity measurements and optical absorption spectroscopy upon picoliter solutions; electrophoretic separation and detection of fluorescent molecules.

The first devices have been fabricated in which electrophoretic migration and separation of fluorescent oligonucleotides are under investigation.

1Division of Physics, California Institute of Technology

54. Premortem molecular markers for the diagnosis of transmissible spongiform encephalopathies

Kelvin H. Lee, Michael G. Harrington

The transmissible spongiform encephalopathies are a class of neurodegenerative diseases characterized by an accumulation of an insoluble form of the PrP protein and spongiform pathology and include Creutzfeldt-Jakob disease in humans, scrapie in sheep, and bovine spongiform encephalopathy (Mad Cow disease) in cows. These diseases are uniformly fatal in all species and no premortem diagnostic test has yet been developed. We have recently identified and characterized a change in the protein composition of the cerebrospinal fluid of clinically-affected humans and animals which correlates with the onset of these diseases. The premortem appearance of the 14-3-3 gamma protein in spinal fluid, as determined by SDS-PAGE immunoassay, has a >96% sensitivity and >99% specificity for these diseases.

In collaboration with the Caltech peptide synthesis group and the Caltech monoclonal antibody production facility we are making monoclonal antibodies against the gamma isoform of the 14-3-3 family. Initial results with our monoclonal antibody demonstrate significantly increased specificity over commercially available polyclonal antisera against a similar epitope. In particular, the 1C1 monoclonal antisera recognizes a single 30 kDa band from spinal fluid while antisera from all other sources immunoreacts with two 30 kDa bands. It appears that our 1C1 mAb can selectively recognize human subjects that are false positive with the standard assay.

We are doing immunohistochemistry to further characterize any role between 14-3-3 and PrP and spongiform pathology. Using antibodies against neuron and glial specific proteins as well as against PrP, 14-3-3 and prostaglandin D synthetase (another molecular marker for disease) we are staining tissues from
patients with Creutzfeldt-Jakob disease as compared to other diseases and controls.

Publications


Britten, R. J. (1997). Rapid exchange of many kilobases of sequence at the opposite ends of all yeast chromosomes. Submitted for publication.


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Gordon and Elizabeth Ross

Summary: Biological imaging has emerged from a confluence of biology, physics, chemistry and engineering, creating a family of new techniques capable of imaging biological structures ranging in size from whole organisms to single molecules. Instead of recording microscopic images on film, these newly-developed digital microscopies can treat image data quantitatively as an array of intensities that can be processed digitally. This processing permits the light microscope to attain resolutions in living cells to be imaged at resolutions previously thought to require electron microscopy. Magnetic resonance imaging (MRI) has become a mainstay for non-invasive imaging of whole organisms to single molecules. Instead of developing digital microscopies can treat image data quantitatively as an array of intensities that can be processed digitally. This processing permits the light microscope to attain resolutions in living cells to be imaged at resolutions previously thought to require electron microscopy. Magnetic resonance imaging (MRI) has become a mainstay for non-invasive imaging of internal structures in humans; recent refinements made here and elsewhere have permitted the development of MRI microscopy, capable of single-cell resolution. An exciting new development is two-photon microscopy, which enables high-resolution imaging deep within tissue with dramatically less photobleaching and virtually no phototoxicity.

The major approach used in this laboratory is to refine the new digital microscopes so that they can be applied to in vivo imaging of living tissues and organisms. A great deal of our effort has gone towards developing the needed techniques and reagents for imaging developing embryonic tissues. Individual projects combine the tools of experimental embryology, cell biology, molecular biology, and chemistry with imaging to examine key events in the patterning of cell migration, the assignment of cell phenotypes, and the patterning of axonal connections. Microinjection techniques, in which single embryonic cells are rendered unique by the introduction of a marker dye, permit individual cells and their descendants to be followed uniquely in the developing embryo. Using this combination of modern and classical approaches, we are determining in intact organisms the roles of the many factors and mechanisms proposed to be critical to normal embryonic development.

55. The role of rhombomeres in neural and cranial development
Paul M. Kulesa, Scott E. Fraser

During the development of the vertebrate nervous system, the neural tube undergoes a period of dramatic cell and tissue movements. In the cranial region of the neural tube, this morphogenesis gives rise to the hindbrain, which is shaped from a cylindrical tube into repeated segments, called rhombomeres. The segments do not appear sequentially, but form in a precise spatial and temporal sequence and are thought to provide a spatial ground plan which guides later development of the hindbrain. Although some molecular data in the hindbrain are known, showing that certain gene expression patterns temporally precede and spatially correlate with the rhombomere segmental boundaries, the cell and tissue mechanisms which sculpt the hindbrain are largely unknown. Several mechanisms have been proposed for the morphogenesis of an individual rhombomere and the subsequent segmentation of the hindbrain, ranging from rapid forward growth and localized expansion of the neural tube to focal proliferation of cells coupled with a constraining structure at the boundaries. Attempts to test these hypotheses or gain further insight into the underlying cellular mechanisms have been hampered by the lack of an assay system to culture a live vertebrate embryo and image cell and tissue dynamics during hindbrain morphogenesis.

To examine the dynamics of cell and tissue movement over time, we have developed a whole embryo chick explant technique (Krull and Kulesa, 1998) and modified a culture system (Krull et al., 1995) for time-lapse video and confocal imaging. The chick embryo offers several advantages, including a rapid hindbrain segmentation process, a relatively simple, 2-D geometry of the embryo during the segmentation process and the ability to grow living tissue outside of the egg. To analyze hindbrain segmentation, we followed the shape changes of the neural tube, the outline of which appeared from a light source shown through the tissue, by recording images in time-lapse video microscopy. Measurements of the data revealed that throughout the segmentation process, the rostrocaudal lengths of each rhombomere remain nearly constant and the rhombomere boundaries form in the same spatiotemporal order as in intact embryos. The lateral width changes of the neural tube in the hindbrain explant during its segmentation show that not all rhombomeres are shaped by an expansion in the lateral width of the mid-rhombomere; either a smaller expansion or a constriction takes place at the rhombomere boundaries. In the caudal hindbrain, the mid-rhombomere expansions are accompanied by nearly equal rhombomere boundary constrictions. Thus, our results suggest that no single, simple mechanism is responsible for the shape changes which define the rhombomeres.

Simultaneous with the segmentation process of the hindbrain, neural crest cells arise at the dorsal midline of the neural tube and migrate to form the peripheral nervous system. The migration pattern follows a well-coordinated spatial and temporal order. Neural crest cells appear to migrate first from the anterior neural tube, followed in time by the migration of more caudally located cells. The groups eventually pattern each of the branchial arches. Whether the migration patterns of the neural crest cells are guided by the segmental ground plan of the rhombomeres is a question which has yet to be resolved. Some contend that the fate of a neural crest cell may be determined by the rhombomeric segment from which it originates, with
some rhombomeres producing very little or no neural crest, while others suggest that all segments give rise to neural crest cells which move rostral or caudal to contribute to adjacent branchial arch populations. To capture neural crest cell dynamics and individual cell movements, we fluorescently label pre-migratory cells in the neural tube and track the precise pathways of neural crest cells from confocal images as they organize, emerge and relocate from the neural tube. Neural crest cell migration shows a broad range of cellular behavior, and the exact trajectories are unpredictable. All segments give rise to neural crest cells and there is a significant amount of mixing of pre-migratory neural crest cells in adjoining rhombomeres, leading to a considerable contribution of neural crest cells to branchial arch populations of neighboring rhombomeres. Neural crest cells migrate in a wide variety of behaviors, ranging from rapid unidirectional motion to stationary and even backwards movement back towards the neural tube. The neural crest cell patterning of each branchial arch is uniquely formed and does not arise from a unique, simple mechanism.

This approach offers the promise of observing local details of individual cell motion during mid-rhombomere expansion and boundary constriction as well as a means of following in time-lapse the effects on the intermediate and final pattern after a perturbation on pre-migratory neural crest cells.

References

56. Neural crest in heart development
David A. Crotty, Scott E. Fraser

Neural crest cells are generated along neural folds where they fuse to form the neural tube. During embryonic development, neural crest cells emigrate from the neural tube throughout the embryo, giving rise to a wide variety of derivatives, including neurons, melanocytes, endocrine cells, and skeletal tissues. Neural crest cells migrate to and populate the walls of the aortic arches (Kirby et al., 1982). The presence of these cardiac crest cells has been shown to be necessary for proper generation of the septum that divides the pulmonary circulation from the aorta (Waldo et al., 1996). Cardiac crest also gives rise to musculoconnective tissue in the large arteries as they emerge from the heart. We are currently employing a variety of imaging and tissue culture techniques to generate detailed maps of the origin of these cardiac crest cells, as well as the pathways through which they migrate to the heart.

Chicken embryos are readily amenable to manipulation and culture techniques. Neural crest cells are labeled with fluorescent dyes in ovo. Intravital microscopy of the labeled cells permits the generation of time-lapse recordings of cell movements and cell differentiation, both in ovo, and in whole embryo explant culture (Figure 1).

The origin and role played by cardiac crest in mammals is not as well characterized as that seen in avian systems. The nature of mammalian development makes it inaccessible for the type of experiments done in chicken. We have developed an embryonic explant technique that allows the growth of embryonic tissues for several days in an observable environment. Again, neural crest cells are labeled with fluorescent dyes and time lapse films are generated to map cardiac crest location and migratory routes (Figure 2). Furthermore, we are taking advantage of several mutant mouse lines

Figure 1. Chicken Neural Crest Migration. The neural tube of this embryo was injected with Dil at the 7 somite stage, and the embryo was grown for two subsequent days. Migratory fluorescent neural crest cells are visible populating and entering the branchial arches.

Figure 2. Mouse Neural Crest Migration. Stills taken from a time lapse recording of a mouse embryonic explant culture. A. shows a phase photograph of the mouse culture with the arrow marking a point just caudal to the otic vesicle. B. shows a fluorescent view of the same culture, with the same arrow marking the base of a stream of migratory neural crest cells.
in which the septum between the aorta and pulmonary artery does not form, resulting in a persistent truncus arteriosus. Time lapse photography in these mutant lines will help determine the role of neural crest migration in the etiology of these phenotypes.

References

57. Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration
Rusty Lansford, Catherine E. Krull, Nicholas W. Gale¹, Andres Collazo, Christophe Marcelle, George D. Yancopoulos¹, Marianne Bronner-Fraser, Scott E. Fraser

In the trunk of avian embryos, neural crest cell migration through the somites is segmental, with neural crest cells entering the rostral half of each somitic sclerotome but avoiding the caudal half. Cues intrinsic to the somites are responsible for the segmental migration of neural crest cells. Little is known, however, about the molecular nature of these cues and how they influence patterned cell migration in vivo.

Several members of the Eph-family of receptor tyrosine kinases (RTKs) and their ligands display intriguing patterns of expression in the developing nervous system, suggesting potential roles in early neural patterning. Based on their binding specificities, the Eph-related RTKs can be divided into two major subclasses: Elk (EphB1)-related receptors, which interact primarily with a transmembrane subclass of ligands, and Eck (EphA2)-related receptors, which interact primarily with a GPI-linked subclass of ligands. Eph receptor/ligand interactions have been implicated in axonal patterning events, such as the topographical organization of the retinotectal projection and axonal fasciculation. Members of this family also are expressed in the developing forebrain and hindbrain and appear to play a role in the regional organization of these regions. During the past year, we investigated whether Eph receptor/ligand interactions influence the patterned migration of avian trunk neural crest cells.

We determined that Eph-related RTKs and their cognate ligands are essential for the segmental migration of avian trunk neural crest cells through the somites. EphB3, a member of the Elk (EphB1) subfamily of RTKs, was localized to the rostral half-sclerotome and to migrating neural crest cells. A cognate ligand of EphB3, ephrin-B1, exhibits a complementary pattern of expression in the caudal half-sclerotome.

We tested the functional significance of this striking asymmetry by culturing neural crest cells on alternating stripes of ephrin-B1/fibronectin versus fibronectin alone. Individual neural crest cells avoided migrating on lanes containing ephrin-B1/fibronectin, and instead migrated on lanes composed of fibronectin alone; addition of soluble ephrin-B1 blocked the repulsion. Time lapse videomicroscopy of migrating neural crest cells showed that upon contact with the ephrin-B1/fibronectin substrates, the lamellipod of migrating neural crest cells underwent collapse and retraction. In contrast, the lamellipod of migrating neural crest cells were not induced to collapse upon contact with control substrates. Our results demonstrate that the ligand ephrin-B1 can directly repulse neural crest migration.

Similarly, in whole trunk explants, the metameric pattern of neural crest migration was disrupted by addition of soluble ephrin-B1, allowing entry of neural crest cells into caudal portions of the sclerotome (see figure). To closely examine the cell migratory defects in the presence of soluble ephrin-B1, Dil-labeled neural crest cells were followed in explants using time lapse videomicroscopy. The trajectories and rates of migration for individual cells in trunk explants treated with soluble ephrin-B1, EphB2, or control conditions were calculated from the time-lapse recordings with XVTrack, an automated cell tracking program (Steve Speicher and Jerry Solomon, manuscript in preparation). Exposure to soluble ephrin-B1 results in neural crest cells emigrating directly from the neural tube into both rostral and caudal somitic halves, in contrast to control explants, where neural crest cells entered only the rostral half-sclerotome. Significant differences were noted between ephrin-B1-treated and mock- or EphB2-treated cells in cell migration. Continuance of cell migration is defined as the net displacement distance of a cell divided by its total distance migrated. In ephrin-B1 treated explants, the migration trajectories of cells in both the rostral and caudal somite halves appeared more erratic and disorganized, thus had a lower continuance, than those in control cultures. However, quantitative measurements of cell migration showed that the mean rates of migration were not significantly different between control and ephrin-B1-treated cells.

Both in vivo and in vitro, we find that addition of soluble ephrin-B1 results in a loss of the metameric migratory pattern and disorganization of neural crest cell movement. These results demonstrate that members of the Eph-family Elk (EphB1)-related RTKs and their corresponding transmembrane ligands are involved in interactions between neural crest and sclerotomal cells, mediating an inhibitory activity necessary to constrain neural precursors to specific territories in the developing nervous system.
Figure 1. Soluble ephrin-B1 disrupts the segmental pattern of neural crest migration. FC alone (Control) or Ephrin-B1-Fc were added to trunk explants and static patterns of neural crest migration were visualized via DII staining.

58. 2-Photon time lapse movies of living dendritic spines

Steve Potter, David Kantor, Scott Fraser, Erin Schuman

It is widely believed that learning induces structural changes in synaptic structures. Most of the evidence for this comes from comparisons between fixed, dead tissue from animals that underwent different learning experiences (in vivo) or brain slices that underwent different electrical stimulation treatments (in vitro) while still alive. Due to the greatly reduced photodamage afforded by 2-Photon microscopy, we now have the potential to observe these changes while they occur, in living specimens.

Experiments from the Schuman Lab have shown that synapses onto pyramidal neurons in area CA1 of the rat hippocampal slice are potentiated by the application of brain-derived growth factor (BDNF), and that this potentiation requires local protein synthesis in the dendrites. It seems likely that the observed electrophysiological potentiation of CA1 synapses is subserved by structural remodeling, such as changes in the shape of dendritic spines.

We have developed methods that allow us to image dendritic spines in CA1 of adult rat hippocampal slices at high resolution for over 12 hours. Slice neurons were labeled either intracellularly with fluorescein dextran or extracellularly with the membrane dye, DiO, and imaged repeatedly at 30-min intervals, using the Biological Imaging Center's 2-Photon Laser-scanning Microscope. Preliminary experiments show numerous rapid (under 30 min) spine changes, including lengthening, shortening and lateral movement. We verified that the slices were healthy after imaging by checking field potentials and inducing long-term potentiation by tetanic stimulation of Schaffer collaterals. We plan to quantitate changes in these baseline spine dynamics when BDNF is perfused across the slice. This would provide further evidence that growth factors are important not only during development, but also during adulthood, as facilitators of synaptic plasticity.

59. Neuronal reorganization in the hippocampus following seizures

Adam N. Mamelak, Steven M. Potter, David Kantor, Scott Fraser, Erin Schuman

The overall goal of this research plan is to better understand the process of neuronal reorganization that occurs following seizure-induced injury to the hippocampus. A stereotypic and robust aspect of this reorganization, termed mossy fiber sprouting (MFS), is the outgrowth of new axon collaterals from hippocampal granule cell axons ("mossy fibers") to the dendritic fields of the granule cells in the Inner Molecular Layer (IML) of the fascia dentata. Because the glutaminergic synapses of granule cell axons appear to preferentially re-innervate their own dendrites, MFS represents an attractive model for seizure-induced formation of a hyper-excitable circuit, which may be a component of epileptogenesis. While MFS occurs in response to a variety of injuries or stimuli, our knowledge of this process is primarily limited to histological and electrophysiological descriptions at relatively early or late time points following injury. The molecular and activity-dependent cues which initiate and guide this reorganization are largely unknown; a detailed anatomical description of the sprouting process is also lacking.

The focus of this research proposal centers around the use of fluorescent tracers and two photon laser scanning microscopy to study MFS in living hippocampal slices, using time-lapse imaging to follow dynamic changes in network reorganization over prolonged periods. We have developed a transportable hippocampal slice chamber apparatus that can maintain adult hippocampal slices in vitro.
for periods of 18 hours or longer at 32C. Hippocampal slices are prepared from rats 10-28 days following pilocarpine-induced seizures and concomitant MFS. Slices are bathed in modified ACSF solution, and the mossy fibers are labeled by extracellular pressure injection of DiO into the granule cell layer. These fluorescently labeled axons are then imaged on the two-photon microscope at 20 minute intervals, for periods of 6-24 hours. Slice viability is measured at the completion of the imaging experiment by measurement of CA1 region field potentials. Time-lapse movies are constructed, and the movies are analyzed for dynamic growth changes. Histological extent of MFS is determined by labeling of the slice with TSQ, a zinc-specific fluorescent dye.

Preliminary data indicate that slices can be maintained in a physiologically viable condition for up to 18 hours. Time-lapse movies reveal some degree of photobleaching over time, but fine details of processes can be followed over several hours or longer. We are currently performing time-lapse experiments in both seizure animals and P10 developing rats to characterize the patterns of MFS in these two states. Alternate labeling methods such as *in vivo* injection of GFP adenovirus are also being initiated. The resultant movies should provide unique insight into the dynamic nature of this growth process, as well as a novel assay system for determining the effects of a variety of biological modifiers.

60. **Image enhancement in two-photon laser scanning microscopy**
   Chun-Ming Wang, Scott E. Fraser

Two-photon laser scanning microscopy (TPLSM) offers many advantages for exploring biological systems, allowing us to image living cells with less photo-bleaching and -toxicity than is possible with confocal laser scanning microscopy (CLSM). We investigated and compared the signal-to-noise ratio (SNR) and the resolution of TPLSM and CLSM on biological specimens. The 3-D point spread functions (PSFs) of TPLSM at different excitation intensities demonstrate that resolution decreases with increased excitation intensity; for example, the lateral resolution is 12% worse and axial resolution is 30% worse at nearly 8-fold more intensity. The 3-D PSFs of CLSM, measured at different pinhole sizes, reveal that resolution improves at the smaller pinhole size, while its optimal SNR lies at 35 µm pinhole. Image-deconvolution techniques improved the resolution of TPLSM nearly two-fold: from 400 nm to 240 nm in the lateral direction, and from 1080 nm to 540 nm in the axial direction. When applied to 3-D neural images acquired with TPLSM, these techniques reduced the blurring to revealed more details of protein distribution in pre-synaptic compartments. Our tests on both synthetic and real control data show accurate and consistent performance, comparable to algorithms that account for noise by regularization. By combining TPLSM with these image-de-convolution techniques, we provide an effective means of *in vivo* imaging with ultra-high resolution and reduced photobleaching, which will yield invaluable insights into the dynamical mechanisms of biological systems.

61. **Molecular tracing of subcellular translocation events during Wnt-signalling**
   Ute Rothbäcker, Micheline Laurent†, Ken W. Y. Cho†, Scott E. Fraser

*In vivo* imaging has been opened to molecular approaches with the cloning of Green Fluorescent Protein (GFP) of the jellyfish *Aequorea* (Chalfie et al., 1994). We use GFP as a molecular tracer to visualize the subcellular localization of ectopically expressed genes in embryos.

The Wnt signaling pathway has been shown to be important during axis formation in insects and vertebrates. Much of its elucidation has profited from genetic studies in *Drosophila* and several molecules of the pathway were shown to be functionally conserved between species (Rothbacher et al., 1995, and for review see Moon et al., 1997). *Xenopus dishevelled* (*Xdsh*) mediates the Wnt signal intracellularly. Understanding its function is important since *dsh* is the molecule closest in the signaling pathway to the recently identified Wnt receptor frizzled and might actually be part of a Wnt receptor complex. This is
supported by recent data that demonstrate a translocation of Xdsh to the cell membrane following over-expression of frizzled. Recent studies from Drosophila further show that upon Wnt signal dsh is hyper-phosphorylated and that this form is enriched in the membrane fractions (Yanagawa et al., 1995). Since dsh is ubiquitously expressed the spatio-temporal regulated of dsh phosphorylation may actually render it in its biologically active form at the right time and place during development. Little is known about the structural and functional domains of this molecule and it therefore remains to be determined which parts of the molecule mediate both its phosphorylation and translocation to the membrane. Additionally it is unknown how the possible subcellular translocations of Xdsh are related to developmental events in various regions at different times during embryogenesis.

To analyze these questions we generated various deletion mutant molecules fused to GFP. These mutants are currently being analyzed for their differential ability to induce a secondary axis (as the wild type Xdsh) or to translocate to the membrane upon frizzled over-expression. Preliminary results show that the ability of the various mutants to differentially induce a secondary axis can be correlated with their translocation to the membrane, as visualized by the subcellular location of GFP in low light microscopy. Biochemical analysis includes the correlation of phenotypic effects with differential degrees of phosphorylation. Experiments are underway to more carefully analyze the concentration dependence of these effects followed by more detailed analysis using Confocal and Two-Photon Laser Scanning microscopes.

We further plan to extend these studies to analyze the distribution of Xdsh in various intracellular compartments during ongoing development. For this we plan to inject mRNA encoding Xdsh-GFP at concentrations low enough to avoid ectopic dorsalizing effects but high enough to detect representative in vivo distribution. The developmental translocation of Xdsh will then be followed in vivo using Confocal and Two-Photon Laser Scanning microscopic imaging.

References

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62. Analysis of spinal cord development in zebrafish embryos and larvae

Mary E. Dickinson, Scott Fraser

We are interested in how fates of individual neurons in the zebrafish spinal cord become specified. Zebrafish are an ideal system for such analysis because they have a relatively simple network of spinal neurons, can be easily manipulated at both embryonic and larval stages and are transparent at these stages so that cells are easily visualized in a light microscope.

This is in contrast to other vertebrates such as chick and mouse which are not as accessible during embryogenesis and even at early stages, the spinal cords of these animals contain a large number of neuronal cell types and develop a very complicated circuitry. In the zebrafish, only eight different cell types, with no more than five of each cell type, are found per hemisegment (with some hemisegments completely lacking certain cell types) (Berhardt et al., 1990). (Hemisegments are reiterated units within the spinal cord that are equivalent to the cervical, thoracic, lumbar and sacral segments in mammals, with hemi referring to either the right or left half of the cord.) Moreover, each of these neurons is quite large (approx. 8-10 µm in diameter of those that have been measured) and each have a very characteristic morphology that can be easily recognized using standard light microscope and labeling techniques. However, these studies have focused on recognizing mature neurons and virtually nothing is known about the timing of development of individual neurons, their lineage relationships with other neurons, or the intermediate steps that each precursor cell takes as it differentiates. Therefore, we would like to investigate early events in spinal cord neurogenesis by labeling cells in groups and individual cells.

We have begun to develop techniques which will allow us to label all of the precursor cells in the spinal cord prior to differentiation. By labeling all of these cells at once and then performing timelapse video microscopy, we hope to establish the birthdates of neurons and identify any neuronal cell types that have not be characterized. This should also allow us to look at the morphologies of non-neuronal cells that form. Precursor cells in chick embryos can be labeled by injecting the lipophilic dye, Dil (Molecular Probes, Eugene, OR) into the brain vesicles and central canal of the neural tube. In both chick and fish, precursor cells lie next to the canal of the neural tube. In both chick and fish, precursor cells lie next to the canal making the canal a convenient way to access these cells. Similarly in zebrafish, we can inject aqueous solutions into the central canal, but Dil (which has to be solubilized in ethanol or DMSO) precipitates out of solution as soon as it is injected and only labels a few cells close to the injection cite. One way to overcome this problem is to solublize the Dil in lipid prior to injecting it with the hope that it will be taken up by the cells in this form by either cell fusion or endocytosis. Small unilamellar vesicles (SUVs) (commonly know as liposomes) have been successfully used by a number of labs as a way of introducing dyes, drugs, DNA or other therapeutic agents into cells (see Lasic, 1997) and there are a number of commercially available liposomes or lipid aggregates that have been marketed. We have been testing a preparation of liposomes made in our laboratory (M. Dickinson, M. Hueber, R. Jacobs, T. Mead and S. Fraser, work in progress) consisting of dimyristoyl phosphotidyl-
choline (DMPC), cholesterol and DiI. Injections of this preparation into zebrafish embryos and larvae showed that some cells were effectively labeled but these cells were located in the brain and no labeled cells were seen in the spinal cord. Thus, our lipid preparation, while effective in solubilizing the DiI, is not incorporated into spinal cord precursor cells. We are currently assaying other preparations to find one that efficiently delivers reagents to these cells.

In addition, we have just begun injecting single cells in various regions of the neural plate and spinal cord to begin to assay the lineage relationships between cells and have also begun to look for genes that can be used as markers for individual spinal cord neurons with the hopes of elucidating part of the molecular events controlling neuron differentiation.

References

63. Hindbrain regionalization in the zebrafish
Katherine Woo, Scott E. Fraser
Fate mapping studies (Biology Annual Report 1995, 1996) showed that progenitors of the different brain regions have coherent order within the presumptive neurectoderm at early gastrula stage. This observation could indicate that regional specification may already be underway at shield stage. In this report, we determined the timing of neural commitment by hindbrain tissue in the zebrafish using microsurgical transplantation. When transplanted at shield-stage to the ventral side of the embryo, presumptive hindbrain cells were not committed, as they could adapt to their environment and gave rise to epidermis. In contrast, when transplanted at 80% epiboly, hindbrain cells retained their neural fate and expressed neural-specific antigens. Moreover, they were able to maintain regional fate, as evidenced by the expression of the hindbrain-specific marker, Krox20. In addition, we observed that committed hindbrain tissues were able to induce presumptive ventral epidermis to form neural crest derivatives, otic vesicles, and neural tissues. We propose that hindbrain progenitors have acquired regional identity as a group at 80% epiboly even before making vertical contact with axial mesoderm. These results suggest that planar induction may constitute a significant component in the zebrafish neural patterning pathway.

64. Specification of zebrafish nervous system by non-axial signals
Katherine Woo, Scott E. Fraser
Our studies in the specification of hindbrain precursors (see previous abstract) suggested the hypothesis that lateral and ventral germ ring tissues may provide patterning cues to the presumptive hindbrain region. Using microsurgery, we generated in vivo evidence for the presence of a spatially distinct "transformer" signal(s) from non-axial (non-notochordal) tissues in the zebrafish. Cells of the germ ring (non-axial mes-endoderm) posteriorize future forebrain progenitors when grafted nearby, resulting in an ectopic hindbrain-like structures, which included ectopic host-derived otic vesicles, pigment cells, and occasionally isthmus-like structures, as well as ectopic Krox20 expression in the forebrain. In contrast, cells of the organizer (axial mes-endoderm) caused no posterior transformation. Studies with amphibian embryos have provide many candidate molecules that may act in this transforming pathway. We tested one, bFGF, by implanting bFGF-coated beads into presumptive forebrain of the zebrafish gastrula. These beads caused malformation of the forebrain, but failed to transform the region into hindbrain. We propose a modified "2-signal" model for the forebrain, in which distinct signals from the shield (organizer) and the germ ring are integrated by the presumptive neurectoderm to generate the complete AF pattern of the neural axis.

65. Bisection of zebrafish unitary eye field by diencephalon precursors revealed by in vivo timelapse microscopy
Katherine Woo, Scott E. Fraser
The mechanisms that pattern and shape the vertebrate forebrain remain largely a mystery. It has been suggested that cells in the ventral diencephalon provide signals that guide forebrain patterning in a process similar to that employed by the notochord and the floorplate to pattern the trunk neuraxis. The fate map of the zebrafish nervous system suggests that the cells that give rise to the ventral diencephalon change position during gastrulation and neurulation, shifting from caudal to the retinal precursors to lie between them. We used time-lapse microscopy to demonstrate that transplanted labeled ventral diencephalon precursors accomplish the predicted "bisection" movement in vivo. This observation was further confirmed using photoactivation to label subsets of retinal precursors and/or diencephalon precursors, proving that the observed cell movements were not a sequence of surgical interference. Consistent with previous observation (Shih and Fraser, 1995 Annual Report), ventral diencephalon precursors extend to form secondary axes after being transplanted to the ventral side of the embryo, suggesting an intrinsic ability to express "convergence and extension" behavior. This indication of intrinsic ability to converge and extend coupled with the rearrangement of the forebrain for fin development suggests that the precursors of the ventral diencephalon play a major role in the patterning and morphogenesis of the rostral nervous system. Some findings that have been interpreted in the past to reflect signaling interactions between cells at the ventral midline of the developing brain may instead reflect morphogenetic events.
66. Egression movements during zebrafish gastrulation  
John Shih, Scott E. Fraser
Involution and ingression are two distinct morphogenetic movements associated with vertebrate gastrulation. Involution is a sheet-like motion of a tissue around a corner, much like a caterpillar crawling over the edge of a table and then walking away from the edge on the underside of the table. Ingression involves more individual cell motions in which cells move from one layer to another by sacrificing contacts with neighbors and adopting new neighbors. Involution is the mode of gastrulation in the Xenopus embryo. Prompted by similarities in cell movement trajectories, it has been proposed that the involution movement that characterizes Xenopus gastrulation also occurred during zebrafish gastrulation. This proposal has fostered speculation that the inductive events underlying neural patterning may be quite similar between the zebrafish and Xenopus. However, the cell trajectories so far described in the zebrafish are consistent with either involution or ingression. In both cases, cells would be expected to move toward the edge of the blastoderm, change layers and then migrate away from the blastoderm. Therefore, we undertook a re-examination of cell movements in the embryonic shield region of the zebrafish gastrula. Using a number of cell-labeling techniques to control for methodological variables, we found that both ingression and egression movements in the zebrafish gastrula. We propose that the cell movement in the zebrafish shield region is of an individual nature that differs from the cells sheet behavior in Xenopus. In some respects, zebrafish gastrulation may more closely resemble that of avian and mammalian embryos than some amphibians. (See Figure, following page)

67. Mechanisms of action for cobalt chelate complexes that irreversibly bind to proteins  
Angie Louie, Thomas Meade
We are studying a family of cobalt complexes that have been shown to have potent anti-viral activity against a number of Herpes viruses (Boettcher et al., 1997). The general structure for the complexes is shown in Figure 1. The complexes are believed to inhibit key viral enzymes by binding to histidine residues in the enzyme active sites. A number of biological structures contain histidine residues critical to function and it should be possible to target cobalt complexes to bind to such structures. For example, we have previously shown that these complexes are able to inhibit the structure and function of zinc finger proteins (Louie and Meade, 1997). These proteins possess domains that include histidine and cysteine residues that tetrahedrally coordinated a zinc atom. As part of these investigations, we have been focusing on the following questions: (1) which amino acids can this class of cobalt complexes bind; (2) characterize the precise location and coordination geometry of the cobalt-protein derivative by preparing model peptides; and (3) what happens to the zinc atom in a zinc finger when cobalt complex is bound.

Cobalt is a "hard" acid, preferentially binding "hard" base ligands such as nitrogen. Therefore, we have proposed that our cobalt complexes should bind strongly to a nitrogen on the imidazole ring of histidine. Cobalt complexes would bind to histidine by a ligand exchange mechanism in which the axial ligand of the complex would be exchanged for the higher affinity nitrogen on the histidine. Although nitrogen is by far the most preferred ligand, cobalt is also capable of binding to sulfur and oxygen groups, and therefore, other amino acids besides histidine may be bound. In order to determine where cobalt complexes are in a protein, we examined their interactions with a number of different individual amino acids by 1H NMR spectroscopy. These studies revealed that, as expected, the cobalt complexes do bind to histidines, but also interact (weakly) with cysteine residues.

We further expanded these studies to investigate model peptide sequences containing potential binding sites. These peptides were allowed to interact with cobalt complexes and then analyzed for the stoichiometry of cobalt binding to the peptide. This work verified that the cobalt complexes bind to histidine and cysteine residues found on peptides. The peptides were then subjected to trypsin digestion and the fragments evaluated by mass spectrometry to verify that cobalt binds to particular residues in the sequence.

![Figure 1 (Louie and Meade). General Structure of the Cobalt Complexes](image)

In order to assess structural perturbations to the protein after the binding of the cobalt complex we have employed Cd-2H Heteronuclear Spin Echo Difference NMR Spectroscopy and (2-pyridylazo)resorcinol (PAR) binding assays. PAR is a zinc specific dye which absorbs at 400nm when unligated to zinc and absorbs at 500nm when zinc is bound. We introduced cobalt complexes to folded zinc finger peptides in the presence of PAR. There is an observable increase in the absorbance at 500nm as cobalt complex is added, indicating that zinc is being released by the peptides and bound by PAR. The HSED experiments should allow direct visualization of precisely which residues, is any, remain bound to metal. By closely evaluating the interactions of these cobalt complexes with
peptides and amino acids we can better predict their inhibitory potential against new targets.

References

FIGURE: Diagram of cell movement during zebrafish gastrulation (See Abstract 66, Shih and Fraser, on previous page).

Diagram of deep-cell movement underlying the segregation of ectoderm (light gray) from mesendoderm (dark gray) progenitors shown as a cross section along the dorsal midline. (Top) At the onset of embryonic shield formation, the progenitors cells of the ectodermal and mesodermal lineages are intermingled within the embryonic shield region (shown here in profile view.) These deep-cell progenitors are overlain by cells of the enveloping layer (light gray) and underlain by the large central yolk cell. In the diagram, the animal end of the embryo is oriented to the right.

(Middle) During gastrulation, the deep cells translocate directly between the hypoblastic layer and the epiblastic layer. The observed egression movements (from the hypoblast to the epiblast) suggest that neural and mesodermal progenitors remain intermingled during gastrulation. Moreover, the observation suggest that neural induction in the zebrafish takes place between individual cells, rather than between separate and distinct cell layers.

(Bottom) At the end of gastrulation, ectodermal progenitors are located in the epilast and mesodermal progenitors in the hypoblast.

68. DNA-mediated electron transfer rates: Single vs. double stranded oligonucleotides
Elizabeth Krider, Thomas Meade

Electronic coupling interactions are significant to understanding electron transfer mechanisms through modified DNA. In order to determine the role of base sequence and r-stacking on electron transfer rates, we have prepared an oligonucleotide which contains two sites for covalent attachment of donor and acceptor complexes. Located at the strand's termini, these attachment sites consist of nucleosides functionalized at the 2' position of the ribose ring. The attachment of the ruthenium complexes, [Ru(NH)_3(pyridine)]^{2+} and [Ru(bpy)_3(imidazole)]^{2+}, does not affect the ability of the oligonucleotide to hybridize with its complementary strand. Therefore, an evaluation of the distance and sequence-dependence on the rate of electron transfer can be conducted. Electron transfer rates through this novel oligonucleotide both in the presence and absence of the complementary strand should yield insight into the nature of the pathway of ET reactions mediated by DNA.

69. Synthesis and in vivo studies of membrane impermeable "Double Score"-MRI contrast agents
Martina M. Hüber, John Shih, Scott E. Fraser, Russ E. Jacobs, Thomas J. Meade

The overall aim of our studies is to develop compounds which can be used to trace cell lineage in vivo for developing embryos simultaneously by light microscopy and by magnetic resonance imaging (MRI). Our specific goal is to modify high molecular compounds (e.g., biopolymers such as polylysine (pdl) or dextran and attach a MRI contrast agent as well as a fluorescent dye). The bifunctional contrast agents described here contain small gadolinium(III) chelates, Gd-diethylentriamine pentaacetic acid (Gd-DTPA), as the MRI component and tetrarmethylrhodamine isothiocyanate as the optically active element.

Images acquired after injection of single cells with these double-score agents enabled us to follow the relative motions and reorganizations of different cell layers during amphibian gastrulation and neurulation. The fluorescent functionality on these agents allowed us to score the accuracy of labeling immediately after injection, and to follow the initial stages of development using epifluorescent microscopy. However, only in combination with analysis by MRI techniques were we able to construct three dimensional images of the developing specimen. Fluorescence microscopy and MRI images were obtained by injecting GRIP (pdl 3.5 kDa) and GRID (dextran 12 kDa) into Xenopus laevis embryos. Close observation of cellular morphology indicated that the compounds do not interfere with the normal development.

70. Magnetic resonance imaging agents that function as enzyme reporters
Martina M. Hüber, Eric Ahrens, Ute Rüthbacker, Russ Jacobs, Scott Fraser, Thomas J. Meade

MRI offers a non-invasive method to map and visualize different tissues in vivo. The observed contrast arise from the intrinsic differences in the T_1 (spin-lattice) and T_2 (spin-spin) relaxation rates
of water protons interacting. MRI agents can enhance the relaxation rates, and hence increase the signal by mediating the interaction of water with unpaired electrons of a paramagnetic metal. The most potent relaxation agent, Gd$^{3+}$ is toxic and has to be chelated in order to use it as MRI agent. An intensively studied chelating macrocyclic ligand with a very large $k_i$ value ($10^{20}$) for Gd$^{3+}$ and extremely small $k_q$ is 1,4,7,10-tetraazacyclododekane-N,N',N'',N'''-tetraacetic acid (DOTA).

The goal of this work is the design and synthesis of a new class of "smart" MRI contrast agents that are able to report on the metabolic state of cells and organs. Our approach is to modify the DOTA ligand by attaching a covalently bound bulky group that sterically blocks the access of water to the Gd$^{3+}$ center. In the presence of a metabolite, the agent will be turned from the "off" state to the "on" state, as viewed by MRI. The first derivative of these enzyme-reporter MRI agents [EGad; (4,7,10-triaceticacid-1-(ethyl-2-oxy-β-galactopyranose)-1,4,7,10-tetraazacyclo-dodecane-gadolinium)] positions a galactopyranose residue at the ninth coordination site of the Gd$^{3+}$ ion. Exposure to the common marker enzyme β-galactosidase removes the galactopyranose from the chelator and causes a transition from the "off" to the "on" state as demonstrated by MRI imaging experiments.

The second generation of these compounds was designed to increase the observed contrast in the "off" and "on" states (i.e. before and after exposure to the enzyme). EGaDMe, (4,7,10-triaceticacid-1-(isopropal-2-oxy-β-galactopyranose)-1,4,7,10-tetraazacyclo-dodecane-gadolinium) introduces a methyl group on the equatorial arm which should further reduce the water accessibility to the Gd$^{3+}$.

While the successful enzymatic cleavage of the galactopyranose residue from EGad was previously shown by MRI experiments in vitro, recent studies demonstrate their in vivo applicability. MRI studies of embryos injected with EGad or EGaDMe reveal a enhanced contrast at regions where the enzyme β-galactosidase is present. Furthermore, preliminary experiments confirm that EGaDMe produces an enhanced MRI signal with respect to EGad after exposure to β-galactosidase.

71. Quenching electronically excited Ru(II) by Co(III): A new approach toward photoactive redox activation.
Ofer Blum, Rose M. Carlos, Jay R. Winkler, Harry B. Gray, Thomas J. Meade
[Co(III)(acacen)L2]+ (acacen = bis-acetylacetonate ethylene-diimine, L = NH$_3$, 2-Me-imidazole) is a proven drug against the ocular occurrence of the herpes simplex virus. Our studies with metmyoglobin and model coordination compounds reveal the rate limiting step of this mechanism is axial ligand exchange of the cobalt complex. We are exploring the use of these complexes as prodrugs for photochemical enzyme inhibitors. Employing an external light source we are generating the very reactive [Co(II)(acacen)]$^+$ (L = NH$_3$, 2-Me-imidazole) which should be avoided and cobalt complexes with higher reduction potentials should be prepared. We currently examining the use of NADH as an photoreductant for our cobalt prodrugs. Myoglobin will be the enzyme target because it reacts quickly with the Co(II) complexes, and provides a spectroscopic handle to monitor the reaction.

72. Selective, high affinity protein binding via cobalt(acacen)-peptide conjugation
Phil Dawson, Toshi Takeuchi, Cindy Quezada, Melvin Simon, Harry Gray, Thomas Meade
The development of new drug candidates is complicated by the necessity to find molecules which are highly specific for a given target but also bind with high affinity. To address this problem, we are investigating an approach for drug design where a cobalt chelate complex that binds tightly to a wide range of proteins is chemically tethered to a peptide with high specificity but low affinity. By linking these two molecules, we are developing a general approach for the design of small molecules that inhibit protein activity irreversibly and selectively.

Recently, Co(acacen) complexes have been shown to inhibit the replication of ocular forms of the herpes simplex virus.
virus. These cobalt complexes bind irreversibly to surface histidine residues of a wide range of proteins including carbonic anhydrase, thrombolysin, thrombin and myoglobin. Although these complexes are very promising for the treatment of ocular herpes, the use of similar compounds to treat other diseases may be limited due to the lack of specificity in their binding.

In order to develop a cobalt complex which is specific for a given protein, we have focused on thrombin, a protease involved in blood coagulation. We are currently investigating methods for the incorporation of the Co(acacen) complex into a peptide sequence which has high selectivity for thrombin. By varying the distance between the high affinity cobalt complex and the high specificity peptide, we hope to be able to create a molecule which can simultaneously bind to a surface histidine (via the Co chelate) and the active site of the target (through the peptide). Straightforward synthetic access to Co(acacen) peptide conjugates should allow us to optimize the chemical linkage in terms of length and composition. In addition, the development of a solid-phase synthesis based approach will allow us to use combinatorial chemistry methods to explore this novel class of potential therapeutic agents.

73. Stretching and breaking duplex DNA by chemical force microscopy

Aleskandr Noy, Dmitri V. Vezhenov, Jon F. Kayyem, Charles M. Lieber, Thomas J. Meade

Specific interactions between complementary strands of DNA and other molecules and macromolecules are central to information storage, retrieval and modification in biological systems. Although the basic structures of duplex DNA and binding energetics have been well characterized in many cases, little information is available about the forces acting in these systems. These forces are of critical importance, since they must be overcome, for example, by protein machines during transcription and repair. Recent developments in atomic force microscopy (AFM) make possible direct measurements of such forces between individual oligonucleotide strands that form duplexes.

We used the chemical force microscopy technique in which oligonucleotides are covalently-linked to the force microscope probe tip and sample surface to measure elongation and binding forces of individual DNA duplexes (Noy et al., 1997). Separation forces between complementary oligonucleotide strands were found to be significantly larger than the forces measured between noncomplementary strands and consistent with the unbinding of a single duplex. With increasing applied force, the separation of complementary strands proceeds in a stepwise manner where B-form DNA is first stretched, then structurally-transformed to a stable form of DNA approximately 2-times longer than the B-form, and finally separated into single-strand oligonucleotides. These data provide a direct measure of the forces required to elastically deform and separate double stranded DNA into single strands.

Force microscopy affords a direct and quantitative measurement of the forces and energetics required to stretch and unbind DNA duplexes. Because these measurements can be readily carried out on synthetic oligonucleotides and in the presence of exogenous molecules and macromolecules, this method affords an opportunity for directly assessing the energetics of distorting and unbinding specific DNA sequences and DNA complexes. Such data could provide unique insight into mechanistic steps following sequence specific recognition by, for example, repair and transcription factors.

References
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74. Mouse brain functional magnetic resonance microscopy

Mark O'Dell, Scott E. Fraser, Russell E. Jacobs

We have been adapting the Beckman Imaging Center high field Nuclear Magnetic Resonance (NMR) Micro-Imaging system (MRI Microscope) to visualize physiological activity in the brains of anesthetized small adult mammals. Our objective is to study experience-induced changes in the primary sensory maps of their tactile and visual systems. The dynamics of these rearrangements reflect the underlying computations used to maintain each representation. They also constrain our understanding of the mechanisms underlying these computations, and provide insight into how these maps are used by the animal to interpret its sensory world.

For some time this project has emphasized technology development. We have been constructing hardware and software to sustain an anesthetized animal subject in the magnet bore, deliver sensory stimuli, and acquire brain or body images. While these efforts are continuing, we are now routinely acquiring high resolution anatomical images of live mice, and more rapidly acquired simple functional images of their brain responses to visual stimulation.

Functional MRI (fMRI) techniques visualize a metabolic reflection of brain activity. Increased cell activity draws more energy and leads to a small but highly local deoxygenation of blood (the initial deoxygen phase). This is followed by vascular dilation that overcompensates and leaves a slightly less-local excess of fresh oxygenated blood (the later vascular dilation phase). These changes in blood oxygenation are detectable with MRI. Iron atoms, used by hemoglobin in blood to carry oxygen, have different magnetic states depending on whether they are binding oxygen. This difference converts local variations in blood oxygenation to changes of magnetic susceptibility within brain tissue. MRI sensitivity to these changes increases with magnetic field strength. When fMRI is performed with humans in clinical imaging systems.
(1.5 - 4 Tesla (T)), timed to use the later vascular
dilation phase of the response, primary sensory stimuli
lead to changes in the detected signal of a few percent.
In a 12 T field, responses extrapolate to tens of percent.
This high sensitivity offers great promise for making
even finer resolution mapping possible with the
smaller initial deoxygenation signal, which produces
changes that are just barely detectable in lower fields.

Brain physiology is fast, so fMRI signal
acquisition must take place quickly. This imposes
restrictions on the spatial resolution of functional
images. We are currently able to acquire 128 x 64 pixel
images in a few seconds, with each point reflecting
signals from a volume element (voxel) of tissue (300µm)²
x 500µm. In order to interpret these pictures, we also
acquire higher resolution spatially-registered
anatomical data. These images are gathered at the
start of each experiment, typically 512 x 256 pixels in
a few minutes with voxels as small as (80µm)² x 250µm.

Figure 1 shows a parasagittal section through the brain
of a live mouse. Figure 2 shows a coronal section. Figure
3 shows functional data superimposed on an image of
the underlying anatomy (a roughly horizontal section).
The stimulus was strobe light illumination of one eye.
The response appears in the gray matter of the
contralateral visual cortex. The small cluster of white
pixels marks an area of locally increased supply of
fresh oxygenated blood which followed elevated cell
activity. By using sequences of more spatially
restricted stimuli, we will soon be producing functional
maps of primary visual and somatosensory cortex. As
technology develops, we will be able to obtain better
spatial & temporal resolution in functional images.
This will allow us to separate the different blood
oxygenation phases, rather than averaging them
together, as we are currently limited to doing.

This functional data was obtained by subtracting
images acquired with and without providing a stimulus
to the animal. The displayed pixels are those that
changed intensity by more than 50%. Such simple data
analysis has been sufficient so far because our observed
fMRI changes are significantly larger than in all
previously reported (lower field) studies. When
hardware improvement makes it possible to gather
data more rapidly and with fewer artifacts (invisible
here due to difference image thresholding for display),
we will apply more sophisticated techniques as well.

Statistical testing, stimulus time course cross­
correlation, and time series analysis are established
methods for detecting subtle changes in functional
images. Calculation and interpretation of functional
data remains an area of some controversy.

Project updates will be available at
http://cns.caltech.edu/~emark/fmri.html

75. Partial-volume Bayesian classification of
material mixtures in MR volume data using voxel
histograms

David H. Laidlaw, Kurt W. Fleischer,
Alan H. Barr

We have developed a new algorithm for
identifying the distribution of different material
types in volume datasets such as those produced with
Magnetic Resonance Imaging (MRI) or Computed
Tomography (CT). Because we allow for mixtures of
materials and treat voxels as regions, our technique
reduces the classification artifacts that thresholding
can create along boundaries between materials and is
particularly useful for creating accurate geometric
models and renderings from volume data. It also has
the potential to make more-accurate volume
measurements and classifies noisy, low-resolution
data well.
There are two unusual aspects to our approach. First, we assume that, due to partial-volume effects, voxels can contain more than one material, e.g., both muscle and fat; we compute the relative proportion of each material in the voxels. Second, we incorporate information from neighboring voxels into the classification process by reconstructing a continuous function, $\rho(x)$, from the samples and then looking at the distribution of values that $\rho$ takes on within the region of a voxel. This distribution of values is represented by a histogram taken over the region of the voxel; the mixture of materials that those values measure is identified within the voxel using a probabilistic Bayesian approach that matches the histogram by finding the mixture of materials within each voxel most likely to have created the histogram. The size of regions that we classify is chosen to match the spacing of the samples because the spacing is intrinsically related to the minimum feature size that the reconstructed continuous function can represent.

Application areas include computer graphics modeling, biological modeling, anatomical and physiological studies, medical diagnosis, CAD/CAM, robotics, and computer animation.

76. Absolute blood pressure maps using magnetic resonance  
J. Michael Tyszka, David H. Laidlaw

The ability to assess and monitor absolute blood pressure non-invasively within the great vessels of the abdomen and in the heart would impact the management of many serious medical conditions that affect millions of Americans each year, including valvular heart disease, hypertension, and atherosclerosis.

We are developing and validating an absolute pressure mapping technique based on magnetic resonance imaging (MRI). Although the relative pressure and the pressure gradient can be measured with ultrasound or magnetic resonance imaging, absolute blood pressure within the aorta and heart chambers can currently only be measured using invasive methods such as catheterization. The technique we are developing will be completely non-invasive and will be capable of generating three-dimensional maps of the variation of absolute pressure throughout the cardiac cycle.

We have implemented an imaging technique for rapidly imaging blood velocity in the cardiac cycle, (see (i) below), and for calculating relative blood pressure maps (see (ii) below).

We are critically assessing the performance of the technique in computational models and in phantoms carefully designed to represent the major anatomical regions involved in the absolute pressure calculation.
One slice of one time step of a cardiac cine volume image. (i) anatomy, (ii) superior/inferior velocity ($-1.0-0.48$ m/s), and (iii) relative pressure map ($0-110$ mm Hg).

77. Goal-directed optimization of magnetic resonance collection parameters

David H. Laidlaw, Mark Montague, Eric T. Ahrens, Russell E. Jacobs, Alan H. Barr

Two challenges in collecting effective magnetic resonance imaging data are choosing among the many collection techniques, or protocols, and selecting values for the collection parameters that control each other. We have developed an interactive computer-assisted process that eases the parameter-setting process by translating user-specified goals for the desired images into an optimization problem, and solving that problem.

The figure shows a screen capture of the interactive MR collection parameter optimization. The user interactively specifies regions of interest and imaging goals for contrast between them, and the optimization process automatically selects collection parameters that achieve the imaging goals in the shortest possible acquisition time.


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Assistant Professor: Bruce A. Hay
Research Fellows: Susannah L. Cole, Christine J. Hawkins
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Summary: We are interested in how cell fate choice is regulated and carried out. A large focus of our work is directed towards understanding the genetic and molecular mechanisms that regulate and bring about cell death. Specifically, we are using *Drosophila melanogaster* as a model system to identify genes that function to regulate cell death, and to identify important roles that cell death plays in normal development. Important cellular regulatory pathways are evolutionarily conserved; thus molecules identified as important regulators of cell death in *Drosophila* are likely to have homologs in vertebrates and the pathways that link these molecules are likely to be regulated similarly. A second set of goals is to take the molecules and pathways uncovered in *Drosophila* and
apply this information to the study of cell death in vertebrates, with the ultimate goal of determining the role that aberrations in this process play in human pathologies. In this context we see Drosophila as a powerful tool for uncovering conserved components and modes of death regulation.

Toward this end we have developed several genetic screening approaches to identify genes important in the regulation of cell death. Our screens take advantage of the power of Drosophila genetics and the biology of the developing eye. During the course of one of these screens we identified a family of Drosophila cell death inhibitors, the Drosophila IAPs (DIAPs), homologous to baculovirus inhibitors of apoptosis (IAPs). We are using genetics and physical interaction screens to begin characterizing the partners with which these proteins interact. We are also exploring the role these molecules play in controlling cell death and how their functions are regulated.

We have also developed a general approach to identify genes that are important regulators of cell fate. This approach takes advantage of the fact that an eye-specific promoter placed in a transposable element can function to drive the expression of genes near the genomic site of insertion. By mobilizing such a transposable element throughout the genome we can search for genes that function to regulate cell death and other developmental processes in the eye based on gene overexpression, as well as a decrease or loss of function.

**Cell death**

Apoptosis is a form of regulated cell death in which superfluous or harmful cells are removed from an organism. Apoptotic cell death is required for many aspects of normal development, tissue size homeostasis, and as a defense against potentially harmful cells, such as self reactive cells in the immune system, virally infected cells, cells that have damaged DNA, or cells that are being induced to proliferate inappropriately (reviewed in Ellis et al., 1991; Vaux et al., 1994; Steller, 1995; Jacobson et al., 1997). Because cell death is widespread during the development and normal function of organisms, de-regulation of this pathway has dire consequences. Inappropriate cell death is associated with degenerative neurological diseases such as Alzheimer's disease and Parkinson's disease; inhibition of normally occurring cell death can contribute to the development of auto immunity, persistent viral infections, and can set the stage for cancer by preventing the death of cells that would normally die, allowing them to undergo mutations that could lead to transformation (reviewed Thompson, 1995).

Although the signals and stimuli that trigger cell death are diverse, once initiated, apoptosis is thought to proceed via a common pathway. The identification in worms, flies and mammals of homologous proteins that function similarly to regulate cell death indicates that, as with other important signal transduction pathways, components and modes of death regulation are likely to be conserved throughout evolution (reviewed in Chinnaiyan and Dixit, 1996). Drosophila is an ideal system in which to do screens for genes important in death signaling because it is a complex organism with multiple life stages in which death plays important roles, it has powerful genetics, and we are able to manipulate death signaling in individual tissues using tissue-specific promoters.

**References**

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**The dominant modifier approach**

Since normally occurring cell death is essential for Drosophila viability, genetic approaches to cell death must take account of this fact as well as the potentially important role of maternal effect gene products. We have circumvented these problems by creating a sensitized system that exploits a tissue dispensable for viability and fertility, the eye. In this system a cell death signaling pathway is made hyperactive (giving rise to flies with small eyes) or partially nonfunctional (giving rise to flies with large, rough eyes). In this sensitized background a decrease in gene dosage of 50% in genes functioning downstream or in parallel to the point at which the signaling pathway has been manipulated might be expected to result in an eye phenotype change, if these components are now rate limiting.

To carry out this approach we use a P element expression vector, pGMR (Glass Multimer Reporter), which drives eye-specific expression of reporter genes (Hay, et al., 1994). To identify genes important in cell death regulation, proteins from Drosophila or other systems that are known to be able to activate or inhibit cell death in some context are expressed in the eye under GMR control, and their capacity to perturb the normal patterns of cell death determined. To the extent that the ability of these proteins to manipulate cell death is conserved, such expression should result in the creation of flies with visible phenotypes (increased cell death=small eye; decreased cell death=large, rough eye). These flies can then be used as screening backgrounds to identify interacting genes.

Several genes have been identified that influence cell death in the fly. The 75C region contains three genes reaper (rpr) (White et al., 1994), hid (Grether et al., 1995, and grim, (Chen et al., 1996) (known as the 75C death activators) that are likely to be necessary for normally occurring cell death in Drosophila. Expression of these genes is largely (though with some exceptions) restricted to cells that have been induced to die, and expression of any of these genes is sufficient to induce the death of at least some normally living cells in which they are ectopically expressed, including cells in the eye (White et al., 1994; Grether et al., 1995; Hay et al., 1995; Pronk et al., 1996; White et al., 1996; Chen...
Normally occurring cell death, as well as cell death due to expression of the 75C death activators, can be prevented by expression of the baculovirus protein p35, which blocks cell death in many organisms (reviewed in Teodoro and Branton, 1997), including Drosophila (Hay et al., 1994).

To isolate new cell death regulators we carried out loss-of-function modifier screens designed to identify enhancers and suppressors of an increase in cell death-induced small eye phenotype due to overexpression of the Drosophila reaper (rpr) gene (GMR-rpr flies). The premise of this screen is that a twofold decrease in signaling strength (making the fly heterozygous at important death regulatory loci) will result in a change in eye phenotype. During this screen we identified the cell death inhibitors DIAP1 and DIAP2 (Hay et al., 1995).

The Drosophila IAP family of cell death inhibitors

Overexpression of either DIAP1 or DIAP2 blocks death in response to multiple stimuli, suggesting that they act on components common to multiple death signals. Though these proteins are homologous, they are different enough from each other in sequence (30% identity), genetic interactions and expression pattern that they probably function in distinct cellular contexts and have preferred substrates of action. DIAPs have two major functional domains: an N-terminal region containing two or three BIR repeats, sufficient for death-preventing activity, and a C-terminal RING finger, which may be functioning to negatively regulate or antagonize this death-preventing activity. Questions we would like to address in the near future are: where are these proteins functioning in the cell, what are the partners that the BIR repeats interact with to mediate death preventing activity, how is the RING domain regulating this activity, and what are the contexts in which these proteins function to regulate death?

Overexpression of the RING domain alone in the eye gives rise to a small eye phenotype associated with excess cell death, suggesting that the DIAP RING domain functions to block or antagonize the death preventing activity associated with the N-terminal BIR repeats. This phenotype provides a starting point for a second round of modifier screening. Modifiers of the RING overexpression-induced small eye phenotype have been isolated in a pilot modifier screen; some of these are distinct from those identified using GMR-rpr, suggesting this screen will isolate additional components of the pathway.

The dominant modifier screen is an attractive approach to identifying components involved in death signaling because large scale screens can be carried out relatively rapidly. But, as with all genetic screens, it has limitations that reflect the biology of the tissue being studied: genes can only be identified as modifiers in a particular screen if the gene is expressed in that tissue, if its activity is rate limiting for the output of the pathway, and if the gene functions downstream or in parallel to the point of intervention. Also, to the extent that specific death signals activate pathways acting in parallel, it may be difficult to identify downstream components of any one sub pathway, since its relative contribution to the observed phenotype may be small. Clearly, no one modifier screen is going to identify all the interesting death regulators or effectors. Multiple screens, in which death signaling is manipulated at distinct points in different tissues, will be necessary to understand how death is regulated and carried out. Thus, a continuing long term interest of the lab will be to create and utilize sensitized genetic backgrounds that model specific cell death signaling pathways.

Gene activation screens

Mutational inactivation is an important approach to understanding the role a gene plays in a specific process. The approach is limited, however, by the facts that many genes do not have an easily assayable loss-of-function phenotype, and that any phenotype that is observed reflects only that aspect of a gene’s function that is not compensated for by other genes and pathways. An alternative approach to understanding gene function is to characterize phenotypes associated with tissue-specific misexpression of genes at elevated levels in tissues where they are normally expressed, or in tissues in which they are not normally expressed. Misexpression may create phenotypes where inactivation does not, providing a powerful approach to identifying genes. Misexpression also allows one to determine if the presence of a specific gene product is sufficient to drive a process.

To carry out misexpression screens for genes important in a particular process one needs to be able to drive the expression, individually, of large numbers of genes in a specific tissue. It is not feasible to individually misexpress known genes because this requires that one have the full length gene in hand, that these be introduced into the germline one at a time, and that one pre-select candidate genes that are likely to be important for the process under study. Misexpression of random genes from their normal genomic location provides a much more general approach for identifying genes that can affect a process without preconceptions about which which genes are likely to be important.

Drosophila is an ideal system in which to carry out such random misexpression screens because transposable elements (P elements) can be mobilized throughout the genome at a high frequency, in a controlled fashion (Cooley et al., 1988), and because mutagenic P elements have a preference for insertion
near the 5' ends of a gene (Spradling et al., 1995). To carry out this approach we designed a P element vector, known as GMREP, that contains an eye-specific promoter near one P element end, as well as sequences sufficient for plasmid rescue of genomic DNA flanking the site of P element insertion. When this P element inserts near the 5' end of a gene it causes the gene to be misexpressed at high levels in the developing eye (Hay et al., 1997). Flies carrying these insertions can then be tested in various ways to identify those that are misexpressing cell death regulators.

References

78. Structure-function analysis of the DIAPs
Susan Wang

Viral and cellular IAPs which prevent cell death, as well as NAIP, contain N-terminal BIR repeats. Removal of the DIAP C-terminal RING finger does not eliminate death preventing activity in response to rpr and hid overexpression in the Drosophila eye. These results suggest that the BIR repeats may be sufficient to mediate DIAP-dependent death blocking activity. One goal of my work is to identify and characterize the domains of the DIAPs that are necessary for DIAP death-preventing activity in the fly. Several approaches are being taken toward this end. We are generating a large number of point mutants in specific, conserved residues within the BIR's, as well as carrying out truncations and domain swaps to ask if specific BIR's play distinct roles in death prevention.

Results from previous truncation and overexpression experiments suggest that the DIAP1 RING finger functions to negatively regulate or antagonize the BIR repeat-dependent death preventing activity. A second goal of our structure-function analysis is to determine the relationship between the the DIAP BIR repeats and the RING finger by generating and testing the activity of a number of proteins that contain mutations in the RING domain, both in the context of the full length protein, and in the context of the isolated RING domain.

79. Cellular localization of the DIAPs.
Susan Wang

Antibodies that are specific for DIAP1 or DIAP2 were generated in the Rubin lab. We are using these reagents to determine the expression patterns of the DIAPs throughout the fly lifecycle. This analysis will tell us where the DIAPS are likely to be functioning to regulate cell death, and thus where loss-of-function phenotypes may be apparent. We are also characterizing the relationships of the DIAP1 and DIAP2 expression patterns to the patterns of cell death, and the expression patterns of Drosophila cell death activators. This analysis may suggest particular mechanisms of regulation of DIAP1 or DIAP2 function. In conjunction with the structure-function analysis described above, we are also determining the intracellular localization of the DIAPs, identifying the domains that mediate localizations to specific cellular compartments, and asking if these localizations change in response to death signals.

80. Loss-of-function phenotypes of the DIAPs
Bruce Hay

We are characterizing the loss-of-function phenotypes of DIAP1 and DIAP2. These phenotypes will identify DIAP gene functions that cannot be substituted for by the activity of other genes or developmental compensatory mechanisms. Homozygous DIAP1 mutant embryos arrest early during embryonic development, and essentially all cells appear to die. Clones of homozygous mutant cells are also not seen in other tissues. These observations all suggest that DIAP1 is required for cell survival, perhaps in all cells. We are characterizing these phenotypes in further detail, focusing particularly on the early embryonic phenotype. By making double mutants with cell death activators in the 75C region, and by expressing the caspase inhibitor p35 in homozygous DIAP1 mutant tissue, we will be able to order DIAP1 function with respect to the other known cell death regulators. We have also identified a P element near the DIAP2 gene and are in the process of determining if this represents a mutant in DIAP2.

81. Proteins that physically interact with the DIAPs
Christine Hawkins

The mechanisms by which the IAP family of cell death inhibitors block cell death is unknown. To identify proteins that are likely to be targets of IAP function or regulators of their activity we are isolating proteins the DIAPs physically interact with. We are using yeast 2-hybrid screens as well as immunoprecipitation of DIAP-associated proteins from embryos, followed by protein sequencing, to identify these proteins.

82. Characterization of interesting cell death related antigens
Christine Hawkins

During a monoclonal antibody screen carried out in the Rubin lab two interesting monoclonal antibodies were identified. We identified one antibody specifically recognizes dying cells in the Drosophila embryo. Based on the size of the antigen seen on Western blots it is unlikely that this antigen corresponds to the known death effectors, REAPER, HID or GRIM. The antigen may correspond to a protein which is transcriptionally induced following receipt of a cell death stimulus, or it may represent a modified form of a protein that is also expressed in cells that normally live. We are using several approaches to clone the gene for this antigen, including expression library screening and immunoprecipitation from embryos.

A second project makes use of a monoclonal antibody we generated that recognizes a baculovirus
protein called p35. p35 is able to block cell death in many organisms, including Drosophila (Hay et al., 1994), probably by inactivating a family of proteases, the caspases, that act to bring about cell death (Bump et al., 1995; Xue et al., 1995). This antibody also recognizes a protein in wildtype Drosophila.

Baculoviruses have probably picked up many cellular genes (Ayres et al., 1994), and the p35 region of the baculovirus genome is not conserved among different strains of baculoviruses (Gombart et al., 1989). Thus it is likely that p35 was picked up from another genome, perhaps a cellular genome. We are interested in determining if this cross-hybridizing signal represents a Drosophila p35 homolog. Expression library screening and protein purification are being used to characterize this antigen.

References
Hay et al. (1994). Development 120, 2121-2129.

83. Gene activation screens
Bruce Hay, Susannah Cole, Christine Hawkins, Susan Wang, Martha Kirouac

The GMREP vector contains an eye-specific promoter near one P element end, as well as sequences sufficient for plasmid rescue of genomic DNA flanking the site of P element insertion. When this P element inserts near the 5' end of a gene it causes the gene to be misexpressed at high levels in the developing eye (Hay et al., 1997). We are mobilizing this P element throughout the genome and characterizing the insertions in several ways to identify cell death regulatory genes. We first score the lines for dominant phenotypes that may be due to increased cell death (a small eye) or decreased cell death (a large, rough eye). We then cross these insertions to lines of flies that express the cell death activator HID or REAPER specifically in the eye (GMR-hid and GMR-rpr flies), and that thus have small eyes. The progeny of these crosses are then scored for the ability of the GMREP insertion to alter the GMR-rpr or GMR-hid phenotype. These modifiers may identify new cell death regulators. Genomic DNA that is likely to contain a portion of the gene being overexpressed can be quickly isolated using plasmid rescue. Because GMREP-dependent phenotypes are primarily due to insertions near the transcription start site, and because GMREP carries the dominant eye color marker white, imprecise P element excision using a genomic source of transposase or X-rays can be carried out to rapidly generate deletions that create loss-of-function phenotypes for the overexpressed gene. We have carried out a pilot screen with this vector (800 insertions) and found a frequency of dominant phenotypes of some sort of about 8%, 0.5% of the total number of lines acted as strong suppressors of GMR-rpr, while 1% acted as GMR-rpr enhancers. We have begun to characterize several of the GMR-rpr suppressors which identify loci not identified in our previous GMR-rpr loss-of-function modifier screen. We are also in the process of carrying out a larger scale GMREP screen for cell death regulators.

Reference

84. echinus, a gene required for cell death in the eye
Bruce Hay

Cell death in the eye removes extra cells between the developing unit eyes, the ommatidia. echinus (ec) mutants have extra interommatidial cells, suggesting that they lack normally occurring cell death in this population (Wolff and Ready, 1991). Since ec animals develop otherwise normally (suggesting that death is not affected in other tissues), and cell fate decisions in the eye involve interactions with neighbors rather than lineage, ec is probably not required for the function of a common death effector pathway. Instead, ec may identify a gene required for sending or receiving a death signal at the plasma membrane, or an intracellular component required for communicating such a signal to the effector machinery. ec cDNAs have been cloned and are being characterized.

Reference

Publications

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Summary: The whole course of development of animals from the lowest worms to human beings is programmed in large part by a tightly linked cluster of genes known as the homeobox-complex (HOX-C). The homeobox (HOX) sequence codes for a DNA-binding region, or "homeodomain," in each of the protein products of the HOX-C. These products, by binding to downstream or target genes, are assumed to act as master regulators of other genes that ultimately execute the final differentiation of the body's structures and organs.

The high degree of conservation of the HOX sequence among the genes of the HOX-C and their tight linkage indicates that the complex evolved by a
process of repeated tandem gene duplication. Whereas in the nematode there are only four genes in the HOX-C, in human beings there are some 13 genes. In vertebrates, commencing with the fish, the HOX-C is present in four semi-redundant copies, each being in a different chromosome in the cases of the mouse and human being.

In *Drosophila melanogaster* the HOX-C is split into two clusters, known as the bithorax (BX-C) and Antennapedia (ANT-C) complexes. We are concerned here with only the BX-C. How it came to be discovered and how the tandem gene duplication hypothesis led to the discovery, by others, of the HOX sequence has been recently reviewed (Lewis, 1996). Once the fundamental plan of the embryo is laid down, the genes of the BX-C control the development of a part of the thorax and all of the abdominal segments of the organism. The three HOX genes of the BX-C are expressed in a colinear manner; namely, the order in which the genes are expressed along the longitudinal axis of the organism parallels their order in the chromosome. This law of colinearity holds almost without exception in all organisms, including human beings in which the HOX-C has been identified. Retention of this law over such a vast evolutionary time scale indicates the importance of proximity of the HOX genes in carrying out their normal functioning. In *Drosophila*, colinearity holds not only for the protein-coding genes of the BX-C but for the immense cis-regulatory regions of the complex as well. These regions constitute over 98% of the entire DNA sequence of that complex. An important property of such regions is that they exhibit transvection, a phenomenon in which gene function depends upon the extent to which cis-regulatory regions in one chromosome are paired with their counterparts in the homologous chromosomes.

We report here on several approaches we are using to study the genes of the BX-C. The first is a study of how transvection appears to be dependent upon binding of the *zeste* protein to certain DNA sequences. The second report concerns how one of the HOX genes of the complex, *Abd-B*, is able to produce two structurally similar proteins with very different functions. And the third is an analysis of cis-regulatory regions controlling the expression of one of these proteins, *ABD-BI*.

**References**


85. Suppression of transvection by mutations of the *zeste* gene

Edward B. Lewis

We now have the complete sequence of the bithorax complex (BX-C) thanks to the work of Michael Palazzolo and his group at the Lawrence Berkeley National Laboratory, Human Genome Center (Martin, et al., 1995; Lewis, et al., 1995). Analysis of that sequence shows a highly non-random distribution of clusters of two or more zeste-binding sites, using the YGAGYG, CRCTCR consensus sequence determined by Benson and Pirotta (1988). *cis*-regulatory regions, *iab*-5 to *iab*-7 inclusive, show no significant increase in the frequency of such clustered sites based on 100 pseudorandom sequences (using a 3rd-order Markov chain method and a program devised by David Mathog); whereas all other major regions of the complex show a highly significant increase in such sites. Transvection in the *Ubx* domain has long been known to be strongly suppressed by the nearly null allele *zeste-ae*<sup>s</sup>. Ian Duncan has reported (Hopmann, et al., 1995) that this allele and other loss-of-function alleles fail to suppress transvection in the *iab*-5 to *iab*-7 region of the *Abd-B* domain. We have tested the effect of the *ae*<sup>s</sup> allele on transvection involving the *iab*-4,5,6<sup>0K</sup> deficiency when *trans* to three deletion mutants, *iab-DI* and two new *iab*-7 deletions induced by P-element excision. Again there was no suppression of transvection by the *zeste-ae*<sup>s</sup> mutant as measured by scoring number of bristles on the male sixth tergite of these genotypes. Thus, consensus zeste binding sites in the bithorax complex correlated with effects of the *zeste-ae*<sup>s</sup> mutant on transvection.

86. Functional studies in the *Abdominal-B* domain

Joanne Topol, John D. Knaufels, William Fisher, Susan E. Celinker

An important and unresolved problem in the field of molecular genetics is how homeotic proteins target the correct sets of downstream genes in order to execute their specific developmental programs. Our research focuses on the homeotic regulatory gene, *Abdominal-B* (*Abd-B*), found within the *Drosophila melanogaster* bithorax complex (BX-C). The work described below involves defining the molecular basis for the different activities exhibited by the two *ABD-B* protein products.

The *Abd-B* domain encodes two proteins, *ABD-BI* and II, which possess distinct and genetically separable functions. A loss of *ABD-BI* protein leads to the transformation of A5 through A8 towards A4, whereas the loss of the *ABD-BII* protein leads to the loss of internal and external genitalia. Since the two *ABD-B* proteins differ only at the amino terminus (*ABD-BI* possesses an additional 221 amino acids) and they share identical homeodomains, the question arises as to how two such structurally related proteins are capable of exerting such different functions. Either of two hypotheses is plausible. The first one maintains that the distinct functions of *ABD-BI* and II are simply due to each protein's unique pattern of expression within the embryo (*ABD-BI* is expressed in parasegments 10-13, while *ABD-BII* is expressed in parasegment 14). More specifically, within each protein's domain of expression, unique cofactors may
reside that, in conjunction with ABD-B, provide the specificity for the expression of the appropriate target genes. The second hypothesis maintains that the two proteins are intrinsically capable of regulating distinct sets of target genes due to their structural differences.

Although ectopic expression studies with ABD-BI and II suggest that the two proteins are functionally similar, it is still unclear whether the proteins are interchangeable in their normal domains of expression. For instance, can ABD-BI substitute for ABD-BII in parasegment 14 and the genital disc? To test this idea, we are using the GAL4 system for targeted gene expression (Brand and Perrimon, 1993), combined with a mutant fly line lacking endogenous ABD-BII function.

At present, fusion genes have been generated in which the yeast GAL4 UAS is placed upstream of the ABD-BI and II coding regions. These fusion genes have been introduced into flies using P element-mediated transformation, and can now be targeted by the yeast GAL4 protein. In order to express the yeast GAL4 in the domain of interest, i.e. parasegment 14 and the genital disc, we have drawn from a result obtained in our study of Abd-BII transcriptional regulation. In this study, a 3.7 kb restriction fragment from the Abd-BII promoter was able to direct lacZ expression in parasegment 14, coinciding with the endogenous expression of Abd-BII transcripts and protein. We have fused the 3.7 kb fragment upstream of the GAL4 protein, and introduced this construct into flies, again using P element-mediated transformation. If these transformed animals express GAL4 where ABD-BII is normally expressed, they will also be able to target ABD-BI to ABD-BII's domain of expression. Consequently, we will determine whether ABD-BI can rescue an ABD-BII mutant.

Reference

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87. Molecular analysis of the Abdominal-BI promoter region
John D. Knafels, Joanne Topol, William Fisher, Susan E. Celniker

The larger of the two Abdominal-B proteins, ABD-BI, is expressed embryonically in parasegments (PS) 10 - 13, in a gradient increasing from anterior to posterior. X-ray induced chromosomal rearrangements indicate that enhancers regulating ABD-BI transcription in PS 10 - 12 are located 3' of the ABD-B coding region. We have cloned regions of the ABD-BI promoter located 5' to the transcription start site into P-element vectors containing β-galactosidase (β-gal) as a reporter, in order to identify sequences necessary for expression in PS 13.

Our basal construct contains 5.9 kb of the promoter region upstream of the ABD-BI start of transcription, the ABD-BI mRNA leader, and the first five amino acids of the ABD-BI coding sequence fused to the coding sequence for β-gal. This ABD-BI promoter fusion gene, known as 5.9cBI, contains several proposed binding sites for candidate trans-regulators of ABD-B expression.

5.9cBI was injected into flies and transformed embryos were assayed for β-gal activity. Staining revealed a repeated pattern with higher expression in the posterior portion of each PS commencing in PS 3 and continuing through PS 13. This pattern resembles other homeotic gene basal promoter patterns.

In an effort to restrict β-gal expression to PS 13, we made two larger constructs. Starting with our basal construct, we added an additional 4.1 kb and 7.1 kb, creating promoter fusion genes containing, respectively, 10 kb and 13 kb of the ABD-BI promoter upstream of the transcription start site. We assume these constructs (10cBI and 13cBI) contain all the 5' regulatory sequences required for ABD-BI expression, based on an analysis of an x-ray induced mutant iab-9~gal~ with a rearrangement breakpoint located approximately 8 kb upstream of the start of transcription. This mutant appears wild type for ABD-BI functions but lacks ABD-BII functions.

10cBI and 13cBI were injected into flies and transformed embryos were assayed for β-gal activity. Both 10cBI and 13cBI still show the basal expression pattern similar to that of 5.9cBI. However, superimposed on this repeated pattern in PS 3 - 13, we observe a significantly elevated level of expression in PS 13. This result suggests that our two larger constructs include at least some of the PS 13 enhancer sequences, and that sequences 3' to the ABD-B translational start site are necessary to repress the basal pattern.

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References

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Arabidopsis thaliana, a single cell that is a fertilized egg leads to marvelous patterns of cell division in shoot apical meristems, and the control of patterns of cell division in shoot apical meristems, and the mechanism by which plant cells perceive plant hormones.

For more than the past decade we have been exploring the specification of floral organ type, and have identified by mutation, and obtained molecular clones of, many key regulatory genes that act in flower development. Among these are several members of the MADS-domain family of transcription factors. The past year we have been concentrating on understanding the DNA-binding and organ-specifying activities of the MADS-domain proteins, and on methods to find genes whose transcription is regulated by the MADS-domain proteins AGAMOUS and APETALA3.

Different MADS-domain proteins have different activities in vivo: activation of AGAMOUS in the first whorl region of flowers, for example, converts sepals to carpels, while activation of APETALA3/PISTILLATA converts sepals to petals. Some of the recent work has shown that this specificity does not result from different DNA binding specificities of the different MADS domains: even when the Arabidopsis MADS domains of several of these proteins are replaced with identical MADS domains taken from the human MEF2 gene, which binds a different optimal sequence than the plant MADS domains, the chimeric plant proteins still have different specificities. This implies that specificity does not reside in the DNA binding domains, but more likely in other domains that bind accessory proteins. Screens, both genetic and biochemical, for these accessory proteins are in progress. In the area of searches for downstream genes of the MADS domain proteins, we have used various gene activation systems along with differential display methods to find several genes upregulated and downregulated by MADS domain organ identity proteins. In addition, we are working to understand the upstream regulators of the organ identity genes. The most important of these appears to be LEAFY, a gene that acts in specification of floral meristem identity, and that is involved in induction of the AP3, PI and AG genes. Genetical and biochemical experiments have been directed toward an understanding of the function of the LEAFY protein in developing flowers.

More recently we have been trying to find and clone genes involved in the patterning of cell divisions, both in developing flowers and in active shoot meristems. Two recent areas of progress are in analysis of genes that regulate cell division in different subdomains of developing flowers, and that regulate numbers of cell divisions in early floral meristems, as well as in shoot apical meristems. We have shown that SUPERMAN, which codes for a zinc-finger protein, is necessary for proper numbers of cell divisions in the third whorl region of flowers, where stamens normally form. Furthermore, we have discovered epigenetic control of SUPERMAN that is associated with non-symmetric cytosine methylation. We also published this year the molecular cloning of the CLAVATA1 gene, which is required for proper regulation of cell number in meristems. It codes for a transmembrane receptor kinase, indicating a new type of signal transduction pathway for cell division control in plants. Additional genes such as WIGGUM, that seem to code for parts of this new control mechanism, are under intense study. Another area of progress is the molecular cloning of the PERIANTHIA gene, mutations in which change the Arabidopsis flower from a 4-fold to a 5-fold symmetry.

Finally, we continue our ongoing studies of hormone action in plants. Earlier we had identified the ethylene receptor family in plants, which are necessary for normal response to that plant hormone. In the past year we have learned of more members of the family, and have produced gain- and loss-of-function mutations in several members, all of which reveals critical aspects of the function of the ethylene signal transduction system.

88. DNA-binding specificity and the organ identity activity of AP1, AP3, PI, and AG proteins

José Luis Riechmann

The MADS domain homeotic proteins APETALA1 (AP1), APETALA3 (AP3), PISTILLATA (PI), and AGAMOUS (AG) combinatorially specify the identity of Arabidopsis floral organs. AP1/AGAMOUS (AG) dimers bind to similar CARG-box sequences; thus differences in DNA-binding specificity among these proteins do not seem to be the origin of their distinct organ identity properties. To assess the overall contribution that specific DNA-binding could make to their biological specificity, we have generated chimeric genes in which the amino terminal half of the MADS domain of AP1, AP3, PI, and AG was substituted by the corresponding sequences of human SRF and MEF2A proteins. In vitro DNA-binding assays reveal that the chimeric proteins acquired the respective, and distinct, DNA-binding specificity of SRF or MEF2A. However, ectopic expression of the chimeric genes reproduces the dominant gain-of-function phenotypes exhibited by plants ectopically expressing the corresponding Arabidopsis wild-type genes. In addition, both the SRF- and MEF2-chimeric genes can complement the pertinent ap1-1, ap3-3, pi-1, or ag-3 mutations to a similar degree as do AP1, AP3, PI, and AG when expressed under the control of the same promoter. These results indicate that determination of floral organ identity by the MADS domain homeotic proteins AP1, AP3, PI, and AG is independent of their
DNA-binding experiments. In addition, the DNA-binding experiments show that either one of the two MADS domains of a dimer can be sufficient to confer a particular DNA-binding specificity to the complex, and that sequences outside the amino-terminal basic region of the MADS domain can, in some cases, contribute to the DNA-binding specificity of the proteins.

89. Determination of petal and stamen identity by the APETALA3/PISTILLATA dimer is independent of its DNA-binding specificity

José Luis Riechmann

APETALA3 (AP3) and PISTILLATA (PI) are MADS domain proteins involved in the determination of petal and stamen identity. Genetic and biochemical analyses suggest that AP3 and PI only act as a heterodimer. Ectopic expression in transgenic Arabidopsis plants of the chimeric genes SRF-AP3, MEF2-AP3, SRF-PI, and MEF2-PI has shown that determination of organ identity by AP3 and PI is independent of their DNA-binding specificity (Abstract M1). However, an unresolved question is whether the chimeric proteins retain the organ identity activity of the corresponding wild-type proteins because they heterodimerize with the endogenous wild-type partner. To address this issue, we have generated strains with the following genotypes: ap3-3 pi-1, 35S::AP3 35S::PI ap3-3 pi-1, 35S::SRF-AP3 35S::SRF-PI ap3-3 pi-1, and 35S::MEF2-AP3 35S::MEF2-PI ap3-3 pi-1. The three doubly transgenic strains showed a partial rescue of the ap3-3 pi-1 mutant phenotype. However, the DNA-binding specificities of the AP3/PI, SRF-AP3/SRF-PI, and MEF2-AP3/MEF2-PI dimers are different. These results indicate that the specificity in floral organ identity determination by the AP3/PI dimer is independent of its DNA-binding specificity. In agreement with this interpretation, two CArG-boxes of the AP3 promoter, that could be involved in the presumed late autoregulation of AP3 expression by AP3/PI, can be substituted for MEF2 binding sites (which are not recognized by AP3/PI in vitro) without abolishing late expression from that promoter.

90. Characterization of the transcriptional activities of MADS domain factors AGAMOUS and APETALA1

Carolyn K. Ohno

The AGAMOUS (AG) AND APETALA1 (AP1) genes encode members of a large family of MADS domain-containing transcription factors in Arabidopsis. The genes were initially identified in homeotic mutants which disrupted correct specification of floral organ identity and aspects of floral meristem identity and determinacy. The AG protein and AP1 transcription factors have been shown to specifically bind to a common consensus sequence element known as a CArG-box as homodimers (Riechmann et al., PNAS 93, 4793-4798, 1996). To further elucidate the mechanism of MADS domain protein function, an in vivo transcriptional assay has been developed in transgenic plants. In order to investigate the transcriptional potential of these MADS domain factors in isolation, the cDNAs have been fused downstream of a cDNA encoding a heterologous yeast GAL4 transcription factor DNA binding domain, thus creating chimeric proteins with the DNA binding specificity of GAL4. Effector genes encoding GAL4 fusions to a series of N-terminally truncated forms of AG or AP1 have been placed under the control of a constitutive CaMV 35S promoter with a tandemly repeated enhancer sequence. Transgenic lines have been generated harboring the 35S::GAL4-AG and GAL4::AP1 effector genes and transgene expression in independent lines will be monitored by immunoblot analysis. A GAL4-responsive reporter gene will be used to detect transcriptional activation by these chimeric proteins. Reporter genes have been constructed which consist of either 3 or 10 copies of a 17 base pair GAL4 DNA binding sequence (UAS) fused upstream of a CaMV 35S minimal promoter directing the expression of either β-glucuronidase (GUS) or green fluorescent protein (GFP) reporter genes. These reporter constructs are essentially silent in wild type transgenic plants as confirmed by the absence of GUS staining in tissues from independent lines. Transgenic lines which harbour both effector and reporter genes have been generated by transformation of the effector construct into stable homozygous reporter lines and as well, by crossing independent transgenic lines. In parallel, these gene constructs are being tested in a transient expression system in Arabidopsis leaf mesophyll protoplasts.

It will be interesting to identify AG and AP1 protein domains that modulate transcriptional activity and as well to determine in which cells these proteins are active. Additionally, dominant negative phenotypes associated with overexpression of these proteins would suggest their interaction with other factors important for their function. Future studies will investigate temporal and cell-specific factors which may be required for this transcriptional activity.

91. Analysis of transcriptional regulation by AGAMOUS

Toshiro Ito

A floral homeotic C function gene AGAMOUS (AG) is considered to be a transcriptional regulator because it has substantial similarity to known transcription factors, binds to specific DNA sequences, termed CArG boxes, in vitro and localizes in the nuclei of the cells of stamen and carpel primordia. AG has several functions such as the determination of floral meristem growth and specification of reproductive organ identity, which are separable by weak loss of function mutations such as ag-4. After the floral organ identity is determined, the expression of AG continues in specific cell types. Antisense analysis has shown that different AG functions may require different amounts of AG activity.
We showed that different AG activity may be also controlled by differently modified AG by four results. First, AG was detected as three bands in a western blot using AG antibody and the ratio of three bands changes as floral development proceeds. Second, the expression level of mutant AG-4 product in ag-4 is almost same as that of wild type AG product in wild type floral buds. Third, a reporter gene driven by tandemly repeated CArG boxes combined with a basal promoter was not expressed in reproductive organs at early stages. Fourth, 35S promoter driven AG chimeric protein with a MADS domain and I domain, which are sufficient for specific binding to CArG boxes, combined with a VP16 activation domain, only partially mimics 35S::AG in transgenic plants. These results suggest that AG activity is controlled not only quantitatively level but also qualitatively, and that AG needs cofactor(s) which facilitate the binding of AG to DNA.

To further investigate transcriptional regulation by AG, we have constructed the following three kinds of AG- VP16 activation domain chimeric genes: full length AG, C-terminal part deleted AG, and half of MADS domain deleted AG. Agrobacterium with these constructs have been infected into ag-1 /+ plants. Phenotypic analysis of these transgenic plants in wild type and ag-1 mutant background will give us a more profound insight into transcriptional regulation by AG.

92. Cofactors of the Arabidopsis MADS domain proteins
José Luis Riechmann

In vitro and in vivo analyses of the activities of AP1, AP3, PI, and AG proteins suggest that selective interactions with additional transcription factors might be essential for these proteins to achieve their biological specificity. The yeast two-hybrid system has failed to uncover any interaction between the plant MADS domain proteins and proteins of a different type, maybe due to the inherent limitations of this approach (fusion proteins in which the bait cannot make direct contacts with the DNA). To overcome these limitations, I have developed a strategy, derived from the one-hybrid approach, in which the native proteins, making direct contacts with the DNA (promoter sequences of genes that are likely to be directly regulated by them), are used as bait for expression library screening in yeast. A binding site for a particular protein is inserted upstream of different reporter genes and the resulting constructs are integrated into the yeast genome. If the reporter genes are silent, this yeast strain is transformed with a plasmid expressing the “bait” protein. If this protein recognizes its binding site but does not activate transcription by itself, the yeast cells are then transformed with a fusion expression library (Arabidopsis floral cDNAs fused to a transcription activation domain). An interaction between the “bait” protein and a library protein could result in transcription activation of the reporter genes. The three components of the system have been prepared. Several yeast strains carrying wt or modified CArG-boxes (binding sites for MADS domain proteins) in the context of different Arabidopsis promoters have been generated. New plasmids to express in yeast one or two proteins at the same time have been constructed, and different versions of AP1, AP3, PI, and AG (wt or modified with regard to DNA-binding specificity) can now be used as bait in the library screenings, which are currently underway.

93. Beyond floral homeotic genes: finding their target genes.
Robert Sablowski, Elliot Meyerowitz

APETALA 3 (AP3) and PISTILLATA (PI) play a central role in the programs of gene expression required for petal and stamen development. To identify which genes are subordinate to the heterodimeric protein AP3/PI, we looked for changes in mRNA populations in floral organs, caused by activation of AP3/PI. To keep mRNA complexity low, flowers were used that produced only sepals (ap3-3; ag-3 double mutants). Transgenes were added, constitutively expressing PI (35S::PI) and a fusion between AP3 and part of the rat glucocorticoid receptor (35S::AP3-GR). Treatment of these flowers with steroid caused formation of petaloid organs instead of sepals, showing that hormone-activated AP3-GR mimicked AP3 function. Steroid activates AP3-GR by allowing nuclear localization of the fusion protein, a process that does not require new protein synthesis. Therefore, AP3-GR could be activated even with protein synthesis inhibited by cycloheximide, which would block indirect target gene activation. Flowers were treated with steroid combined with cycloheximide, or cycloheximide alone, and mRNA populations were compared by differential display. A cDNA was cloned, corresponding to a mRNA consistently up-regulated by steroid treatment. Northern blots and in situ hybridization comparing wild-type and ap3-3 flowers showed an expression pattern consistent with activation by AP3/PI. The target gene showed homology to a petunia gene (NO APICAL MERISTEM) which is necessary for meristem function and for setting boundaries between floral organs. Overexpression of the target gene or antisense inhibition caused defects, respectively, in petal and stamen formation. The results start revealing the genes and functions regulated by a homeotic gene during the morphogenesis of floral organs.

94. Identification of a gene positively regulated by the floral homeotic gene AGAMOUS
Xuemei Chen, José Luis Riechmann, Elliot Meyerowitz

The Arabidopsis floral homeotic gene AGAMOUS (AG) codes for a MADS domain DNA-binding protein and specifies the identities of stamens and carpels. In order to study the organogenesis of floral organs after their identities have been specified, we set out to look for genes that are regulated by AG. We expressed AG
under the control of a heatshock promoter, and used differential display to identify genes whose RNA levels were affected by AG induction. One such gene, A6a, was isolated and found to be more abundant in wild-type inflorescences than in those of ag-3 mutant plants. *In situ* hybridizations showed that A6a RNA is most abundant in developing stamens and carpels, consistent with it being positively regulated by AG. It is also expressed at lower levels in the inflorescence meristem and in petals, suggesting that it is not solely regulated by AG. Its expression was examined in various mutant backgrounds and the findings strongly suggest that AG positively regulates A6a, although *APETALA3 (AP3)*/*PISTILLATA* may be responsible for its expression in the petals. The promoter of A6a contains a "CArG" box, a known binding site of MADS domain proteins. When fused to the reporter gene uidA, this promoter conferred the correct expression patterns, and responded to the ectopic expression of AG in 3SS:AG lines. A6a codes for a leucine rich repeat protein and is likely to be the orthologue of the *Fil2* gene in *Antirrhinum*. *Fil2* was isolated in a differential screen aimed at identifying genes regulated by *DEFICIENS*, the *Antirrhinum AP3* orthologue. Although *Fil2* expression in stamens is decreased in some *def* mutants, its expression in carpels is unaffected, suggesting that *DEFICIENS* cannot be the only regulator of *Fil2*. Our data indicate that A6a may be regulated by both AG and AP3, with AG being the major regulator.

95. Identification of a gene negatively regulated by the floral homeotic gene AGAMOUS

Xuemei Chen, Elliot Meyerowitz

A38a2 was isolated in a differential display experiment aimed at identifying genes regulated by the floral homeotic gene AGAMOUS (AG). It codes for a small, possibly secreted protein of no obvious homology to known proteins. Heatshock induction of AG caused a reduction in the abundance of its RNA, suggesting that AG may negatively regulate A38a2. RNA filter hybridizations showed that it is four times more abundant in ag-3 mutant flowers than in wild-type flowers. The promoter of this gene was isolated and shown to confer the correct expression pattern to a reporter gene. When this promoter-reporter construct was crossed into lines expressing *APETALA1 (AP1)* from the 3SS promoter (3SS:AP1), ectopic expression of the reporter gene in the carpels was detected. Since AG is known to negatively regulate AP1, it is possible that AG exerts its effect through AP1, which positively regulates A38a2. *In situ* hybridizations showed that A38a2 expression is flower specific and L1-layer specific. It is most strongly expressed in petals starting from around stage 8, and weakly expressed in stamen filaments and some cells of the sepal. A striking feature of the expression pattern is that A38a2 RNA first appears in cells at the apical end of petals at around stage 8, and the domain of expression gradually expands basally as the petal matures until it occupies the entire petal blade. This gene was previously isolated as a gene induced by the plant hormone GA, which is known to promote petal growth. Our preliminary data suggest that the expression pattern of A38a2 may correlate with cell division patterns in expanding petals.

96. Activation screen for downstream genes of AG

Toshiro Ito

AGAMOUS (AG) is a floral C function gene that specifies reproductive organ identity. AG has another function, to determine floral meristem identity. AG protein belongs to the MADS box family of transcription factors, localizes in the nuclei of specific cell types and binds DNA in vitro. AG protein is suggested to control several genes, corresponding to several functions, as a transcription factor. Although extensive screening for downstream genes of AG has been done, no target genes of AG with clear functions have yet been isolated.

In order to clarify the molecular cascades of determination of floral organ identity and of organ formation, we have started a new genetic screen to find downstream genes of AG. We are using an activation tagging vector (the gift of Dr. Detlef Weigel, Salk Institute), in an ap2-1 background, where ap2-1 is a weak loss of function mutation of the AP2 gene. The 1st whorl of ap2-1 has no functional ABC genes and shows leaf-like structure. We are expecting some downstream genes activated by the 3SS enhancer in the activation vector will bypass the molecular cascade of floral organ development and induce (a part of) floral organ specification in the 1st whorl of ap2-1.

97. splayed specifically abolishes organ identity in leafy mutant flowers

Doris Wagner, Elliot Meyerowitz

leafy (lfy) mutants cause axial meristems (stem cells) in Arabidopsis to continue generating indeterminate inflorescences instead of determinate flowers. The flowers that do eventually form are abnormal in organ number, position, and identity (Weigel et al., 1992). To identify other components in the LFY signaling pathway, we mutagenized a weak *lfy* allele, which has flowers consisting of all four floral organ types and is fully fertile, chemically and by creating insertions with foreign pieces of DNA (T-DNA mutagenesis).

We identified several classes of enhancers in both screens. One class causes *lfy* flowers to be more severely abnormal, yet does not affect the switch from inflorescence to flower formation. Among these *splayed*(spl) mutants in the *lfy* mutant background have "flowers" consisting of leaf/sepal like organs only, that are initiated in a more spiral than whorled pattern. In the wild-type background, spl is characterized by splayed open floral buds. Flowers show abnormal organ number, position and identity, as well as organ fusion and the formation of filamentous
98. Biochemical characterization of flower mutants. and determining its role within the genetic hierarchy organs. In addition the carpel fuses incompletely and the flower is female sterile. The primary inflorescence in spl terminates in sepal/carpel-like structures after forming 6 to 15 flowers. We are currently mapping spl and determining its role within the genetic hierarchy that controls flowering in Arabidopsis by analyzing additional double mutants with spl and relevant flower mutants.

Reference

99. Interaction of Late Flowering Mutants with the meristem identity genes LEAFY and APETALA1 Thomas D. Tubman, Steven E. Jacobsen
Late flowering mutants (LFMs) of Arabidopsis thaliana, as a class, all confer a similar phenotype: an increased number of rosette leaves produced and a proportional increase in growth time before the plant switches from vegetative growth to reproductive growth. Double mutants between 11 LFM loci and strong and weak mutant alleles of the leafy and apetala1 loci have been constructed, providing some evidence as to how the LFMs interact with these meristem identity genes.

The severity of the double mutant phenotypes was quite unpredictable, however the phenotypes suggest that a few LFM loci appear to play a role in meristem identity themselves. fd, ft, and fwa, when crossed to different leafy alleles, produce plants with no reproductive structures at all. Those same LFMs, when crossed to apetala1-1 plants, produce a double mutant which produces no flowers from the shoot apical meristem. Thus it appears that FD, FT, and FWA may play a role in meristem identity, possibly affecting the expression of either LEAFY or API proteins.

To analyze this further, API in situ were performed in various ft, fd, leafy-6, and double mutant backgrounds. The API RNA expression pattern was not found to be altered in either of the LFM single mutant backgrounds, and it was present at a reduced level in the leafy-6 sections, which has been shown in previous experiments. API RNA expression was also present at a similar reduced level in the ft leafy-6 and ft leafy-6 double mutants, and was limited to the axils of cauline leaves. This expression pattern is not significantly different from any of the other genotypes, in which the pattern is localized to the axils, pedicels, and flowers. The double mutants do not contain flowers, so the expression pattern is limited to the only structures which are still present in those plants. This indicates that these genes, FT and FD, do not function upstream of APETALAl, and do not regulate it.

In addition, LEAFY immunolocalizations are being performed in ft, fd, and double mutant backgrounds to determine whether LEAFY expression is altered in the genetic background of interest.

100. Towards the cloning of late flowering genes in Arabidopsis thaliana
Thomas D. Tubman
Arabidopsis plants which are mutant for any of the late flowering class of genes all display the same phenotype: an increase in the length of the vegetative phase of the plant life cycle, an increase in the number of leaves and second-order branches produced by the plant, and an overall increase in the lifespan of the plant. Since all of the aerial structures and organs of the plant are produced by the shoot apical meristem (SAM), late flowering mutants (LFMs) must alter the wild type function of the SAM. When double mutants were constructed between the LFMs and plants mutant for either leafy or apetala1 (two other genes which affect wild type meristem identity), the LFMs fd-1 and ft-1 showed the strongest double mutant phenotypes. They virtually eliminated the process of flower production when doubly mutant with different leafy alleles. In order to increase the understanding of the process of flower production, a chromosome walk is proceeding to clone FD and FT.

The first step has been to generate 4 separate doubly mutant lines between ft-1 and the following a number of linked visible marker lines. Double mutant lines were also generated between ft-1 and different linked visible marker lines. All of these lines were made in the Landsberg erecta (L-er) background. These lines were crossed to a Columbia (Col) line which is wild type for both loci of interest. The F2 plants are
being screened for ones carrying one of the mutations of the ancestral double mutant but not the other. This indicates that a recombination event has occurred in the region between the LFM and the visible marker. The resulting chromosomes are hybrids of the L-er and Col backgrounds. The many lines, each with a different visible marker, are being used to characterize the area of the chromosome around the target LFM locus.

Restriction Fragment Length Polymorphism (RFLP) clones in the respective regions around FD and FT will be tested on the recombinants, followed by YAC identification and DNA sequencing in the area of both genes.

In addition, a pool of 26 T-DNA insertion LFM in the Wassilewskija ecotype (Ws) have been made available. These 26 lines, along with another isolated in this group, are the focus of a T-DNA cloning effort. The 27 lines are being backcrossed to wild type Ws and are being assayed for resistance to Kanamycin (the Kan resistance gene is contained within the T-DNA insertion). Each line in which the LFM phenotype cosegregates with the drug resistance will be crossed with each of the known LFM loci to determine if the lines are allelic with any of these loci or if the T-DNA LFM locus is heretofore unknown.

λ genomic libraries will be made with each of these resistant lines and will be screened with the T-DNA sequence. Clones containing both plant DNA and T-DNA from the insert will be subcloned and used to screen a cDNA library in order to isolate a cDNA which corresponds to genomic sequences flanking the T-DNA insert. If this is successful, the other mutant alleles (if any) will be sequenced to confirm the cloned gene is the target LFM locus.

101. The functional analysis of TERMINAL FLOWER1 in Arabidopsis

Shosaku Ohshima, Hajime Sakai

TERMINAL FLOWER1 (TFL1) plays an important role in regulating the transition between vegetative and reproductive growth and in allowing inflorescence meristems to grow indeterminately. In tfll mutants, growth of the inflorescence stem is determinate, and the plant flowers earlier than wildtype. Several genetic analyses of tfll and other mutants indicate that TFL1 interacts with other genes, particularly floral meristem identity genes, LEAFY (LFY) and APETALA1 (AP1). LFY and AP1 are ectopically expressed in shoot apical meristems of tfll mutants and transgenic plants which constitutively express LFY or AP1 show the tfll phenotype. Therefore, it has been suggested that TFL1 is a negative regulator of LFY and AP1 (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Simon et al., 1996; Bradley et al., 1997; Ohshima et al., 1997).

In order to gain insight into TFL1 function, the TFL1 gene was isolated using the T-DNA tagged tfll mutant (tfll-15). The putative TFL1 gene product partly shares similarity with a phosphatidylethanolamine-binding protein, indicating that TFL1 is associated with signal transduction.

We have further analyzed TFL1 expression by in situ hybridization methods. TFL1 expression is detected in the corpus region of shoot apical meristems soon after germination. After transition of the vegetative meristem into an inflorescence meristem, TFL1 is strongly expressed below the L2 layer of the inflorescence meristem. It is also detected in the developing vascular system of the inflorescence stems. TFL1 is not expressed in apical meristems of lfy mutants, but is expressed in ap1 mutants. These results indicate that TFL1 is regulated by LFY. To examine the effect of ectopic TFL1 expression in plants, we produced transgenic plants (35S-TFL1) that constitutively express TFL1 with the cauliflower mosaic virus 35S (CaMV35S) promoter. In some flowers of 35S-TFL1 plants, carpels in the fourth whorl are converted into a new inflorescence. This indicates that ectopic expression of TFL1 in the floral meristem causes loss of floral meristem identity. Moreover, since LFY and AP1 are expressed in 35S-TFL1 plants, TFL1 does not appear to be the negative regulator of LFY and AP1. We are planning further genetic analyses of 35S-TFL1 to elucidate the function of the TFL1 gene.

References
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102. Identification of new genes required for specifying the identity of stamens in Arabidopsis flowers

Xuewei Chen, Elliot Meyerowitz

The homeotic gene AGAMOUS (AG) specifies the identity of stamens in Arabidopsis flowers together with the B function genes APETALA3 (AP3) and PISTILLATA (PI). In severe AG loss-of-function mutants, the third whorl organs are completely converted to petals, but they remain stamens in a weak ag allele, ag-4. In order to identify other genes that work in the AG pathway, or in parallel pathways, to specify the identity of the third whorl organs, we carried out a generic screen for new mutations that enhance the ag-4 mutant phenotype.

Five independent enhancer lines were isolated: m7, m15, m186, m187 and m188. All except m187 are known to be extragenic enhancers, while m187 could be another ag allele. m15 is the most severe of all, showing a complete conversion of anthers to petal blades. In m7, m186, m187 and m188, the anthers are flat and petaloid, but still have recognizable features of anthers. Complementation tests are being carried out to determine how many genes these enhancer lines represent.

m15 alone may contain mutations in two genes, one causing an enhancement of ag-4 like the other lines and one causing an even weaker enhancement, yet the combination of the two causing complete conversion of anthers to petal blades. Each mutation alone does
not show any phenotype, but plants containing both mutations have carpels that bulge at the tips. Plants containing both mutations and also heterozygous for ag-4 have petaloid stamens and additional flowers inside carpels, suggesting a loss of determinacy. So these enhancer genes are like AG in that they are required not only for stamen identity but also for floral determinacy.

103. fused floral organ (ffo) mutants disrupt early floral patterning events in Arabidopsis

Jennifer C. Fletcher, Joshua Z. Levin, Xuemei Chen

Local control of cell proliferation during plant development is necessary to establish and maintain boundaries between incipient vegetative and floral organs. We have identified mutations in three independent Arabidopsis thaliana genes that cause the inappropriate fusion of floral organs within and between whorls. Mutations at the FUSED FLORAL ORGAN1 (FFO1) locus cause intrawhori sepal/sepal and stamen/stamen fusions, as well as inappropriate attachment of the margins of cauline leaves to the meristem. The ffo3 mutant has similar but slightly weaker floral and cauline leaf defects, and also displays reduced meristem intermode elongation. ffo2 mutants have variable organ number and organ fusion defects in all four whors, and appear to be semi-dominant. At least one allele of each FFO locus was identified in a screen for enhancers of the ufo mutant phenotype, in which a flower occasionally does not develop and a filamentous structure is formed in its place. ffo ufo double mutants generate predominantly filamentous structures, indicating that these genes share a common function in controlling cell proliferation during floral meristem initiation. Further double mutant analysis indicates that the ffo alleles interact with a subset of floral meristem identity and floral organ number mutants, as ffo ify-6 but not ffo clo3-1 double mutants produce filamentous structures. Thus, like UFO, the FFO genes seem to be required for proper control of cell proliferation during early floral patterning events, and for establishing and/or maintaining boundaries between floral organs within and between whors.

104. Characterization of TARANTULA, a gene controlling flowering time, floral organ patterning, and cell determinacy in leaves

Eva C. Ziegelhoffer, Jennifer C. Fletcher

A recessive, EMS-induced mutation of the TARANTULA (TAR) gene of Arabidopsis confers unusual vegetative and floral phenotypes. Isolated in the Nossen ecotype, tar plants are late flowering, bolting approximately two weeks later than wild-type with nearly double the number of rosette leaves. tar rosette leaves are long and narrow, with long petioles, and produce small ectopic leaves on the abaxial surface at the base of the midvein. The ectopic growth on tar leaves is somewhat reminiscent of ectopic meristems produced, near veins, on the adaxial surface of 35S::KNAT1 leaves. Leaf phyllotaxy in tar plants is also affected such that leaves are not produced at stereotypical angles to one another. Other phyllotaxy defects in tar plants include multiple flowers or higher-order inflorescence meristems produced at or nearly at the same node. tar flowers, although essentially normal with respect to organ identity and organ number, have narrow sepals and an overall disorganized appearance. The position of perianth organs in tar flowers often does not conform to the stereotypical cruciform pattern of wild-type flowers, resulting in flowers in which petals are not always alternate to sepals. The severity of these tar phenotypes is dependent on environmental conditions - in general, the healthier the plant, the more severe the tar phenotypes. Further analysis of tar under a variety of environmental conditions is currently being pursued. Additionally, potential genetic interactions between tar and other developmental mutants of Arabidopsis are also currently under investigation.

105. Molecular characterization of PERIANTHIA, an Arabidopsis gene involved in the determination of floral organ number

Chiou-Fen Chuang

The PERIANTHIA (PAN) gene has been isolated from a T-DNA tagged mutant line. Three lines of evidence suggest that the cloned gene corresponds to PAN: (1) Mutations in the cloned gene are found in both pan-1 and pan-2 alleles; (2) RFLP analysis of the cloned sequences are consistent with the previously-defined map position of the pan locus; and (3) A 7-kb of genomic DNA fragment containing the cloned gene can complement the pan mutant phenotype.

To mimic a gain-of-function phenotype for PAN, we have constructed transgenic lines which ectopically express the gene under the control of the 35S constitutive promoter. Overexpression of PAN can rescue the phenotype of pan mutant plants but does not confer any phenotype in wild-type plants. One possibility is that PAN needs to function with other factors in a complex, and consequently, overexpression of PAN alone is not enough to cause phenotypes. I am using two approaches, protein-protein interaction screening and genetic screens for enhancers and suppressors, to isolate potential partner proteins of PAN.

We have also placed a GUS (β-glucuronidase) reporter gene under the control of the PAN promoter and transformed this construct into plants. The expression pattern has been determined by GUS assay and is similar to that found by in situ hybridization analysis. To investigate the subcellular localization of the PAN protein, we have overexpressed the PAN gene in E. coli and then produced polyclonal antibodies recognizing the gene product in rabbits. Western blot analysis of plant proteins from wild-type and transgenic lines over-expressing PAN show that the antibodies can recognize plant proteins of the expected
size of the PAN protein, and the signals at that size were stronger in the transgenic lines compared to wild-type. Immuno-localization assays on sections of different tissues are under way.

106. Studies of PERIANTHIA gene activity
Mark P. Running, Bobby W. Williams

The PERIANTHIA (PAN) gene is required for proper positioning of floral organs, with mutants showing defects in both number and pattern of floral organs. Mutations in PAN lead to flowers most commonly with 5 sepals, petals, and stamens, arranged in a regular pattern resembling pentameric flowers of distantly related dicotyledonous species. Slight defects in carpel development are also seen in pan mutants. We cloned the PAN gene, which encodes a protein resembling bZIP transcription factors, by virtue of T-DNA tagging. In order to better understand the role of PAN in organ initiation, we studied the RNA expression pattern of PAN in Arabidopsis.

Expression is detected in cells of L1, L2, and the upper L3 in the apical meristem, though expression is not uniform in this region. Expression is also present in the upper cell layers of floral meristems. Expression is detected throughout the regions where floral organs initiate. At stage 3, after sepals have initiated, expression is restricted to cells interior to the sepals. At stage 5, after all organs have initiated, expression in the floral meristem ceases. Expression in developing floral organs is not detected, except in the septum of the carpel, which shows slight developmental defects in pan mutants.

There is some evidence that expression in the floral meristem is restricted to cells with the potential to form floral organs. First, expression is present only in regions where organs will form, and absent after they have formed. Second, expression is not found in deeper cells, only in L1, L2, and L3 cells close to the surface, which are likely to be able to take part in organ initiation. Third, distinct, circular regions of expression are detected in transverse sections of stage 4 flowers; these patches of expression predict the future sites of petal initiation. These expression studies support a role of PAN in organ initiation, with expression in cells that have the potential to form floral organs, and expression becoming more restricted as development proceeds.

107. Isolation of PAN homologues
Chiou-Fen Chuang

In an attempt to isolate homologues of PAN, I used the PAN cDNA at moderate stringency to probe a λ phage genomic library of Arabidopsis DNA, yielding many positive clones. Among ten clones analyzed, nine clones represent two previously isolated genes, and only one clone, PANH5c1b, was a previously unidentified gene. The corresponding cDNA was cloned and the full length open reading frame determined by 5'RACE-PCR has the potential to encode a protein of 401 amino acids. The predicted proteins of PANH5c1b and PAN show 63% identity and 89% similarity over a 284-aa region. In particular, the basic regions are 100% identical, and the leucine zipper regions are 85% identical and 100% similar.

The expression pattern of the PANH5c1b transcript was analyzed by isolating poly(A) RNA from various tissues. Unlike the PAN transcript, which is detected only in young flowers, appreciable levels of the 1850-bp PANH5c1b transcript was found in different tissues including roots, leaves, stems, and young flowers. These tissues have been fixed for in situ hybridization. Since both genes are expressed in young flowers, interaction between these two proteins will be tested.

I am using multiple approaches to determine the panh5c1b mutant phenotype, thereby facilitating an analysis of its function. First, an antisense strategy can be used to yield a hypothesis about null and/or hypomorphic phenotypes for PANH5c1b. Second, pooled DNA of T-DNA transformation lines can be used to screen mutants with an insertion in PANH5c1b by PCR. Third, determination of the panh5c1b mutant phenotype should be facilitated by identifying mutations that map to a similar location. The PANH5c1b genomic sequences were mapped to the top arm of chromosome 1, close to four embryo-defective (emb) mutations. Two strategies could be used to determine whether the PANH5c1b gene represents the wild-type EMB allele. The first strategy is to generate transgenic plants carrying PANH5c1b and cross these plants with plants heterozygous for each of these emb mutations. The frequency of defective seeds produced following self-pollination should decrease when the correct EMB gene is introduced. The other strategy involves PCR amplification of sequences from mutant plants using primers based on PANH5c1b cDNA and genomic sequences, and directly sequencing the amplified products. Mutations should be found if PANH5c1b corresponds to the EMB genes analyzed.

108. SUPERMAN function in cell proliferation of floral meristems is distinct from the class B gene function.
Hajime Sakai

Based on previous data, we postulated two models for the possible function of SUPERMAN (SUP) in maintenance of boundaries between the stamen and carpel whorls. One model predicts that SUP prevents continuous spreading of expression of the class B homeotic genes, AP3 and PI. The other model predicts that SUP controls cell proliferation in floral meristems, restricting growth of the stamen whorl, and, at the same time, stimulating growth of the carpel whorl. The results obtained from SUP ectopic expression and cell division analyses supported the latter model for SUP function.

To examine these models, we further analyzed genetic interactions of SUP and the class B homeotic genes, AP3 and PI. Ontogenic studies of sup ap3 as well
as sup/pi revealed that the flowers of double mutants develop differently from those of the single mutants ap3 and pi, while the overall appearance is very similar to the single mutant flower, forming carpels in the center of the flower. In ap3 and pi, carpels and carpelloid structures are formed in whorls 3 and 4. In double mutants, however, carpels arise from one whorl and are composed of five to six primordia.

Furthermore, nectaries, which are associated with whorl 3, develop in double mutants extensively, whereas they are rarely seen to mature in single mutants. These results show that the sup mutation causes overproliferation of whorl 3 and reduction of whorl 4, even in the ap3 and pi mutant background, and that SUP is functional in these mutant backgrounds. SUP function is thus not dependent on AP3/PI function, excluding the first model which postulates that SUP function is to control the class B genes.

The genetic analysis further indicates requirement for SUP in a very short period of flower development. Being a target gene of AP3 and PI, SUP is strongly downregulated in ap3 and pi mutants, so that SUP RNA is detected only in the stage 4 floral meristem. This SUP expression reduced in quantity and duration is, however, sufficient for function, as is shown in the double mutant analysis. SUP thus seems to establish the balance of cell proliferation between whorl 3 and whorl 4 by acting very briefly at the stage prior to the development of stamen and carpel primordia.

109. SUPERMAN expression is regulated by at least three distinct pathways

Hajime Sakai, Beth Krizek

Previous studies showed that the SUPERMAN (SUP) gene is involved in the developmental process which determines proper size of stamen and carpel whorls. SUP is initially expressed in a part of the whorl 3 floral meristem. In order to investigate genetic circuits controlling the meristem proliferation, we analyzed SUP expression in various genetic backgrounds. A series of in situ hybridization experiments revealed that the homeotic genes specifying stamen identity, AP3, PI and AG, positively control SUP gene expression. AP3 and PI are necessary for SUP expression from the initial stage, whereas AG is required for the maintenance of SUP expression in later stages. These homeotic genes, however, are not sufficient to regulate SUP in the stamen whorl: constitutive expression of these homeotic genes in all four whorls could not induce SUP expression ectopically. This shows the activity of other gene(s) is required for induction of SUP in the stamen whorl.

The other gene which controls SUP is LFY. The LFY gene is involved in specification of the floral meristem from the very beginning of its development. Loss of function of this gene causes complete loss of SUP expression in the stamen whorl. The loss of SUP expression is, however, partly recovered by transgenically induced expression of AP3 and PI. This further shows that the regulation by LFY of SUP expression is mediated by at least two distinct pathways, one through the class B homeotic genes and another through (an) as yet unidentified gene(s). Since this transgenic background in the lfy mutant is still able to induce SUP expression in the proper region of the stamen whorl, the genetic pathway delimiting SUP expression appears not to be regulated by LFY. As no other previously characterized mutant alters the SUP expression pattern, this pathway displays a new genetic circuit, required for the proper floral meristem development.

Furthermore, studies on the DNA methyltransferase gene brought an unexpected finding of the sup gene regulation to light. In collaboration with Jean Finnegan and Liz Dennis at CSIRO in Australia, we analyzed the transgenic plants expressing antisense RNA of the DNA methyltransferase gene. These plants very often exhibit a phenotype very similar to sup, especially when the overall cytosine methylation decreases to below 30% of the wildtype level. In situ expression studies revealed that the SUP phenotype is accompanied with the decrease of SUP expression to an undetectable level. This result strongly indicates that DNA methylation is one of the factors regulating SUP gene expression.

110. Evolutionary study of the SUPERMAN gene

Hajime Sakai, Leonard J. Medrano

In Arabidopsis, the SUPERMAN (SUP) gene has two functions, one in flower development, to regulate the size of the stamen and carpel whorls, and another in ovule development, to regulate cell proliferation of the outer integument. We are further interested in evolutionary aspects of the SUP gene, asking questions such as how strongly SUP is conserved during angiosperm evolution, and whether SUP is involved in the diverse floral structures found in flowering plants. To start answering these questions, we isolated SUP homologs from a molecularly well studied dicot plant, snapdragon, or Antirrhinum majus. Two homologous genes were isolated through low-stringency screening of a lambda genomic library and degenerate inverse PCR of the genomic DNA. The expression study revealed that one gene, Am9, is exclusively expressed in developing pollen cells in anthers, while the other gene, Am1, has an expression pattern parallel to that of SUP in Arabidopsis. Furthermore, our collaboration study with Enrico Coen at John Innes Institute in England identified that the disruption of Am1 in Antirrhinum results in a phenotype very similar to the sup mutant phenotype. This strongly indicates that Am1 is the SUP ortholog. Through further investigation, we are trying to isolate SUP homologs from more distantly related plants such as maize, water lily and magnolia.

Another question we are interested in is whether there is another SUP-like gene in Arabidopsis, having its function in a different process of plant development.
The isolation of the homolog gene \((Am9)\) in Antirrhinum suggested the presence of such genes in Arabidopsis. Through low stringency screening of a lambda genomic library using \(Am9\) as a probe, we were able to obtain three homologous genes, and one more homologous gene was identified by sequencing the 42kb-long \(SUP\) genomic region. Expression study revealed that each of the homologs has a distinct expression pattern. For instance, the \(SPL1\) gene (\(SUP\)-Like gene 1) is exclusively expressed in developing embryo sac, and its expression ceases after fertilization. The \(SPL3\) gene has an expression pattern very similar to that of the class B floral homeotic genes, however, it is also expressed in outer integuments and very young vegetative leaf primordia. Further study of \(SPL\)s in Arabidopsis and also from other plant species may help to understand some of the evolutionary processes involved in plant development.

111. Polycomb relatives and other proteins interact with \textit{SUPERMAN}

Hajime Sakai, Leonard J. Medrano, José Ruiz Riechmann

\textit{SUPERMAN} (\textit{SUP}) encodes a protein with one C2H2-type zinc finger, basic and leucine-zipper-like domains. The combination of these domains is unique among the known zinc finger proteins. In order to investigate function of the \(SUP\) protein, we started analyzing other proteins potentially interacting with \(SUP\). For isolation of such proteins we applied the yeast two-hybrid system, using the full-length \(SUP\) protein fused to the GAL4 DNA binding domain as bait. Through screening over ten million flower cDNAs which are fused to the GAL4 activation domain, we obtained approximately 140 positive clones which could activate two reporter genes in the presence of the bait construct in yeast. These positive clones were further grouped into 23 different genes through hybridization, and the longest representative clone for each gene was sequenced to determine its encoded protein.

To identify which region of the \(SUP\) protein is interacting with each of these 23 different proteins, the bait construct was shortened to four different sizes and we analyzed the activation of reporter genes with these cDNAs. Interestingly, all except one appeared to interact with the 24 aa-long C-terminus, which contains a highly conserved region among the \(SUP\) homologs. 5 of 22 still activate the reporter genes even when the \(SUP\) portion of the bait constructs is replaced with \(SUP\) homologs, indicating that the interaction site is the conserved region, with 8 amino acid residues. 4 of these 5 are highly homologous with each other and contain one WD-40 motif. Although this motif is thought to function as a protein-protein interaction, it does not serve as the interaction site for \(SUP\). 7 of 17 which specifically interact with the \(SUP\) carboxyl-terminus exhibit interaction specific to a certain conformation of \(SUP\). The interaction of these proteins with the C-terminus is interrupted when the neighboring basic region is added in the bait construct. Since full-length \(SUP\) shows interaction with these proteins, the basic region appears to form a different steric structure in the shortened proteins, which perhaps inhibits the interaction with these proteins. One of the proteins whose interaction is influenced by the basic domain contains a so-called chromo domain, which in animals is found in the large group of Polycomb proteins. The Polycomb proteins are often associated with heterochromatin structures are thought to be involved in long-term gene regulation of homeotic genes. We are interested in investigating whether this Polycomb-like protein, discovered in plants for the first time, has a similar function, and whether the role of \(SUP\) in cell proliferation of floral meristem is mediated through gene regulation by the Polycomb-like protein.

112. \textit{SUPERMAN} epi-alleles reveal a novel methylation system in plants

Steven E. Jacobsen, Elliot M. Meyerowitz

Seven unstable alleles of the \textit{Arabidopsis} \textit{thaliana} flower development gene \textit{SUPERMAN} (\textit{SUP}) have been isolated. These epi-alleles (the \textit{clark kent} alleles) are associated with nearly identical patterns of dense cytosine methylation of the \(SUP\) gene, and are accompanied by a decreased \(SUP\) RNA expression level. Phenotypic reversion correlates with a restoration of \(SUP\) RNA levels and loss of hypermethylation. The \(SUP\) hypermethylation phenomenon is unique in that a reproducible pattern of dense non-symmetric methylation is faithfully inherited through mitosis and meiosis, and involves only single copy \(SUP\) genomic sequences. Surprisingly, a recently reported antisense methyltransferase transgenic \textit{Arabidopsis} line, which shows an overall decrease in cytosine methylation and which also has a \(SUP\)-like phenotype, fails to complement \(SUP\) and exhibits a nearly identical methylation defect at the \(SUP\) locus. This shows that at least some of the developmental abnormalities associated with the antisense methyltransferase lines are due to hypermethylation.

113. KAPP may function in the \textit{CLV1} signaling pathway

Robert Williams, Jeanne Wilson

The \textit{Arabidopsis} \textit{CLAVATA1} (\textit{CLV1}) gene encodes a potential receptor involved in apical and floral meristem structure. The protein contains a putative extracellular domain that consists of tandem leucine-rich repeats and a cytoplasmic kinase domain. Mutations in the \textit{CLV1} gene result in plants that have enlarged apical and floral meristems, leading to stem fascination and extra floral organs. The \textit{CLV1} mRNA expression pattern is consistent with a model where \textit{CLV1} plays a role in regulating proliferation of the central undifferentiated cells of the meristems.

The \textit{CLV1} kinase domain, when expressed in \textit{E.coli} as a GST-fusion protein, autophosphorylates on serine residues. Wild-type \textit{CLV1} protein can also...
phosphorylate a catalytically inactive mutant form of the CLV1 protein, indicating that, in vitro, auto-
phosphorylation takes place by an intermolecular
mechanism.

Kinase Associated Protein Phosphatase (KAPP)
was identified by its ability to interact with RLK5, a
protein very similar in sequence and organization to
CLV1 (Stone et al. 1994). Similarly to RLK5, we have
shown that KAPP can bind to the CLV1 kinase domain
and this interaction is greatly enhanced by the
phosphorylation state of CLV1. In vitro, the KAPP
protein can also dephosphorylate CLV1 in a Mg²⁺/Mn²⁺
dependent manner.

In situ hybridization shows that KAPP mRNA
is expressed throughout the apical and young floral
meristems, and later in floral development, in pollen
and ovules. CLV1 is expressed in a subset of KAPP-
expressing cells. When KAPP is placed under the
control of the strong constitutive 35S promoter, the
resulting transgenic plants have a phenotype similar to
weak clv1 mutants. These data, taken together, suggest
a role for KAPP as a negative regulator of the CLV1
signaling pathway.

Reference

114. Functions of the LRR-kinase gene family in
Arabidopsis
Bobby Williams
We are interested to see what other aspects of
plant development CLV1-like genes may affect. We
have isolated several genes that hybridize to CLV1
under low stringency, two of the genes, CLL2 and CLL4
(for CLavatal-1 like), have been partially sequenced.
They both contain putative serine/threonine kinase
domains and extracellular leucine rich repeat (LRR)
domains. Many other LRR and/or kinase genes similar
to CLV1 are in the EST or Genbank databases.

The kinase domain from CLL2 is 49% identical and
75% similar to CLV1 at the amino acid level.
Although, both CLV1 and CLL2 have introns in the
same position, the intron is much larger in CLL2. The
putative extracellular domain of CLL2 has not been
completely sequenced but it does contain at least two
LRRs of the same length and consensus as those in
CLV1. In situ hybridization shows that the CL2 gene
is expressed in developing anthers, in particular in the
tapetum and in the locules where pollen is being
formed. A genomic clone containing the CLL2 gene was
placed on the existing RFLP map and lies on the top
arm of chromosome 1. No known pollen defective
mutants map near CLL2.

The kinase domain from CLL4 is 70% identical and
85% similar to CLV1 at the amino acid level. The
intron in CLL4 is approximately the same size as in
CLV1. Thus, CLL4 is much closer to CLV1 than is CLL2.
CLL4 contains at least 13 extracellular LRRs very
similar in length and structure to those of CLV1. CLL4
has not yet been mapped. In situ hybridization shows
that CLL4 mRNA is expressed on the flanks of the
apical and floral meristems, in a pattern
complementary to CLV1.

115. Toward Cloning CLAVATA3
Jennifer C. Fletcher, Mark P. Running
The regulation of cell proliferation is crucial for
obtaining proper organ number and position in
developing flowers, yet the molecular mechanisms
responsible for generating these stereotypical patterns
are poorly understood. The Arabidopsis thaliana
CLAVATA1 (CLV1), CLV2 and CLV3 genes have been
shown to play important roles in the control of
meristem size and floral organ number. Mutations in
any of these three genes cause similar phenotypes:
apical meristem overgrowth, the formation of extra
organs in all whorls and the development of additional
whorls of carpels. Confocal microscope studies have
revealed that clv1 and clv3 mutant plants have
enlarged apical and floral but not root meristems,
indicating that CLV1 and CLV3 are specific regulators
of cell proliferation in shoot and flower meristems.
Genetic interactions between clv1 and clv3 mutants
suggest that CLV1 and CLV3 function in the same
pathway to control meristem development, perhaps by
promoting meristic stem cell differentiation and
incorporation into organ primordia.

The CLV1 gene encodes a transmembrane receptor
serine/threonine kinase, suggesting that the CLV1
product may play a role in meristem signal transduction
pathways. To better understand the developmental
role of the CLV3 product, we are cloning the CLV3 gene.
We have isolated a T-DNA-induced allele, clv3-3,
in which the mutant phenotype cosegregates with a
marker linked to clv3 and with a single drug resistance
 locus contained within the T-DNA sequence. Having
been unable to recover flanking plant genomic DNA
using inverse PCR or plasmid rescue, we have instead
screened a lambda library made from clv3-3 mutant
plants with probes specific to the T-DNA borders.
We have identified numerous positive clones, and
are currently screening them to determine which give
a polymorphism between wild-type and clv3-3 mutant
DNA. The T-DNA-flanking genomic sequences from
the polymorphic clones will then be used to isolate
cDNAs from a flower-specific library, and in rescue
experiments to determine whether these sequences
correspond to the CLV3 gene.

116. The effects of WIGGUM on plant development
Mark P. Running, Jennifer C. Fletcher
We have been studying the effects of WIGGUM
(WIG) on the development of Arabidopsis. Mutations
in WIG were identified based on its effect on floral
organ number. All three independent mutant lines
analyzed so far produce extra floral organs, especially
sepalas and petals, though all four whorls of the flower
are affected to some extent. Like CLAVATA1 (CLV1)
and CLAVATA3 (CLV3) mutants, WIG regulates floral
meristem size, with larger floral meristems at the
early stages of organ initiation leading to the visible
phenotype. While clavata1 and clavata3 mutants have taller floral meristems, leading to more inner whorl organs (stamens and carpels), wig mutants have wider floral meristems, leading to more inner whorl organs.

All three wig mutants affect other aspects of plant development as well. Germination time is lengthened and germination rate is reduced in all 3 alleles. wig mutants produce their first flowers about one week later than wild type, with one additional leaf on average, and the wig total lifespan is considerably lengthened. Both the aerial portion of the plant and the roots are shorter in length. In rare instances fasciation of the main stem is observed, showing that wig affects apical meristem structure as well as floral meristem structure.

Flowers in wig mutants show a number of defects in addition to aberrant organ numbers. SEM studies show that sepals arise in irregular positions, often not equidistant to each other. Sepals largely lose positional identity; that is, unlike in wild type, sepals arising closest to the medial positions are often not larger than those arising close to lateral positions. Petal and stamen position and number show no dependence on sepal position or number. In these ways wig most resembles cle1 and cle3 mutants, suggesting that a loss of control in cell division in the meristem leads to defects in floral organ patterning and growth. These studies also support the idea that one mechanism of control of floral organ number is by regulation of floral meristem size.

117. Genetic studies of WIGGUM mutants

Mark P. Running, Jennifer C. Fletcher

Studies of wiggum (wig) mutants have shown that WIG is necessary for the control of cell proliferation in apical and floral meristems. In order to better understand the role of WIG in the context of previously characterized genes, we constructed double mutants of wig and other mutations showing defects in flower and inflorescence development.

The most striking double mutant phenotypes are seen in combinations of wig and certain mutations affecting floral meristem identity. For instance, wig leafy double mutants fail to form flowers or flower parts, except for occasional abnormal carpels. In the place of flowers, many thin filaments are formed, and the meristem terminates very early. Similar but less dramatic effects are seen with wig and filamentous flower. In contrast, wig doubles with mutations specifically affecting flower development, such as mutations affecting floral organ number (superman, perianthia) and floral organ identity (agamous, apetala3) are additive. Taken together, these double mutant studies support the idea of the importance of WIG in the maintenance and control of cell division in the apical and floral meristem, acting in a separate pathway from previously characterized genes.

118. Toward the cloning of WIGGUM, a gene controlling meristem proliferation

Eva C. Ziegelhoffer, Mark P. Running

The WIGGUM (WIG) gene of Arabidopsis controls meristemetic cell division in both the shoot apical meristem and floral meristems. Mutants of wig result in extra floral organs, particularly in the outer whorls, consistent with an increase in cell number across the width of young floral meristems. wig mutants also have an increase in cell number across the width of the apical meristem. In order to understand WIG's role in the control of meristem development, molecular cloning of the WIG gene using map-based cloning techniques has been initiated. Preliminary mapping data using Simple Sequence Length Polymorphism (SSLP) markers in addition to observed linkage to fns2 places wig in the middle of chromosome 5. To obtain more precise mapping information, wig-1, isolated in the Landsberg erecta (L-er) ecotype has been crossed with several nearby visible markers in a 10 cm span to generate double mutants. The doubly mutant L-er lines will then be crossed with a wild-type line in the Columbia (Col) ecotype and screened for mutants which retain only one of the two visible phenotypes, indicating that a recombination event occurred between wig and the other mutant locus. Due to the scarcity of ecotype-specific Restriction Fragment Length Polymorphism (RFLP) markers in the middle of chromosome 5, markers traditionally used for fine scale mapping of genes, a high density set of molecular markers will be generated using the PCR-based Amplified Fragment Length Polymorphism (AFLP) technique. Ecotype-specific AFLP analysis of recombinant pools representing recombination above or below the wig locus will provide a high resolution map of the region surrounding wig. The nearest AFLP markers can then be cloned directly and used as probes to identify
YAC and cosmid clones containing this region. Cosmid clone complementation of wig-1 and subsequent DNA sequencing of the wig region will follow.

119. Searching for genes controlling cell proliferation in meristems

Eva C. Ziegelhoffer

Control of meristematic cell division is vital to proper plant growth and reproduction. Several Arabidopsis genes involved in the control of shoot apical meristem and floral meristem structure and function have been identified in recent years. One class of these genes, comprised by the CLAVATA genes (CLV1, CLV2, and CLV3) and WIGGUM (WIG), limit the number of cell divisions in apical and floral meristems. The control by CLVs and WIG in the floral meristem helps to produce the typical four-whorled Arabidopsis flower composed of four sepals, four petals, six stamens, and two carpels from outer to inner whorls, respectively. Specifically, CLVs seem to be primarily responsible for maintaining the correct number of inner whorl organs (stamens and carpels) while WIG seems to affect the outer whorls (sepals and petals) more than the inner ones. Both the CLV genes and WIG result in increased cell number in young floral meristems but they do so in different patterns; clv mutants result in taller meristems whereas wig mutants have wider meristems, consistent with cell proliferation in either the center or periphery of the flower, respectively. clv wig double mutants result in an increasingly synergistic effect in which the apical meristem overproliferates at the expense of flower development. In order to identify other genes involved in apical and floral meristem control, a search for suppressors and enhancers of wig has been initiated. Two mutagenesis schemes are being employed: chemical mutagenesis using ethyl methanesulphonate (EMS) and Agrobacterium-mediated transferred DNA (T-DNA) insertion. Screening only a small fraction of the total number of M2s from the EMS mutagenesis has already yielded both putative suppressors and enhancers of wig. One enhancer, in particular, is quite dramatic in that the apical meristem is so severely disrupted that initially only a mass of filaments appears across the entire surface of the enlarged apical meristem, and ultimately a large, unruly mass of carpel tissue and filaments persists beyond the normal wig life span. This mutant and other putative mutants suggest that this approach will be useful in identifying new genes involved in meristem control.

120. Cloning the fifth member of the putative ethylene receptor gene family

Jian Hua, Elliot M. Meyerowitz

A family of homologous genes (ETR1, ETR2, EIN4, and ERS) have been shown to be involved in ethylene perception in Arabidopsis. We have now cloned the ERS2 gene, the fifth member of this family, by its cross-hybridization with the ETR2 gene. As it has sequence homology to the other four members, we tested whether it is also involved in ethylene sensing by a reverse genetics approach. Two mutations were introduced into the ERS2 genomic DNA fragment. One results in an Ile to Phe change similar to that in etr1-D. The other results in a Pro to Leu substitution similar to the etr2-1 mutation. The two mutated ERS2 genes and the wild-type ERS2 gene were introduced to wild-type Arabidopsis by Agrobacterium-mediated transformation. Both of the mutated ERS2 transgenes conferred ethylene insensitive phenotypes to wild-type Arabidopsis, while the wild-type ERS2 transgene did not. This indicates a role of ERS2 in ethylene perception. Double mutants were made between transgenic ers2 ethylene-resistant lines and the clv1-1 mutant, which has constitutive ethylene responses. These double mutants largely resembled the clv1 single mutant, indicating that ERS2, like the other four genes, acts upstream of CTR1.

Several features are noticed from the sequence comparisons of the five genes. Firstly, they seem to belong to two subfamilies. One consists of ETR1 and ERS, and the other consists of ETR2, EIN4, and ERS2. Members in the same subfamily have higher amino acid sequence similarity to each other than to members in the other subfamily. Moreover, genes in the same subfamily have conserved intron positions which are not shared between the two subfamilies. Secondly, the receiver domain is only present in ETR1, ETR2, and EIN4, but not in ERS and ERS2. Thirdly, although they all show sequence homology to the bacterial histidine kinases, the autophosphorylation site histidine residue is absent from ETR2 and ERS2 proteins. It will be interesting to see whether these features have any functional relevance.

121. Isolation of loss-of-function mutants of the putative ethylene receptor genes

Jian Hua, Elliot M. Meyerowitz

Five related genes (ETR1, ETR2, EIN4, ERS, and ERS2) in Arabidopsis encode putative ethylene receptors, as their dominant mutants have ethylene-resistant phenotypes. No recessive mutations in these genes were isolated from genetic screens for ethylene response mutants. As the mechanism for the dominance of these mutations is not known, it is difficult to infer the wild-type functions of these genes. We therefore attempted to isolate loss-of-function alleles.

We hypothesized that the loss-of-function alleles might come out of a mutagenesis as intragenic suppressors of the dominant alleles, if they are not lethal. Indeed, three etr1 loss-of-function alleles were obtained from suppressor screens of dominant etr1-1 and etr1-2 mutants, as reported last year. Using the same approach, we EMS mutagenized about 15,000 plants each of etr2-1 and ein4-1 mutants. From about 150,000 M2 plants screened for each mutant, we isolated three intragenic suppressors of etr2-1 and eight intragenic suppressors of ein4-1. Sequencing the corresponding genes in these lines revealed that the suppression
resulted either from nonsense mutations, or from destruction of the consensus sequences of intron splicing. At least one etr2 allele and five ein4 alleles are likely to be molecularly null.

As dominant ers and ers2 mutants were only available as transgenic lines, it is not feasible to isolate loss-of-function ers or ers2 alleles by the above approach. To obtain such alleles, we screened DNA pools representing 7,000 T-DNA inserted lines for T-DNA insertions in the ERS or ERS2 genes. One line gave positive PCR product when an ERS2-specific primer and a T-DNA specific primer were used. This line has a T-DNA insertion in the putative histidine kinase domain of the ERS2 gene, and it is likely to be a loss-of-function ers2 mutant.

In summary, we have obtained loss-of-function mutants of four genes out of five in this receptor family. We are now characterizing their phenotypes while cleaning up the possible background mutations.

122. Toward understanding the functions of a family of putative ethylene receptor genes
Jian Hua, Elliot M. Meyerowitz
Loss-of-function mutants of four putative ethylene receptor genes were isolated (see the last abstract). No obvious growth or developmental defects were observed in these mutants. Neither did they have dramatic ethylene response defects, which explains why no such alleles were isolated in the genetic screens for ethylene insensitive or constitutive ethylene response mutants.

Detailed analysis revealed a unique function of the ETR1 gene. Ethylene dose responses were tested on etiolated etr1, etr2, and ein4 loss-of-function alleles. Wild-type seedlings had increasingly reduced hypocotyl and root length as the ethylene concentration increased. etr2 and ein4 mutants behaved similarly to the wild type in this response. etr1 mutants, however, had shorter hypocotyls and roots than the wild type at the same ethylene concentration. This defect was due to smaller cells rather than fewer cells in the seedlings, which indicates a unique role of ETR1 in promoting cell elongation. We are further analyzing this defect to see whether it is ethylene-concentration dependent.

To reveal the functions that might be masked by redundancy, we are constructing double loss-of-function mutants. etr1;ein4 but not etr1;etr2 double mutants have smaller leaves and shorter stature than either the wild type or single mutants. One interpretation is that etr1;ein4 double mutants grown in air behave like wild-type plants grown in the presence of a low concentration of ethylene. This suggests that there is functional redundancy between ETR1 and EIN4 genes and that they might normally act as negative regulators of ethylene response. Analyses of other double, triple, and quadruple mutants are likely to lead to better understanding of the functions of this gene family.

123. An ABC model-based hypothesis for the origin of the floral homeotic phenotype of Lacandonia schismatica
Francisco Vergara Silva, Elena Alvarez-Buylla
Lacandonia schismatica (Lacandoniaceae: Triuridales) is a myco-heterotrophic endemic monocot from Mexico whose reproductive structures constitute the only known example of a spatial inversion of the almost universal location of the inner whorls in a bisexual flower; that is, it has a group of carpels surrounding three central stamens, instead of the opposite order that is observed in Arabidopsis, for example, whose perfect, disymmetric flower has a central gynoecium and a peripheral whorl of male organs. This morphological character makes L. schismatica a very interesting instance of an escape from a developmental-genetic constraint that was probably established millions of years ago, during the early evolutionary history of the land plants.

Our general explanatory hypothesis for the homeotic phenotype observed in the flowers of L. schismatica is based on the ABC model of floral organ identity determination, and postulates that the molecular basis of the described reproductive anatomy is concerned with a regulatory change of the spatial expression domains of the genes orthologous to AP3 and PI, the well-characterized MADS-box genes that represent the B function in the model. We are starting to test this hypothesis by screening genomic libraries for homologues of the B function genes, in an attempt to compare them later with other members of their corresponding clade in the multigenic family. After the cloning of the putative orthologues is completed, we pretend to study their mRNA expression patterns with in situ hybridizations in young floral buds. In the long term, we will try to frame the data obtained in a taxonomical-systematic framework, by assessing the phylogenetic relationships between L. schismatica and other closely related Triuridales using rDNA as a source of characters, a task that is almost finished. If possible, we will extend the characterization of MADS-box gene expression patterns to those related species, since that would be the best way to substantiate this example as a general lesson about the role of regulatory changes in the evolution of complex structures in multicellular organisms.

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Publications


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**Summary:** Our laboratory is studying the molecular and cellular events involved in the early stages of T-lymphocyte differentiation in the thymus. Increasingly, we have become interested in the developmental choices these cells make as they progressively become committed to the T-lineage pathway. The process these cells go through is the outcome of a complex interplay between environmental signals impinging on the cells and the changing sets of transcription factors that are present within the cells at successive stages. We ultimately wish to explain the process of lineage commitment in terms of the expression of specific genes and their regulation by specific transcription factors.

To study these events we have first established in our laboratory several biological systems that give us access to cells in the earliest stages of T-lineage commitment. Three different strains of immunodeficient mice, RAG2–/– mice and scid/scid mutants on two genetic backgrounds, have thymus populations with an initial 100-fold enrichment of early precursors. In abstract (1), we describe the fine-scale dissection of the cell types present in these enriched populations. Although aspects of the cellular characterization are still continuing, we have already established the main properties of three main subsets: one clearly progressing toward T-lineage differentiation, one stem cell-like population that is probably not yet committed to the T lineage, and one population that appears to be undergoing differentiation along a related, but divergent, developmental pathway, toward natural killer cells. To get a more complete look at the transitions from hematopoietic stem cell to effector T cells of different subsets, we supplement these cell populations with later-stage thymic T-cell precursors and mature T cells; with selected cell lines that represent various stages in lymphoid and myeloid hematopoietic differentiation; and most recently, with fetal thymus organ cultures, in which precursors can differentiate in vitro under controlled conditions. Characterization of the lineage-specific gene expression in these cells by RT-PCR has given us landmarks to stage the distinct events that occur as the cells progress towards maturity.

The first question is exactly how the expression of particular T-lineage genes (T-cell receptor [TCR], CD3 signaling components of the TCR, functional response genes associated with T cells) is related to developmental specification and ultimate commitment. From cell-transfer studies by other groups, we know approximately at what stage the cells lose the ability to give rise to erythrocytes and neutrophils; at what stage they lose the ability to become B cells; and finally, at what stage they lose the ability to become natural killer cells or thymic dendritic cells. It is interesting that several T-lineage genes are turned on before the last two stages have been passed, i.e. while the cells are still not completely committed (Abstract 125). Functional response genes, which mature cells turn on in response to signals transduced by the TCR, are precociously turned on in development as well (Abstract 126). Conversely, we have obtained striking evidence that at least one B-cell gene is turned on in early T-cell development, and then "negligently" left on until the T-cell differentiation program is virtually complete (Abstract 125). These results are strong evidence against the notion that expression of these lineage-specific genes depends on the action of some lineage-determining "master" transcription factor that is activated only as the cells undergo commitment.

The second major question is what the regulators are that act in these early stages. The promoter and enhancer regions of many T-cell genes have been studied enough to identify likely motifs for
transcription factor binding, and to correlate at least some of these with functional roles in gene expression. However, mammalian transcription factors active in hematopoiesis are almost invariably members of multifactor transcription factor families, all of which share DNA sequence specificity but which have different in vivo roles. To add to the complexity, in several documented cases the transcripts of the gene encoding a single factor can initiate at different promoters and/or undergo differential splicing, leading to products which can have diverse or even opposing effects on gene expression. We have undertaken three different approaches to identify factors which may actually be responsible for the changes in gene expression we observe at key transitions. First, we are constructing arrayed cDNA libraries from scid/scid thymocyte RNA and screening them with transcription factor family-specific probes to identify the mRNAs that are actually present (Abstract 127). This "top-down" approach is providing us with a large number of interesting candidates that can be tested for activity on known T-lineage promoters that are activated at the developmental stages when these transcription factor RNAs are expressed. Second, focusing on a single family (CBFα, also called AML or PEBP2α) that is essential for all TCR enhancer activation, we have undertaken a detailed exon-by-exon characterization of the forms of these factors that are available in different early lymphoid cells, which may distinguish them from their myeloid cousins (Abstract 128). Third, we are using a variety of biochemical approaches to identify the factors that may be responsible for the developmental onset of expression of the IL-2 gene, an important T-cell-specific response gene that is also turned on at the earliest stages of T-lineage specification (Abstracts 129 & 130). For the IL-2 gene, we have previously shown a programmed downregulation of inducibility during a later stage when pre-T cells undergo one last fate determination process, and we identified the bZIP factor family AP-1 as a key component of this downregulation. In current work (Abstract 131), we are dissecting the regulation of distinct AP-1 family members to explain which one(s) control IL-2 inducibility during these stages.

Two other projects are extending our investigation of lineage choices to subsets within the T-cell lineage. CD4+ and CD8+ cells have very different roles in immune responses, reflecting their preferential uses of different response genes and their different growth regulation properties. Conservatively starting with the IL-2 response gene, which is differentially expressed between these two subsets, we have already identified several enhancer-binding factors that may distinguish IL-2 producing CD4+ cells from non-IL-2 producing CD8+ cells (Abstract 132). We also plan to embark on a broader project to search for genes that may directly affect the choice of a cell to differentiate or survive as a CD4+ cell or a CD8+ cell (Abstract 133).

124. Different arrest points for RAG2-/− and SCID thymocytes: a foundation for studying developmental choices and mechanisms before TCR gene rearrangement

Rochelle Diamond, Susan B. Ward, Kyoko Owada-Makabe1, Hua Wang, Ellen Rothenberg

For several years we have been characterizing the different developmental arrest points in RAG2-/− and two strains of SCID mouse thymocytes. When normal mice successfully rearrange their first T cell receptor genes, they pass a crucial checkpoint at which they are allowed to proliferate and continue to differentiate along their maturation pathway. Mutant SCID and RAG2-/− mice, however, are prevented from rearranging their T cell receptor genes and therefore arrest at or before this crucial checkpoint. The SCID mutant thymocytes arrest at diverse stages prior to this checkpoint because the mutation prevents DNA from being repaired once any strand breaks are made. The RAG2 gene knockout thymocytes are lacking the recombinase activating gene 2 and therefore arrest fairly uniformly at the stage where recombination is necessary.

We have utilized multiparameter flow cytometry and cell sorting to dissect out various stages of maturation present in 4-6 week old mutant thymuses in order to characterize phenotypes and markers for each subpopulation. The RAG2-/− thymocytes accumulate as quiescent cells by cell cycle analysis, with the predominant phenotype of heat-stable antigen (HSA)+ CD25+ CD44+ ckitlow, resembling normal cells just prior to selection for T-cell receptor β-chain expression. The SCID thymocytes accumulate at diverse stages up to this stage as mentioned above and maintain more active cycling cells than the RAG2-/− cells. We have previously reported that two surface markers in the HSA1 subset, an adhesion molecule, CD44, and a growth factor receptor, CD17, downregulate in a highly correlated manner along this pathway until they reach their minima at this crucial checkpoint. The genetic backgrounds are unrelated to this down regulation which serves as a developmental time clock for stages up to the checkpoint regardless of T-cell receptor gene rearrangement. Other markers like Sca-1 are also downregulated but only in some strain backgrounds. Experimental crosses of the two SCID backgrounds to get F1 thymocytes demonstrated that Sca-1 is controlled by a recessive trait in one but not the other background.

The SCID thymocytes die as they fail to repair their DNA. This death appears to be correlated with the up regulation of the "suicide" receptor Fas on the cell surface. The C57BL/6-SCID thymocytes appear to die at earlier stages than the C.B-17 SCID thymocytes. Natural Killer(NK)-like cells are also found in these thymuses. RNA analyses by reverse transcription-dependent polymerase chain reaction (RT-PCR) to measure IL-12 and perforin expression were
undertaken to look at IL-12 induced NK maturation and the NK-cell product perforin. The RT-PCR analysis did show differences in SCID vs. RAG2-/- thymuses, however, the relative amounts of NK-like cells and products were the same between all the immunodeficient thymuses when cell number and dilution factors were taken into consideration.

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125. Ig-β(B29) expression in thymocytes: cross-lineage gene regulation in lymphocyte development

Hua Wang, Rochelle A. Diamond

In the mammalian hematopoietic system, cells differentiating from the same precursor can adopt at least eight distinct developmental fates. The development of B and T lymphocytes from their respective progenitors is an ordered and coordinated process. Choice of a developmental lineage involves the activation of lineage-appropriate genes and the repression of inappropriate genes.

B-lineage progenitor cells express a set of four genes: mb-1, B29, A5 and Vpre-B. The mb-1 and B29 genes encode the Ig-α and Ig-β components respectively. They are required for cell-surface transport and signal transduction by membrane-bound Ig. The A5 and Vpre-B genes encode surrogate light chains. T-lineage progenitor cells express genes encoding auxiliary components of the TCR, which include subunits of the CD3 complex that are required for cell-surface assembly and signaling by the TCR. Both B and T progenitor cells express genes encoding the recombination apparatus, Rag-1, Rag-2 and TdT which are required for antigen receptor gene rearrangement and diversification.

To understand how the changes in developmental potential are related to changes in actual patterns of gene expression in the differentiating cells, molecular indices of lineage commitment were studied in immature thymocytes by analyzing mRNA expression as a function of developmental stage.

We sorted immunodeficient thymocytes into four fractions according to the cell surface marker Sca-1 and HSA expression and compared them with fractions from normal E 14.5 fetal liver which were sorted based on their B220, Sca-1, and Gr-1 expression. RT-PCR assays on these cells suggest that TCRβ germline transcription is activated first in T-lineage precursors, followed by CD3ε expression, then by the expression of pTα and TdT, then finally by Rag-1. The expression of pTα and TdT seems to be blocked or down regulated in the NK-like HSAlowSca-1- population, and Rag-1 expression is induced exclusively in those cells that have attained the HSA+ stage. Only at the stage when Rag-1 is turned on are the cells thought to be committed to the T-cell lineage. In contrast to the low level or absence of A5 and mb-1 expression in thymocytes, Ig-β was unexpectedly found in not only the HSAlowSca-1- thymocytes but also the more advanced HSA+ thymocytes that have completely lost B cell developmental potential. Only the HSAlowSca-1- thymocytes expressed this gene at lower levels.

To verify that the Ig-β primers were really amplifying the specific product that has previously been reported to be B cell specific, normal adult tissues were tested for expression. Mature T cells and nonlymphoid cells do not express Ig-β RNA.

To define the stage of thymocyte development at which Ig-β expression might be turned off, normal C57BL/6 thymocytes were fractionated to separate cells all through different stages of positive selection. Ig-β was found throughout the CD4+CD8+ stage and was only downregulated during positive selection. Thus this aspect of the B cell developmental program is not repressed until after extensive T-lineage differentiation.

126. An activation event marked by interleukin-2 induction may precede lineage commitment in early T cell development

Hua Wang, Rochelle A. Diamond

As described in last year’s Annual Report, we have found IL-2 gene expression is independent of TCR expression in thymocytes. IL-2 transcripts were detected not only in the most immature fraction (HSAlowSca-1+) but also in the NK-like HSAlowSca-1low fraction.

To establish the developmental status of the cells expressing IL-2, additional genes implicated in T cell development were monitored. RAG-1 and TdT which are involved in the rearrangement of TCR genes were both expressed abundantly in the HSA+ cells but very few in the HSAlowSca-1low fraction. Although the HSAlowSca-1+ fraction has no detectable RAG-1 expression, it has a significant amount of TdT expression. CD3ε follows a pattern similar to TdT except that it was also expressed at a low level in the HSAlowSca-1low fraction. These analyses support the interpretation that the HSAlowSca-1+ cells include T cell precursors but have not yet begun TcR gene rearrangement. By contrast, the HSAlowSca-1low cells may be either exceptionally early T cell precursors or non-T lineage altogether. These results also indicate that the cells which express IL-2 in vivo are not found in the subsets most definitively associated with progression to TcR gene rearrangement, but rather in those cells which have not--or have not yet--turned on RAG-1 expression.

Transcripts of the B lineage genes mb-1 and A5 are essentially undetectable in the IL-2 expressing HSAlow fractions. By contrast, perforin expression was readily detectable in the HSAlowSca-1low fraction. Thus although the cells expressing IL-2 in the thymus could not be shown to possess molecular features of pre-B cells, they could be shown to be present in a population expressing a prominent NK-associated response gene.

To test whether a specific activation event in the thymus was involved in eliciting IL-2 expression, we sought evidence for such an activation event using the
activation marker CD69. Our data shows that a significant proportion of these thymocytes do indeed express CD69, and that they reside primarily in the HSA<sup>+</sup> populations. Higher levels of IL-2 expression were found in the CD69<sup>-</sup> populations.

All together, our results show that an important population of cells expressing IL-2 in vivo stands at the earliest cusp of T lineage specification, possibly including cells with dual T/NK precursor roles and their earliest NK-like progeny.

127. A global search for transcription factors active in early stages of T-cell development

Michele Anderson

While many aspects of T cell differentiation and activation have been described in detail at the molecular level, early molecular events leading to the divergence of the T cell lineage from their hematopoietic precursors have not been satisfactorily defined. Many of the transcription factors which appear to be involved in regulation of T cell-specific gene expression are present in many different cell types, suggesting that a specific combination of factors rather than any one master control gene is responsible for the acquisition of T cell identity. In order to identify the population of transcription factors which are active during T cell specification, we are using the immunodeficient SCID mouse, in which thymocyte development is halted at an early stage due to an inability to rearrange the T cell receptor genes. Degenerate PCR primer sets complementing a wide variety of transcription factor families, including the Ets, POU domain, nuclear receptor, BTB, PAX, HMG box, HOX, GATA, and basic helix-loop-helix families, have been used to amplify portions representing multiple family members from SCID mouse first strand cDNA. These PCR products, as well as an oligonucleotide complementing C<sub>2</sub>H<sub>2</sub> zinc finger containing genes, are being used as probes for low stringency screening of arrayed SCID thymocyte cDNA. The hybridizing clones will be rearrayed and subjected to differential expression analysis. Those clones which appear to be preferentially expressed in SCID thymocytes will be selected for transfection experiments using a fetal thymic organ culture system to assess their effects on hematopoietic lineage determination and T cell development. Minimal analysis of a small percentage of the cDNA clones thus far identified have yielded both genes previously known to be important in early stages of T cell development, such as TCF-1 and SOX-4, and several new genes which are currently under investigation.

128. CBFα transcription factors in lymphoid development

Janice C. Telfer

T cells, critical components of the immune system, develop from multi-potential precursor cells in the bone marrow which migrate to the thymus and acquire the capabilities of adult cells. The regulation of the development of these vital cells is little understood, however. At which point, and by what mechanism, the precursor-derived cells irrevocably commit to becoming T cells is not known. We are interested in studying the regulation of transcription factors which in turn regulate the expression of those molecules which indicate T cell commitment, in order to trace back the T cell lineage.

The transcription factors CBFα1 and CBFα2 are expressed in T cells and are thought to be important for the expression of many of the cell-surface molecules by which T cells are identified: CD2, CD8, CD3ε, CD3δ and the T cell receptor chains α, β, γ and δ. The mechanism of regulation of their expression is not known. We have cloned genomic DNA encoding CBFα1 and CBFα2 in order to characterize the promoter sequences necessary and sufficient for appropriate expression. In analyzing the pattern of mRNA transcript expression of CBFα1 and CBFα2, we have found that while both CBFα1 and CBFα2 are expressed in T cells, CBFα2 appears to be more restricted to cells of the hematopoietic lineage than CBFα1. The picture is made more intriguing by the prevalence of multiple splicing isoforms of CBFα2, which we are characterizing by cloning and sequencing cDNA clones and by exon-mapping via reverse-transcriptase PCR. The latter technique is sensitive enough to examine the earliest thymocytes and fetal hematopoietic cells. We are presently looking at the occurrence of an alternatively spliced 5' region in CBFα2, which may use a T-cell specific promoter. We are also developing an antisense or dominant negative viral infection system to differentiate the in vivo importance of the various splicing isoforms in thymocytes and T cells.

129. Functional analysis and determination of the transcription factors that bind to the upstream DNase I hypersensitive site in the IL-2 promoter

Susan Ward

Using in vivo genomic footprinting on DNase I treated EL4 T cells, we discovered a tissue specific, stimulation independent DNase I HS site extending from -450 to +350 in the IL-2 promoter. Since this site is present in T cells before they express IL-2, it may be responsible for establishing tissue specific IL-2 expression. Extensive in vivo footprinting of this region revealed that a single nucleotide at position -410 is susceptible to DMS footprinting, which directly maps protein/DNA interactions. To determine the function of this footprinted region, we used site directed mutagenesis to mutate the -410 site in the context of the entire IL-2 regulatory region. Using transient transfection assays and a luciferase reporter gene, we will compare the activity of this construct to that of the wild type -410 site. One potential caveat of the transient system is that it will not be useful in detecting alterations in chromatin structure. If the -410 site is involved in chromatin structure, we will not see an
overwhelming effect of the mutation in a transient (ie, non-integrated) assay. It will then be necessary to generate stable cell lines containing this mutated site to see if there is any effect on transcription. Although this site is found in a cell line that represents immature T cells, it is possible that the importance of this site is different in different stages of T cell development. It will also be useful then to assay the effect of the -410 mutant, as well as other IL-2 promoter mutants, in primary immature and mature T cells.

We are also very interested in identifying the factors that interact with this potentially important site. The region surrounding and including the -410 site is extremely AT rich. In addition, it contains two non-consensus binding sites for Octamer protein and up to four potential HMG binding sites. Gel mobility shift assays using an oligo that spans the -410 site indicate that there are at least six different shifted species in unstimulated EL4 T cells. Upon stimulation, an additional very high molecular weight species is also shifted. Importantly, some of these shifted bands are tissue specific. We are now in the process of trying to identify all of the components of these protein/DNA complexes. A subset of the bands does super-shift when antibody to the transcription factor Oct-1 is added to the binding reaction. Under appropriate conditions (ie, when dGdC is added), other bands co-migrate with HMG/DNA complexes.

In addition to super-shift experiments, we are also starting to perform Shift-Westerns. Using this technique, we can determine which combination of factors is present in the higher molecular weight complexes. By determining if the tissue specific bands are comprised of bonafide tissue specific components or of a tissue specific combination of more general factors we hope to elucidate the function and possible mechanism of this region of the IL-2 gene.

130. Investigation of additional transcriptional control regions in the IL-2 gene

Sudipta Bardhan, Susan Ward, Gabriela Hernandez-Hoyos, Robert Chen

In the past, transient transfection assays have provided valuable information about the position and the relative importance of transcriptional regulatory regions in the IL-2 gene. The focus of these early experiments was on the role of the region 5' to the transcriptional start site. Recent sequence analysis of the rest of the IL-2 gene, including the second and the third introns, revealed that there are extensive regions of homology between the murine and the human IL-2 gene within both introns.

In an attempt to determine if these conserved regions also contain potentially important regulatory information, we are analyzing their function in two ways. First, by using a transient transfection assay system which utilizes the luciferase reporter gene, we have initiated a comparison of the full length IL-2 genomic sequence with that of a sequence that does not contain any introns. To circumvent any inherent problems with this transient assay system, we are using three different methods — electroporation, SuperFect (Qiagen), and DEAE-dextran – to introduce the modified DNA into T cells. Our initial results indicate that the intronic regions as a whole do have an effect on transcription of the reporter gene, but more work must be done to define the particular regions responsible.

A second approach is to look at the chromatin structure of these introns as they exist in their native conformation in cells that express IL-2. Differences in chromatin structure are often indicative of important regulatory regions. HSE-3 was recently mapped to the second intron. Unlike the distal HSE-1 region, this intronic site is not altered with stimulation. This implies that this site is fundamentally different than the upstream site and that it may play a different role in IL-2 gene regulation. At present, we are investigating the tissue specificity and the protein/DNA interactions at this site. In addition, we are trying to determine the contribution that each different HS region makes to IL-2 gene expression (see Abstract #). The presence of these HS sites indicates that IL-2 regulation is more complicated than originally thought. It is possible that there are even more sites in the IL-2 locus and that each one has a separate yet vital role in gene regulation. The insights that we gain by unraveling this complexity will help to further our understanding of transcriptional regulation in general.

A third approach is to use long-range in vivo DNase hypersensitive site mapping to define regions of chromatin distortion or transcription factor binding that may be located anywhere within Southern blotting range of the IL-2 gene. This work was originally initiated by Fei Chen, and recent optimizations have confirmed the presence of a strong, stimulation-independent hypersensitive site in the second intron of the gene. In addition to looking for DNase 1 sensitive sites in the intronic regions, this genomic analysis will also enable us to analyse regions outside of the cloned IL-2 sequence. This is particularly important because correct developmental regulation of many hematopoietically expressed genes has recently been found to depend on sequence up to 30 kb away.

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131. Regulation of AP-1 transcription factors during thymocyte development

Fei Chen

The transcription factor AP-1 is subject to sharp regulation during thymocyte differentiation. AP-1 DNA-binding activity is inducible in immature, CD4 CD8 (DN) T-cell receptor (TCR) - negative thymocytes, but not in their CD4 CD8 (DP) TCR+ cortical descendants. However, in the rare cells that mature to become TCR+CD4 CD8 or CD4 CD8+ (SP) thymocytes, AP-1 DNA binding activity becomes inducible once more. To determine whether AP-1...
activity is regulated in development at the level of subunit synthesis, the induction of jun and fos family mRNAs after stimulation was compared in immature (RAG2), cortical, and mature thymocyte subsets. Both c-jun and junB mRNAs could be detected after stimulation, and there was no significant difference in their expression among the three populations. However, the induction of c-fos and fosB mRNA was much lower in cortical thymocytes than in immature DN and mature SP cells. fra-2 expression followed yet a third pattern, with induction-independent expression at different levels in the various subsets. Consistent with these results, severely decreased Fos family protein induction was also observed in cortical thymocytes. The c-Fos and FosB proteins participate in the majority of AP-1 DNA binding complexes present in activated immature and mature thymocytes, as shown by antibody supershift experiments. These results imply that AP-1 activity in thymocytes is regulated, at least in part, by developmental restrictions on induction of c-fos and fosB RNA. The effect of FosB on T-cell development will be further studied using FosB knockout mice.

132. Characterization and comparison of IL-2 regulatory factors within T helper cells and cytotoxic T lymphocytes
Ray Holz

IL-2 expression is stringently regulated by several trans-acting nuclear factors which bind to specific regulatory elements 5' of the IL-2 coding sequence. When a helper T cell is activated, nuclear factors cooperatively bind to specific regulatory elements and induce transcription from the IL-2 promoter. While cytotoxic T lymphocytes also contain the same 5' regulatory sequence, no IL-2 mRNA can be detected within these cells after activation. Effort is underway to characterize the similarities and differences between the nuclear factors responsible for IL-2 inducibility within these two T-cell lineages.

Nuclear extracts from EL4 helper cells and CTLL-2 cytolytic cells have been isolated and used for mobility shift assays. Similarities are observed in the binding pattern of Octamer and NFAT proteins in both cell types. A difference has been observed in the binding pattern at the NF-κB site. Proteins in CTLL-2 extracts showed a decreased capacity to bind the NF-κB site. Stimulated EL4 extracts present a reproducible, induction-dependent NF-κB site binding pattern that has never been observed from CTLL-2 extracts.

A difference was also observed in the AP-1 site binding pattern of the two cell types. EL4 extracts form only one complex at the AP-1 site. CTLL-2 extracts form the same complex, but also form a second very distinct protein/DNA complex. The second complex migrates more rapidly in native gel electrophoresis and apparently does not require induction. Discovery of the components of this second complex may lead to an explanation of why cytolytic cells are unable to transcribe from the IL-2 promoter.

133. Searching for genes responsible for CD4/CD8 lineage commitment
Gabriela Hernandez

During their development in the thymus, T cell precursors are subject to serial windows of selection, one of which is positive selection. Positive selection is the series of events that induces immature CD4+CD8+ thymocytes to become mature functional CD8+ or CD4+ single positive cells, depending on whether their T-cell receptor rearrangements have affinity for class I or II major histocompatibility molecules (MHC), respectively. These two cell lineages not only differ in their phenotype, but also in the functional capacities they are able to exert. Thus, at this stage of development, cells not only continue a developmental program, but also make a lineage-commitment choice.

How these cells transduce a signal that will eventually lead to the lineage-commitment is something we still do not know. We are particularly interested in understanding the early transcriptional events involved in the making of this choice. More specifically, we would like to identify key genes, the expression of which is essential following the onset of positive selection to induce the CD4+CD8+ thymocytes into either developmental pathway. In order to do this, we would ideally require isolating a sufficient number of cells that have received positively selecting signals at a known time, and synchronously. It would prove difficult to achieve this by sorting cells from a normal thymocyte population because of the relatively scarce number of such cells that would be present in this population, plus the additional problem imposed by the fact that cells at slightly different developmental stages might share the same phenotype. To overcome this problem we are working on setting up a recently described organ-free positive selection system, where purified immature CD4+CD8+ thymocytes stimulated with various concentrations of phorbol esters and calcium ionophores can give rise to either functional CD4+ or CD8+ single positive cells (Iwata et al., 1996), with no cell proliferation. To ensure our starting CD4+CD8+ cell population has never received positively selecting signals, we are planning to use thymocytes from MHC-deficient mice. These thymocyte populations will be negatively selected using magnetic columns against CD44+ / CD25+ to get rid of thymocytes at earlier stages of development. Once the conditions for obtaining mainly CD8 or CD4 single positive cells are known, we will determine the earliest timepoints following the initial activation event at which transcription is detected, by staining cells with acridine orange an analysing their RNA vs. DNA content by flow cytometry. When these two goals are achieved, we will then obtain mRNA populations from CD4+CD8+ thymocytes induced to become CD4, or
CD8 or uninduced, and compare them using subtractive hybridization of cDNA libraries, and/or other molecular approaches used by other members of this group.

Reference

Publications


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Summary: The work in our laboratory continues in two main areas; one is Signal Transduction and the other is designed to advance the goals of the Human Genome Project.

Signal Transduction
We made a number of important advances this year in the genetic analysis of G-protein function in mammalian organisms. We and our collaborators generated deficiencies in G protein genes and in the genes corresponding to effectors that respond to G protein activation. We worked with two gene families, the Gq family and the G13/G12 subfamily. Almost nothing is known about the function of Gα12 and Gα13. There is evidence that suggests that they activate a rho mediated pathway that leads to changes in cytoskeletal structure. However, the precise nature of the activation mechanism is not known. We found that overproduction of Gα12 in the activated form can lead to oncogenesis. More recently, Henning Althoefer and Pamela Eversole-Cire in our laboratory found that overproduction of activated forms of Gα13 induce apoptosis. However, the basic signaling pathway is not well defined. Stefan Offermanns found that Gα12 and Gα13 are phosphorylated through a PKC activated pathway during platelet activation. This occurs within 10 seconds after platelet activation is initiated and leads to stoichiometric phosphorylation. However, the function of the phosphorylated Gα protein is not known.

To get a better idea of Gα12 and Gα13 function we generated gene deletions in the mouse. The Gα13 knockout resulted in embryonic lethality. We were able to trace the defect to the day 8.5 embryo and to demonstrate that the difficulty was a result of failure of the formation of the vascular system. Endothelial cells can be seen in the yolk sac but vasculogenesis appears to be blocked. Primary fibroblasts were defective in chemokinases and these results suggest that Gα13 is required for appropriate cell movement and perhaps for interactions with chemoattractants or other growth factors at some early stage in vasculogenesis.

The phenotypes of deficiencies in Gα12 are still unclear. Initial experiments suggested that deficiency leads to embryonic lethality within the first day in the pre-implantation embryo. In order to confirm that this is not a result of an artifact introduced in generating the animals we made a second independent line of deficient mice using completely different deletion techniques and this line is currently being propagated. This work is being carried on by Stefan Müller.

We have also made progress in understanding the physiological function of the Gqq family. We have eliminated both Gqq14 and Gqq. Gqq14 deficiencies show no phenotype. However, the Gqq mice demonstrate a number of specific effects of the absence of the gene. First, the animals are ataxic. In collaboration with Dr. Masanobu Kano in Japan,
Stefan Offermanns has shown that this deficiency is the result of persistence of multiple climbing fibers in Purkinje cell development. Interestingly, this same phenotype is shown by PLC β4 deletion mice (made in collaboration with Dr. Diaqing Wu and Huiping Jiang, University of Rochester). They show ataxia and persistence of multiple climbing fiber innervation of cerebellar Purkinje cells. Dr. Kano and others have shown similar effects in mGluR1 and PKC γ deletion mice. We postulate that Gαq, PLC β4, the mGluR1 and the PKC γ gene products all assemble a signal transducing complex that localizes to the postsynaptic regions of the Purkinje cell dendrites. This complex controls synaptic function and also modulates the second phase of the development of cerebellar Purkinje cell innervation which occurs 10-20 days after birth. At this time multiple climbing fibers are eliminated and almost all of the Purkinje cells in normal mice retain interaction with only a single climbing fiber. In the mutants most of the cells have multiple persistent climbing fiber synapses.

The Gαq -/- mice also have a deficiency in platelet aggregation. Gαq plays a pivotal role in aggregation and degranulation. Its activation is absolutely required for the process to proceed. On the other hand, other G protein mediated processes such as platelet shape change are independent of Gαq. We postulate that Gαq initiates a series of events that leads to integrin receptor activation and platelet aggregation and degranulation. We are currently exploring these pathways.

In a continuing effort to understand the precise details of the mechanisms involved in visual system signal transduction, we have collaborated with Dr. Denis Baylor (Stanford University) and Dr. Jeannie Chen (University of Southern California), and concentrated our studies on the mechanisms involved in signal "turn off", i.e. the events that occur after the photolyses of rhodopsin. Dr. Jun Xu in our laboratory deleted the arrestin gene and the results of this change led to persistent activation and slow "turn off" of the signaling process. Taking together the results that we obtained over the last few years from deletions of the C terminus of rhodopsin, the deletion of arrestin and of the recoverin gene, we can begin to generate a picture of the contribution that each of these elements makes to the "turn off" process. To complete the picture we continue to generate deficiencies in other signaling components, e.g. rhodopsin kinase and these animals are currently being studied.

In addition, our examination of the "turn off" process in the visual system has led us to look for other proteins that might be involved in catalyzing GTPase activity. In *in vitro* reconstitution systems the GTPase activity of transducin alone is much too slow to account for the termination of the visual signal which occurs in just a couple of hundred milliseconds. Dr. Jason Chen found a protein RGSr that is highly specific for the retina. Together with Thomas Wieland he showed that this protein is part of a family of genes that bind to the configuration of the G protein that is involved in stabilizing the transition state for the hydrolysis of GTP. They thus speed up GTPase activity. Thomas Wieland and Jason Chen have done careful analysis of the kinetics of RGSr and its relationship to the γ subunit of phosphodiesterase in reconstituted systems and we suggest that both proteins interact with a partially overlapping site on transducin. RGS binds very tightly only to the intermediate transition state form of the G protein while PDE γ binds most tightly to the GTP form of the protein. The two proteins may play sequential roles in the cycle of G protein activation and inactivation. We are continuing our examination of the role of RGSr in the visual process.

Finally we have found that many of the mutations that we made, deleting phototransduction genes lead to apoptosis in the retina and retinal degeneration. We are studying the effects of introducing anti-apoptotic genes and testing for rescue of the photoreceptor cells from light and gene modification induced cell death.

Extensive genetic analysis of G protein mediated pathways may be easier to do in complex model systems. We began a collaboration with Paul Sternberg's laboratory a number of years ago to work on G-protein function in *C. elegans*. Lorna Brundage and Carol Bastiani have been doing suppressor analysis of activated G proteins of the Gq family and the G12 family in *C. elegans*. We expect that these systems will behave in a way analogous to the mammalian systems.

We continue to work on the structure and function of histidine kinases in signal transduction in bacteria and in fungi. Lisa Alex is exploring the pathway that incorporates Nik-1 and Nik-2, two histidine kinase genes in *Neurospora*. She has also found homologs of these genes in *Candida*.

We are working on experiments designed to elucidate the atomic structure of bacterial histidine kinases.

**The Human Genome Project**

Our group has contributed to the Human Genome Project by innovating approaches for generating libraries to maintain and clone large fragments of mammalian DNA. We continue to develop new ways to make BACs (bacterial artificial chromosomes) to add to the world's supply and to generate arrays of subsets of BACs that belong to different chromosomes and that generate contiguous overlapping regions corresponding to interesting and important parts of the genome. Thus, we are engaged in a process of trying to find sets of BACs for each of the genes that have been defined by ESTs (5'-end sequence tags). We plan to expressed generate contigs in both human and mouse that will be useful resources for studies of control of gene expression. We are also working with The Institute for Genome Research (TIGR) led by Mark Adams and Craig Venter on a project devoted to sequencing chromosome
16. We are providing arrayed BACs with minimal overlap to use as substrates for the sequencing project. We are collaborating with Dr. Leroy Hood at University of Washington, Seattle, and TIGR to sequence the ends of BACs to provide a resource for a variety of human genome studies. We are also developing new methods for manipulating these clones once they have been obtained so that regions of any clone can be removed or new components can be inserted. This will make these clones ideal for functional genomic studies.

134. Double mutants with disruption of both Gq and Gxi11 genes in the mouse

Stefan Offermanns, Masanobu Kano, Tom Wilkie, Valeria Mancino, Yi-Hui Hu, Susumu Minamisowa, Illdigo Sarosi, Melvin I. Simon

The Gq family of the heterotrimeric guanine nucleotide binding proteins (G proteins) consists of four members Gq, Gxi11, Gxi14 and Gxi15/16 which are differentially expressed. The Gq and Gxi11 subunits are 88% homologous in amino acid sequence and appear to be expressed ubiquitously. They are primarily responsible for coupling receptors, in a pertussis toxin insensitive manner, to phospholipase C β-isoforms. Receptors activating the Gq/11 family members do not discriminate between Gq and Gxi11 and in similar fashion Gq and Gxi11 indistinguishably activate the β1, 3 and 4 isoforms of phospholipase C and are poor regulators of PLC β2. In order to study their role in development and function in the whole organism, we have generated mice in which either the Gq or Gxi11 subunits have been disrupted.

The Gq homozygous deficient mice are born alive, they show increased postnatal mortality and develop progressive ataxia with impaired motor coordination. Anatomical study of the peripheral and central nervous system did not show obvious morphological changes. However, it has revealed that approximately 40% of adult Gq mutant mice cerebellar Purkinje Cells remain innervated by multiple climbing fibers, in contrast to normal mice where at about postnatal day 20 most Purkinje Cells are innervated by a single climbing fiber.

In contrast the Gxi11 homozygous deficient mice are born alive and appear to be perfectly normal and healthy.

We have crossed the g and 11 mice and observed that the lack of both genes results in embryonic lethality at approximately day 10.5, when the double knock-out embryos appear to be visibly smaller than the wild type and to have a very thin myocardium compared to the wild type. We have observed 100% postnatal mortality within the first few hours in pups whose genotype is Gq-/-Gxi11-/- and 96.4% postnatal mortality in pups that are Gq-/-Gxi11-+-1. In both these cases histological analysis done on the hearts of the pups has shown anomalies ranging from massive cardiac hypertrophy to thinner heart walls, to the presence of a common ventricle. The double heterozygous mice look normal as expected, so do all the other genotypic combinations, except in the case of Gq-/ mice when ataxia is present.

We are now in the process of taking a closer look at the hearts of the pups of day 18 pc embryos by scanning microscopy after fixation or by in vivo physiological function analysis.

We are also establishing primary and immortalized fibroblast cell lines as well as primary cardiomyocyte cultures from the double knock out embryos and wild type to study in vitro response to a variety of stimuli.

135. Defective platelet activation in Gq deficient mice

Stefan Offermanns, Christopher F. Toombs, Yi-Hui Hu, Melvin I. Simon

Platelets are small disc-shaped cell fragments which undergo a rapid transformation when they encounter vascular damage. They become more spherical and extrude pseudopodia, fibrinogen receptors are activated leading to aggregation, they release their granule contents and eventually form a plug responsible for primary hemostasis. In contrast to most mammalian tissues that express Gq together with its close structural and functional homologue Gxi11, platelets contain only Gq.

To define the role of Gq in platelet activation we examined both in vivo and in vitro platelets from Gq deficient mice and showed that they are unresponsive to a variety of physiological platelet activators. As a result, Gq deficient mice have increased bleeding times and are protected from collagen/epinephrine induced thromboembolism.

We think that Gq plays an essential role in signaling processes employed by diverse platelet activators and that the requirement for Gq cannot be replaced by the presence of Gxi or the βγ-subunits of the heterotrimeric G-proteins. Gq represents a new target for therapeutics which could block platelet activation.

136. Phosphorylation of G-protein activated phospholipase C isoforms by cGMP-dependent protein kinases

Mingyao Liu, Osamu Nakanishi, S. Lohmann1, U. Walter1, Melvin I. Simon

Phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to generate diacylglycerol and inositol 1,4,5-triphosphates (IP3), leading to the activation of protein kinase C (PKC) and the mobilization of intracellular calcium. The various PLC isoforms appear to be activated by different receptors and in some cases by different G-protein components. There are four well-characterized PLC isoforms, all of them are activated to various extents by the Gq family proteins. Specific activation of PLC isoforms (β2 and
β3) by Gβγ subunits has also been observed. Increase in intracellular cGMP concentration has been known to inhibit the mobilization of calcium in a number of cells, presumably by activating cGMP-dependent protein kinases (PKGs). To understand the molecular mechanism underlying these phenomena, we investigated the effects of three PKG isoforms on the activation of PLC-β2 and PLC-β3. We found that both purified PLC-β2 and PLC-β3 are phosphorylated in vitro by PKG isoforms. Phosphopeptide analysis indicated that four or more peptides are phosphorylated and different phosphorylation sites exist compared to the phosphorylation of PLC by cAMP-dependent protein kinase (PKA). Phosphoamino acid analysis showed that both serine and threonine residues are phosphorylated. Cotransfection of PKG isoforms with cDNAs encoding PLC-β2 and PLC-β3 in Cos-7 cells shows that PKG inhibited the activation of PLC by Gβ1γ2 subunits. These results provide a mechanism for understanding the effects of cGMP in regulating intracellular calcium and other functions.

137. The Ca²⁺-dependent binding of Calmodulin to a N-terminal motif of the heterotrimeric G-protein β subunit

Mingyao Liu, Bo Yu, Osamu Nakanishi, Thomas Wieland, Melvin Simon

Ca²⁺ ion concentration changes are critical events in signal transduction. The Ca²⁺-dependent interactions of calmodulin (CaM) with its target proteins play an essential role in a variety of cellular functions. In this study, we investigated the interactions of G-protein βγ subunits with CaM. We found that CaM binds to known βγ subunits and these interactions are Ca²⁺-dependent. The CaM-binding domain in Gβγ subunits is identified as Gβ residues 40-63. Peptides derived from the Gβ protein not only produce a Ca²⁺-dependent gel-mobility shift of CaM, but also inhibit the CaM-mediated activation of CaM Kinase II. Specific amino acid residues critical for the binding of Gβγ to CaM were also identified. We then investigated the potential function of these interactions, and showed that binding of CaM to Gβγ inhibits the pertussis toxin (PTX)-catalyzed ADP-ribosylation of Gαo subunits, presumably by inhibiting heterotrimer formation. Furthermore, we demonstrated that interaction with CaM has little effect on the activation of PLC-β2 by Gβγ subunits, supporting the notion that different domains of Gβγ are responsible for the interactions of different effectors. These findings shed light on the molecular basis for the interactions of Gβγ with Ca²⁺-CaM and point to the potential physiological significance of these interactions in cellular functions.

138. Expression of constitutively active GaQ and Ga13 induce apoptosis

Henning Althofer, Pamela Eversole-Cire, Rochelle Diamond, Melvin I. Simon

Programmed cell death or apoptosis is an intrinsic genetic program within multicellular organisms which when activated causes a variety of morphological changes including cell shrinkage, nuclear condensation, activation of specific proteases and endonucleases, and DNA fragmentation. Programmed cell death has been implicated in many important biological processes such as immune defense, control of cell growth, and development. The programmed cell death genetic pathway is activated by intrinsic signal transduction events which link physiological changes to the cell death machinery. Signal transduction through seven pass membrane receptors is a common mechanism of eukaryotic signaling and physiological control. Heterotrimeric G-proteins are responsible for transducing the signals from the membrane receptor into the cell. Normally the G-protein signal transduction pathway is tightly regulated since aberrant signaling may lead to certain pathological states.

We have studied the effect of expression of constitutively active Gα-mutants on cell survival. Transfection of constitutively active GaQ and Ga13 in two different cell lines resulted in condensation of genomic DNA and nuclear fragmentation. Endonuclease cleavage of genomic DNA was confirmed by labeling the DNA fragments and subsequent analysis using flow cytometry. The signaling pathways involved in this cellular function were investigated to distinguish between the cell death inducing ability of these two constitutively active Gα subunits. GaQ was found to induce apoptosis via a PKC-dependent pathway whereas Ga13 induced programmed cell death through a pathway involving activation of the small G-protein rho. Both of these pathways leading to apoptosis were shown to be blocked by overexpression of bcl-2, whose gene product has been shown to protect many cell types from cell death induced by a variety of insults. In contrast to other cell death inducing systems, expression of constitutively active GaQ and Ga13 induced apoptosis in high serum conditions as well as in defined medium. Our results demonstrate that pathological constitutive expression of certain Gα subunits can lead to programmed cell death. It will be interesting to determine if pathological sustained expression of GaQ or Ga13 are involved in the etiology of any human disorders.

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139. **gpa-12**, a gene encoding a G12 alpha subunit

Lorna Brundage, Hendrik C. Korswagen1, Ronald H.A. Plasterk1, Paul W. Sternberg, Melvin I. Simon

Heterotrimeric G proteins are classified into four families, Gi/Go, Gq, Gs and G12, based on the similarity of their sequence and function. In contrast to the other G protein families, the receptors and the second messengers involved in transduction of signals via the G12 family are largely unknown. To elucidate the G12 pathway in *C. elegans*, we are studying the gene *gpa-12*, encoding a G12 alpha subunit we identified by screening the sequence generated by the *C. elegans* genome project. We have engineered a constitutively active allele of *gpa-12* under hsp-16 control. When this allele is induced by heat shock during embryogenesis, animals hatch into starved, developmentally arrested and uncoordinated larvae which eventually recover. We plan on exploiting this phenotype to identify *gpa-12* effectors in a genetic screen. We have also obtained a presumptive null allele of *gpa-12*, via the transposon insertion/deletion method, which removes 90% of the *gpa-12* coding sequence. Animals homozygous for this allele exhibit no obvious developmental or behavioral defects, a surprise since we have found that *gpa-12::gfp* is expressed broadly in *C. elegans*.

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140. **Genetic analysis of Gqα mediated signaling pathways in C. elegans**

Carol A. Bastiani, Lorna Brundage, Paul W. Sternberg, Melvin I. Simon

*egl-30* encodes a protein with more than 82% amino acid sequence identity to mammalian heterotrimeric G protein alpha subunits of the Gq family. The mammalian Gq family can be activated by at least thirty different receptors, including receptors which respond to the neurotransmitters acetylcholine, serotonin, and glutamate. The Gq family represents a major component of G protein mediated signaling through phosphoinositides by direct activation of phospholipase Cβ (PLCβ) isoforms. Previously characterized reduction-of-function mutations have implicated *egl-30* in the regulation of behaviors such as egg-laying, coordinated movement, and viability in *C. elegans* (Brundage et al., 1996).

We have engineered a constitutively active allele of *egl-30* fused to the hsp-16-2 promoter element and integrated this gene fusion into the *C. elegans* genome. Within two hours after a heatshock regimen at 32°C for thirty minutes, these worms hypercontract and become paralyzed. Interestingly, this effect closely mimics the initial response of wild-type worms to arecoline, an acetylcholine agonist. To define downstream components of *egl-30* mediated signaling pathways in *C. elegans*, we are screening for mutations which can suppress the paralysis which results from overexpression of the constitutively active allele of *egl-30*. In a screen of ~20,000 haploid genomes, we have identified 12 mutations that suppress both hypercontract and paralysis. These mutations are defined by two complementation groups. We are currently in the process of carrying out additional mutagenesis screens and of mapping and further characterizing these suppressor genes.

Reference

141. **Characterization of empty G protein α subunits**

Bo Yu, M. I. Simon

We have engineered a xanthine nucleotide binding Gqα double mutant D273N/Q205L (GooX) that can be regulated by xanthine nucleotides instead of guanine nucleotides. GooX binds βγ only in the presence of XDP, and retains the receptor specificity for wild type Goo. XTP promotes the conformational change of GooX and the dissociation of βγ trimeric complex. In cells, XMP is an intermediate in the biosynthesis of GMP, however the steady state concentrations of XDP and XTP are relatively low. Therefore GooX behaves as an empty form of the G protein α subunit without exogenous XDP or XTP. The empty state of Gα has long been proposed as an important intermediate in ligand stimulated G protein receptor activation; activated receptors and empty Gα subunits form stable complexes. In transfected COS-7 cells, we found that GooX inhibited PLCβ activity stimulated by thrombin receptors. The thrombin receptor activates the Gq pathway. Since GooX has no effect on the βγ stimulated PLCβ2 activity, it suggests that GooX competes with endogenous Gq for the thrombin receptors. In order to further characterize the interaction between GooX and receptors, we purified His6-tagged GooX from *E. coli* and examined its interaction with M2 receptors expressed in sf9 cells. We found that GooX bound to M2 receptors and stayed on the membrane in the absence of βγ subunits, while wild type Goo interacted with the receptors poorly. In the presence of XDP or XTP, GooX dissociated from the receptors and was released from the membrane. In order to study empty forms of other G protein α subunits, we also made similar DN mutations in G11α and G16α. These mutants also exhibited dominant negative activity toward their respective receptor stimulated pathways. These experiments support the hypothesis that empty Gα subunits form stable complex with the receptors, and that their dominant negative effects on receptors may be used to shut down specific signal transduction pathways.

142. **Structure-function analysis of alpha-factor receptor**

Tao-Mu Yi, James U. Bowie, Melvin I. Simon

Alpha-factor receptor (Ste2p) is a G-protein-coupled receptor (GPCR) from *S. cerevisiae*. The goal of this research is to investigate the structure and function of α-factor receptor. More specifically, my efforts have focused on two areas: (a) performing structural studies...
on peptide fragments derived from Ste2p bound to the yeast G-protein; and (b) fine-mapping of the functional features of Ste2p using exhaustive mutagenesis.

a. **Galpha/receptor-peptide complex**

As an intermediate step in determining the structure of the receptor as a whole, I have sought to study the properties of fragments of the receptor bound to the G-protein. Through a close collaboration with Bo Yu in the Simon lab, I have created an "empty" version of yeast Gα, Gpa1p<sup>C391N/Q323L</sup>, that does not bind guanine nucleotide. Instead, this mutant is able to interact with alpha-factor receptor even in the presence of GDP or GTP. I believe that this double mutant will serve as an effective platform on which to dock the Ste2p cytoplasmic loop peptides.

I have attempted to establish a two-hybrid system for measuring the interaction between Gpa1p<sup>C391N/Q323L</sup> and the receptor peptides. More importantly, this system can be used to screen for variations in the peptides or the Gα that further enhance this interaction. In this manner, I can define more precisely which regions of the loops actual bind to Gpa1p.

b. **Mutagenesis of alpha-factor receptor**

The analysis of mutations in GPCRs has yielded a wealth of information on residues that make critical contacts to ligand or G-protein. As I describe below, by monitoring the yeast pheromone response which is triggered by the binding of alpha-factor to alpha-factor receptor, I can test the function of large numbers of Ste2p mutants.

I have redesigned the Ste2 gene so that there is a unique restriction site every 300 base pairs (bp) in the gene. In this manner, after mutagenesis, 300 bp fragments spanning the whole gene can be individually subcloned into a new background. I am able to select for mutations that disrupt the activity of alpha-factor receptor by spreading cells transformed with mutagenized Ste2 on plates containing alpha-factor initiated. RGS-r can then accelerate PDE deactivation by competing with PDE and removing it from TDα as well as by increasing P, release and the rate at which TDα can recycle. In conclusion, the data argue for an important role of RGS-r in the fast termination of TD activation observed in vivo.

**References**


**143. A retinal specific RGS protein binds transducin and facilitates signal termination**

**Thomas Wieland, Ching-Kang Chen, Melvin EI. Simon**

In vertebrate photoreceptor cells, the heterotrimeric guanine nucleotide binding protein transducin (TD) mediates signaling between rhodopsin and cGMP phosphodiesterase (PDE). It relieves the inhibitory effect of the γ-subunit of PDE by transiently binding the γ-subunit. One important mechanism involved in the termination of signaling is the hydrolysis of GTP by the inherent GTPase of the TD α subunit (TDα). Evidence has been presented that this reaction can be accelerated by retinal specific GTPase activating proteins (GAPs). One protein exhibiting GAP activity is the effector itself, i.e. the γ-subunit of PDE (PDEγ). In addition, we cloned the cDNA corresponding to a thus far unknown protein termed RGS-r, which belongs to the GAP superfamily of RGS (Regulator of G protein Signaling) proteins. RGS-r is most abundantly expressed in the retina. When expressed and purified from _E. coli_ recombinant RGS-r was found to be a highly potent and efficient GAP for TD. Besides accelerating the single turnover GTPase rate of TDα, it also catalytically increased the multiple turnover GTPase, thus the recycling of TDα. Most interestingly, RGS-r specifically interacts with TDα in the so called transition state when the hydrolysis of GTP is initiated, but not with TDα in its pure GTP- and GDP-bound form which most likely accounts for its catalytic role in recycling.

When the GAP function of RGS-r and PDEγ were compared, both proteins stimulated single turnover GTPase of TDα. However RGS-r was more effective than PDEγ. When added together, PDEγ competitively inhibited the RGS-r stimulated GTPase. Therefore, the interaction of TDα in all three states, the GTP-bound form (TDα<sub>GTPγS</sub>), the transition state (TDα<sub>GDPγS</sub>) and the GDP-bound form (TDα<sub>GDP</sub>), with RGS-r and PDEγ was measured by surface plasmon resonance. PDEγ displayed highest affinity for TDα<sub>GTPγS</sub>, weaker affinity for TDα<sub>GDPγS</sub> and weakest affinity for TDα<sub>GDP</sub>. RGS-r exhibited only high affinity for TDα<sub>GDPγS</sub>, while the affinity for the two other nucleotide stabilized forms was low. Thus, the observed competition between RGS-r and PDEγ for TDα most likely occurs when the hydrolysis of GTP is initiated. RGS-r can then accelerate PDE deactivation by competing with PDEγ and removing it from TDα as well as by increasing P, release and the rate at which TDα can recycle. In conclusion, the data argue for an important role of RGS-r in the fast termination of TD activation observed in vivo.
recovery phase, suggesting that arrestin is required for complete and timely recovery. The normal early recovery observed in A-/- mouse rods suggests the presence of an arrestin independent recovery mechanism. We seek to investigate the role of RK in this early recovery by targeting murine RK gene. Recording from RK-/- mouse rods showed unquenched and prolonged photoresponse, similar to that observed in RhoACT rods. These results show that both arrestin and RK are required for the recovery of vertebrate phototransduction. RK functions in the early phase of recovery and arrestin is responsible for the late phase of recovery.

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145. Interference with programmed cell death to delay retinal degeneration

Pamela Eversole-Cire, Jeannie Chen', Jean Edens, Melvian I. Simon

Retinitis Pigmentosa (RP) refers to a group of inherited retinopathies which affect approximately 1 out of every 3000 individuals. Degeneration of rod photoreceptors initially results in night blindness and ultimately leads to a generalized loss in tissue affecting overall vision. Recent evidence indicates that several components of the visual transduction pathway may be involved in the etiology of this disease. Mutations within the genes encoding rhodopsin and retinal cGMP-phosphodiesterase have been linked to RP as well as genes encoding structural components of the photoreceptor cell, such as peripherin and rom-1. Retinal degeneration may also result from cellular insults such as constant light exposure. Regardless of the nature of the primary genetic defect or insult, retinal degeneration appears to be a direct result of programmed cell death.

Programmed cell death or apoptosis is a physiological process which normally controls and stabilizes cell populations in multicellular organisms. Several cellular genes have been identified which are involved in regulating the genetic pathway leading to programmed cell death. The Bcl-2 gene product protects several human cell types, including neurons, from cell death induced by a variety of insults. Bcl-2 functions as a complex with Bax or BAG-1. In addition, genes with anti-cell death activity, such as p35, are encoded by viral genomes to inhibit host cells from undergoing apoptosis during infection.

Ectopic expression of genes involved in regulating programmed cell death in photoreceptors of mice with RP may serve as a potential means to treat retinal degenerative diseases. An expression vector using the 4.4 kb rhodopsin promoter was used to target the expression of the human Bcl-2 gene to photoreceptors of mice with retinal degeneration to study the effect of Bcl-2 expression on programmed cell death in rod cells. Results show that retinal degeneration is temporarily delayed by the ectopic expression of the Bcl-2 gene (Chen et al., 1996). Bax and BAG-1 are now being coexpressed with Bcl-2 to determine if their functional complexes will enhance the protective effect against cell death afforded by Bcl-2 alone. p35 is also being ectopically expressed to determine if expression of this gene will be effective in the prevention of photoreceptor cell death. Results from these experiments may help to elucidate the role of programmed cell death in retinal degeneration and perhaps other neurodegenerative disorders.

Reference
Chen et al. (1996). PNAS 93, 7042-7047

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146. Two-component histidine kinases in Neurospora crassa

Lisa A. Alex, Melvin I. Simon

Two-component signal transduction pathways mediate many cellular responses to a variety of environmental stimuli such as nutrient availability, cell density, and osmolarity. These signalling systems are prevalent in bacteria but have recently been found to exist in eukaryotes such as fungi, plants, slime molds, and yeasts. In its most simple form, the first component of the two-component pathway is an auto-phosphorylating histidine kinase whose activity is regulated by some stimulus. Phosphate is subsequently transferred to a downstream protein termed a response regulator. Differential phosphorylation of a response regulator controls its activity and allows a desired response to occur. Two-component signalling proteins are most easily recognized by their amino acid sequence homologies.

Our lab has identified several histidine kinases from Neurospora crassa, Aspergillus nidulans, Dictyostelium discoideum, and Candida albicans. We have concentrated on understanding the function of the kinases from N. crassa and C. albicans. N. crassa has two genes encoding histidine kinases, nik-1+ and nik-2- Def. Deletion of nik-1+ results in organisms that exhibit aberrant hyphal and conidial structure and exhibit sensitivity to growth under conditions of high osmolarity. Calcofluor staining indicates the abnormality may lie in a defective cell wall structure. We are currently trying to identify suppressors of this mutant by insertional mutagenesis/plasmid rescue. In addition we are planning to analyze the possible protein-protein interactions of Nik-1 using the two-hybrid method. The nik-1 gene has also been determined to be the os-1 locus of N. crassa by Selitrennikoff's group at the University of Colorado Health Sciences Center.

Deletion of nik-2- shows no large alteration from the wild-type phenotype. When Δnik-2 cells are grown at elevated temperature however, they begin to elaborate large swellings in their aerial hyphae. The molecular basis for this is not understood at present. We hope to delineate the signalling pathway involving Nik-2 using the two-hybrid approach.
147. Two-component histidine kinases in pathogenic fungi
Lisa A. Alex, Jacqueline Agnan1, Claude Selitrennikoff2, Melvin I. Simon
We have cloned two histidine kinase encoding genes from the opportunistic pathogen Candida albicans. One of these, cos-1, encodes a homolog of Nik-1 (also known as Os-1) from Neurospora crassa. The second gene is unique. Currently we are working to understand the function of Cos-1 in Candida to determine if it is involved in pathogenicity. A knockout is under construction using the ura blaster technology developed for Candida albicans gene replacements. We are also trying to determine the cellular location of this protein with epitope tagging. Recently, we have been able to express GST fusion proteins of the kinase domain in E. coli and preliminary experiments indicate that phosphorylation occurs. We are also trying to express the full length protein and response regulator domains to assess their biochemical function. Ultimately our goal is to find potential inhibitors that may have antifungal activity. The histidine kinases of bacteria and fungi are attractive drug targets as they have not been found to exist in mammalian cells.

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148. BAC library construction
Yuling Sheng, Yu-Jian Chen, Hiroaki Shizuya
Human and mouse BAC clones have been extensively used for a variety of research areas because of their stability in E. coli, easy handling, and relatively large insert size. Mapping by BACs is one area focused on finding genes responsible for a variety of cancers and genetic diseases. More recently, when various genome centers all over the world began to sequence human genomic DNA at a large scale, BACs have been used extensively as sequencing template materials. We have generated over 300,000 BAC clones from two individuals, and arrayed them in 384-well microtiter plates to organize these clones. They were spotted on filters at very high density (40,000 clones / filter) to screen them by hybridization, and pooled in an appropriate configuration to do PCR based screenings. However, more BAC libraries are needed in order to be more cost effective, and accelerate the progress of entire sequencing efforts, and to finish by 2005, the scheduled completion date of the Human Genome Project. By increasing the coverage of the human genome with deeper libraries, larger number of BACs in a given region will be found, leading to minimal overlapping, and to constructing more accurate maps. We initiated a new round of BAC library construction. For this purpose, we made a new generation BAC vector, plndigoBAC45 which gives much darker blue colored colonies on the X-gal plates. This feature enables us to identify clones with inserts more accurately, resulting low percentage of empty clones, and to shorten the time required for library construction. With this vector we are now constructing new BAC libraries using two additional restriction enzymes to ensure the complete coverage of the human genome, and plan to generate more than 300,000 additional BAC clones in next 6 months.

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150. Completing the BAC-based physical map of human chromosome 22

Holger Schmidt1, Hiroaki Shizuya

To initially demonstrate the usefulness of the BAC system for long range physical mapping a scaffold integrated contig map of the entire long arm of chromosome 22 was constructed. Individual clones were ordered into contigs by fingerprint analysis and placed along the genomic stretch according to their content of genetic anchorpoints. The map consists of more than 600 BAC clones and spans the length of approximately 45 megabase pairs. Each clone is being further characterized by fluorescence fingerprinting and end-sequencing the inserts. Aligned clones from this map are currently being used by the Sanger Center, Cambridge, U.K., and University of Oklahoma for sequencing the chromosome arm.

Although the map already provides about 90% coverage of 22q, there are many gaps remaining. Sequence information of the clone ends is presently being used to select new clones from the 15x coverage BAC library, which extend contigs and close the gaps. Our ultimate goal is to generate an accurate set of BAC clones, which provides complete physical coverage of the entire long arm of chromosome 22 with minimal overlaps. This map will serve as an excellent resource to discover any sequence present on the chromosome, and can readily be used for cost-efficient genomic DNA sequencing with minimal redundancy. Additional information on the localization of new transcriptional units will be obtained by the identification of BACs using clones from a chromosome 22-specific hncDNA library. Individual clones have been partially sequenced and so far no homology to entries in the database could be found.

151. Construction of genome-wide physical BAC contigs using mapped cDNA as probes

Steve C. Mitchell, Diana Bocskai, Robert X. Xu, Yicheng Cao, Melissa M. Natividad, Enrique Colayco, Yan Ding, Christie Gomez, Gabriella Rodriguez, Annabel Echeverria, Melvin I. Simon, Ung-jin Kim

The goal of the human genome project is to characterize and sequence the entire genomes of human and several model organisms, thus providing complete sets of information on the entire structure of transcribed, regulatory and other functional regions for these organisms. In the past years, a number of useful genetic and physical markers on human and mouse genomes have been made available along with the advent of BAC library resources for a number of organisms. These advances in technology and resource development made it feasible to efficiently construct genome-wide physical BAC contigs over the human and other genomes. Currently, over 30,000 mapped STSs and 27,000 mapped ESTs (Expressed Sequence Tags derived from corresponding cDNA) are available for human alone. ESTs or cDNA are excellent resources for map building for two reasons. First, they exist in two alternative forms - as both sequence information for PCR primer pairs, and cDNA clones - thus making library screening by colony hybridization as well as pooled library PCR possible. We are now able to screen genomic libraries for large numbers of DNA probes with high efficiency by combining over 100 cDNA probes for each hybridization. Second, the linkage and order of expressed sequences, or genes are rather conserved among human, mouse and other model organisms. Therefore, mapping with cDNA markers rather than random anonymous STSs will facilitate comparative genomic studies as well as physical map building. This "BAC-EST" project is currently generating lists of thousands of cDNA-positive BAC clones each month. The clone end sequences are also being determined from each of the clones. BAC-end sequence (BES) information can be extremely useful to precisely align these mapped BAC clones against any known sequence contigs by direct sequence comparison. These BAC clones with end sequence information and precisely known genomic location with respect to expressed genes will provide highly efficient source of material for future genomic studies.

152. Physical mapping and sequencing of human chromosome 16

Diana Bocskai, Steve C. Mitchell, Robert X. Xu, Yicheng Cao, Melissa M. Natividad, Jun-Ryul Kim1, Byeong-Jae Lee1, Enrique Colayco, Gony H. Kim, Christie Gomez, Igor Vivanco, Gabriella Rodriguez, Annabel Echeverria, Robin Hua Li, Mark D. Adams2, Melvin I. Simon, Ung-jin Kim

Extensive physical mapping efforts and standard automated sequenced technology have matured to the extent that large-scale sequencing of large human chromosomal regions has now become feasible. Currently, NIH and DOE are supporting several centers in the U.S. to begin sequencing the genomes of human and other model organisms systematically and in massive scale. In the past 1.5 years, Caltech has been mapping and sequencing a 20 Mbp region on human chromosome 16p arm (16p13.1-11.2) jointly with TIGR (The Institute of Genomic Research) as a pilot experiment to sequence chromosome-sized regions under the auspices of NCHGR/NIH. At Caltech, we have been generating large sequenceable BAC clones by rapidly developing BAC contigs in the targeted region using the STS and other ordered markers available from the YAC-STS chromosome 16 map previously constructed by Los Alamos National Laboratory. Currently, 12X coverage human BAC libraries (A, B & C) are available for screening by both high density filter hybridization and pooled library PCR approaches. Thus far we have identified over 2,000 BAC clones that are positive to STS markers and cDNA clones from the 16p13.1-11.2 region. Clones that
have been mapped and thoroughly characterized (sized, fingerprinted, FISH mapped and compared with genomic DNA by Southern blot hybridization) have been sent to TIGR, where the clones are sheared, subcloned and sequenced redundantly in random shotgun fashion. The random sequences are then assembled and gaps and ambiguous regions are resolved by local resequencing. Over 31 non-overlapping BAC clones (3.5 Mbp in total length) have been completed and 40 more are in queue at TIGR. Progress in mapping and sequencing chromosome 16p is being posted in two WEB sites: http://www.tree.caltech.edu, and http://www.tigr.org/tdb/humgen. We are currently using BAC-end sequencing (BES) technology to rapidly and precisely determine the extent of overlaps between completely sequenced BACs and other neighboring BACs. The sequences of both ends of all chromosome 16 BAC clones are being determined and compared against completely sequenced BACs to obtain precise overlap information. Restriction fingerprint analysis techniques are being used to confirm the overlaps determined by sequence alignment.

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153. Fluorescent fingerprinting of bacterial artificial chromosome (BAC) clones
Yan Ding, J. Enrique Colayco, Marty Johnson, Yu-Juin Chen, Jim Melnyk, Holger Schmitt, Hiroaki Shizuya

High resolution fluorescent fingerprinting of BAC clones is under development for applications to uniquely identify individual BAC clones and to detect overlapping pairs of BACs. The technique involves a double digest of BAC clones with one 6-base and one 4-base restriction endonuclease and labeling of 6-base ends with fluorescent dye-attached deoxy terminators in a single step. The restriction fragments are then separated on an ABI Sequencer 377 and sized with an internal standard labeled with different dye. Fragments size ranging from 75 bp to 55 bp are identified by software GeneScan (ABI). Contigs can be then built by finding overlapping BACs and incorporating other marker data using software FPC (Fingerprinted Contig, The Sanger Center).

Currently, ddATP and ddCTP, both labeled with three distinct mobility-matched dyes are available for restriction fragment labeling. For each BAC clones, we plan to analyze three sets of fragments generated by three pairs (one 6 base cutter with one of three 4 base cutters) of restriction endonucleases, each of which is labeled with different dye and combined into a single lane. Data from three sets of restriction digest are then analyzed independently in order to eliminate false overlap which might be resulted from coincidental fragment matching between pairs of BACs. Current effort is concentrated on (1) examination of band distribution produced by different restriction endonuclease pairs; (2) determination of sizing variation among different runs; (3) calculation of fragment size matching by chance among randomly chosen BACs; (4) feasibility test of FPC to generate accurate and minimally overlapped contigs using this fluorescent fingerprinting technique.

154. Human transcript mapping on bacterial artificial chromosome (BAC) clones
Yan Ding, Stephen Mitchell, Ung-Jin Kim, Melvin I. Simon

Over 10,000 human transcripts representing a set of unique genes will be mapped onto subsets of BAC clones, which can be used as substrates for sequencing as well as for functional analysis. A pooling strategy is being developed to screen BAC libraries gridded as high density arrays (HDG) on Nylon membranes using hybridization. cDNAs are pooled as (1) plate-pools, and (2) well-pools contain cDNAs from one particular well of each plate. So that a plate-pool has one cDNA overlap with a well-pool. Any positive clones that are identified with both plate-pool and well-pool are assumed to be detected by the cDNA which is present in both probe pools. Under this scheme, BAC clones positive or ten thousand individual cDNAs can be identified with 200 hybridizations, if 100 cDNAs are pooled in one hybridization, instead of 10,000 individual hybridizations for each cDNA.

To validate and establish the procedure, a pilot study has been carried out to test purity, identity, and sequence redundancy of cDNA clones, to determine an optimal number of cDNAs to be pooled as probes, and to cross-exam BAC positives identified by pooled cDNA probes with corresponding EST primers and restriction endonuclease fingerprinting. Preliminary results suggest that (1) 100 cDNAs can be pooled as probe to screen HDG, producing clean and unambiguous positives at a number equivalent to genome coverage of a library; (2) bad HDG filters account for most of the missing positives, sometimes resulting in more than 50% reduction of expected number of positive BACs; (3) EST primers confirm 80% (32/40) of positives with a few exceptions, possibly due to absence of one or both primers or cross-hybridization of homologous sequences. The pooling strategy has been used to map 343 chromosome 16 specific cDNAs on an human BAC library with 9 x coverage, identifying over 1500 BAC clones. They will be fingerprinted in order to verify and integrate these data into an existing chromosome 16 map.

Publications


Associate Professor: Paul W. Sternberg
Senior Research Fellow: Jane E. Mendel
Visiting Associate: Mark Viney
Research Fellows: Anna Newman, Maureen M. Barr, Marie-Anne Félix, René Garcia, Neil Hopper, Robert Palmer
Graduate Students: Aidyl Gonzalez-Serricchio, Charles Yoon, Chris Lacenere, Giovanni Lesa, Jing Liu, Keith Brown, Lily Jiang, Minqin Wang, Thomas R. Clandinin, Wen Chen, Chieh Chang

Research and Laboratory Staff: Gladys Medina, Lynn K. Carta, Yvonne Hajdu-Cronin, Shahla Gharib, Helen Walsh, Jeff Copeland, John DeMolena
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Summary: Our laboratory uses a molecular genetic approach to study basic questions in developmental biology, neurobiology and evolutionary biology: What are the molecular mechanisms of intercellular signaling? How is the fate of a cell specified in response to several intercellular signals? How do neurons control complex behavior? What types of changes in development occur during evolution? Our developmental studies focus on induction of the Caenorhabditis elegans hermaphrodite vulva and its connection to the uterus, and morphogenesis of the male copulatory spicules. Our behavioral studies focus on male mating behavior and sinusoidal movement. Our major strategy is to identify genes that control development or behavior by mutations that cause cells or animals to misbehave.

We have taken two main approaches to study behavior. In one approach, we are dissecting a complex behavior, mating, by cell ablations and genetic analysis (Abstracts 155, 156). We have found mutations that cause some aspects of behavior to occur inappropriately (Abstract 156), and this allows us to analyze behavioral pathways using the logic we have successfully applied to developmental pathways. In the second approach, we started with particular signaling proteins, heterotrimeric G proteins, and used molecular genetics to define their roles in nematode behavior including movement and egg-laying. (Abstracts 157, 158). Once we found phenotypes for mutant G proteins, we began to use extragenic suppressor screens to identify new components of G protein mediated signaling (Abstract 158).

Many of our studies address how certain cells signal other cells during development. We are trying to understand how the same molecular pathway acts in several different examples of induction. The development of the vulva, which allows hermaphrodites to release eggs and to copulate with males, provides an opportunity to study at single cell resolution the problem of induction. During C. elegans vulval induction, the anchor cell induces three epidermal precursor cells (the VPCs) to proliferate...
and generate vulval cells. The VPCs are of three types: 1° and 2° VPCs, which generate specific subsets of vulval cells, and 3° VPCs, which generate non-vulval epidermis. We have analyzed a number of genes that are required for vulval induction. lin-3 encodes a growth factor precursor comprising a single EGF repeat and a transmembrane domain; we have shown that the EGF repeat of lin-3 is the signal that induces vulval differentiation and that it can act in either a graded or sequential manner (Abstract 159). The two forms of LIN-3 are possibly responsible for the two phases of vulval induction in other species of nematodes which we have discovered (Abstract 160). We found that the VPCs lose their ability to respond to LIN-3 at about the time they undergo their first division (Abstract 161); we are beginning to explore the factors that limit VPC competence. We have identified new genes that regulate lin-3 by screening for extragenic suppressor mutations (Abstracts 162, 171). After vulval induction, the progeny of the three VPCs differentiate as vulval cells (of which their are 7 types), and undergo morphogenesis to form the mature vulva. Meanwhile, the ventral portion of the developing uterus similarly gets patterned by the anchor cell (Abstract 163). The uterus then connects to the vulva. We have studied genes necessary for this connection (Abstracts 164, 165). We are continuing to screen for additional genes necessary for vulval pattern formation and morphogenesis (Abstract 166).

let-23 encodes an EGF-receptor-like transmembrane tyrosine kinase and is likely the LIN-3 receptor. We have discovered that a number of genes negatively regulate this inductive signaling pathway, and are screening for more (Abstracts 167, 171). slt-1 encodes a homolog of the c-cbl oncogene of mammals (Abstract 168). rol-1 encodes a novel tyrosine kinase (Abstract 169). The lin-15 locus encodes two products that act upstream of let-23 to prevent vulval differentiation in the absence of inductive signal (Abstract 170). LET-23 is required for other aspects of development, including specification of cell fates during male spicule development and ovulation in the adult hermaphrodite. We have been studying male spicule development because it provides an opportunity not only to study the role of LIN-3 and LET-23 in another example of organogenesis, but also because of the crucial role spicules play in mating behavior. We have searched for genes that regulate spicule neuron specification (Abstract 172), and studied other genes that are involved in both spicule development and vulval formation (Abstract 173). The P12 neuroblast is specified by interactions among LIN-3/LET-23, HOM-C genes and a Wnt signaling pathway (Abstract 175). We have analyzed regions of LET-23 involved in its regulation and tissue-specific action (Abstract 174). This year we discovered the nature of the RAS-independent pathways from LET-23 involved in ovulation: this pathway involves regulation of intracellular calcium (Abstract 176). We have begun to search for new genes that act in this pathway (Abstract 23).

In confronting the post-genomic era of C. elegans biology, we are working on additional molecular genetic methods to analyze gene function in vivo. We are using C. elegans to try to understand the functions of genes associated with human cancer but for which the function is unknown (Abstract 178). We have adapted a method to visualized chromosomes using green fluorescent protein for C. elegans, and are working on adapting site-specific recombination systems to activate and inactivate genes at particular times in development (Abstract 179). Finally, we are using C. elegans vulval patterning as a proving grounds for computational models in collaboration with Professor Bruck's laboratory (Abstract 180).

155. Hermaphrodite signals for male mating. A cellular and genetic approach

Maureen M. Barr

The C. elegans male possesses a rich mating behavior repertoire. He responds to contact with a hermaphrodite by switching from forward to backward motion, turns at her ends (head or tail), stops moving upon contacting the vulva, inserts his spicules, and proceeds to transfer sperm. Male neurons necessary for the stereotypic mating ritual have been identified (Liu & Sternberg, 1995). Although the hermaphrodite is a behaviorally passive mating partner, her vulva and uterus provide cues to the male. The vulva may signal the male hook sensillum, causing the male to stop backing and to attempt spicule insertion. The uterus may signal male sperm release. We are interested in the cellular and genetic basis of both hermaphrodite cues and male responses.

To elucidate the nature of vulva and uterine cues, intact or neuron-ablated males were paired with ablated or mutant hermaphrodites. We found that the 2° vulva cells are both necessary and sufficient for vulva location. The male hook and post-cloacal sensilla may be involved in sensing and discriminating 2° cell characteristics. Males can transfer sperm to hermaphrodites with a defective vulval-uterine connection, suggesting the uterine signal may be diffusible. SPY-ablated males can transfer sperm to uterine-ablated hermaphrodites, supporting a role for SPY as a negative regulator of sperm transfer and sensor of an inhibitory uterine signal. We are now carrying out more refined ablation studies and behavioral analysis to limit these hermaphrodite cues to a single cell or minimal group of cells. We hope to determine whether chemosensation, mechanosensation, or both are involved in male behavioral responses. Additionally, we are performing a behavioral screen to identify mutants that are defective in vulva location or sperm transfer. Molecular cloning and characterization of genes required for mating behavior will address the
question of how an organism intercepts, interprets, and responds to various environmental stimuli.

Reference

156. Analysis of a substep involved in C. elegans male mating behavior
L. René García

We are interested in understanding the mechanisms that regulate complex behaviors. To achieve this goal, molecules involved in integrating information from several neuronal circuits must be identified and their functions determined. Male-mating behavior of Caenorhabditis elegans is an excellent model to identify components involved in coordinating complex behavior. The mating sequence consists of several substeps, and the male-specific sensory and motor neurons involved in initiating each one have been identified. To identify principles of behavioral regulation, aspects of mating behavior are being dissected and individually analyzed. We are initially focusing on defining a genetic pathway involved in executing the insertion of the males spicules into the hermaphrodite vulva. Our goal is to determine how this behavior is carried out at the molecular level, and how it is coupled to the preceding and following mating substeps.

Mutant males that have difficulty in inserting their spicules during mating have been isolated, and several cod (copulation defective) genes involved in this sub-behavior have been defined: cod-1, cod-3, cod-4, cod-7, cod-8, and cod-9 (Liu et al, in preparation). To facilitate the genetic analysis of these cod genes, we have isolated male mutants that protract their spicules in the absence of mating stimulus. From a preliminary screen of 4,000 EMS-mutagenized gametes, 3 mutant lines have been identified. 2 of the mutations are semi-dominant, and one is recessive.

To understand how these genetic lesions affect the ability of males to penetrate hermaphrodites, we are continuing our characterization of the sensory and motor neurons that function in this sub-behavior. From laser ablation analysis, we currently believe that the neurons involved in locating the hermaphrodite vulva also trigger the spicules to attempt insertion. Upon vulva stimulation, the spicules repeatedly prod the vulva until they partially penetrate through the vulval lips. We postulate that once partial penetration has occurred, the spicule motor neuron then facilitates full spicule extension into the hermaphrodite.

Reference
Liu et al., manuscript in preparation

157. Two C. elegans G proteins mediate neurosensory information that is transduced into a developmental program
Jane E. Mendel, Richard R. Zwaal, Ronald H. A. Plasterk

We have been examining the roles of several G protein α subunits in C. elegans. Heterotrimeric G proteins, composed of α, β, and γ subunits, couple to seven-transmembrane receptors, which are activated by several types of ligands including neurotransmitters, odorants, and pheromones. We have determined that two G protein α subunits, encoded by gpa-2 and gpa-3, function in a signalling pathway that leads to a developmental switch. Under conditions of overcrowding nematodes can form an alternate third larval stage called the dauer larva. Dauers are formed in response to a pheromone and to starvation. Both signals are detected by ciliated neurons involved in chemosensory. Worms transgenic for activating mutations in either gpa-2 or gpa-3 form constitutive pheromone-independent dauers, while those having deletions in either gene show a reduced response to pheromone. Animals deficient for both genes show a further reduction, but still form dauers in response to pheromone indicating additional pathways of pheromone response. Mutants of all classes respond normally to food under most conditions. Wild-type dauer larvae and those lacking gpa-3 cannot resume development in the presence of dauer pheromone. However, dauers lacking gpa-2 are able to recover in the presence of pheromone, indicating an additional role for gpa-2. Reporter-gene fusions indicate that both gpa-2 and gpa-3 are expressed in sensory neurons including those involved in chemosensation, and also in interneurons receiving synaptic input from sensory neurons. Interactions between mutations in gpa-2, gpa-3 and other genes involved in dauer formation indicate that both genes function in ciliated neural endings, and thus act in signal transduction events preceding the dauer decision. These two G proteins appear to act in parallel. However, transgensics bearing both activated gpa-2 and activated gpa-3 show mutual suppression rather than additivity, suggesting common negative regulation.

Reference
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158. Suppressors of activated C. elegans Goα
Wen Chen, Yoosem M. Hajdu-Cronin

Goα is involved in several C. elegans behaviors such as egglaying, movement and feeding (Mendel et al., 1995; Segalat et al., 1995). We isolated 24 independent mutations that revert the phenotype of syis17 (a Goα gain-of-function transgenic integrant under the control of a heat shock promoter). 21 alleles are from 21,000 EMS-mutagenized gametes and 3 alleles are from 11,000 psoralen-UV-induced gametes. These define 8
complementation groups and can be divided into 3 classes according to their effects. Class 1 includes sag-1 X and sag-2 I. They are hyperactive, egg-laying constitutive and have empty uteri; as are Goa(lf) mutants. Class 2 mutants, sag-3 I, sag-5 III and sag-7 X, mimic the phenotype of Goa activated in the egg-laying deficiency and drug resistance. Class 3 includes sag-4, V and sag-6. They are nearly wild type in appearance and behavior. The phenotype of sag-8 is under characterization. Double mutants between different classes suppress syis17 better than when they are alone, indicating that the three classes of genes act in different aspects of Goa regulation. Class 1 may encode components of the Goa pathway in the tissues where Goa is usually expressed since they suppress syis9, an activated Goa mutant under its own promoter. Class 2 and 3 may function in other tissues which, when Goa is misexpressed, causes lethality and a more severe defect in movement and feeding than syis9. We are mapping these genes further and we are in the process of cloning sag-1, sag-2 and sag-4.

References

159. lin-3 encodes one short and one long range signaling molecule
Jing Liu
lin-3 function is required for viability, fertility, vulva and male spicule development in C. elegans, and it is under tissue-specific regulation. We have been interested in how lin-3 is regulated at its protein level to accommodate the whole spectrum of its functions. Two LIN-3 proteins exist due to alternative splicing, one has 15 extra amino acids between the EGF motif and the transmembrane domain. Since growth factor precursors are cleaved in this region to generate secreted signals, we studied whether these two proteins have different cleavage properties. We constructed transgenic animals carrying either the short or the long form of LIN-3 (LIN-3S and LIN-3L, respectively). Genetic and laser-ablation experiments show that LIN-3S can induce vulva at a distance, but LIN-3L induces vulva only when the VPCs are in close proximity to AC. It is possible that LIN-3S is a diffusible signal and LIN-3L is membrane-anchored. The cytoplasmic domain is required for the long-range signaling of LIN-3, presumably by facilitating interactions between LIN-3 and its protease. A truncated LIN-3 in the cytoplasmic domain can still induce vulva but functions as a short-range signal. The far-acting LIN-3 can induce vulva independent of the LIN-12-mediated lateral signaling pathway, presumably inducing each VPC directly; the short-range signal, on the other hand, only induces one VPC which is closest to AC and this VPC will induce its neighboring VPCs using the LIN-12 pathway.

The two signals may provide two somewhat redundant modes of vulval induction to ensure the formation of an invariant vulval pattern. They may also have unique functions in other tissues. A mutation that deletes most of the cytoplasmic domain results in animals that are sterile but only weakly defective in other tissues, raising the possibility that the long-range signal is absolutely required for fertility, but not the other aspects. This may provide an interesting example of how a ligand in the signaling pathway is regulated to generate signaling specificity.

160. Evolutionary variations in nematode vulva patterning mechanisms
Marie-Anne Félix, Mark Viney
To understand how developmental mechanisms vary during evolution, we compared the mechanism of vulva induction of different nematode species and have initiated genetic analysis in one species other than C. elegans. During vulva development, Pn.p precursor cells acquire one of three fates in a symmetric pattern centered around the gonadal anchor cell: non-vulval fates at the periphery, outer and inner vulval fates towards the center. In C. elegans, the three fates are specified around the same time by cell interactions: a graded induction by the anchor cell and lateral signaling of P6.p to P5.p and P7.p. We find that instead, in three other nematode species spanning two families, a first gonadal induction specifies vulval vs. non-vulval fates, and a second induction, localized to a subset of the Pn.p daughters, specifies inner vs. outer vulval fates.

To understand the molecular nature of the 2 signals from the anchor cell and their relation with the pathways known in C. elegans, we have started a genetic and molecular analysis of 2-step vulva induction in Oscheius n. sp. CEW1. So far, we have isolated in a random F2 screen several vulva mutations and 35 marker mutations (a spectrum similar to C. elegans). The first characterized vulva mutation (sy447) affects the first induction only: the outermost cells adopt the outer vulval fate instead of a non-vulval fate. We are also developing molecular tools in this species, such as a cDNA library and transformation, and cloning homologs of molecules involved in vulva induction in C. elegans, such as LIN-3 and Ras.

161. Competence of VPCs during C. elegans vulval induction
Minqin Wang
The C. elegans vulva is induced by a lin-3-encoded EGF-like growth factor produced by the anchor cell (AC). Each vulval precursor cell (VPC) is competent to respond to LIN-3 by adopting an either 1° or 2° fate during late L2 to early L3 stage. The AC is necessary for wild-type vulval induction until about one hour before the VPCs divide. By expressing the EGF domain of LIN-3 under the control of an hsp16 promoter, we found that the VPCs lose their competence to respond to LIN-3 at or about their first division. The LIN-12 protein promotes differentiation of the 2° cells. To test the effect of activated LIN-12, we did a similar
expression of a lin-12 gain-of-function background. Based on observation of characteristic $1^\circ$ lineages, expression of a $1^\circ$ specific marker and adult vulval morphogenesis, we conclude that LIN-12 activity extend the end of the VPC competence after their first division.

One model to explain this result is that LIN-12 delays cell commitment to the $3^\circ$ fate, and maintains competence of uncommitted VPCs to respond to later signals. A second model suggests that LIN-12, like its homologs, might promote cell proliferation. By stimulating the cell cycle, LIN-12 might drive VPCs along what is at first a common pathway, and thus remain competent to respond to LIN-3. To examine the issue of whether the effect of LIN-12 in extending the window of VPC competence is due to an effect of LIN-12 stimulating the cell cycle, we are testing the effect of simply driving cell cycle using a cul-1 mutation. A third model proposes that LIN-12 has dual roles of both promoting the $2^\circ$ fate and inhibiting the $3^\circ$ fate by preventing VPC fusion with hyp-7. To test this model, we are examining the competence of VPCs when their fusion has been inhibited by overexpression of a HOM-C gene using a hs-LIN-39 construct.

**162. Further Analysis of slm-1, a transcriptional regulator**

Chieh Chang, Jing Liu

The C elegans vulva is induced by the anchor cell and requires expression of lin-3 within the anchor cell. lin-3 encodes a transmembrane protein with one EGF motif. syis1 has multiple copies of the lin-3 gene integrated into genome resulting in a multivulva phenotype. The slm-1 locus was identified in a screen for suppressors of the syis1 Muv phenotype. Since slm-1 does not suppress mutations in let-23 but does affect the anchor cell expression of lin-3::lac-Z when expressed under lin-3 cis-regulatory region, we inferred that slm-1 works upstream of let-23, potentially as an anchor cell specific transcription factor. More recently, we have made a longer lin-3 construct containing approximately 2 kb 5' to the transcriptional start site and fused it with lacZ cassette in the first cytoplasmic domain. This construct is expressed in several tissues as we mostly expected from previous genetic analysis. We find that slm-1 is not only necessary for detectable lin-3::lacZ expression in the anchor cell but also in other tissues. To test whether slm-1 is specific to lin-3 we tested other lacZ constructs and find that slm-1 mutant lacks lag-2::lacZ expression in the gonad as well as the ventral cord. Furthermore, slm-1 mutant lacks lin-11::lacZ expression in the gonad as well as in the vulva. These results demonstrate that slm-1 does not encode an anchor cell specific transcription factor but rather a more general regulator of gene expression.

**163. Morphogenesis of the C. elegans hermaphrodite uterus**

Anna Newman, John White, Paul W. Sternberg

We have undertaken electron micrographic reconstruction of the Caenorhabditis elegans hermaphrodite uterus and determined the correspondence between cells defined by their lineage history and differentiated cell types. In this organ, many cells do not move during morphogenesis and the cell lineage may function to put cells where they are needed. Differentiated uterine cell types include the toroidal ut cells that make structural epithelium, and specialized utse and uv cells that make the connection between the uterus and the vulva. A cell fate decision in which the anchor cell (AC) induces adjacent ventral uterine intermediate precursor cells to adopt the $\pi$ fate, rather than the ground state $\rho$, has profound consequences for terminal differentiation: all $\pi$ progeny are directly involved in making the uterine-vulval connection whereas all $\rho$ progeny contribute to ut toroids or the uterine-spermatic valve. In addition to specifying certain uterine cell fates, the AC also induces the vulva. Its multiple inductions thereby function to coordinate the connection of an internal to an external epithelium. The AC induces the $\pi$ cells and ultimately fuses with a subset of their progeny. This is an example of reciprocal cell-cell interaction that can be studied at single cell resolution. The AC is thus a transitory cell type that plays a pivotal role in organizing the morphogenesis of the uterine-vulval connection.

**164. The PAX gene egl-38 mediates developmental patterning in Caenorhabditis elegans**


Mutations in the C. elegans gene egl-38 result in a discrete set of defects in developmental pattern formation. In the developing egg-laying system of egl-38 mutant hermaphrodites, the identity of four uterine cells is disrupted, and they adopt the fate of their neighbor cells. Likewise, the identity of two rectal epithelial cells in the tail is disrupted, and one of these cells adopts the fate of its neighbor cell. Genetic analysis suggests that the egl-38 functions in the tail and the egg-laying system are partially separable, as different egl-38 mutations can preferentially disrupt different functions. We have cloned egl-38 and shown that it is a member of the PAX family of genes, which encode transcription factors implicated in a variety of developmental patterning events. The predicted EGL-38 protein is most similar to the mammalian class of proteins that includes PAX2, PAX5, and PAX8. The sequence of egl-38 mutant DNA indicates that the tissue-preferential defects of egl-38 mutations result from substitutions in the DNA-binding paired domain of
the EGL-38 protein. egl-38 thus provides the first molecular genetic insight into two specific patterning events that occur during C. elegans development and also provides the opportunity to investigate the in vivo functions of this class of PAX proteins with single cell resolution.

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165. cog-1 encodes a homeodomain protein

Robert Palmer, Lily Jiang, Paul Sternberg

We discovered the C. elegans cog-1 locus because it is necessary to form the connection between the gonad and the outside. In the hermaphrodite, cog-1 is required for the anchor cell to invade the vulval epidermis. In the male, cog-1 is required for the gonadal linker cell to reach and attach to the developing proctodeum. Because cog-1 is necessary for two analogous processes, connection of tissues during organogenesis, we have cloned molecularly the cog-1 locus. We found that it encodes a homeodomain protein related to human Gtx-1, defining a divergent subfamily of these putative transcriptional regulators. Using a GFP reporter construct that rescues the cog-1(sy275) mutant, we found that cog-1 is expressed in the vulval epidermis but not in the anchor cell; in the male, cog-1::GFP is expressed in the developing proctodeum but not the linker cell. Thus, it is likely that cog-1 controls genes involved in signals from the vulva to the anchor cell and from the proctodeum to the linker cell, as well as being involved in the differentiation of these non-gonadal tissues. We previously found that cog-1 is required for some aspects of the 2° VPC lineage.

166. Genes involved in commitment and execution of VPC fates

Minqin Wang

The development of the hermaphrodite vulva involves several steps. One relatively late step independent and genetically separable from cell fate specification is execution of terminal vulval cell fates. I am interested in identifying components of this pathway with 1° or 2° specific functions. Candidate genes should be those that specifically affect either 1° or 2° fate and make VPCs adopt a similar but defective fate, compared with a canonical fate, when mutated. To isolate such genes, I started with a wild-type strain with a reporter gene specific for vulval cell fates: egl-17::gfp that expresses the green fluorescent protein under the control of the egl-17 control region. This marker can be used to indicate both 1° and 2° fate, since GFP expression is detectable in all 1° VPC progeny during L3 and in a subset of 2° VPC progeny (N and T) during L4. I have performed a genetic screen looking for mutants with protruding vulva (Pvul) and abnormal expression pattern of egl-17::gfp. I expect two classes of mutants to be recovered, including mutants of 1° or 2° specific defects. From 12,500 haploid genome screened, I have found four mutants of the second class, all with similar phenotype. They have egl-17::gfp expression missing in 2° cells, abnormal vulval morphology and disrupted connection between vulva and uterus. Two of these mutations are alleles of egl-29 and the other two are alleles of lin-11. Interestingly, the double mutant displays severe defects of 1° cells based on cell lineage, expression pattern of another 1° specific marker and late morphogenesis, while null alleles of the single mutants show only subtle defects of 1° cells if there is any. This result suggests that the functions of egl-29 and lin-11 in execution terminal 1° fate might be partially redundant, although both genes are required for terminal 2° fate. To further understand the pathway leading to the final 1° and 2° fates, I am currently examining the possible functions of other genes, including egl-38 and cog-1, that might be involved in this pathway.

167. Negative regulation of vulval development

Chris Lacenere, Jeffrey Copeland, John DeModena, Charles Yoon, Neil A. Hopper

Several negative regulators of vulva induction have been identified. These include sli-1, sli-2, rok-1, rok-2, and unc-101. Mutations in a single gene causes little or no vulval phenotype. However, in general, any combination of two or more of these mutants causes a synthetic multivulva (Muv) phenotype. A general strategy for identifying new negative regulators of vulval induction has involved the mutagenesis of a single mutant, such as sli-1, and screening for multivulva animals. We have taken a novel approach to perform a saturation screen for negative regulators of vulval induction while reducing the frequency of lin-2, lin-7 and lin-10 mutations. lin-2, lin-7, and lin-10 activity is required in the vulva precursor cells for proper processing of the anchor cell signal. It has recently been reported that overexpression of the receptor, let-23, rescues the vulvalsless phenotype of lin-2 and lin-7 mutations (Simske et al., 1996). We hypothesized that introduction of a high copy let-23 transgene into a sli-1 background would eliminate or reduce the frequency of lin-2, lin-7, and lin-10 mutations in a screen for Muv animals. lin-2; sli-1 is not Muv in a let-23 overexpression background. Three mutations have been isolated and are currently being characterized. We are in the process of cloning sli-2 based on its genetic map position.

We are also interested in the mechanisms of EGF receptor endocytosis and sorting and the effects of receptor transport on signaling. The hermaphrodite vulva offers a convenient system to study signaling from an EGF receptor, LET-23. Genetic screens are being pursued in order to dissect these pathways. We are also using the two-hybrid system to test interactions between proteins potentially involved in receptor transport.

Reference

168. Genetic and molecular studies of SLI-1, a negative regulator of the LET-23-mediated vulval induction

Charles H. Yoon

SLI-1 is a negative regulator of the LET-23 receptor tyrosine kinase (RTK) mediated vulval signaling (Jongeward et al., 1995). Reduction-of-function mutations in sli-1 can suppress the vulvaless phenotype associated with non-null, reduction-of-function mutations in let-23, a homolog of the mammalian EGFR receptor. SLI-1 has sequence similarity to the mammalian proto-oncoprotein, c-Cbl (Yoon et al., 1995). Oncogenic mutations in Cbl are thought to be involved in pre-B and pro-B lymphomas. We have shown that in the vulva, sli-1 mutations fully suppress severe reduction-of-function and null mutations of sem-5. SEM-5 is a homolog of the mammalian adaptor Grb2. We have also found however, that wild-type sem-5 is required for sli-1's suppression of the Vul phenotype associated with receptor mis-localization. sli-1 mutations do not suppress the severe reduction-of-function and null mutations of let-60, which encodes the nematode homolog of the mammalian Ras protein. Yeast two-hybrid assays suggest that SLI-1's C-terminal proline domains can interact with SEM-5's C-terminal SH3 domain. Structure-function studies of SLI-1 show, however, that the domains which interact with SEM-5 are not essential for SLI-1 wild-type function in vulval differentiation. Our results suggest that SLI-1 negatively regulates a SEM-5-independent signaling pathway between LET-23 RTK and LET-60 Ras and that this alternate pathway requires wild-type LET-23 receptor localization. We suggest that SLI-1/c-Cbl class of proteins can restrict RTK-mediated signaling to a subset of potential effectors used in Ras activation.

References

169. The tyrosine kinase ROK-1 negatively regulates LET-23 mediated signalling

Neil A. Hopper

The let-23 gene (a member of the EGFR family of receptor tyrosine kinases) encodes the receptor for the signal produced from the anchor cell that induces vulval differentiation. Genetic analyses have identified several negative regulators of let-23 mediated induction. The rok-1 locus was identified in a screen for mutations that result in excessive vulval differentiation in a sensitized background carrying sli-1(sy143) (mutations in the sli-1 locus alone are silent). A loss of function mutation in the rok-1 locus (sy247) in the absence of other mutations is phenotypically silent at 20°C; however, at 25°C some animals with excessive vulval differentiation are observed. Likewise, rok-1(sy247) in trans to a deficiency results in a low penetrance hyperinduced phenotype at 20°C. rok-1(sy247), to varying degrees, is able to suppress the vulval differentiation defect observed in animals carrying non-null mutations in lin-3, let-23 and sem-5 but is unable to suppress mutations in let-341 and let-60 ras. Thus, we believe that rok-1 (for regulator of kinase mediated signalling) acts upstream of ras. We have cloned the rok-1 locus and found it to encode a single polypeptide of 1043 amino acids predicted to contain tyrosine kinase activity, an SH3 domain and a proline rich carboxy-terminus. ROK-1 interacts with SEM-5 in the yeast two hybrid system. This interaction has been mapped to a 50 amino acid region at the C-terminus of ROK-1 and the C-terminal SH3 domain of SEM-5. We speculate that this interaction mediates recruitment of ROK-1 into the receptor complex whereby negative regulation occurs by some other means.

170. The function of the LIN-15 proteins

Aidyl S. Gonzalez-Serricchio

In Caenorhabditis elegans the lin-15 locus encodes two proteins (LIN-15A and LIN-15B) from non-overlapping transcripts, each of which encodes a redundant but novel protein involved in negatively regulating vulva development. Genetic epistasis experiments revealed that the proteins act at the LET-23 receptor level, parallel to the LIN-3 signal (Huang et al., 1994). Antibody experiments showed that the LIN-15B protein is a nuclear protein expressed everywhere except pharyngeal cells and the developing germline (Huang, 1995). Initial antibody work for the LIN-15A protein indicates that it is nuclear and its expression sites are similar to those of LIN-15B. My research is focusing on how lin-15 negatively regulates the let-23-mediated signal transduction pathway. I will determine which cells require either lin-15A or lin-15B activity by mosaic analysis. I will also localize the expression of each transcript by removing its native promoter and replacing it with tissue-specific promoters. Another focus of my research is how lin-15 locus affects fertility. The brood size of the lin-15 null allele is more than 50% smaller than the brood size of wildtype worms at 20°C. The lin-15 null alleles are sterile at 25°C. I have not yet determined whether the low brood size is due to the number of sperm, the inability of the oocyte to be fertilized, or is it due to malformed spermathecae? I will screen for mutations that suppress the sterility to define another pathway regulated by lin-15.

References

171. The vulval induction pathway is regulated negatively at multiple steps

Jing Liu

The precise pattern of the vulva is crucial in determining a hermaphrodite's brood size and therefore one aspect of its evolutionary fitness. The activity of the vulval induction pathway is under heavy regulation to ensure that it is not excessive nor insufficient. Several negative regulators of the pathway have been identified which act at the level
of let-23. By screening for suppressors of severe loss-of-function of lin-3, I found three new genes, nrl-1(IV), nrl-2(X) and nrl-3(II), that negatively regulate the vulval induction pathway. Each of these negative regulators only suppresses a subset of lin-3 phenotypes and are hence tissue-specific. nrl-1 functions upstream of let-23, nrl-2 appears to function on let-60 Ras, and nrl-3 exerts its negative regulation downstream of lin-25, which is a transcription factor very downstream of the pathway. It is interesting to find that although the negative regulators don’t function at the same level in the pathway, they have synergistic effects, for example, nrl-3 interacts with sli-1 and rkt-1, which function at the level of let-23, to result in a Muv phenotype. This suggests that the negative regulation of the pathway works in a dosage-sensitive fashion.

172. Study of neurogenesis in male spicule development

Lily Jiang

SPD, SPV and SPC are three spicule associated neurons in C. elegans males responsible for multiple steps during male mating behaviors including spicule insertion and sperm transfer. These three neurons are generated from β and ζ sublineages, descendants of the male B blast cell. β and ζ sublineages share similarity with the Drosophila sensory organ precursor lineage, where one cell divides twice to generate neuron and non-neuronal structural cells, sheath and socket. We are interested in how these neuronal precursors are generated, and how the neuronal vs. non-neuronal fates are determined.

Previous studies have shown that β and ζ fates are specified via multiple cell-cell interactions. We are using a spicule neuron-specific molecular marker, gpa-1, to further analyze these lineages. Cell ablation experiments have shown that gpa-1::lacZ is expressed in SPD neurons specifically, while gpa-1::GFP is expressed in all three spicule associated neurons. Using this molecular marker, we have started an F1 clonal screen to look for mutants with misexpression of this marker. From a pilot screen of 3,200 gametes, several classes of mutants have been isolated, including those that lack gpa-1 expression and those with additional expression specifically in the spicule region. One mutant, 3J7, loses the gpa-1::lacZ expression in SPD neurons. Although it has various lineage defects in F, U, and β lineages, ζ lineage seems to be normal except the axis of the final cell division is different from wild type. This mutant fails to complement a lin-48 allele. Another mutant, 2M10, shows extra staining of the gpa-1::lacZ marker in the spicules, 2 cells per spicule instead of 1. Lineage analysis did not reveal any abnormality and the spicule morphology appears to be normal. These mutants could be candidates for terminal cell fate specification defects. Further analysis of these mutants using other molecular markers, vital dye and behavior assay are undergoing. Characterization of other mutants will be pursued.

173. Cell-cell interaction and cell fate specification in the C. elegans male

Keith B. Brown

I have been working to elucidate signal integration as it applies to cell specification during development of the male tail. Many signals act concurrently to generate the copulatory structures of the male. The anterior daughter of the C. elegans B cell (B.a) generates the copulatory spicules of the male tail by producing 8 cells to form four equivalence groups (dorsal, ventral, and two lateral pairs). The cells communicate through a signal transduction system which governs the B.a cell progeny fate. Three more male-specific blast cells provide signals to help pattern the system (F, U, and Y). I have been studying two such genes.

One such mutant (sy287) has a 100% penetrance of crumpled spicules in the male with no lethality or egg-laying defects. After, mapping the mutation to a small region on the LG IV, I performed complementation tests and found it to be an allele of egl-38, a pax2/pax5/pax8 homologue (see abstract 10). sy287, cells F and U are misspecified. Since these cells provided a key signal for the B.a progeny, this signal is most likely lost. Furthermore, the B.a progeny fail to migrate to their proper pattern at the late L2 stage of larval development. It remains to be seen if this is directly or indirectly. Since cells F and U are born before the larva hatches, it is possible that egl-38 plays a key role in the early patterning of the system.

Another mutation, vex-1(sy207) was first identified due to its affect on primary-fated vulva precursor cells. The vulva lineage defect is failure to complete the third round of division of any cell assuming a primary fate. Development of the spicules and male hook involve equivalence groups similar to the vulva and are subsequently affected in vex-1(sy207) mutants. We have mapped vex-1(sy207) to LG II to the right of rol-6. Furthermore, sy207/Df has a similar phenotype as the mutant homozygote, consistent with it decreasing vex-1 function.

174. In vivo signaling by LET-23, a C. elegans Epidermal Growth Factor receptor

Giovanni M. Lesa

The major determinant of receptor tyrosine kinase (RTK) signaling specificity have been proposed to be Src Homology 2 (SH2) binding sites, phosphotyrosine-containing oligopeptides in the cytoplasmic domain of the receptor. The C. elegans Epidermal Growth Factor receptor (EGFR) homolog, LET-23, has multiple functions during development and has eight potential SH2 binding sites in a region carboxyl terminal to its kinase domain. By analyzing transgenic nematodes for three distinct LET-23 functions, we have shown that six of eight potential sites function in vivo and that they are required for most, but not all, of LET-23 activity. A single site is necessary and sufficient to promote wild-type fertility. Three other sites activate the
RAS pathway and are involved only in viability and vulval differentiation. A fifth site is promiscuous and can mediate all three LET-23 functions. An additional site mediates tissue-specific negative regulation. Putative SH2 binding sites are thus key effectors of both cell specific and negative regulation in an intact organism. We suggest two distinct mechanisms for tissue-specific RTK-mediated signaling. A positive mechanism would promote RTK function through effectors present only in certain cell types. A negative mechanism would inhibit RTK function through tissue-specific negative regulators.

175. Interactions between EGF, Wnt, and HOM-C genes specify P12 neuroblast fate in C. elegans.
Lily Jiang, Paul W. Sternberg
Temporal and spatial interactions between multiple intercellular and intracellular factors are important to control cellular behaviors. Here we investigate how these factors act together to specify P12 neuroblast fate in C. elegans. P11 and P12 are the most posterior pair of postembryonic ventral cord precursors in C. elegans. They form an equivalence group: when either one is ablated before it enters the ventral cord, the other one expresses the P12 fate. Three classes of genes are involved in the specification of P12 fate: genes of the lin-3/let-23 epidermal growth factor signaling pathway, a Wnt gene lin-44 and its putative receptor gene lin-17, and a homeotic gene egl-5. We show that LIN-3 is an inductive signal sufficient for the P12 fate. Overexpression of LIN-3 leads to P11 to P12 fate transformation, whereas inactivation of the pathway results in P12 to P11 transformation. Epistasis analysis indicates that the lin-3/let-23 pathway specifies the P12 fate the same way as it does in vulval induction. HOM-C gene egl-5 is a downstream target of the lin-3/let-23 pathway in executing P12 fate. egl-5 is epistatic to the lin-3/let-23 pathway, overexpression of EGL-5 suppresses the P11/P12 defect caused by let-23 mutation, and egl-5 transcription can be turned on in P12 lineage upon activation of the lin-3/let-23 pathway. Temporal control of egl-5 expression is crucial for its function. Wnt signal LIN-44 acts through its putative receptor LIN-17 in P12 fate specification. The lin-44/lin-17 Wnt signaling pathway acts synergistically with the lin-3/let-23 EGF signaling pathway but at a different developmental time. It might regulate egl-5 expression at early stage thereby regulate the competence of the cell to the lin-3 signal indirectly.

176. Tissue specific suppressors of lin-3
Tom Clandinin
Many of the genes required for vulval induction act in other tissues; mutations in these genes can affect development of the male tail, the hermaphrodite gonad and the early larva. How is the activity of this highly conserved signal transduction cascade interpreted differently by different cells? I have taken a genetic suppression approach to these questions. Starting with relatively pleiotropic mutations that affect many cell fate decisions, I have screened for second site suppressors that revert some but not all of the defects caused by the original mutation. Such mutations should identify cell-type specific targets or regulators of the conserved signal transduction cascade. The let-23 receptor couples to distinct downstream effectors in different tissues. In particular, it appears that one set of downstream effectors is used by the receptor to mediate its gonad function while a separate set, including RAS and RAF, mediate vulval development. I am interested in identifying components of the hermaphrodite fertility pathway.

To identify such components, I began with a reduction of function mutation affecting the lin-3 gene. lin-3 (n1058) is a mutation in the vulval induction signal that also causes defects in the hermaphrodite gonad. By screening for suppressors that cause lin-3 (n1058) animals to become fertile, I have identified seven tissue specific suppressors that define two loci, designated ife-1 and ife-2. These loci function downstream of let-23 by genetic criteria. I have cloned both ife-1 and ife-2 and shown that they encode gene products involved in regulating intracellular calcium. Our current hypothesis is that the normal function of the LIN-3/LET-23 pathway in the gonad is to control the muscle contractions involved in ovulation through direct manipulation of the intracellular calcium concentration in the somatic gonad.

177. Screen for genes involved in let-23-mediated fertility
Giovanni M. Lesa, Thomas R. Clandinin
let-23 encodes an Epidermal Growth Factor Receptor homolog required for at least five functions in C. elegans: viability, vulval development, fertility, specification of the P12 neuroblast, and formation of specialized sexual structures in males. let-23 is known to mediate all these functions except fertility employing a pathway highly conserved in evolution which includes let-60 ras. Classical and molecular genetic experiments have suggested that let-23-mediated fertility employs a set of genes different from the ras pathway. Indeed, two suppressor loci of sterile lin-3 (the LET-23 ligand) and a sterile let-23 allele have been identified that do not interfere with the ras-mediated functions, ife-1 and ife-2 (ife=Let-23 fertility effector). Moreover, distinct let-23 domains are required to activate fertility and the ras-mediated functions. To find additional, positively acting components of the let-23 fertility pathway (gene "X"), we screened for sterile mutations that can be suppressed by ife-2. To this end we mutagenized a strain of the relevant genotype ife-2; syEx[ife-2(+), rol-6] where syEx represents an extra-chromosomal array. We screened F2 progeny for presence of animals that roll (Rol phenotype conferred by rol-6) and are sterile ["X" not suppressed because of ife-2(+)] and absence of animals that do not roll and are sterile ["X" suppressed
because there are no copies of \(lfe-2(+)\). We screened ~8,000 mutagenized gametocytes and found one putative candidate mutation, sy499. Animals homozygous for sy499 are sterile. sy499 is almost completely suppressed by mutations in both \(lfe-1\) and \(lfe-2\). We hypothesize that the sy499 mutation defines a gene required for LET-23-mediated fertility.

**178. Molecular genetics of cancer genes of unknown function**

*Jane Mendel, Paul Sternberg, Shahla Gharib, L. René Garcia*

An increasing number of genes whose expression is up or down regulated in the progression from normal to cancer cells are being identified. One challenge is to embed these genes into the context of a pathway. *C. elegans* molecular genetics is a useful tool towards this goal. It is relatively easy to obtain transgenic worms, and more than one gene can be included in one transgene. We have begun screening for functions of cancer-associated human genes. We express human cDNAs under the control of a strong inducible promoter/enhancer in transgenic *C. elegans* and examine the strains after induction of expression for defects in growth, development and behavior. So far we have seen only minor defects. Now that over 75% of the *C. elegans* genome is sequenced, a second approach is to study the *C. elegans* homologs of human genes associated with cancer. We will continue the cDNA expression approach for genes with no matches to *C. elegans* genes, and begin to analyze the *C. elegans* genes with reasonable degree of similarity at the amino acid level.

**179. Conditional transgene expression in *C. elegans***

*John DeModena*

The ability to have tight control over individual genes being studied would greatly facilitate analysis of gene function. To date, inducible expression of transgenes in *C. elegans* has been limited to heat shock promoters. While a very powerful tool, they are rather limited in that they allow us to turn on genes for a (often) variable amount of time, are not always at reproducible, or can have undesirable pleiotropic effects on the worms. The aim of this project is to develop a system allowing for conditional expression of transgenes in *C. elegans*. One way to lock in transient expression is to alter the DNA sequences using a site-specific recombination system. We are therefore attempting to develop a similar system in *C. elegans* which has been proven effective in other complex eukaryotic systems such as *Drosophila* and mammalian cells. Our method is to adapt the FLP recombination system from the yeast *S. cerevisiae* to function in worms. FLP is a site specific recombinase that acts on two copies of a specific target sequence (FRT). These can be in direct or inverted orientation. Recombination between direct repeats will cause excision of the intervening DNA and one of the two FRTs. This allows us to regulate gene activity by being able to remove regions of DNA which we have engineered to inhibit transcription by simply inducing the FLP recombinase. Conversely, gene activity can be stopped by recombination which removes promoters or other regulatory regions, quickly and effectively. We are first trying to control FLP recombinase activity using a heat shock promoter, as it is a well proven inducible promoter.

**180. Modeling vulval pattern formation**

*Paul Sternberg, Mingqin Wang, Martha Kilowac*

*C. elegans* vulval fate patterning involves several intercellular signaling systems that combine to specify precisely a spatial pattern of cell fates: \(3°-3°-2°-1°-2°-3°\). These signals include: LIN-3 and LET-23 mediated inductive signaling from the anchor cell to the vulval precursor cells (VPCs), LIN-12-mediated lateral signaling among the VPCs, and a negative signal mediated by lin-15 and related genes. Information concerning the pattern formation process has gone beyond intuitive models, and we have therefore initiated a computational approach in collaboration with Michael Gibson and Jehoshua Bruck in the CNS program. The challenge is to devise an appropriate representation of vulval patterning that will allow exploration of particular models without exact description of all parameters. We are using an efficient molecular marker of VPC fate to allow us to generate sufficient quantity of experimental data, in the form of distribution of fate patterns after particular perturbations such as varying the strength and duration of the LIN-3 inductive signal, to allow some parameter fitting and the testing of predictions. For example, in a gonad-ablated lin-15 mutant, the pattern of VPC fates are stochastic in a spacing pattern that depends on LIN-12-mediated lateral signaling (e.g., \(2°-1°-2°-1°-2°-1°\) or \(1°-2°-2°-1°-2°-1°\)).

**181. Evolution of the neuronal control of nematodes male mating behaviors**

*Alan C. Cheng*

The objective of this project is to study the neuronal control behavior of the species PS1010 and compare it with that of *C. elegans*. These are closely related species, yet their mating behaviors are very different: *C. elegans* mates with partner aligned in parallel, while PS1010 mates with the male perpendicular to the female. We have characterized PS1010 mating behavior: the male responds to contact with the female, backs underneath or above her body, coils around her body, searches for the vulva by moving circularly around the female, locates the vulva, inserts the two copulatory spicules into the vulva, and transfers sperm. Many of the neurons that control mating behavior in *C. elegans* have been identified by killing individual neurons or their precursor cells using a laser microbeam. We used this technique to identify the neurons that control the unique mating behavior of PS1010. Ablation of B.a and of P11.p results in similar behavioral defects in both PS1010 & *C. elegans* males. Ablation of B.a in both species does not impair vulva
also observed evolutionary divergence of the function of P9.p and P10.p. In C. elegans P9.p & P10.p generate the hook sensillum and are essential for effective vulva location, but in PS1010, males with ablated P9.p & P10.p demonstrated no behavioral or mating efficiency defects. This implies that P9.p and P10.p may have a different function and that other neurons may control vulva location behavior. These results demonstrate that both similarities and differences in how sensory structures control mating behavior between PS1010 and C. elegans.

Publications

Summary: We are interested in just about everything. But the brevity of life being what it is compels selectivity. Hence our current subject — the ubiquitin system — chosen partly by preference and partly by accident. During the 1980's, my colleagues and I produced the first evidence that ubiquitin is required for protein degradation in living cells. We also discovered the first degradation signal of the ubiquitin system, its first physiological functions, and its crucial mechanistic attributes such as the multi-ubiquitin chain and subunit selectivity of proteolysis. Our present work continues this line of studies. In addition, we are exploring new ways to improve the selectivity of drugs against currently intractable diseases. The abstracts below reflect this spectrum of interests.

The effect of a given intracellular protein on the rest of a cell is determined in part by the concentration of active protein molecules. The latter is determined by the rate of synthesis of the protein in relation to the rates of its degradation, inactivation by other means, or export from the compartment. The in vivo half-lives of proteins in a cell range from a few seconds to many days. Over the last decade, a number of regulatory circuits were shown to involve metabolically unstable proteins. A short in vivo half-life of a regulator provides a way to generate its spatial gradients and allows for rapid adjustments of its concentration (or subunit composition) through changes in the rate of its synthesis. A protein can also be conditionally unstable, i.e., long-lived or short-lived depending on the state of a cell. One example of the latter class is cyclins — a family of related proteins whose destruction at specific stages of the cell cycle regulates cell division and growth.

Features of proteins that confer metabolic instability are called degradation signals, or degrons. The essential component of one degradation signal is a destabilizing N-terminal residue of a protein. This signal is called the N-degron. Within a species, the set of different destabilizing residues yields a rule, termed the N-end rule, which relates the in vivo half-life of a protein to the identity of its N-terminal residue. In eukaryotes, the N-end rule pathway is a part of the ubiquitin (Ub) system. Ub is a 76-residue protein whose covalent conjugation to other proteins plays a role in a legion of processes, including cell growth, differentiation, and responses to stress. In many of these settings, Ub acts through routes that involve the degradation of Ub-protein conjugates by the 26S proteasome — a multisubunit, ATP-dependent protease.

Biochemical and genetic studies of the N-end rule pathway and other aspects of the Ub system in yeast and mouse continue to be a major theme of our work.
In addition, we are interested in employing the increasing understanding of degradation signals and proteolytic pathways to devise new biochemical and genetic methods, as well as new classes of therapeutic agents.

182. A mouse amidase specific for N-terminal asparagine: the gene, the enzyme, and their function in the N-end rule pathway

Sergei Grigoryev¹, Yong Tae Kwon, Alfred Stewart², Stuart Arfin³, Ralph Bradshaw³, Neal Copeland⁴, Nancy Jenkins⁴, Alexander Varshavsky

We isolated and analyzed a mouse cDNA and the corresponding gene (termed Ntan1) that encode an amidohydrolase (termed Ntn¹-amidase) specific for N-terminal Asn in polypeptides (Grigoryev et al., 1996). The ~17 kb Ntan1 gene was sequenced on both strands. Ntan1 is located in the proximal region of mouse chromosome 16, and contains 10 exons whose lengths range from 54 to 177 bp. The ~1.4 kb Ntan1 mRNA is expressed in all of the tested mouse tissues and cell lines, and encodes a 310-residue (35 kD) protein. The Ntan1 promoter is located ~500 bp upstream of the Ntan1 start codon. The deduced amino acid sequence of mouse Ntn¹-amidase is 88% identical to the sequence of its previously characterized porcine counterpart (Stewart et al., 1995), but bears no significant similarity to the sequence of the 52 kDa Nta1p, the NTA1-encoded Ntn¹-amidase of the yeast S. cerevisiae (Baker and Varshavsky, 1995) (Abstract 2). In contrast to mouse Ntn¹-amidase, the yeast Ntn¹-amidase can deamidate either N-terminal Asn or Gln.

The expression of mouse Ntn¹-amidase in ntaΔ S. cerevisiae was used to examine the in vivo selectivity of this enzyme and its ability to implement the Asn-specific subset of the N-end rule. Mouse Ntan1 is the first cloned mammalian gene whose role appears to be confined to the N-end rule pathway. Biochemical and genetic dissection of Ntan1, including its null phenotype analysis, are under way (Abstract 2).

References


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183. Construction and analysis of mouse mutants that lack the Ntan1-encoded, asparagine-specific N-terminal amidase (Ntn¹-amidase)

Yong Tae Kwon, Natalie Barteneva, Alexander Varshavsky

Previous work (Abstract 1) described the isolation and analysis a mouse cDNA and the corresponding gene (Ntan1) that encode an Asn-specific N-terminal amidase (Ntn¹-amidase). The ~17 kb Ntan1 gene is located in the proximal region of mouse chromosome 16, and contains 10 exons ranging from 54 to 177 bp in length. To address the function of Ntan1, we constructed Ntan1 (-/-) ("knockout") mice, using targeted mutagenesis in mouse ES cells, blastocyst injections, and matings of heterozygous Ntan1 (+/-) mice. The Ntan1 (-/-) mice are alive, fertile and outwardly normal. Embryonic fibroblasts derived from Ntan1 (-/-) mice lack the enzymatic activity of Ntn¹-amidase but retain the enzymatic activity of a Gln-specific Nt¹-amidase (Ntn²-amidase), indicating that the still uncloned Ntn²-amidase is encoded by a distinct locus (instead of, for example, a differentially spliced variant of Ntan1 mRNA). Phenotypic and biochemical studies of Ntan1 (-/-) mice, including their neurological, behavioral, and immunological characterization, as well as a search for physiological ligands of Ntn¹-amidase, are under way (Y. T. Kwon, N. Barteneva, and A. Varshavsky, unpublished data).

184. Identifying physiological substrates of the S. cerevisiae N-end rule pathway

Youming Xie, Alexander Varshavsky

The currently known physiological substrates of the N-end rule pathway are RNA polymerase of the Sindbis virus and Go subunit of the heterotrimeric G protein in S. cerevisiae (reviewed by Varshavsky, 1996). These substrates were identified using strategies that are unlikely to encompass many different N-end rule substrates. A complementary and more systematic way to identify N-end rule substrates is to screen for random translational fusions to a reporter protein that are short-lived in the presence but not in the absence of the N-end rule pathway. We carried out a screen based on a library of S. cerevisiae DNA fragments containing quasi-random fusions to E. coli lacZ. This library (a gift from M. Snyder, Yale University) was constructed in E. coli using a Tn3-derived transposon, which can be used to insert the ATG-lacking lacZ gene into a gene of interest. The library was used to integrate lacZ-yeast DNA fusions into S. cerevisiae genome, producing a collection of yeast transformants some of which expressed β-gals whose N-termini were fused to amino acid sequences encoded by yeast DNA. The S. cerevisiae strain used for integration had been designed to express UBR1 from the galactose-inducible, glucose-repressible P_CALI promoter. We screened for integrants that formed blue colonies on X-Gal plates containing glucose (inactive N-end rule pathway), but formed white or light-blue colonies on galactose-containing X-Gal plates. One premise of this screen is that, for many of the (unknown) physiological substrates of the N-end rule pathway, the joining of their N-terminal regions to a downstream reporter moiety would not preclude the in vivo processing that normally generates destabilizing residues at the N-termini of these substrates. Several β-gal-expressing transformants that form blue colonies on X-Gal plates in the absence but not in the presence of the N-end rule
pathway have recently been identified, and are being analyzed.

Reference

185. N-terminal sequences that confer UBR1-dependent metabolic instability in S. cerevisiae

Michel Ghislain1, Jurgen Dohmen2, Alexander Varshavsky

The N-end rule was discovered in experiments that involved the expression, in yeast, of a fusion protein containing Ub and a reporter such as E. coli βgal. In this and other eukaryotes, Ub-X-βgal is rapidly (cotranslationally or nearly so) cleaved at the Ub-protein junction regardless of the identity of a residue X at the C-terminal side of the cleavage site, proline being the single exception. By allowing a bypass of the “normal” N-terminal processing of a newly formed protein, this finding yielded an in vivo method for generating different residues at the N-termini of otherwise identical proteins — a technical advance that led to the N-end rule (Bachmair et al., 1986). Since nascent proteins bear N-terminal Met (a stabilizing residue), the N-degron of an N-end rule substrate must be produced from a pre-N-degron. The design of a pre-N-degron in Ub fusion-based, engineered N-end rule substrates such as Ub-X-βgal is unlikely to be relevant to physiological N-end rule substrates, because natural Ub fusions (including the major precursors of Ub) either contain a stabilizing residue at the Ub-protein junction or bear a mutant Ub moiety that is expected to be retained in vivo (reviewed by Varshavsky (1996)).

Endoproteolytic cleavages of viral precursor polyproteins have been found to produce N-end rule substrates (deGroot et al., 1991). However, since most nonviral proteins are not synthesized as polyproteins, the knowledge of a polyprotein-based route to an N-end rule substrate does not, by itself, address the problem of how a destabilizing residue could be generated at the N-terminus of a cytosolic protein. Most of the directly sequenced cytosolic and nuclear proteins lack destabilizing residues at their N-termini. This bias stems in part from properties of the known Met-aminopeptidases, which remove N-terminal Met if and only if the second residue is stabilizing in the yeast N-end rule. Another likely reason for this bias is an underrepresentation of short-lived proteins in the current set of directly determined N-terminal sequences.

A destabilizing residue at the N-terminus of an N-end rule substrate must be generated through a proteolytic cleavage (or cleavages) of a pre-N-degron-containing protein by an unknown protease(s) at some unspecified distance from the protein’s initial N-terminus. Could this distance be just one or a few residues? If so, a short (≤ 10 residues) N-terminal sequence might contain both the recognition motif and the cleavage site(s) for a relevant (unknown) processing protease. On this assumption, we constructed a library of random 10-residue sequences fused to the N-terminus of the Ura3p reporter protein, and screened, in S. cerevisiae, for the fusions that were short-lived in the presence but not in the absence of the N-end rule pathway (Ghislain et al., 1996). To enable the use of an N-end rule substrate as a conditional marker, a S. cerevisiae strain, termed JD54, was constructed that expressed UBR1 from a galactose-inducible, glucose-repressible promoter. JD54 cells expressing Arg-Ura3p were Ura+ in the presence of glucose but Ura- in a galactose-containing medium, owing to the induction of the N-end rule pathway by galactose.

The ~65 N-terminal, 10-residue extensions identified by this screen were largely dissimilar to each other. However, certain residues appeared to be excluded from some of the 10 positions, and in addition, 85% of these deduced sequences bore a destabilizing residue at position 2 (after the deduced N-terminal Met), while the remainder bore Val at this position. In a screen similar to the one above, Sadis et al. (1995) have used a library of 11-residue N-terminal extensions fused to a βgal reporter, and screened for the extensions that conferred metabolic instability on βgal. A large fraction of the sequences thus identified targeted βgal for degradation by the N-end rule pathway. None of the N-terminal sequences identified by Sadis et al. (1995) were evidently similar to the sequences identified in our screen. While this lack of sequence similarities may stem in part from differences in the screens’ design (randomization immediately after N-terminal Met in our screen but after position 2 in the screen by Sadis et al. (1995)), it is also likely that a large number of 10-residue N-terminal sequences can produce the N-degron in vivo, perhaps analogous to a large number of N-terminal sequences that can function as signals for protein translocation across the endoplasmic reticulum (ER) membrane. Furthermore, both screens explored at most 0.00001% of the a priori available sequence space (there are ~1 x 1013 distinct 10-residue amino acid sequences, and ~1 x 106 of these could be, in principle, present in a library of the type described above). One N-terminal sequence identified by this screen was used in another screen, resulting in the isolation of the UFD3 gene (Abstract 5).

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186. Cdc48p interacts with Ufd3p, a WD-repeat protein required for ubiquitin-mediated proteolysis in S. cerevisiae

Michel Ghislain1, Jurgen Dohmen2, Frederic Levy, Alexander Varshavsky
A library of random 10-residue peptides fused to the N-terminus of a reporter protein was screened in
the yeast *S. cerevisiae* for sequences that can target the reporter for degradation by the N-end rule pathway (Abstract 8). One of N-terminal sequences identified by this screen was used in a second screen — for mutants incapable of degrading the corresponding reporter fusion. A mutant thus identified had an abnormally low content of free Ub. This mutant was found to be allelic to a previously isolated mutant in a Ub-dependent proteolytic system distinct from the N-end rule pathway (Johnson et al., 1995). We isolated the gene involved, termed **UFD3**, which encodes an 80 kD protein containing tandem repeats of a motif that is present in many eukaryotic proteins and called the WD-repeat. Both immunoprecipitation and two-hybrid assays demonstrated that Ufd3p is an in vivo ligand of Cdc48p — an essential ATPase required for the cell cycle progression and the fusion of endoplasmic reticulum membranes. Further, we showed that, similarly to Ufd3p, Cdc48p is also required for the Ub-dependent proteolysis of test substrates (Ghislain et al., 1996).

### References

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### 187. Targeting complex of the N-end rule pathway

Ailsa Webster, Fangyong Du, Michel Ghislain, Alexander Varshavsky

The known components of the *S. cerevisiae* N-end rule pathway that mediate steps prior to the actual proteolysis of a substrate are N-terminal amidase (Nt-amidase), encoded by the NTA1 gene (Baker & Varshavsky, 1995); Arg-tRNA-protein transferase (R-transferase), encoded by the ATE1 gene (Balzi et al., 1990); N-recognin, encoded by the UBR1 gene (Bartel et al., 1990); the Ub-conjugating (E2) enzyme, encoded by the UBC2 gene (Madura et al., 1993); and the Ub-activating (E1) enzyme, encoded by the UBA1 gene (McGrath et al., 1991). In addition to a direct (immunoprecipitation-based) evidence for the physical association between N-recognin and Ubc2p (Madura et al., 1993), there is also a circumstantial (overexpression-based) evidence for the existence of a complex between N-recognin, R-transferase and Nt-amidase (Baker and Varshavsky, 1995). More recently, we obtained direct evidence for specific interactions between Nta1p and Ate1p, and also data suggesting that both Nta1p and Ate1p interact with Ubr1p. Specifically, all three proteins were tagged with the FLAG or ha epitopes, then overexpressed in *S. cerevisiae*, singly or in combinations, and immunoprecipitated from cell extracts with anti-FLAG or anti-ha monoclonal antibodies. In a quaternary complex between Ubc2p, Ubr1p, Nta1p and Ate1p that is suggested by these data, Ate1p and Nta1p interact with each other and with Ubr1p. Further characterization of the targeting complex is under way.

Proteins that participate in a multistep metabolic transformation are often physically associated. Advantages of such assemblies stem from their increased fidelity due to often present editing capabilities, and also from the properties of processivity and channeling that are central to the functioning of multienzyme machines. Expression of genes that encode interacting proteins is often coregulated to maintain correct stoichiometries of the complex's components. The effect of UBC2 expression on the level of **UBR1** mRNA (Madura et al., 1993) is a likely example of such a regulation. The expression of *S. cerevisiae* genes encoding Nt-amidase (Nta1p), R-transferase (Ate1p) and N-recognin (Ubr1p) appears to be coregulated as well, presumably to maintain correct stoichiometry of these interacting enzymes. Specifically, promoter regions of NTA1, ATE1 and UBR1 contain two sequence elements common to these promoters but not encountered together in other sequenced yeast genes (Baker and Varshavsky, 1995), suggesting that these elements are recognized by transcription factors whose combination is specific for genes that encode targeting components of the N-end rule pathway.

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### 188. Biochemical and genetic dissection of N-recognin

Ailsa Webster, Michel Ghislain, Fangyong Du, Alexander Varshavsky

In *S. cerevisiae*, the targeting machinery of the N-end rule pathway comprises Ubr1p (the E3, or N-recognin) in a complex with Ubc2p (a specific E2 enzyme) (Madura et al., 1993). The pathway's other targeting components are Arg-tRNA-protein transferase, Ate1p, and N-terminal amidase, Nta1p, which are required for the degradation of proteins bearing, respectively, the secondary (Asp, Glu) and tertiary (Asn, Gln) destabilizing residues. Earlier dipeptide inhibition experiments identified two distinct substrate-binding sites of the 225-kD Ubr1p (Gonda et al., 1989; Baker and Varshavsky, 1991). Type 1 site recognizes polypeptides bearing positively charged N-terminal residues (Arg, Lys and His). Type 2 site recognizes polypeptides bearing bulky hydrophobic N-terminal residues (Leu, Ile, Tyr, Phe and Trp). We employed genetic and biochemical methods to map the substrate-binding sites of Ubr1p, and to define the molecular partners of Ubr1p in the targeting complex.

The **UBR1** gene was mutagenized *in vitro*, and genetic screens were designed to identify **ubr1** mutants that are selectively impaired in the degradation of proteins bearing either type 1 or type 2 N-terminal destabilizing residues. Sequencing of these mutants
identified Cys-145, Gly-173 and Asp-176 as critical residues for binding of the type 1 substrates, and Val-314, Asp-319, His-322, Pro-406 and Glu-559 as essential residues of the type 2 binding site.

Recently, Cup9p, a homeodomain-containing transcriptional repressor, has been shown to be degraded in a Ubr1p-dependent manner (Abstract 8). Using the type 1 and type 2 ubr1 mutants, we showed that degradation of Cup9p requires the type 2, but not the type 1, substrate-binding site of Ubr1p. Some of the ubr1 alleles that were defective in the degradation of both type 1 and type 2 substrates were found to be dominant-negative: their introduction into a UBR1 background inhibited the degradation of engineered N-end rule substrates and the physiological substrate Cup9p. Deletion analysis showed that the dominant-negative effect required a C-terminal region of Ubr1p, but not the ~50-kDa N-terminal domain which contains the type 1 and 2 substrate-binding sites. Ubr1p and Ubc2p are known to form a stable complex. Using GST fusion proteins we confirmed that the 20-kD Ubc2p binds to a C-terminal region of the 225-kD Ubr1p. One interpretation is that dominant-negative alleles of Ubr1p compete with the wild-type Ubr1p for binding to Ubc2p, thereby reducing the concentration of the Ubr1p-Ubc2p targeting complex.

Recently, we have overexpressed Ubr1p, Ubc2p, Ate1p, Nta1p, the Ub-activating enzyme Uba1p, and several N-end rule substrates, using E. coli, yeast and baculovirus expression systems. The purified proteins will be used to address the architecture of the targeting complex and the mechanisms of targeting and ubiquitylation by the N-end rule pathway.

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189. The N-end rule pathway controls the import of peptides through degradation of a transcriptional repressor

Christopher Byrd, Glenn Turner, Alexander Varshavsky

While engineered N-end rule substrates have been extensively characterized, little is known about their physiological counterparts. The few identified physiological substrates of the N-end rule pathway include the GPA1-encoded Ga subunit of the S. cerevisiae heterotrimeric G protein which mediates the pheromone response in this fungus, and RNA polymerases of alphaviruses whose hosts include mammalian and insect cells (Madura and Varshavsky, 1994; deGroot et al., 1991). Functions of the instability of these proteins remain to be understood. Inactivation of the N-end rule pathway in S. cerevisiae — through a deletion of the UBR1 gene that encodes the pathway's recognition component — resulted in cells that grew slightly slower than their wild-type counterparts, had a sporulation defect (increased frequency of asci containing fewer than 4 spores), but appeared to be normal otherwise (Varshavsky, 1996).

Alagramam et al. (1995) have found that ubr1Δ cells are deficient in the import of di- and tripeptides, suggesting that this process — universal among living cells — requires the presence of the N-end rule pathway, in particular the presence of Ubr1p. We recently found that Ubr1p regulates the import of peptides in S. cerevisiae through the degradation of Cup9p, a 35 kD homeodomain protein. Cup9p was identified using a screen for mutants that bypass the requirement for Ubr1p in the import of peptides. We showed that Cup9p is a short-lived protein (t1/2 of ~5 min) whose degradation requires Ubr1p.

We also showed that Cup9p acts as a repressor of PTR2, a gene that encodes the transmembrane peptide transporter. In contrast to engineered N-end rule substrates, which are recognized by Ubr1p through their destabilizing N-terminal residues, Cup9p is targeted by Ubr1p through an internal degradation signal. The Ubr1p-Cup9p-Ptr2p circuit is the first example of a physiological process controlled by the N-end rule pathway. An earlier study (Knight et al., 1994) identified Cup9p as a protein required for an aspect of resistance to copper toxicity in S. cerevisiae.

The discovery that Ubr1p controls the import of peptides through degradation of the Cup9p repressor identifies the first clear physiological role for the N-end rule pathway. Whether Cup9p is constitutively short-lived or whether its stability is regulated in a manner dependent upon external conditions such as, for example, different nitrogen sources, remains to be understood. The existence of mammalian, plant, and bacterial homologs of the yeast Ptr2p transporter suggests that the import of peptides in these organisms may also be regulated by the N-end rule pathway. Another interesting aspect of Cup9p is the multiplicity of its functions: it is also required for resistance of S. cerevisiae to copper ions' toxicity in the presence of lactate, a nonfermentable carbon source (Knight et al., 1994). Copper homeostasis is mediated by a complex set of circuits some of which regulate iron metabolism as well. The double role of Cup9p in regulating both peptide import and copper homeostasis may signify a physiological connection between these seemingly unrelated processes.

The finding that Cup9p lacks a destabilizing N-terminal residue indicates that Ubr1p, the recognition component of the N-end rule pathway, is able to target substrates bearing either internal degradation signals or N-degrons. Ga — the other known physiological N-end rule substrate in S. cerevisiae — also lacks a destabilizing N-terminal residue (Madura and Varshavsky, 1994) Thus, a substantial fraction of naturally short-lived proteins targeted by the N-end rule pathway may bear internal degrons rather than the N-degrons. The exact location
and structure of these degradation signals remain to be determined.

References

190. Using ubiquitin to follow the metabolic fate of a protein
Frédéric Lévy1, Nils Johnsson2, Tillmann Rümenap3, Alexander Varshavsky
We devised a method that can be used to produce equimolar amounts of two or more specific proteins in a cell. In this approach, termed the UPR (ubiquitin/protein/reference) technique, a Ub moiety is placed between polypeptides A and B within a linear fusion. In vivo, this tripartite fusion is cleaved, cotranslationally or nearly so, at the Ub-B junction, yielding equimolar amounts of two separate polypeptides, A-Ub and B (Lévy et al., 1996). More generally, by positioning n Ub moieties within a fusion, one can produce equimolar amounts of n + 1 separate polypeptides in a cell. In applications such as pulse-chase analysis, the UPR technique can compensate for the scatter of immunoprecipitation yields, sample volumes, and other sources of sample-to-sample variation. In particular, this method allows a direct comparison of proteins’ metabolic stabilities from the pulse data alone.

The development of UPR was prompted in part by insufficient reproducibility of conventional pulse-chase assays in studies of the N-end rule, and also by the necessity of estimating the degradation of N-end rule substrates and other short-lived proteins during the pulse. The utility of UPR was tested by using it to determine the N-end rule in L-cells, a fibroblast-like mouse cell line (Lévy et al., 1996). The increased accuracy afforded by the UPR technique underscored insufficiency of the current “half-life” terminology, since the in vivo degradation of many proteins deviates from a first-order kinetics. This is so because a protein molecule in vivo is not a fixed structural entity. For example, the probability of degradation of a nascent, partially unfolded, chaperonin-associated protein should be, in general, different from the probability of degradation of a folded counterpart of this protein at a later time in the same cell. In addition, most proteins undergo covalent modifications and associate with other molecules (including other proteins) in a cell, the fraction of a modified or a complex-associated protein being typically less than unity. Some of these modifications and associations are relevant to the protein’s function, while the rest are caused by a variety of quasi-random events that include protein damage. Thus, an in vivo ensemble of protein molecules encoded by one and the same ORF is inhomogeneous structurally and/or conformationally, and therefore may not decay exponentially. For some short-lived proteins, e. g., S. cerevisiae Mata2p, deviations from a first-order decay (at times comparable to a half-life) appear to be small. However, many other short-lived proteins, including the engineered N-end rule substrates, decay with a pronounced nonexponential kinetics. Lévy et al. (1996) consider this problem and discuss other applications of UPR.

References
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191. Detecting and measuring cotranslational proteolysis in vivo: the double-ubiquitin technique
Glenn Turner, Frédéric Lévy1, Alexander Varshavsky
A nascent polypeptide chain emerging from the ribosome may present many degradation signals before it assumes its final conformation. Similarly, protein folding may not be 100% efficient, and some polypeptides may reach “dead-end” conformations in the folding pathway, never progressing to the mature conformation. Both of these arguments suggest that a fraction of proteins may be degraded as nascent chains emerging from the ribosome. This long-standing problem can now be experimentally addressed using the double-ubiquitin technique. The idea of this method is to establish a kinetic competition between the two events: processive proteolysis of a nascent polypeptide chain by the proteasome or, alternatively, a cotranslational, site-specific cleavage of the same chain as it emerges from the ribosome. The polypeptide to be examined for cotranslational degradation is linked to a reporter protein by a junction which is cleaved as soon as that junction unfolds. This ensures that the only time the fusion’s two domains will be degraded together is when the degradation occurs before the cleavage junction folds and is cut. The Ub moiety in a fusion provides this “folding-sensitive” junction. When a linear Ub fusion is made, it is rapidly cleaved at the C-terminus of Ub by Ub-specific proteases (UBPs), but only if the Ub moiety is properly folded (Johnsson and Varshavsky, 1994). A fusion protein AUb-BUb-CUb is constructed, where the polypeptide region B carries the “cotranslational” degradation signal, and A and C are reporter domains. In the absence of cotranslational degradation, the molar yield of A and C (upon the cleavages of the fusion by UBPs) must be equal. However, if the A Ub moiety emerges and is cleaved off, then the moiety B emerges and is immediately available to processive degradation, a significant fraction of BUb-CUb may be degraded as a unit — in the absence of further cleavages by UBPs. This outcome will result in the yield of C being lower than A, the difference being the measure of cotranslational proteolysis.
Using this technique we have found that proteins possessing a strong N-terminal degradation signal such as the N-degron can be degraded cotranslationally. The extent of cotranslational proteolysis depends on both the size of the target protein and the time it takes for the ribosome to produce the protein. In the absence of a strong degradation signal the extent of cotranslational degradation appears to be low but still detectable. Specifically, a fraction of the wild-type E. coli β-galactosidase, a protein known to fold in an hsp60-independent manner, may undergo cotranslational proteolysis.

Experiments are planned to test whether there may be a quality control mechanism for protein folding that functions to degrade, cotranslationally, the proteins which fail to fold correctly in a “sufficiently” short time. This question can be addressed using a series of mutant proteins with a defined range of folding defects as protein domain B, and measuring the C/A ratio to determine the extent of cotranslational proteolysis associated with a given folding defect.

References

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192. Using split-ubiquitin sensor to detect transient in vivo interactions between a signal sequence and components of the translocation apparatus
Nils Johnsson1, Alexander Varshavsky

We devised a method, ubiquitin-based split-protein sensor (USPS), which makes possible assays for kinetic and equilibrium aspects of protein interactions at their natural sites in living cells (Johnsson and Varshavsky, 1994a). In eukaryotes, newly formed Ub fusions are rapidly cleaved by Ub-specific proteases (UBPs) after the last residue of Ub at the Ub-polypeptide junction. We found that the cleavage of a Ub fusion by UBPs requires the folded conformation of Ub (Johnson and Varshavsky, 1994b). We also found that when a C-terminal ‘half’ of Ub (residues ~36 to 76; denoted as CUb) is expressed as a fusion to a reporter protein, the fusion is cleaved only if an N-terminal fragment of Ub (residues 1 to ~36; denoted as NUb) is also expressed in the same cell. This reconstitution of native ubiquitin from its fragments, detectable by the in vivo cleavage assay, is not observed with a mutationally altered NUb. However, if CUb and the altered NUb are each linked to polypeptides that interact in vivo, the cleavage of the fusion containing CUb is restored. This result yielded a generally applicable assay (USPS) for kinetic and equilibrium aspects of in vivo protein interactions (Johnsson and Varshavsky, 1994a).

Features of USPS that distinguish it from the two-hybrid technique (Fields and Song, 1989) include the possibility of monitoring a protein-protein interaction as a function of time, at the natural sites of this interaction in a living cell.

Recently, we used USPS to detect and analyze transient interactions of S. cerevisiae Sec62p and the signal sequences of either the SLIC2-encoded invertase or the MFA1-encoded precursor of a-factor, a mating pheromone. Sec62p is an integral membrane protein and essential component of the translocation complex which mediates the transport of proteins bearing signal sequences across the endoplasmic reticulum membrane (Deshaies and Schekman, 1990). The USPS assay detected specific, transient interactions between Sec62p and the signal sequences; it also made possible a kinetic analysis of these in vivo interactions, which were demonstrated previously using photocrosslinking in a cell-free system. The transient proximity between a signal sequence and the membrane-embedded Sec62p could be detected using several NUb and CUb arrangements in Sec62p and signal sequence-bearing test proteins. These findings illustrate the versatility of USPS, its sensitivity to relatively weak and transient protein interactions, and the remarkable flexibility of ‘allowed’ NUb and CUb configurations within test proteins.

References
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193. Cleavage-regulated conditional toxins
Ilia Davydov, Tetsuro Suzuki, Alexander Varshavsky

A major limitation of the present-day antiviral therapies is either an insufficient selectivity of a drug, or the emergence of drug-resistant viral mutants, or (most often) both. These difficulties account in part for the fact that there are no curative drugs against a majority of pathogenic viruses.

We are interested in developing new antiviral strategies. In particular, we are working on a class of conditional toxins whose metabolic stability (or intracellular localization) can be altered in response to the presence of a virus-encoded processing protease (Varshavsky, 1996). In one of these designs, a degron-based toxin against a virus (whose reproduction cycle must include a virus-specific processing protease (PR)) is a protein fusion that contains: (1) a C-terminal effector domain, for example, a cytotoxic domain such as the A-chain of ricin or diphtheria toxin; (2) an N-terminal domain bearing a degradation signal, for example, the N-degron; and (3) a domain located between the two aforementioned domains and bearing the recognition/c cleavage site for a viral PR.

(The problem of delivering a toxin into cells is common to all protein-based drugs and is discussed elsewhere (Varshavsky, 1995, 1996).) In uninfected cells, the N-degron-bearing toxin would be short-lived and therefore relatively nontoxic. By contrast, in infected cells the same fusion would be cleaved by a viral PR at
the fusion's recognition/cleavage site, resulting in physical uncoupling of the N-degron from the fusion's toxic domain. The net result would be an increased metabolic stability of the toxic domain in virus-infected but not in uninfected cells, and hence a selective toxicity of the toxin against virus-infected cells. One advantage of this strategy is that the probability of emergence of a toxin-resistant mutant virus is expected to be negligible. Indeed, since the cleavage site of an antiviral sitoxin is functionally indistinguishable from the natural cleavage sites in a viral polyprotein, and since the processing of polyprotein is essential for infectivity of the virus, mutations of viral PR (including those that make it resistant to protease inhibitors) cannot produce a toxin-resistant virus without rendering it uninfectious as well.

We are testing the mechanistic feasibility of cleavage-mediated conditional toxins using the processing protease of the human immunodeficiency virus (HIV PR) as a target, and either the ricin's A-chain or the diphtheria toxin's A-chain as toxic protein domains.

References

194. Codominance-mediated conditional toxins
Lawrence Peck, Tetsuro Suzuki, Alexander Varshavsky
A strategy for designing macromolecular drugs that are sensitive to the presence or absence of more than one target at the same time has recently been proposed (Varshavsky, 1995, 1996). A key feature of these reagents, termed comtoxins (codominance mediated toxins), is their ability to utilize codominance, a property characteristic of many signals in proteins, including degrons and nuclear localization signals (NLSs). In the case of degrons, codominance refers to the following property of these signals: each degron in a protein bearing two or more degrons can target the protein for degradation independently of other degrons in the same protein, i.e., multiple degrons of a protein can be codominant. The crucial property of a degron-based comtoxin is that its intrinsic toxicity is the same in all cells, whereas its half-life (and, consequently, its steady-state level and overall toxicity) in a cell depends on the cell's protein composition, specifically on the presence of proteins that have been chosen to define the profile of a cell to be eliminated. A comtoxin designed to kill cells that express intracellular proteins P1 and P2 but to spare cells that lack P1 and/or P2 is a multidomain fusion containing a cytotoxic effector domain (Abstract 20) and two degrons placed within or near two domains P1* and P2* that bind, respectively, to P1 and P2. In a cell containing both P1 and P2, these proteins would bind to the P1* and P2* domains of the comtoxin and sterically mask the nearby (appropriately positioned) degrons, resulting in a long-lived and therefore toxic drug. By contrast, in a cell lacking P1 and/or P2, at least one of the comtoxin's degrons would be active (unobstructed), yielding a short-lived and therefore nontoxic drug. These and related ideas (Varshavsky, 1995, 1996) should enable the construction of protein-based reagents (comtoxins) that possess multi-target, combinatorial selectivity, in that they would be toxic exclusively to cells whose content (presence or absence) of predetermined, multiple molecular targets matches the specificity of a given comtoxin. We are verifying the mechanistic feasibility of these designs, using degradation signals and toxins mentioned in Abstract 12. One aim of these projects is to convert the N-degron into a conditional degradation signal that can be regulated through the binding of a protein ligand.

References

195. Using differential hybridization techniques to identify new functions of the N-end rule pathway
Hai Rao, Alexander Varshavsky
The aim of these experiments is to identify S. cerevisiae mRNAs whose concentrations differ significantly between wild-type cells and their congenic counterparts lacking a component of the N-end rule pathway. The power of this approach stems in part from efficiency of the recently developed methods for identifying differentially expressed genes, and also from the fact that a near-absence of a certain mRNA species from mutant cells should be easy to detect, whereas the mutant phenotype caused by this absence may be far from obvious and therefore difficult to come across. Our first target are ubr1Δ cells, which lack the main recognition component of the N-end rule pathway. Using either subtraction-suppression hybridization (SSH) or, in collaboration with P. Brown's laboratory at Stanford University, a version of an array hybridization technique, we have detected a number of yeast genes whose expression differs from 2- to 10-fold between congenic UBR1 and ubr1Δ strains. We are verifying these preliminary results using direct, Northern hybridization-based comparisons of the putative differentially expressed genes. To identify specific substrates of the N-end rule pathway that underlie differences in the expression of specific genes between UBR1 and ubr1Δ backgrounds, we shall carry out suppressor screens analogous to those that yielded Cup9p as a Ubr1p substrate and the regulator of peptide import in S. cerevisiae (Abstract 9).

196. The N-end rule pathway in Xenopus egg extracts
Ilia V. Davydov, Debabrata Patra1,
William G. Dunphy1, Alexander Varshavsky
Purified, DHFR-based, radioactively labeled N-end rule substrates were added to Xenopus egg extracts, and kinetics of their degradation was assayed. Xenopus egg extracts were found to contain a highly active N-end rule pathway. Cytostatic factor-
arrested, interphase-arrested and cycling egg extracts showed similar rates of degradation with test N-end rule substrates. Degradation of either type 1 or type 2 N-end rule substrates could be efficiently and specifically inhibited by dipeptides bearing destabilizing N-terminal residues. This selective inhibition of the N-end rule pathway had at most minor effects on the course of apoptosis in a cell-free system produced from aged Xenopus eggs.

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197. Analysis of mitochondrial function of the Nta1p NT-amidase in S. cerevisiae
Hoi-Rung Wang, Alexander Varshavsky

The NTA1-encoded N-terminal amidohydrolase (NT-amidase) of S. cerevisiae deamidates N-terminal Asn or Gln but not internal Asn and Gln in polypeptides. Another budding yeast, K. lactis, contains a sequence homolog of S. cerevisiae Nta1p (D. Grigoryev and A. Varshavsky, unpubl. data), whereas mammals contain two distinct Nt-amidases specific, respectively, for N-terminal Asn and Gln (Abstracts 1 and 2). nta1Δ S. cerevisiae cannot degrade N-end rule substrates bearing N-terminal Asn, but appear phenotypically normal otherwise. Our experiments were initiated by the observation of two in-frame ATG start codons in the NTA1 ORF (Baker and Varshavsky, 1995). These codons are separated by a region that resembles a mitochondrial translation signal. Specifically, the 5′ mapping showed that a significant fraction of NTA1 mRNAs has its 5′ ends immediately upstream (or even downstream) of the inferred (+1) NTA1 start codon. Thus, there exist NTA1 mRNAs that either lack the (+1) start codon or contain it too close to the 5′ end of the message for efficient initiation of translation at that position. An in-frame ATG is present 30 bp downstream of the (+1) NTA1 start codon. Initiation of translation at this (+31) ATG should yield a protein that lacks the first 10 residues of the inferred Nta1p. The (+31) ATG lies within a favorable context for translation initiation, with A’s in positions -3 and +4 (AAGATGA), whereas the (+1) ATG is located in a less favorable context, with pyrimidines at -3 and +4 (TGAATGC). The amphipathic 29-residue region between Asp-4 and Asp-34 in the larger Nta1p strongly resembles mitochondrial translocation signals (Baker and Varshavsky, 1995).

To address the possibility that Nta1p is present in both the cytosol and mitochondria, plasmids that expressed FLAG-tagged Nta1p-GFP (green fluorescent protein) fusions from the natural PNTA1 promoter were constructed, in which either the "upstream" (+1) or the "downstream" (+31) ATG codon of NTA1 was converted to another codon. The Nta1p-GFP derived from the unaltered NTA1 ORF was found to be located in both mitochondria and the cytosol, with the mitochondrial species being apparently predominant. The GFP alone (without Nta1p) was located in the cytosol (H. R. Wang and A. V., unpubl. data). We are continuing this work, whose aim is the understanding of Nta1p function in mitochondria.

Reference

Publications


Professor: Barbara J. Wold
Research Fellows: Sonya D. Zabludoff Palmer, Jeong Kyo Yoon
Graduate Students: Dawn Cornelison, Marie Csete, Susan Wang, Kyuson Yun
Research and Laboratory Staff: Shuling Wang, Xin Yu
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Summary: In the Wold group we are interested in the problem of how mammalian cell fates are specified and executed. A major challenge is to understand at molecular and cellular levels the series of developmental decisions that lead from multipotential precursor cells to their specialized, differentiated products. We use the mouse as our primary experimental animal and the specific lineages we study are mesodermal; they ultimately produce skeletal muscle, bone and skin. Skeletal myogenesis is governed by both positive- and negative-
acting regulatory factors. In this process, coordination of cell cycle regulation with regulation of differentiation is a hallmark of the system. A family of four closely related, positive-acting transcription factors are central to muscle development. They are MyoD, myogenin, myf-5 and MRF4/herculin. Upon transfection into many types of non-muscle cells, each of these has the capacity to drive recipient cells into and through the myogenic pathway. Such transformants, under appropriate culture conditions, differentiate into skeletal muscle cells that express a complex battery of muscle-specific proteins. Given their extraordinary power to drive or even redirect a cell fate decision, one experimental goal is to understand how MyoD family regulators function. Projects by Xin Yu, Jeong Yoon, Roger Wagner, and Dee Cornelison focus on different aspects of this problem, emphasizing new approaches to study their activities in in vivo settings. At the level of the whole organism, this includes gene disruption and gene transplacement by targeted homologous recombination to study the phenotypes of null mutations in muscle regulators, controlled ectopic expression in transgenic animals, the development and characterization of cell cultures and lines from early embryonic muscle precursors, and experiments to trace the fates of cells that have, at some earlier time in development, expressed key regulators. At the molecular level, this involves application of a novel strategy for probing the MyoD regulatory network (forced dimer polyproteins), further development and application of in vivo footprinting, and protein complex retrieval to help understand how protein:DNA complex formation is regulated and related to gene activity in the cell.

**Negative-acting regulators of muscle differentiation.** At cellular and molecular levels, it is clear that negative regulators of skeletal myogenesis are probably just as important for regulating the outcome as are the positive MyoD and MEF2 family regulators. The interaction between the two sets of regulators is of particular interest. Negative regulators of skeletal muscle differentiation that we find especially interesting are those that are expressed in multipotential mesodermal precursors or in proliferating muscle precursors (myoblasts). It is postulated that some of these are important for specifying their precursor status and/or maintaining precursor cells in an undifferentiated state. Other negative regulators appear to have a role in driving proliferation of the precursor compartments at the same time that they mitigate against execution of the muscle differentiation program. Regulators in these classes include twist, myc, Max/myb, id, c-jun, and protein kinase C. Sonya Zabludoff Palmer, Kyuson Yun, and Marie Csete are studying different aspects of this problem.

**Cell cycle progression and its roles in development.** A major problem in development is controlling cell proliferation and coordinating it with cell fate decisions so that the proper number of cells of each type are ultimately provided. In many cell types the function of the final differentiated cell depends on its cell-cycle status. For skeletal muscle, differentiation is tightly linked with entry into a G0 arrested state. Work by Sonya Zabludoff, Marie Csete and Susan Wang is directed at understanding regulation and execution of the entry into the a G0-state and the role, played by that c-myc, G1 cyclins, their cognate cdks, and their recently discovered inhibitory proteins play in directing or inhibiting that progression. A new and related thrust is our interest in myogenic satellite cells. These cells are muscle stem cells of the adult and they are responsible for all muscle regeneration. We want to understand their lineal origin, cell cycle regulation, and regulation of their myogenic status. Dawn Cornelison is studying the expression and function of growth and trophic factor receptors, cdk/cyclin inhibitory proteins, and cdk/cyclin complexes in the context of mouse regenerating.

**What regulates the regulators?** This is an inevitable question when a set of developmental regulators is discovered. In this case, the specific problem is to learn what turns on and then maintains expression of MyoD family members during development. Jeong Yoon is studying this problem with special attention to MRF4 and myf5. Studies of other regulators that appear important in the mesodermal lineages prior to expression of MyoD family members or in non-myotominal portions of somites focus on mouse twist, previously cloned and characterized in the laboratory. Kyuson Yun, Shuling Wang, and Marie Csete have looked at different aspects of its activity. 198. **Different MRF4 knockout alleles differentially disrupt Myf-5 expression: cis-regulatory interactions at the MRF4/Myf-5 locus**

Jeong Kyo Yoon, Eric N. Olson1, Hans-Henning Arnold2, Shirley Pease3

Three different null alleles of the myogenic bHLH gene MRF4/herculin/Myf-6 were created recently. The three alleles were similar in design but were surprisingly different in the intensity of their phenotypes which ranged from complete viability to homozygotes to complete lethality. One possible explanation for these differences is that each mutation altered expression from the nearby Myf-5 gene to a different extent. This possibility was first raised by the observation that the most severe MRF4 knockout allele expresses no Myf-5 RNA and is a developmental phenocopy of the Myf-5 null mutation. Furthermore, initial studies of the two weaker alleles had shown that their differences in viability correlate with the intensity of rib skeletal defects, and the most extreme version of this rib defect is the hallmark phenotype of Myf-5 null animals. In the present study we tested this hypothesis for the two milder MRF4 alleles. By analyzing compound heterozygous animals carrying either the intermediate or weakest MRF4 knockout
allele on one chromosome 10 and a Myf-5 knockout allele on the other chromosome, we found that both of these MRF4 alleles apparently downregulate Myf-5 expression by a cis-acting mechanism. Compound heterozygotes showed increased mortality of the normally viable MRF4 allele, together with intensified rib defects for both MRF4 alleles and increased deficits in myotomal Myf-5 expression. The allele-specific gradation in phenotypes also suggested that rib morphogenesis is profoundly sensitive to quantitative differences in Myf-5 function if Myf-5 products drop below hemizygous levels. The mechanistic basis for cis-interactions at the MRF4/Myf-5 locus was further examined by fusing a DNA segment containing the entire MRF4 structural gene, including all sequences deleted in the three MRF knockout alleles, with a basal promoter and lacZ reporter. Transgenic embryos showed specific LacZ expression in myotomes in a pattern that closely resembles the expression of Myf-5 RNA. Cis-acting interactions between Myf-5 and MRF4 may therefore play a significant role in regulating expression of these genes in the early myotomes of wildtype embryos.

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199. Targeted disruption of mouse M-cadherin gene
Jeong Kyo Yoon, Shuling Wang

Cell adhesion proteins (cadherin family) have been suggested to play a key role in cell-cell communication and cell migration during development. One cadherin family member, M-cadherin, is exclusively expressed in the skeletal myogenic lineage during mouse development. Little is known about the role of M-cadherin in skeletal myogenesis though it is reasonable to postulate that it has some role in the myoblast alignment and cell-cell interactions required for cell fusion and myotube formation. To investigate in vivo role of M-cadherin in skeletal myogenesis, we are disrupting the mouse M-cadherin gene by targeted homologous recombination. We are creating two independent mutant alleles. For the straight-forward knockout allele, exons (from exon 4 to exon 10) encoding EC domains that participates in homophilic interaction of M-cadherin are deleted and replaced with pgkneo selection marker. We are also creating another mutant allele in which the lacZ gene has been inserted into the M-cadherin locus. LacZ in this allele is designed to recapitulate the endogenous M-cadherin expression pattern. This mutant allele will provide a tool for marking and isolating the cells expressing M-cadherin. Concurrently, we are generating ES cell lines homozygous for mutated M-cadherin using a second selection marker, hygromycin gene. Since ES cells can be differentiated into various differentiated cell types including skeletal muscle, the role of M-cadherin in skeletal myogenesis will also be examined in tissue culture.

200. Isolation and culture of mouse skeletal muscle fibers and their associated satellite cells
Dawn Cornelison

Skeletal muscle in the adult mouse is composed of bundles of muscle fibers arranged longitudinally through the muscle. Each differentiated fiber contains several hundred myonuclei, and the majority of these are in the fiber syncitium. This syncitium formed during embryonic and fetal development from mononucleate muscle precursor cells. These cells originate in the somite from which they emigrate, subsequently differentiate, and finally fuse. These fiber nuclei are permanently postmitotic and these fibers preeminently express the myogenic regulatory factor (MRF) MRF4.

A small fraction of myonuclei in adult muscle have not fused into muscle fibers but remain mononucleate and reside between the basal lamina and the sarcolemma of mature muscle fibers. In intact healthy muscle they are in a G0 mitotically quiescent state, but under conditions of injury or disease, they are stimulated to enter the cell cycle and divide vigorously. In the resting state, they express no MRFs, but upon stimulation they commence expression of MRF family members and eventually differentiate and fuse to form new myofibers or fuse into existing fibers. In this manner, satellite cells are the sole source of muscle regeneration in humans and in mice.

Despite the importance of satellite cells in muscle regeneration and their potential as vehicles for gene therapy in inherited diseases of skeletal muscle, very little is known about the mechanisms that control their development, maintenance in the resting state, stimulation, regulation of proliferation and differentiation, and possible return to the resting state. Reasons for the gap in molecular genetic knowledge of these cells comes from their rarity, difficulty of isolation, and lack of molecular markers that can identify the cells prospectively i.e., in their unstimulated state.

One method which can be used to identify satellite cells is the isolation and culture of single living muscle fibers, together with their associated satellite cells; this has been best executed and characterized by Bischoff (1986), who prepared fibers from the very small, tendonous muscles of rat foot. In culture, satellite cells will enter the cell cycle and can be identified by 3H thymidine uptake and later by upregulation of the MRFs. A key problem with this preparation was its technical difficulty and the fact that it did not generalize well to all skeletal muscles.

I have adapted this method to allow isolation of single myofibers from any mouse skeletal muscle; I have found that in culture the satellite cells associated with these fibers proliferate and differentiate to form multinucleate, contractile myotubes. In combination with a method which exploits the cytotoxic effects of the anesthetic Marcaine on myofibers (but not satellite cells) and the live cell-specific dye calcein AM, I have
also been able to identify satellite cells on freshly isolated fibers, before they begin to proliferate or express MRFs.

These preparations are now being used to analyze potential molecular markers for satellite cells, to examine their proliferation and differentiation in culture at the single-cell level, and to screen for genes expressed in quiescent satellite cells which may mediate the maintenance of the quiescent state and/or response to stimulation.

201. The c-met receptor is a marker for quiescent and proliferating skeletal muscle satellite cells

Dawn Cornelison

The receptor tyrosine kinase c-met is a proto-oncogene that acts as a mitogen, a motogen and/or a cellular morphogen, depending on the cell environment in which it is expressed and activated. It is expressed in multiple, diverse contexts during embryonic mouse development; it appears to be involved in epithelial-mesenchymal signaling and is required for proper liver, kidney, and tooth morphogenesis, neural crest migration, and muscle development. In developing skeletal muscle, c-met is expressed in the myotome and in the migratory myoblasts which will populate the limb. Its functional significance in skeletal muscle development has been emphasized by the lack of appendicular muscles in homozygous c-met null mice. In these mutant embryos, the cells of the somite that are precursors of the limb muscles fail to migrate into the limb buds. The result is failure to form any limb muscle.

Skeletal muscle satellite cells are mononucleate cells that are committed to a muscle fate but express no known markers of skeletal muscle differentiation. They are mitotically quiescent and express no myogenic regulatory factors (MRFs) prior to stimulation by injury or damage to the adjacent muscle fiber. When stimulated through signaling mechanisms that are presently poorly understood, they re-enter the cell cycle and provide for the repair and/or regeneration of the damaged muscle. Despite their biological importance, satellite cells have been difficult to study due to their rarity, difficulty in isolation and especially due to the lack of molecular markers that would permit their identification prospectively i.e., before they are stimulated to divide and to differentiate. Since satellite cells seem to resemble the migratory muscle precursor cells of the limb in several respects, we reasoned that they might also express some of the same genes and that these might then be used as markers; using a working protocol that I previously developed for isolation and culture of whole myofibers and identification of satellite cells (see previous abstract), I have examined c-met expression in satellite cells.

When freshly prepared fibers from muscles of either the appendicular lineage (i.e., soleus) or axial lineage (i.e., latissimus dorsi) are killed with the myotoxic agent Marcaine and their satellite cells marked with the live-cell specific dye calcein AM, the labeled satellite cells are all positive for c-met immunostaining. This staining persists in satellite cells of fibers allowed to live in culture, where it defines the population of mononucleate cells that re-enter the cell cycle and express the myogenic regulatory factors MyoD and myogenin.

Thus, c-met is expressed in satellite cells in all states. Other mononucleate cells in it can now be used as a marker to identify satellite cells on live fibers at any given time point. The next important step is to use it as a vital stain for satellite cell selection from the general population of myonuclei. It may also prove useful in tracing satellite cells during earlier developmental timepoints to better understand their lineage and relationship to conventional myoblasts.

202. Analysis of regulatory gene expression in single skeletal muscle satellite cells by multiplex RT-PCR

Dawn Cornelison

Skeletal muscle satellite cells are mononucleate, mitotically quiescent muscle precursor cells which are associated with mature myofibers. They mediate muscle regeneration in response to injury and disease. When stimulated by damage to the adjacent muscle fiber, they proliferate and differentiate to mediate repair and/or regeneration. However, due to difficulties in obtaining pure and/or unstimulated populations and lack of reliable molecular markers, almost all aspects of their biology remain only poorly understood. This includes knowledge of their developmental origins, the mechanisms for maintaining their committed but quiescent state, the nature of the stimuli to which they respond, as well as the details of that response.

To approach these problems I began by developing a method for examining single living satellite cells in situ on cultured myofibers and identified a reliable molecular marker for satellite cells (see previous two abstracts). Recently, I have used these tools to survey satellite cells, especially freshly isolated satellite cells, for expression of genes which may be important in these processes. Keiko Kato in the Fraser Laboratory has developed a protocol for single-cell RT-PCR using a patch-clamp pipette to harvest cytoplasm from a single cell for analysis; I have adapted this technique for use on satellite cells from fiber explants. I consistently see c-met expression in cells chosen on the basis of Marcaine survival or morphological criteria to be satellite cells. Among the genes I have assayed for so far is m-cadherin, a homotypic adhesion molecule which is known to be expressed in stimulated satellite cells but whose expression in quiescent satellite cells is debated. I have found that m-cadherin is expressed in over 90% of c-met positive satellite cells in culture for 48 hours
or more that I have assayed, but in only about 25% of freshly prepared c-met positive satellite cells.

From these results, and from the observation that muscle derived fibroblasts do not express detectable c-met or m-cadherin, I conclude that c-met but not m-cadherin can be used as a molecular marker of satellite cell identity in the context of muscle fiber explants. I also concluded that the population of satellite cells is physiologically heterogeneous, even at time zero.

I recently extended the study of regulatory gene expression in individual satellite cells to include the four MRF regulators measured simultaneously with m-cadherin and c-met. These were measured in groups at individual satellite cells over a 96 hour time course. From this study, I was able to generate a new model for the course of satellite cell activation and differentiation.

203. Optimizing and applying jellyfish green fluorescent protein (GFP) as a vital marker for transgenic mice

Jeong Kyo Yoon, Dan Eckstein

In our studies of somitogenesis and myogenesis, it would be highly desirable to be able to nondestructively identify cells that are expressing key regulatory genes in the process or that are expressing genes that act as molecular markers of progenitor cell identity. Cloned GFP from the jellyfish has been very useful for this purpose in the invertebrates, especially Drosophila and C. elegans, but it has not been so successful in mammalian cell systems or in transgenic mice where we need a strong vital marker. Over the past few years we have made and tested a number of variant GFPs designed to localize the protein to different subcellular compartments, to improve its translation in mammalian systems by altering codon usage patterns (in collaboration with scientists at AMGEN), and to improve its excitation/emission properties with standard FITC optics. Each of these changes improved vital fluorescent detection as reported in the literature or as postulated in postmitotic myocytes and myotubes. However they were, to varying extents, either ineffective, cytostatic or cytotoxic when tested in rapidly dividing precursor cells (myoblasts), rapidly growing fibroblasts, ES cells, or the blastomeres of early mouse embryos. The basis for this toxic or cytostatic effect remains uncertain, but may come from misfolding of GFP at 37°C. Recently reported mutations in GFP that apparently improve its thermostability were therefore introduced into the background of our GFP that already contains the other optimizing changes and is being tested for its efficacy and hoped for compatibility with active cell division. This vital marker is then to be "knocked in" at several loci that identify myogenic precursor cells at various stages of their developmental progression. We will then use mice containing the marked cells and the isolated cells to test the effects of mutations in other regulators in the pathway and to test the effects of various candidate inducing agents.

<undergraduate, California Institute of Technology>

204. Multiple mechanisms of action for the bHLH regulator twist in myogenesis

Kyuson Yun, Lawrence Schaufler

We have examined the molecular and functional interactions of murine twist with other positive and negative basic-helix-loop-helix (bHLH) regulators. Consistent with its expression pattern in somites, twist is a powerful negative regulator of MyoD family function. In a manner similar to the HLH negative regulator Id, twist can efficiently inhibit MyoD DNA binding in vitro. Twist also robustly inhibits myogenesis initiated by MyoD family regulators in vivo. Unlike Id, however, twist also forms sequence specific DNA binding complexes with E-proteins, and these bind preferentially to a non-muscle class E-box sequence, CATATG. This suggests a second function for twist: E-protein complexes as direct regulators of downstream genes containing twist class DNA binding sites. Based on these observations, it is proposed that twist inhibits myogenesis by a dual molecular mechanism in which the myogenic phenotype is repressed through inhibition MRF activity, while one or more alternate phenotypes (precursor state or sclerotomal/dermatomal fates) are supported by the DNA binding action of twist:E-protein factors. This mechanism obligatorily links positive and negative regulation through the properties of twist and therefore appears well suited for regulating the establishment or maintenance of mutually exclusive cellular phenotypes. An additional possible mechanism of action for twist-mediated suppression of myogenesis is twist binding and sequestration of other myogenic auxiliary factors including MEF2 and p300. The roles played by each are under study.

<undergraduate, California Institute of Technology>

205. Searching for BRCA1 function

Sonya D. Zabludoff

The 17q-linked breast and ovarian cancer susceptibility gene (BRCA1) functions as a tumor suppressor gene. Mutations in the BRCA1 gene are responsible for a large fraction of familial breast cancers and may also be directly or indirectly involved in sporadic breast cancers. In addition to suppressing malignant transformation of cells, recent observations suggest that wild type BRCA1 may also promote differentiation. In order to understand these disparate activities, we have analyzed BRCA1 mRNA expression in adult mouse tissues with detailed attention to its expression in prepuberal and adult testis. From expression studies on highly purified germ and somatic cells of the mouse testis, we found the highest level of BRCA1 mRNA expression in germ cells and not somatic cells. Within the germ cell lineage, the highest level of expression was in cells which are in meiosis, pachytene spermatocytes and...
their downstream products, round spermatids, which are post-meiotic cells undergoing differentiation. This is in contrast to premeiotic germ cells which express little or no BRCA1 mRNA. This set of observations, considered together with recent data on the expression in breast epithelium, argues against a function for BRCA1 in early progenitor cells and calls attention instead to a role intimately associated with terminal differentiation and final rounds of cell division.

At present, the specific molecular function of BRCA1 is still unknown. We are currently identifying and analyzing genes whose protein products interact physically with BRCA1 in the testis. Our working hypothesis is that other proteins interact with BRCA1 and that such interactions will be important for its action and/or regulation. This idea finds general support from studies of other tumor suppressors and oncogenes, and more specific support from the presence in BRCA1 of evolutionarily conserved protein motifs that mediate protein:protein interactions in other better understood genes. BRCA1 interacting proteins we expect to find may include other previously unknown genes important in the disease and we will use novel functional analysis to analyze them. They fall into several important classes; genes that promote BRCA1 tumor suppressor function in breast may themselves be previously unknown tumor suppressor genes; interacting proteins that negatively regulate BRCA1 function in breast may independently act as breast oncogenes; interacting proteins that differ according to cell type or cell state may specify differently the nature of BRCA1 action. Finally, some BRCA1-IPs may be genes that are already known and whose molecular function is well-described. Such information about a BRCA1 binding protein would immediately help us to generate more specific and testable hypotheses concerning BRCA1 function.

206. Mouse embryonic expression of p27, a cyclin dependent kinase inhibitor

Sonya D. Zabludoff, Roger Wagner, Xin Yu, Marie Csete

We have examined the in vivo expression pattern of p27 during mouse development. Using immunocytochemical techniques and confocal microscopy, we demonstrated that p27 accumulates specifically in nuclei of the early developing myotome which is the first site of muscle differentiation in the embryo. Co-staining of p27 with either myogenin, myoD or α-actinin demonstrated that p27 is expressed in the myogenic lineage. Somitic expression of p27 was first detected at e9.5 followed by maximal expression at 10.5 and limited to no detectable expression by e11.5. We did, however, detect p27 in neuronal cells of the dorsal root ganglia at e11.5. By examining the expression pattern of parasagittal sections, it appeared that p27 was expressed in a rostral to caudal gradient and a dorsal to ventral gradient expression pattern. These results suggest that p27 expression is linked directly to the development and differentiation of the myotome. However, since we observed cells within the early myotome the express myogenin only and conversely express p27 only, we can not determine whether p27 expression precedes or follows myogenin expression. The appearance of detectable nuclear p27 reactivity did, however, precedes expression of downstream muscle differentiation genes. Two models therefore remain plausible: either p27 and other CDI family members initiate the withdrawal from cell cycle before a cell expresses myogenic regulatory factors or alternatively aids the myogenic regulatory factors in withdrawal from cell cycle following myogenic determination.

207. The effects of G1 cyclins and cyclin dependent kinase inhibitors on in vitro myogenesis

Sonya D. Zabludoff, Marie Csete

During development withdrawal from cell cycle is exquisitely controlled and coordinated with cellular differentiation. The mechanisms of these controls, however, are poorly understood. Skeletal myogenesis is a useful in vitro (see below) and in vivo model to examine these control mechanisms. To determine if critical cell cycle regulators such as G1 cyclins and cyclin dependent kinase inhibitors (CDIs) are likely to be involved in regulating cell cycle withdrawal, we have examined G1 cyclin and CDI levels in proliferating and differentiated myogenic cell lines and adult muscle tissue by ribonuclease protection assays. We observed that cyclin E mRNA levels were consistently down regulated in all differentiated myogenic cell lines examined and cyclin D1 was down regulated in a subset of differentiated myogenic cell lines. p21 mRNA, a CDI, on the other hand, was up regulated in the cell lines upon differentiation and p27 mRNA, another CDI, remained constant. Interestingly, however, we and others have found the p27 is under posttranscriptional regulation and at the protein level is up regulated upon differentiation. We next determined whether ectopic expression of cyclin D1 or cyclin E can inhibit myogenesis and whether ectopic expression of p21 or p27 can enhance myogenesis. Interestingly, the G1 cyclins can inhibit myogenesis and the CDIs can enhance myogenesis. Since the G1 cyclins are also capable of inhibiting a tethered dimer between MyoD and E47 it is unlikely that they mediate their effects by affecting the ability of MyoD to dimerize with binding partners.

We are currently trying to determine how these cell cycle regulators mediate these effects. We believe that cell cycle regulators effect myogenesis by regulating the cell cycle and perhaps by modifying the activity of the myogenic regulators. We have shown that in our model system p27 expression causes efficient withdrawal from the cell cycle. We are currently trying to determine if the myogenic regulatory factors and their co-factors are targets of the G1 cyclin kinase
complexes and therefore subject to phosphorylation. For these studies, we used in vitro phosphorylation model systems to demonstrate that a subset of MRFs and their cofactors can be phosphorylated. We are now trying to demonstrate that these phosphorylations are indeed regulated by the cdk activity. Finally, using myogenic extracts from undifferentiated and differentiated cells and reticulolysate expressed protein, we are examining protein complexes in order to identify putative cell cycle regulators which may be a member of the MRF complexes.

208. P16^INK4a/p19^ARF in mouse development: Expression and gene disruption
Susan L. Wang, Shuling Wang, Shirley Pease

INK4a and INK4b are tandemly linked genes encoding p16 and p15, inhibitors of the G1 cyclin D-dependent kinases cdk4 and cdk6. Studies indicate that these genes are specially expressed in a limited number of cell types. We believe that these regulatory molecules may play a key role in coordinating the transition of a dividing precursor cell to a noncycling terminally differentiated cell. With this project I am investigating the role of p16/arf during development. Three main approaches are being pursued: (1) Analysis of the INK4a/ARF and INK4b expression patterns in mouse tissues. (2) Generation and study of mice lacking p16/arf. (3) Study of double-knockouts of INK4a/ARF with other CdkIs that may have redundant function or interact in the pathway as p16.

I have characterized the expression pattern of p16 in mouse embryos and adult tissues by RT-PCR and ribonuclease protection experiments. Results indicate that p16/arf is expressed specifically in a limited number of cell types. To determine cells where p16 arf may be functionally important, I am analyzing expression patterns in more detail via in situ hybridization and immunohistochemical assays.

The mouse homolog of INK4a was isolated from the 129 genomic library. After mapping of a INK4a clone to determine exon/intron structure, a deletion vector was constructed to remove exon, 2, replacing it with a PGK-neo cassette. Exon 2 encodes the ankyrin repeat motifs and putative functional domain of p16. It also constitutes a major portion of the coding region of ARF, a protein utilizing a separate exon 1 spliced to exon 2 of p16. Thirty-six independent ES cell lines were generated and confirmed by PCR and Southern blotting analysis to carry the expected modification of the INK4a/ARF locus. From these we have generated a germline-transmitting chimeric founder by blastocyst injection from which several INK4a/ARF homozygous mutant mice have been derived. These mice are adult viable and fertile, indicating that if p16 has a critical function, it must be compensated by the action of related genes.

The anticipated p16^-/- tumor suppressor function is prominent in these animals, with spontaneous tumors of diverse types arising in virtually all animals by nine months of age. Lymphomas were most prominent among the tumors. The age of onset for detectable tumors appears, in a still small numeric sampling, to be elevated in p16^-/- mice that also lack one or both copies of p27. p27 is a Cdi of the second type (p21n p57), and these preliminary data suggest that deficits in it can intensify the p16 null tumor suppressor phenotype, even though p27 nulls have not shown an elevated level of transgenesis on their own.

Developmental efforts of p16 and p27 mutation on myogenesis and on neural crest pathways are under investigation, as is the ability of p15 to substitute for p16 functions in vivo.

Publications
Molecular Biology and Biochemistry

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Summary: Our laboratory is concerned with the role of RNA in the transmission of genetic information. Following transcription in the nucleus most RNA molecules are processed in a number of steps including capping, polyadenylation, splicing and base modification. The RNA molecules are then transported to the cytoplasm where they are translated or in the case of rRNA and tRNA mediate translation. Although RNA molecules generally function in conjunction with proteins, in a number of cases evidence is accumulating that the RNA plays a catalytic role. One such RNA mediated reaction that our laboratory has studied for some time is pre-mRNA splicing. Pre-mRNA splicing is the process by which non-coding sequences (introns) are removed from eukaryotic messenger RNA. Pre-mRNA splicing involves two distinct transesterification reactions. In the first step of splicing, the mRNA is cleaved at the 5' splice site between the upstream exon (exon 1) and the intron concomitant with the formation of a phosphodiester bond at the 2'-5' branch point in the intron. This reaction leads to the formation of two specific intermediates, the exon 1 and a lariat structure of the intron and the downstream exon (exon 2). In the second step, the lariat intermediate is cleaved at the 3' splice site, with a transfer of the phosphodiester bond to the 3' end of the free exon 1, leading to the formation of the spliced mRNA and the excised intron. This process has been shown to occur in a large RNA/protein complex dubbed the 'spliceosome'. The spliceosome is a complex structure composed of five small RNAs (the snRNAs U1, U2, U4, U5, and U6), more than 30 polypeptides, and the precursor mRNA. Each snRNA is found in an RNA/protein complex, or snRNP. In addition, a number of proteins have been identified which interact transiently with the apparatus at specific stages in splicing. Due to a similarity of the intermediates of pre-mRNA splicing and those of group II self-splicing RNAs it is believed that the catalytic activity for pre-mRNA splicing resides in RNA rather than protein moieties (most likely regions of the U6 and possibly the U2 and U5 snRNAs).

In the past year we have continued our study of the role of the snRNAs in splicing using two biochemical assays that we have developed in our laboratory. These are a complementation assay in which the endogenous U6 or U2 snRNA in the extract is specifically degraded inactivating the extract and a trans-splicing assay. In the case of the complementation assay, splicing activity is restored by addition of synthetic U6 or U2 RNA to the extract. Remarkable progress in the chemical synthesis of RNA has allowed us to do the complementation with completely synthetic U6 RNA. Specific bases can be altered to include photo crosslinking agents or base analogues. In this way the function of specific bases in splicing and their proximity to other RNA molecules or to proteins can be probed. Advances in our study of the role of the snRNAs are reported in the abstracts by Kim, Ryan and Balakin. In analogous fashion, a completely synthetic 5'-splice site leader can be used in the trans-splicing assay and Johnson describes an elegant new assay in which inclusion of a biotinylated nucleotide at the 5' or 3' end of the 5'-splice site leader allows for an affinity selection of the products of trans-splicing, greatly increasing the sensitivity of the assay.

The in vitro splicing assay requires hydrolyzable ATP, thought not to be required in the splicing reaction itself, but rather in a series of enzyme catalyzed assembly steps leading to the formation of the active spliceosome. The enzymes which catalyze these steps are a family of RNA dependent ATPases related to the translation initiation factor, eIF4A. These proteins, called DEAD box proteins for a conserved sequence in the ATP binding site, are generally thought to be involved in structural changes in the spliceosome mediated by changes in RNA-RNA or RNA-protein interactions. We have been working on Prp5 which acts at an early stage of spliceosome assembly, probably mediating a structural isomerization of the U2 snRNP and Prp22 which acts at a late stage, after splicing is completed, releasing the mRNA product from the spliceosome. Wagner has now provided compelling evidence that Prp22 has helicase activity, one of several different RNA structural changes one can imagine these proteins doing. This promises to bring us closer to the ultimate goal of understanding precisely what is the biological role of any one of these proteins. Finally we are continuing our efforts to determine the structure of one member of this class of proteins, universally conserved in all three lines of descent. Story reports our efforts to obtain crystals of DbpA, a well characterized E. coli DEAD box protein and also another member of the family from M. jannaschii, a thermophyllic Arche bacterium.
A second RNA processing reaction which has been of interest to us is tRNA splicing. By contrast to the complex process of pre-mRNA splicing described above, eukaryotic tRNAs are removed by a splicing mechanism catalyzed by just three enzymes. In the archael kingdom there is a similar protein-based enzymatic mechanism for tRNA splicing, but this system is distinct by a number of functional criteria. All known prokaryotic tRNA introns are self-splicing. So although tRNA splicing occurs across all three major lines of descent, the mechanism for this process is not conserved. Major questions regarding the origin of tRNA introns and the relationship between the disparate enzymatic machineries required for their removal therefore remain. Do the different mechanisms reflect independent origins or a single origin and later divergence of a common splicing mechanism? This year decisive progress in a 15 year project in our laboratory, to understand the yeast tRNA splicing endonuclease, together with work from the laboratories of Charles Daniels at Ohio State and Glauco Tocchini-Valentini in Rome have led to answers to these questions.

Much of our knowledge of the mechanism of eukaryotic tRNA splicing comes from studies in Saccharomyces cerevisiae. The S. cerevisiae genome contains 272 tRNA genes of which 59, encoding ten different tRNAs, contain introns. These introns are 14 to 60 bases in length and interrupt the anticodon loop immediately 3' to the anticodon. Splicing of introns from precursor tRNA (pre-tRNA) is accomplished through the action of a site-specific endonuclease, an RNA ligase, and a phosphotransferase. The tRNA splicing endonuclease, the sole determinant of splice site selection, cleaves pre-tRNA at the 5' and 3' splice sites to release the intron. The products of the endonuclease reaction are two tRNA half-molecules bearing a 2'->3' cyclic phosphate and 5'-OH termini. tRNA ligase covalently joins the half-molecules through a complex series of ATP- and GTP-dependent reactions. After ligation, a 2' phosphate remains at the splice junction and is removed by a phosphotransferase which transfers this phosphate to NAD.

The mechanism of intron removal in tRNA splicing has been investigated through purification of the endonuclease enzyme from yeast. Endonuclease behaves as a membrane-bound protein present in a low abundance of ~100 molecules per cell. The purified enzyme contains three subunits of 54, 44 and 34kD and 15kD. The molecular weight of the holoenzyme as judged by gel exclusion chromatography implies that the enzyme is an abgd tetramer. In work described by Trotta we have now determined the nucleotide sequences of the genes for all four of these subunits. Each of these genes proved to be essential for cell viability. The predicted amino acid sequence of each of the four subunits provides clues as to their potential roles in tRNA splicing. Each of the subunits contains a canonical nuclear localization sequence. Two subunits of the endonuclease enzyme, Sen2 and Sen34, contain a homologous domain of approximately 100 amino acids in length. Apparent homologs of this domain occur within the gene for the archael tRNA splicing endonuclease of Halobacterium volcanii recently cloned by Daniels and co-workers and in the potential endonuclease homologs found in the sequenced genomes of Methanococcus jannaschii and Methanobacterium thermoautotrophicum. This suggests that the yeast tRNA splicing endonuclease contains two distinct active sites for cleavage of precursor tRNA. This hypothesis is strongly supported by the finding that a mutation in Sen2 blocks 5' splice site cleavage and that a mutation in Sen34 blocks 3' splice site cleavage. Thus one is led to the model of endonuclease shown in Trotta's abstract in which the substrate recognition by the two active site subunits is modified by their interaction with Sen54 and Sen15. Further it appears that the endonuclease is an ancient enzyme, present in the common ancestor of eukaryotes and archa and that since the divergence a gene duplication together with the acquisition of two new subunits has led to an enzyme that employs an identical biochemical mechanism but different substrate recognition properties.

Our work on tRNA recognition has also led us into the fascinating area of the Archea. With the completion of the entire sequence of the M. jannaschii genome we realized that this organism lacks the genes for four of the amino acyl tRNA synthetases: gln, asn, lys and cys. We were particularly interested in the possibility that the cysteinyl tRNA synthetase function is assumed by seryl tRNA synthetase. Preliminary experiments by Saks support that idea. Thus it is possible that SerRS acylates M. jannaschii tRNA^{37} and that a separate enzyme then modifies the acylated SertRNA^{37} to CysrRNA^{59}. If this model turns out to be true it may suggest steps in the early pathway to the modern genetic code.

209. Probing the active site of the spliceosome
Chang Hee Kim

U6 snRNA is thought to be a key component of the dynamic network of RNA-RNA interactions which compose the catalytic core of the spliceosome. In order to probe the interactions of U6 with pre-mRNA (the substrate for splicing reaction) and the four other essential snRNAs (U1, U2, U4, U5), we have introduced a single photochemical crosslinking reagent (4-thiouridine) into an extensive number of specific sites in the conserved nucleotides of U6. Synthetic U6 RNA containing the single 4-thioU modification can reconstitute splicing activity in extracts depleted of endogenous U6 snRNA. Upon photoactivation with low energy UV (>300nm), 4-thioU on U6 can form covalent bonds with the bases of an RNA molecule in close proximity.

Recently, positions 87 and 88 at the base of the conserved intramolecular helix at the 3'-end of U6
were determined to be crosslinked to U2 snRNA by sensitivity of the crosslinks to RNaseH digestion with oligonucleotides complementary to sequences in U2 snRNA. This may be a particularly important interaction since current models for the active site of the spliceosome suggest that U6 interacts with U2 in concert for at least the first chemical step of pre-mRNA splicing. In addition, these two positions in U6 are analogous to the 4-thioU nucleotides where Peebles (personal communication) obtained crosslinks from domain V in Group II intron self-splicing ribozymes. This analogy may prove to be especially useful in showing that the active site of the spliceosome is composed of RNA since Group II ribozymes can catalyze the removal of their introns in the absence of any proteins. Two other nucleotides in U6 -- 62 and 64 also crosslink to U2 snRNA. This region is adjacent to base mutations in U6 which cause an arrest of the second step of splicing, and also to the "catalytic AGC triad" of nucleotides as named in domain V of Group II ribozymes. All of the crosslinks to U2 are dependent on pre-mRNA and ATP, suggesting that they are dependent on the formation of spliceosomes. The exact location of the crosslinks on the U2 snRNA are currently being mapped using primer extension analysis. We have also found high-yield ATP-dependent crosslinks between U6 and U4 from positions 51 (invariant nucleotide in the ACAGA motif) and 80 (a conserved bulged nucleotide in the 3' intramolecular helix). However, these crosslinks are not dependent on pre-mRNA suggesting that they form prior to the assembly of spliceosomes. In order to determine and define the order of these crosslinks in the spliceosome we are also doing the crosslinking experiments in ts yeast mutant splicing extracts arrested at sequential stages in spliceosome assembly.

210. Investigating the roles of specific sequences of U6 snRNA

Dan Ryan

The 3' terminal domain of yeast U6 snRNA, downstream of the highly conserved central domain (nts 47-85), includes a putative U2-interaction domain (for U2/U6 helix II) at nts 92-102 and half of a proposed U6 pseudoknot motif at nts 86-95 (base-paired with upstream nts 36-43 of U6 in the proposed pseudoknot). We have prepared yeast U6 RNAs that are truncated or mutated at the 3' end to define the sequence requirements of the 3' domain for splicing activity in yeast extracts. The results of these studies demonstrate that neither U2/U6 helix II nor the U6 pseudoknot are required for splicing activity in vitro. We are testing whether the U6 pseudoknot mutations block the association of U4 and U6 snRNPs, a spliceosome assembly step that is thought to be mediated by Prp24p protein. Genetics studies have suggested that the U6 pseudoknot may be a binding site for Prp24p protein; therefore, we are trying a crosslinking approach (as below) to identify any contacts between Prp24p and the pseudoknot sequences of U6 snRNA.

Other sequences of U6 snRNA had previously been identified as possible sites for interactions with spliceosomal proteins, e.g., nts 40-46 of yeast U6 may interact with Prp16p protein, a member of the DEAD/H-box family of putative RNA helicases (Madhani and Guthrie, Genetics, 137, 677-687, 1994). We have prepared U6 RNAs substituted at nts 40-46 and 53-58 with a single iodouridine. These are used to reconstitute U6-depleted prp16-1 mutant extract in which prp16-1p has diminished ATP hydrolysis activity that almost completely blocks the Prp16p-dependent step. Thus prp16-1p may bind its RNA substrate(s) for a prolonged period, and we may be able to crosslink prp16-1p to iodouridine-substituted U6 snRNA if U6 is a substrate for Prp16p. For a set of U6 RNAs with a single iodouridine at position 40, 41, 42, 43, 44, 45, or 46, each iodouridine-substituted U6 produced a UV-dependent crosslink to a 50-55 kDa protein (perhaps Prp24p, MW 51 kDa). Experiments are underway to try to determine whether the crosslink is dependent on ATP, prp16-1 versus wt extract, and the iodo-U versus wt U6 RNAs. To determine if the crosslinked protein is Prp24p, we will test whether the crosslink is specifically immunoprecipitated with anti-Prp24 antibodies from RNase T1 digested samples. Further attempts to crosslink prp16-1 in the mutant extract involve using the highest specific activity possible for the 32P-labeled iodouridine-substituted U6 RNAs and irradiating with a UV lamp as well as our He-Cd laser (325 nm line). A set of U6 RNAs with a single iodouridine at each site from 53-58 (as suggested by a recent genetics study: D. McPheeters, RNA 2, 1110, 1996) will also be tested in the prp16-1 extract.

211. U2 RNA interactions in the spliceosome:

The crosslinking approach

Andrey G. Balakin

U2 snRNA is a crucial player in pre-mRNA splicing. At an early stage of spliceosome assembly, ATP-dependent interaction of U2 with pre-mRNA defines the intron branch point nucleotide. Following this recognition event U2 actively participates in both catalytic steps of splicing through multiple interactions with other molecules. At least nine proteins have been identified as specific components of U2 snRNP in mammals and homologues for most of them are known in yeast. In yeast, U2 is also believed to be a substrate for a putative RNA helicase, Prp5. U2 RNA-RNA interactions include its base-pairing with the pre-mRNA branch point site and the 3' region of U6 snRNA. The ultimate goal of this study is to identify physical contacts of U2 with components of the spliceosome during assembly and catalysis. We are using the yeast cell-free system, where splicing activity can be efficiently and reproducibly reconstituted with in vitro synthesized U2 RNA.
We tested a number of mutant U2 RNAs containing large deletions in the non-essential central domain for their ability to substitute for endogenous U2. One RNA (260 nt) was found to be more efficient than a full-length U2 T7 transcript (1175 nt) or any mutant RNA tested. This molecule was constructed from five fragments: four synthetic oligoribonucleotides representing the first 118 nucleotides of U2, and a T7 transcript corresponding to the rest of the molecule. For crosslinking experiments one of the oligonucleotides will be chemically synthesized to contain a single photoreactive 4-thiouridine substitution. The molecules assembled will be UV irradiated in U2 depleted extracts and RNA-RNA and RNA-protein photo adducts will be analyzed. In preliminary studies the applicability of the approach has been demonstrated with commercially available 4-thiouridine phosphoramidite.

212. Probing 5' splice site interactions using a yeast trans-splicing system

Tracey L. Johnson

RNA-RNA and RNA-protein interactions at the 5' splice site are quite likely to be important for accurate and efficient splicing. Studies of these interactions can be facilitated by utilizing a trans-splicing system with splicing extracts from Saccharomyces cerevisiae (Ghetti & Abelson, 1995). In these reactions, the RNA contributing the 5' splice site is a 20-nucleotide, chemically-synthesized RNA, while the 3' splice site is contained on an in vitro synthesized RNA. We now show that the trans-splicing reaction proceeds normally when the 5' synthetic RNA is biotinylated either at the 3' end, the 5' end, or both.

The RNA that is precipitated from the splicing reaction is incubated with streptavidin-coated paramagnetic beads and, hence, any biotinylated reaction product can be separated from unbiotinylated RNAs. This system allows easy isolation of the splicing products and intermediates for subsequent studies of RNA requirements at the 5' splice site as well as RNA-RNA and RNA-protein interactions. By using this approach, we are able to substitute photoreactive crosslinkable groups within the 5' splice site-containing, synthesized RNA to identify the RNA-RNA and RNA-protein interactions that occur during splicing. Additionally, these studies will be facilitated by the use of temperature sensitive mutant strains which can, by heat inactivation of particular PRP proteins, be used to halt splicing at discreet steps. This should allow us to follow the interactions at the 5' splice site through the splicing reaction.

References

213. PRP22p, a DEAD-box splicing protein, catalyzes RNA duplex unwinding

John D. O. Wagner

The conformation that RNA molecules adopt is of central importance in defining their capacity to act as catalysts in biochemical reactions, as well as to interact specifically with other cellular components. Therefore, proteins that define or modulate RNA conformation are likely to be central to both determining and regulating the ordered progression of RNA-related processes. However, very little is known mechanistically about how proteins modulate RNA conformation. The members of a large group of proteins related by a conserved central domain, the "DEAD/H-box," are good candidates for study in this respect. DEAD/H-box proteins are involved in nearly every aspect of RNA metabolism characterized to date, including RNA synthesis, processing and maturation, transport and localization, translation, and degradation. Where characterized, these proteins have been shown to be RNA-dependent ATPases, that is, that they consume energy-rich chemical bonds in a reaction that is linked to an RNA interaction. It has been suggested that these proteins may act as "molecular motors" that drive energy-dependent conformational alterations in RNA-protein complexes.

Our studies have focused on examining the activity of Prp22p, a DEA/H-box protein required for the later stages of pre-mRNA splicing. In splicing, a number of the DEAD/H-box proteins are required, each at specific, ATP-requiring steps. Indirect evidence would suggest that several of these proteins are involved in gross morphological alterations in the ribonucleoprotein pre-mRNA splicing complex, the spliceosome. However, no evidence that the reactions catalyzed by these proteins involve direct RNA structural rearrangements is available. We have purified recombinant Prp22p to near homogeneity. The recombinant protein is functional in an in vitro pre-mRNA splicing assay. The complementation of the prp22-specific splicing block by the recombinant protein is specific, efficient, and ATP requiring, as expected. In an exciting result, we have shown that the recombinant protein can catalyze the disruption of intermolecular RNA duplexes, that is that Prp22p exhibited RNA unwinding activity in vitro. We have shown that RNA unwinding was dependent on ATP and, most likely, ATP hydrolysis. Specific mutation of the putative ATP phosphate binding motif in the recombinant protein eliminated the RNA unwinding capacity, demonstrating that the recombinant protein is responsible for the activity and providing further evidence that ATP is a cofactor for the unwinding reaction. These results are significant because they suggest that the DEAD/H-box proteins directly act on RNA substrates within the spliceosome. It is anticipated that the system will provide the basis for probing the mechanism of the RNA-unwinding
reaction, with the aim of better understanding how proteins modulate RNA structure.

214. Dominant negative mutants in the search for PRP5 function
   Imre Barta

   The PRP5 gene of the yeast Saccharomyces cerevisiae encodes one of several structurally related proteins that are believed to mediate ATP dependent conformational rearrangements during pre-mRNA splicing. Prp5p is a member of the DEAD-box subfamily of putative RNA-dependent ATPases and ATP-dependent RNA-helicases and has been implicated in pre-spliceosome formation, the first ATP requiring step in splicing. Although the specific substrate of Prp5p has not yet been identified numerous data have indirectly linked Prp5p to U2 snRNA. Our goal is to obtain direct evidence for an interaction between Prp5p and other components of the spliceosome that would help us identify the function of the protein.

   Previous experiments have suggested that Prp5p is not an integral part of the spliceosome but its interaction with splicing components is only transient. To circumvent this problem we attempted to generate mutants that would stall the assembling spliceosome at the Prp5p-dependent step. The finding that PRP5 is essential for viability made it possible to set up a genetic system that allowed us to test phenotypes of PRP5 mutants. We have identified three prp5p mutants that upon overexpression inhibited growth even in the presence of a functional PRP5 allele. This dominant negative growth phenotype is consistent with a scenario in which mutant prp5p is still able to interact with its substrate but is unable to carry out one or more of its putative enzymatic activities such as ATP binding, ATP hydrolysis and/or RNA unwinding. We are planning to isolate and analyze complexes containing mutant prp5p.

215. Affinity purification of yeast splicing complexes
   Scott Stevens

   Purification of yeast splicing complexes has remained an elusive task due to their low abundance in the cell. Purification of the individual yeast snRNPs as well as splicing complexes assembled on pre-mRNA will allow the immediate identification of the components involved in yeast splicing. Last year, the yeast genome sequence was completed and now all of the yeast genes are immediately identifiable. Purification and identification of the protein components involved in splicing in yeast will allow us to identify the gene from protein sequence or mass spectrometry data which will speed genetic and biochemical characterization of the genes and gene products involved.

   Biochemical affinity purification of proteins and protein complexes can be achieved using epitope tagging techniques and/or metal affinity tagging techniques. Affinity purification of RNA and RNA complexes can be achieved by using aptamer technology which involves using a sequence that has evolved to bind a ligand either naturally or in the laboratory. For the purification of the assembled yeast spliceosome, I have chosen to use the RNA hairpin II from the human U1 snRNA which binds specifically and with high affinity to the human U1A protein. By including tandemly repeated copies of the hairpin in the pre-mRNA, it should be possible to assemble complexes in active yeast splicing extracts and purify them using immobilized human U1A protein. Separation of the resulting polypeptides on 2-D SDS-PAGE gels will allow their characterization by tandem mass spectrometry. Because the yeast splicing system can be genetically or biochemically arrested at various stages in the splicing reaction, we can observe snapshots of the players at any one point in time.

   To purify the individual snRNPs, I have chosen to use protein tags on a protein known to bind to a particular snRNP. Using an octahistidine sequence followed by an epitope for a commercially available antibody, I plan to use antibody affinity chromatography in serial with metal affinity chromatography to purify the snRNPs from splicing extracts. The same techniques used for the spliceosome complexes will be employed to identify the genes and gene products from the yeast genome database. Since the individual snRNPs are contained in the spliceosome, these techniques will involve some overlap of proteins in the snRNP set and the spliceosome set, but will show if any of the proteins involved leave the spliceosome upon assembly as well as showing which non-snRNP associated factors bind and at what stage in the reaction.

216. Structural studies of DEAD box proteins
   Randall M. Story, Hong Li

   DEAD box proteins form a large family of ubiquitous proteins that are utilized in processes involving RNA. These proteins share a series of amino acid motifs, and are observed to be RNA-dependent ATPases in vitro. Some have been shown to be RNA helicases as well. We are interested in X-ray crystallographic studies of these proteins so as to further our knowledge of how these proteins couple ATP binding and hydrolysis to biological processes involving RNA. Extensive trials with a number of these proteins have not yielded crystals. Our current efforts focus on two proteins: (1) E. coli DbpA, in complex with RNA or Fab, and (2) a 38 kDa protein from Methanococcus jannaschii.

   DbpA is a 50 kDa protein with an unknown yet essential function. It is unique in that its ATPase activity has been shown to be activated by a specific RNA, namely a region of 23S rRNA (Nicol and Fullerpace, 1995). The laboratory of Fuller-Pace has overexpressed DbpA in a T7.7 vector. Using this construct and a modification of their protocol, we can rapidly purify roughly 70 mg of protein from 4 liters of cells. We have made several T7 constructs from
which we can synthesize 5-10 mg RNA from a 10 ml reaction. Several constructs approximately 75 nt long have been purified. We have developed a gel shift assay that shows binding of such RNAs at nanomolar concentrations of protein. Recently, Chris Tsu, in Uhlenbeck's laboratory at CU-Boulder, has shown activation of the ATPase with a much shorter RNA of 32 nucleotides. We have cloned this fragment for T7 transcription, and will use this RNA in future experiments. RNA-protein cocrytallization experiments are being attempted in the absence of added nucleotide, or with ADP, ATP, or analogs. Additional experiments are underway to crystallize a DbpA-Fab complex, in collaboration with Fullers-Pace's laboratory.

We have turned to the hyperthermophile Methanococcus jannaschii as a source for a DEAD box protein that might be particularly stable and suitable for crystallographic studies. We can purify 35 mg of protein, tagged with 10 histidines at the amino terminus, from 4 liters of cells. We are attempting to crystallize this protein, either alone or with nucleotide cofactors. Additional experiments will be performed to assay the biochemical properties (ATPase, helicase) of this protein.

Reference

217. Initial studies of the coupling of transcription and splicing in yeast
Tracy L. Johnson

Recent studies of various nuclear processes (such as transcription, RNA 3'-end processing, and splicing) indicate that these reactions are likely to be temporally as well as spatially coordinated. However, the mechanism of this coordination remains unclear.

Work from a number of labs indicates that mammalian splicing factors can associate with RNA polymerase II, particularly the hyperphosphorylated (elongation-competent) I10 form of the polymerase. This has led to a model in which the polymerase, via its hyperphosphorylated C-terminal domain, recruits transcriptional, splicing and cleavage-polyadenylation machineries, and mature message production is enhanced by channeling the newly-synthesized RNA directly to the processing apparatus. One might also imagine that regulatory events that occur during transcription elongation such as transcriptional pausing and premature termination may also affect subsequent events such as splicing.

We are interested in examining the coupling between transcription and splicing using yeast splicing extracts. Western blot analysis indicates that the standard splicing extracts contain RNA polymerase II. Furthermore, a monoclonal antibody directed against the C-terminal domain of the RNA polymerase can inhibit splicing in yeast extracts.

We also show that the extract sustains transcriptional activity. RNA polymerase II is capable of promoter-independent transcription from a template containing a single-stranded poly dC extension. Using such a dC-tailed template derived from the Histone H3.3 gene (which contains several well-characterized blocks to Pol II elongation), we demonstrate that the extract has α-amanitin sensitive transcriptional activity and that it efficiently responds to elongation blocks. Finally, we have identified conditions in which both transcription and splicing can occur using the yeast extracts.

These preliminary experiments support the idea of a coupling between transcription and splicing and allow us to begin to look at the factors that may be involved in coupling the two processes in yeast. Furthermore, these experiments open up the possibility that templates can be designed which can be transcribed by polymerase in the splicing extracts to produce splicing substrates, thus allowing direct examination of splicing to changes in transcription elongation.

References
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218. The yeast tRNA splicing endonuclease
Christopher Trotta

In yeast, there are 269 tRNA genes of which 59 genes encoding tRNAs from ten different isoaccepting groups, contain short intervening sequences. These introns must be removed to produce functional tRNA molecules. Intron removal requires the function of three enzymes: a tRNA splicing endonuclease, a tRNA splicing ligase and a 2' phosphotransferase. Our work has been directed at understanding the composition and function of the tRNA splicing endonuclease of Saccharomyces cerevisiae. Purification of the low abundance, membrane-bound enzyme has shown it to be composed of four distinct protein subunits of 54, 44, 34, and 15 kDa. The genes encoding each of the four subunits have been identified through a combination of genetic and biochemical approaches (Trotta et al., 1997). Each subunit is essential for viability. All four subunits contain nuclear localization sequences, supporting the notion that the endonuclease is a nuclear membrane-bound protein. Further analysis of the sequence features of the subunits of endonuclease has revealed several interesting features. The gene for the 44kDa subunit, SEN2, contains a potential membrane spanning domain and a possible antiparallel coiled-coil, a sequence feature used for protein-protein or protein-RNA interactions. The gene for the 15kDa subunit, SEN15, contains a potential mitochondrial import sequence suggesting this subunit may have dual function. The gene for the 54kDa subunit, SEN54, is a highly positively charged protein and as such may interact with the negatively charged phosphate backbone of nucleic acid, specifically tRNA. Most importantly, there is a homologous domain of approximately 130 amino acids found in the SEN2 and
 SEN34 genes suggesting that the proteins encoded by these genes perform a similar function in endonuclease.

Two pieces of information have helped reveal the function of these two subunits of endonuclease. First, a mutation isolated in the SEN2 gene, sen2-3, produced endonuclease enzyme which failed to cleave pre-tRNA at the 5′ splice site, suggesting that SEN2 carries an active site for the cleavage reaction (Ho et al., 1990). Second, this domain of 130 amino acids is also found in the endonucleases from organisms of the Archaea. Archael tRNA splicing is rather different than eucaryal splicing. Recognition of the intron by the archael endonuclease requires a very defined structural element whereas the eucaryal endonuclease relies on recognition of the mature domain of tRNA and not the intron (see abstract by Hong Li). However, the cleavage reaction is identical with both reactions resulting in a 2′3′ cyclic phosphate and 5′OH. Taken together these two pieces of information strongly support the notion that the homologous domain encodes the active site for the cleavage reaction. Unlike the tetrameric yeast endonuclease, the archael endonuclease behaves as a homodimer with each monomer containing a single active site. This suggests that both SEN2 and SEN34 contain an active site for cleavage. Given the phenotype of sen2-3 (failure to cleave at the 5′ splice site) this suggests that SEN34 contains the active site for cleavage of the 3′ splice site. To test this we generated a mutant in the homologous domain of SEN34 and demonstrated that the mutation impaired cleavage at the 3′ splice site only (Trotta et al., 1997). This leads us to the following model for the yeast endonuclease:

![Diagram of SEN34 genes suspecting the proteins encoded by these genes perform a similar function in endonuclease. Two pieces of information have helped reveal the function of these two subunits of endonuclease. First, a mutation isolated in the SEN2 gene, sen2-3, produced endonuclease enzyme which failed to cleave pre-tRNA at the 5′ splice site, suggesting that SEN2 carries an active site for the cleavage reaction (Ho et al., 1990). Second, this domain of 130 amino acids is also found in the endonucleases from organisms of the Archaea. Archael tRNA splicing is rather different than eucaryal splicing. Recognition of the intron by the archael endonuclease requires a very defined structural element whereas the eucaryal endonuclease relies on recognition of the mature domain of tRNA and not the intron (see abstract by Hong Li). However, the cleavage reaction is identical with both reactions resulting in a 2′3′ cyclic phosphate and 5′OH. Taken together these two pieces of information strongly support the notion that the homologous domain encodes the active site for the cleavage reaction. Unlike the tetrameric yeast endonuclease, the archael endonuclease behaves as a homodimer with each monomer containing a single active site. This suggests that both SEN2 and SEN34 contain an active site for cleavage. Given the phenotype of sen2-3 (failure to cleave at the 5′ splice site) this suggests that SEN34 contains the active site for cleavage of the 3′ splice site. To test this we generated a mutant in the homologous domain of SEN34 and demonstrated that the mutation impaired cleavage at the 3′ splice site only (Trotta et al., 1997). This leads us to the following model for the yeast endonuclease:

The interactions depicted in this model between Sen34 and Sen15 and between Sen54 and Sen2 were confirmed by a two-hybrid analysis. Interaction between these two heterodimers will be investigated in a biochemical approach involving reconstitution of the enzyme from purified protein subunits. Another aspect of this model which we are interested in investigating is the interaction of the enzyme with tRNA. As modeled, we suggest Sen54 because of its highly charged nature interacts with the phosphate backbone of the mature domain. Through its interaction with an active site containing subunit, Sen2p, it then measure the distance from the mature domain to the 5′ splice site and subsequently the 3′ splice site.

References

219. Crystal structure determination of tRNA endonuclease from Methanococcus jannaschii
Hong Li

The native tRNA endonuclease from Methanococcus jannaschii shares sequence homology with the 34 and 44 kDa subunits of yeast tRNA endonuclease, particularly in the hypothesized catalytic domains (see the abstract by C. Trotta). While archael and eucaryal enzymes may share the same catalytic reaction mechanism, their substrate recognition is significantly different. We have initiated an effort to obtain crystal structures of the tRNA endonuclease from Methanococcus jannaschii and its complex with a minimum RNA substrate. This should allow us to make direct comparisons to the yeast system. The native methanogen endonuclease is a 20.6 kDa protein. It has a high content of hydrophobic residues (Leu: 13%, Val 10%) that are regularly spaced, which is reminiscent of leucine zipper motif found in a wide variety of proteins, including tRNA synthetases, and could be involved in protein-protein or protein-RNA interactions. It is also rich in aromatic and glycine residues that are found in the center of the conserved region. These residues are also candidates for sites of RNA-protein interaction as observed in crystal structures of the mRNA splicing protein U1-A and other members of RNP family. A recombinant form of endonuclease from Methanococcus jannaschii with His/FLAG tag at N-terminal end is highly expressed in E coli and is active on archael pre-tRNA as judged by an in vitro splicing assay. Crystals of the endonuclease in this form have been obtained. The smallest dimension of the crystals range from 0.1 to 0.25 mm. The crystals are in an orthorhombic space group and their cell dimensions are a = 62 Å, b = 80 Å, c = 194 Å. With the assumption that there are four endonuclease molecules in an asymmetric unit, the solvent content of the crystals is 54%. Endonuclease crystals diffract to 2.8 Å at a rotating anode X-ray source and to at least 2.4 Å at a synchrotron X-ray source. Native data sets have been collected to 2.5 Å resolution at beamline X4A of the National Light Source at Brookhaven National Laboratory. We are currently looking for heavy atom derivatives in order to solve the structure by isophormous replacement methods.

220. Diversity in seryl-synthetase function?
Margaret E. Saks

Most organisms have twenty aminoacyl-tRNA synthetases. Each synthetase forms an amino acid adenylate from one of the twenty amino acids and subsequently transfers the amino acid to the 3′-terminus of its cognate tRNAs. However, there are exceptions. In the gram positive bacteria, glutamyl-synthetase
(GluRS) transfers glutamic acid to not only tRNA^{Glu}, but also to tRNA^{Gln}. This "misacylation" is then corrected by a transamidase which converts the Glu to Gln. These observations raise the possibility that there may be other exceptions to the "twenty synthetase" rule. Indeed, analysis of the complete genome sequence of *Methanococcus jannaschii* suggests that this archaeon apparently lacks genes encoding GlnRS, AsnRS, LysRS and CysRS. Since the codons for Gln, Asn, Lys and Cys exist in *M. jannaschii* genes, there must be some means for correctly aminocylating the corresponding tRNAs.

Analysis of the amino acid sequence of SerRS from *M. jannaschii* (Mj_SerRS) indicated that it may be a dual function enzyme capable of recognizing tRNA^{CyC} as well as tRNA^{Ser}. Moreover, analysis of the nucleotide sequences of *M. jannaschii* serine tRNAs indicated that the mechanisms involved in the recognition of these tRNAs may differ from those of the *E. coli* system. Thus SerRS from *M. jannaschii* was of interest with respect to problems related to the evolution of aminocyl-synthetases as well as with respect to problems of RNA-protein recognition.

The genes encoding Mj_SerRS as well as serine and cysteine tRNAs were obtained by PCR of the *M. jannaschii* chromosome (kindly provided by Carl Woese). During the PCR step, a 17 promoter was added to each tRNA gene and, in two separate constructs, a sequence encoding six His residues was added to either the 5' or the 3'-terminus of the gene for Mj_SerRS. The tRNA genes were transcribed, in vitro, using T7 RNA polymerase. Mj_SerRS was obtained by over-expression in an *E. coli* strain (BLR(DEL)) which was additionally over-expressing tRNA^{Asn(UCU)} and tRNA^{Ser(CAU)} which are in low abundance in *E. coli* but which proved helpful for the efficient expression of Mj_SerRS.

In vitro aminocylation assays were carried out using purified Mj_SerRS having the tag at the N-terminus as well as purified *E. coli* SerRS. Whereas Ec_SerRS is competent to aminocylate *E. coli* tRNA^{Ser}, it is not competent to aminocylate either tRNA^{Ser} or tRNA^{Ser} from *M. jannaschii*. In contrast, Mj_SerRS is competent to aminocylate serine tRNAs from not only *M. jannaschii* but from *E. coli* as well. Given the different sequences of the *E. coli* and *M. jannaschii* serine tRNAs, it seems likely that details of the mechanisms involved in tRNA^{Ser} recognition differ in the *E. coli* and *M. jannaschii* systems. Moreover, whereas Ec_SerRS had no activity with respect to *E. coli* tRNA^{Glu}, initial rates of aminocylation by Mj_SerRS for *M. jannaschii* tRNA^{Ser} and tRNA^{Ser} differed by only about a factor of five. Further studies are in progress to more completely elucidate the aminocytosylation specificity of Mj_SerRS. However, the preliminary data suggest that Mj_SerRS may join GluRS as an exception to the "twenty synthetase" rule.
to gain new insights into basic questions concerning mitochondrial function and genetics. Thus, the very rare occurrence of complementation between a recessive mtDNA mutation (frameshift mutation or tRNA gene mutation) and the corresponding wild-type gene co-existing in the same cell within distinct organelles has clearly pointed to the fundamental genetic autonomy of mitochondria in cultured mammalian cells. Furthermore, the importance of a synergism between different mtDNA mutations in the same molecule in the manifestation of a mtDNA mutation-induced biochemical defect has been demonstrated in the case of the non-syndromic deafness associated with the 7445 mutation which affects the synthesis of the subunits of NADH dehydrogenase act in concert with the 7445 mutation in bringing about a reduction in oxidative phosphorylation activity. Also, the analysis of transmitochondrial cell lines derived from maternally-related symptomatic or asymptomatic individuals carrying the 12S rRNA 1555 mutation associated with non-syndromic deafness has provided evidence for nuclear gene involvement in the manifestation of the mutant phenotype.

Studies based on the use of a novel method for the measurement of mtDNA mutations in single neurons, isolated by microdissection from cryostat sections, have again highlighted the importance of the nuclear background in the phenotypic manifestation of the mitochondrial tRNA<sup>35S</sup> mutation associated with the MERRF encephalomyopathy. In particular, the role of the nuclear background has been revealed by the lack of correlation between proportion of mutant mtDNA and degree of cell loss in different CNS regions. The recent introduction in our laboratory of a new approach for the in vivo analysis of the control that cytochrome c oxidase exerts on the respiratory flux and its application to the analysis of mutant cell lines carrying the MERRF mutation have opened exciting new perspectives for understanding the pathogenetic role of mtDNA mutations and the striking tissue specificity of the mutation-associated phenotype. Similarly, the development of a new methodology for the analysis of post-transcriptional modifications in the mitochondrial tRNAs has opened the way to the investigation of the role that such modifications have on the synthesis and function of these tRNAs and of the effects that mtDNA mutations may have on such modifications.

Substantial progress has also been made in the investigation of the contribution of the accumulation of mtDNA damage to the aging processes. A previous analysis of ~360 transmitochondrial cell lines constructed by introducing into <sup>p</sup> cells mitochondria from fibroblasts of 21 individuals spanning a large range of ages, from 20 weeks (fetal) to 103 years, had revealed a very significant decrease in the oxygen consumption rate of the transformants with the donor's age. Evidence that this decrease in respiration rate reflected a progressive mtDNA damage, most likely due to oxygen radical effects, has been provided by a recent analysis of mtDNA by a modified DGGE technique, which has revealed an age-related occurrence of heteroplasmy in segments of the D-loop and adjacent region of mtDNA. In the past year, this analysis has been extended to ~280 transformants derived from 4 previously analyzed and 16 additional individuals ranging in age between 65 and 101 years. This new set of measurements has shown a very significant trend towards higher respiration rates and higher activities of NADH dehydrogenase with the increasing age of the mitochondria donors. These results have pointed to an age-related increase in resistance of mtDNA to oxygen radical damage, resulting presumably from more effective defense mechanisms in individuals who have reached a very old age.

The molecular mechanisms underlying the transcription processes in human cells continues to be another area of major interest in our laboratory. The cloning and expression of the transcription termination factor (mTERF) have opened many avenues of investigation. A significant finding has been the demonstration that the recombinant mTERF, although it produces a footprint on the target DNA sequence very similar to that produced by the native protein, lacks a transcription termination promoting activity in an in vitro system. This result points to another component(s) and/or possibly to secondary modifications of the protein being required for making mTERF termination competent. The mTERF has been expressed in bacteria and in insect cells infected with recombinant baculovirus, opening the way to large-scale production of the protein for X-ray crystallographic studies and antibody production. In other investigations, the mouse mTERF cDNA has been cloned, revealing a 72% amino acid identity of the coding region to that of the human mTERF, and a perfect conservation of the modular structure of the protein, with three leucine zippers and two widely spaced basic domains. A 12 kb genomic DNA containing the majority of the mouse mTERF gene has also been cloned, and its organization is presently being analyzed, in preparation for knock-out experiments. In other studies, in organello footprinting of the promoter region of human mtDNA has revealed ATP- and ethidium bromide-dependent modifications that could be correlated with changes in the rate of rRNA but not of mRNA synthesis, strongly supporting the model of independent regulation of transcription of the rRNA genes and of the protein coding genes located downstream.
221. Very rare interorganelar complementation of mitochondrial frameshift and tRNA mutations in cultured human cells

Juan Cabezas-Herrera, José A. Enríquez

Since the discovery of mtDNA-linked diseases, several potential gene therapeutic approaches have been suggested: (i) Allotopic expression of a mtDNA protein coding gene in the nuclear-cytosolic compartment and import of the gene product into the organelles; (ii) DNA-mediated gene transfer; and (iii) mitochondria-mediated mtDNA transfer. The first two approaches, successfully tried in yeast, have remained elusive in mammalian cells. Although a new technique to introduce DNA into isolated mitochondria has been reported (Siebel et al., 1995), we still lack yet a definitive confirmation of the in vivo applicability of this technique.

Mitochondria-mediated transfer of wild-type mtDNA into cells carrying mtDNA with a recessive mutation can have two potential outcomes: the intermixing of the two mtDNAs and functional complementation of the mutant mtDNA by the wild-type form (Hayashi et al., 1994, 1997), or lack of complementation and cell death, under conditions requiring mitochondrial function, because no or very limited interactions occur between mtDNAs carried in separate organelles (Yoneda et al., 1995). Which of these two alternatives will prevail will depend on the nature of the interactions between mitochondria and mtDNA molecules within mammalian cells. We have directed our efforts towards developing conditions for isolating cells which carry two mtDNAs with non-allelic mutations, introduced into the same cell within separate organelles, and which have thereby recovered their mitochondrial function, and to estimate the frequency with which this recovery occurs.

To achieve this goal, we fused pT1 cytoplasts, carrying the MERRF tRNA$^{5\text{C}}$ 8344 mutation (Shoffner et al., 1990), with C47 cells, carrying a frameshift ND4 mutation (Hofhaus and Attardi, 1993), and vice versa. The recessive nature of both MERRF and ND4 mutations suggested that, if mitochondrial fusion would occur, the C47 mtDNA could supply the wild-type tRNA$^{5\text{C}}$ and the pT1 mtDNA could supply the wild-type mRNA for ND4 gene. We took advantage of the selective pressure for mitochondrial function provided by growth of the cells in medium containing galactose instead of glucose.

We were able to isolate clones in both types of fusion experiments. The mitochondrial transformants able to grow under selective conditions contained both mutant mtDNAs and depended on mitochondrial function for their survival. All these clones exhibited the expected electrophoretic patterns of the in vivo synthesized proteins. Evidence of recovery of the respiratory activity in these clones was obtained by measurement of oxygen consumption. The fusion efficiency was estimated to be 2.9% and, under selective conditions, we were able to isolate up to 14 clones from ~1.66x10$^9$ acceptor cells. These data allowed us to estimate, for the first time, the complementation frequency as ~3x10$^3$. This very low frequency of complementation reinforces the idea that, in human cultured cell lines, interactions between mitochondrial genomes carried in distinct organelles occur very rarely.

It is clear that the clarification of this aspect of mitochondrial genetics is extremely important for understanding the phenotypic manifestations, the temporal progression and the susceptibility to genetic therapeutic approaches of disease-causing mtDNA mutations.

References


222. Pathogenetic mechanisms of the mitochondrial tRNA$^{3\text{C}}$ 7445 mutation and coexisting Complex I subunit mtDNA mutations associated with non-syndromic deafness

Min-Xin Guan, José Antonio Enríquez, Nathan Fischel-Ghodsian

Deafness is the most common inherited human sensory defect, affecting one in 2000 children. However, very little is known about the molecular events leading to deafness and about the genes that control normal hearing. Recently, a homoplasmic A to G transition at position 7445 of mtDNA that is immediately adjacent on 3'-end side to the tRNA$^{3\text{C}}$ coding sequence was discovered in two unrelated pedigrees in Scotland and New Zealand, which exhibit maternally inherited non-syndromic sensorineural deafness. In the mtDNA of the New Zealand pedigree, the 7445 mutation coexists with several Complex I subunit mutations.

To understand the pathogenetic mechanism of the 7445 mutation and coexisting Complex I subunit mutations which are associated with non-syndromic deafness in the New Zealand family, a biochemical analysis has been carried out of lymphoblastoid cell lines derived from four individuals of this family and from four control individuals. A significant decrease in the amount of the tRNA$^{3\text{C}}$ and in the rates of mitochondrial protein synthesis, total O$_2$ consumption as well as Complex I-dependent respiration was observed in the four mutant cell lines, when compared to the four control cell lines. These observations strongly suggest that a synergism of the biochemical defects resulting from the mitochondrial tRNA$^{3\text{C}}$ precursor 7445 mutation and the Complex I mutations is responsible for the development of deafness.

In order to test whether there is any nuclear gene involvement in the phenotypic manifestation of non-syndromic deafness associated with mtDNA mutations in the New Zealand pedigree, as suggested by the age difference of the defect, we have used a mitochondrial transformation approach developed in this laboratory.
which utilizes human mtDNA-less (p0) cells as recipients (King and Attardi, 1989). In particular, p0206 cells were fused with enucleated cells derived from four control individuals and four individuals of the New Zealand family. The analysis of transformants is presently being performed, and it is hoped that will lead to a better understanding of the pathogenetic mechanisms of the mtDNA mutations occurring in this family.

References

223. The contribution of a nuclear gene(s) to the biochemical defects underlying the non-syndromic deafness associated with the mitochondrial 12S rRNA 1555 mutation
Min-Xin Guan, Nathan Fischel-Ghodsian

As previously reported, a biochemical analysis of the human mitochondrial 12S rRNA gene mutation at position 1555 associated with maternally inherited non-syndromic deafness and sensitivity to aminoglycoside-induced deafness was carried out on 25 lymphoblastoid cell lines derived from members of a large Arabic-Israeli family carrying this mutation in homoplasmic form and from control individuals. The severity of mitochondrial dysfunction in the mutant cell lines was correlated with the presence or absence of hearing loss in the donor individuals. Furthermore, a considerable variability in the intensity of the biochemical and cell growth defects was observed among the cell lines derived from either the symptomatic or the asymptomatic group of individuals. These observations strongly suggested a role of a nuclear factor(s) in the phenotypic manifestation of the mutation.

The above suggestion led to the prediction that transfer of mtDNA from the mutant cell lines into a constant nuclear background would eliminate the phenotypic difference between the cell lines from symptomatic and those from asymptomatic individuals and reduce the intragroup variation. To verify this prediction, we have used a mitochondrial transformation approach developed in this laboratory utilizing human mtDNA-less (p0) cells as recipients (King and Attardi, 1989). In particular, p0206 cells were fused with enucleated lymphoblastoid cells derived from three controls, three asymptomatic individuals and five symptomatic individuals of the Arab-Israeli pedigree. The analysis of the transformants was performed by measuring the rates of mitochondrial protein synthesis, the doubling times in glucose-containing medium and galactose-containing medium, and the O2 consumption rates. Unlike the original proband-derived cell lines, a similar degree of biochemical dysfunction, as compared to the control transformants, was observed in the transformants isolated from cell lines derived from family members carrying the mutation, whether symptomatic or asymptomatic. These results strongly support the previous conclusion that a nuclear gene(s) contributes to the phenotype of non-syndromic deafness associated with the mitochondrial 12S rRNA mutation.

References

224. Cell loss in nervous system of MERRF patient does not correlate with proportion of tRNA137 mutation
Li Zhou1, Anne Chomyn, Giuseppe Attardi, Carol Miller1

Selective vulnerability of subpopulations of neurons is a striking feature of neurodegeneration. Mitochondrially transmitted diseases are no exception. In this study, CNS tissues from a patient with MERRF syndrome, which results from an A to G transition of nucleotide (nt) 8344 in the mitochondrial tRNA137 gene, were examined from the proportion of mutant mtDNA. Either individual neuronal somas or the adjacent neuropil and glia were microdissected from cyostat tissue sections of histologically severely affected brain regions, including dentate nuclei, Purkinje cells and inferior olivary nuclei, and from a presumably less affected neuronal subpopulation, the anterior horn cells of the spinal cord. The proportion of mutant DNA was quantified by restriction digestion of DNA after PCR amplification using a mismatched primer. The neurons, and the surrounding neuropil and glia from all the CNS regions analyzed exhibited high proportions of mutant mtDNA ranging from 97.6 ± 0.7% in Purkinje cells to 80.6 ± 2.8%, in the anterior horn cells. Within each neuronal group analyzed, neuronal soma values were similar to those in the surrounding neuropil and glia or in the regional tissue homogenate. Surprisingly, neuronal loss varied greatly among the different CNS regions analyzed, ranging from 7% of the Purkinje cells to 46% of the neurons of the dentate nucleus in MERRF cerebellum. Thus, factors other than the high proportion of mutant mtDNA, in particular nuclear-controlled neuronal differences among various regions of the CNS, appear to contribute to the mitochondrial dysfunction and ultimate cell death.

References

225. In vivo control of respiration by cytochrome c oxidase in wild-type and mitochondrial DNA mutation-carrying human cells
Gaetano Villani

The metabolic control of respiration is still poorly understood, due mainly to the lack of suitable approaches for studying it in vivo. Experiments on isolated mammalian mitochondria have indicated that a relatively small fraction of each of several components of the electron transport chain is sufficient to sustain a normal O2 consumption rate. These experiments, however, may not reflect accurately the
in vivo situation, due to the lack of essential cytosolic components in the mitochondrial fraction and to the use of excess of substrates in the in vitro assays. In the present work, an approach has been developed whereby the control of respiration by cytochrome c oxidase (COX) was analyzed in intact cultured human osteosarcoma 143B.TK cells and other wild-type cells and in mitochondrial DNA mutation-carrying human cell lines. Surprisingly, in wild-type cells, only a slightly higher COX capacity was detected than required to support the endogenous respiration rate, pointing to a tighter in vivo control of respiration by COX than generally assumed. Cell lines carrying the MERRF mitochondrial tRNA\(^{\text{Lys}}\) gene mutation, which causes a pronounced decrease in mitochondrial protein synthesis and respiration rates, revealed, in comparison, a significantly greater COX capacity relative to the residual endogenous respiration rate, and, correspondingly, a higher COX inhibition threshold above which the overall respiratory flux was affected. The observed relationship between COX respiratory threshold and relative COX capacity and the potential extension of the present analysis to other respiratory complexes have significant general implications for understanding the pathogenetic role of mutations in mtDNA-linked diseases and the tissue specificity of the mutation-associated phenotype.

References
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226. Role of post-transcriptional modifications in the structure and function of wild-type and MERRF A8344G mutation-carrying human mitochondrial tRNA\(^{\text{Lys}}\)

Mark Helm

Recently, the pathogenetic mechanism of the MERRF encephalomyopathy-associated A8344G mutation in the human mitochondrial tRNA\(^{\text{Lys}}\) gene has been closely examined in this laboratory by experiments carried out in vivo (Enriquez et al., 1995). As a result, the MERRF syndrome is probably the best understood in the constantly increasing list of mitochondria-related inherited pathologies. In particular, in vivo studies utilizing mtDNA-less cell transformants expressing the MERRF-associated mutation and wild-type cell lines have demonstrated that the MERRF mutation affects protein synthesis by lowering both the steady-state level of the mutant tRNA\(^{\text{Lys}}\) and the aminoacylation efficiency of this tRNA, as compared to the wild-type counterpart.

Structural studies have been performed in vitro on human mitochondrial tRNA\(^{\text{Lys}}\) at the Institut de Biologie Moléculaire et Cellulaire in Strasbourg, France. The native mitochondrial tRNA\(^{\text{Lys}}\) has been purified from human human placenta, sequenced, and its structure in solution probed. A comparative structural work carried out on the in vitro transcribed version of the mitochondrial tRNA\(^{\text{Lys}}\) has revealed that modified bases are very important in the correct folding of the native tRNA. Moreover, a comparative aminoacylation analysis of the two types of tRNAs has underlined the crucial role of modified bases in the achievement of functional properties by the tRNA\(^{\text{Lys}}\). Thus, a methylation at position N1 of adenosine 9 acts as a structural chaperone, and its absence would be responsible for a functionally invalid folding, whereas modified bases in the anticodon stem and loop would be important as aminoacylation identity elements, as well as for structural stabilization of this region. These results have led to the conclusion that modified bases have an especially important role in the production of a functional mitochondrial tRNA\(^{\text{Lys}}\).

Base modifications of tRNAs are post-transcriptional events based on the recognition of particular signals within the naked tRNAs by specific modification enzymes. Thus, a single point mutation in the substrate tRNA, such as the one associated with MERRF, may perturb any of these signals and hinder the occurrence of the correct modification pattern. According to our data, the alteration of the post-transcriptional modification pattern may affect the structural and functional properties of the tRNA.

The aim of the present project was to examine if post-transcriptional modifications are involved in the pathogenetic mechanism of the A8344G mutation in the human mitochondrial tRNA\(^{\text{Lys}}\) gene. For this purpose, the extent of post-transcriptional modifications in vivo was quantified both in the wild-type tRNA and in its mutant counterpart. A protocol for the qualitative and quantitative analysis of modified bases in a specific human tRNA was developed, using the mt-tRNA\(^{\text{Lys}}\) as a test material. The first step consisted of culturing several human cell lines in the presence of \(^{32}\)P-orthophosphate, in particular HeLa cells and 143B.TK cells (wild-type cell lines) and R1C3 cells (carrying the MERRF A8344G mutation in homoplasmic form). The isotopic dilution and the final concentration of phosphate in the medium were optimized for in vivo labeling. After cell harvesting and breakage, the mitochondria were isolated according to procedures well established in this laboratory. Total mitochondrial RNA was extracted using a modified guanidinium thiocyanate method. Preparation of total tRNA from the total mitochondrial RNA by differential salt elution from DEAE columns was optimized using commercially available QIAGEN-2-tips.

Purification of tRNA\(^{\text{Lys}}\) from total mitochondrial tRNA was performed with an oligodeoxyribonucleotide probe (Mör et al., 1995) complementary to a 3'-end-proximal segment of the tRNA\(^{\text{Lys}}\). The biotinylated probe was fixed on streptavidin-coated magnetic beads. After hybridization, the probe retained the tRNA\(^{\text{Lys}}\) on the magnetic beads, which could thus be easily separated from the bulk tRNA. This technique has
been previously applied in Strasbourg for the purification and sequencing of tRNA\textsuperscript{59} from human placenta, and was downscaled for the present purpose. In the final step, the tRNA\textsuperscript{59} was submitted to complete digestion by nuclease P1, yielding nucleotides with a 5'-phosphate. These were then separated by two-dimensional thin-layer chromatography, and quantified by phosphorimaging and/or conventional autoradiography (reviewed in Keith, 1990).

The modification patterns of mitochondrial tRNA\textsuperscript{59} from the different human cell lines were analyzed both in log phase and stationary phase cells. The observed patterns were identical to that obtained from placental tRNA\textsuperscript{59}, with one exception. In particular, nucleotide 37 was found to be modified to t6A in the cell lines, whereas it is hypermodified to m52t6A in placenta. Interestingly, a first quantitative comparison of the yield of modified bases in the tRNA\textsuperscript{59} from the wild-type cell line 143B.TK\textsuperscript{+} and in that from the MERRF cell line R1C3 showed some differences. These have to be verified by controlling thoroughly the different parameters of the tissue culture conditions.

An interesting question opened by the present results is whether the hypermodification at position 37 in placenta is a kinetic or rather a tissue-specific phenomenon. The preliminary results of the quantitative comparisons are consistent with an involvement of post-transcriptional modifications of tRNA\textsuperscript{59} in the phenotypic expression of the A8344G mutation. If confirmed, these results may turn out to be important for understanding the pathogenesis not only of the disease under investigation, but also of other pathologies related to point mutations in tRNA genes.

References


227. Further analysis of the contribution of mitochondrial DNA mutations to the aging process

Kenneth Laderman, Laurie Thomas

In previous work from this laboratory, an analysis of ~360 transmitochondrial cell lines constructed by introducing into human mtDNA-less (p\textsuperscript{e}206) cells mitochondria from fibroblasts of 21 human individuals spanning a large range of ages, from 20 weeks (fetal) to 103 years, had revealed two independent functional alterations of mtDNA resulting in a very significant decrease with donor age in the ranges and averages of respiration rates and in a statistically unrelated decrease with donor age in the ranges and averages of the cellular mtDNA contents. In the past year, these investigations have been extended to ~280 transformants derived from 4 previously analyzed and 16 additional individuals ranging in age between 65 and 101 years. Contrary to the previous observations, the new measurements, carried out on transformants derived entirely from fibroblasts of older individuals, have revealed a statistically very significant trend towards higher respiration rates with increasing age. This result is not unexpected, pointing to an age-related increased resistance to, or an increased repair of, free radical damage in the donor cells. An in vitro selection, which removed cells with the greatest mtDNA damage from the primary fibroblast population, or an in vitro selection, in which cells with high mtDNA damage were removed during the transformation procedure, may have taken place. Evidence supporting the occurrence of an in vitro selection has been provided by the observation of a statistically significant decrease in transformation efficiency, which was independent of the passage number of the primary fibroblast cultures, with the increasing age of the mitochondrial donors.

In contrast to the behavior of the respiratory capacity, the measurements of the mtDNA content in the new set of transformants derived from older individuals confirmed the trend of the age-related decline observed in the previous analysis of clones derived from individuals 20 weeks (fetal) to 103 years in age. The mtDNA content again showed no correlation with oxygen consumption, supporting the conclusion that the low mtDNA content is the result of a mtDNA damage independent of the damage which affects the respiration rate.

During the past year, extensive use has also been made of new rapid screening methods developed in this laboratory for the analysis of the enzymatic activities of Complex I (NADH:ubiquinone oxidoreductase) and Complex IV (cytochrome c oxidase), both of which contain mtDNA-encoded subunits, in a microtiter plate format. Thus, analysis of transformants derived from the older age group of mitochondrial donors revealed a strongly positive correlation between Complex I activity and age of the mitochondria donors. By contrast, no significant correlation was observed between Complex IV activity and mitochondria donor age or oxygen consumption of the transformants, strongly suggesting that the cytochrome c oxidase activity is to a large extent independent of these parameters.

In other studies, to support the conclusion that the mitochondria donor age-related decline observed in the respiratory capacity of the p\textsuperscript{e} cell transformants reflected an accumulation of mtDNA damage, rather than being the result of epigenetic factors, second-stage mitochondria-mediated transformation experiments were carried out. For this purpose, cytoplasts from a respiratory deficient and a fully respiratory competent clone derived from a 101 year-old individual and a respiratory competent clone derived from a 10 year-old individual were fused to cells of a p\textsuperscript{e} cell line different from the primary p\textsuperscript{e} cell line, which was isolated from an 8-azaadenine-resistant derivative of the 143B.TK\textsuperscript{+} cell line. The results clearly showed that the
transformants derived from the mutant primary clone exhibited a much lower average oxygen consumption rate than the transformants derived from the age-matched and the young control clones, as well as a lower average Complex I activity. These results were thus consistent with the genetic basis of the observed defective phenotype of the mutant primary transformant.

Reference

228. Structural analysis of mitochondrial DNA in transmitochondrial cell lines derived from fibroblasts of human individuals covering a broad range of ages.

Yuichi Michikawa, Asad Aman¹, Rachel Steinberger³, Kenneth A. Laderman

In a previous study from this laboratory, a large number of transmitochondrial cell lines were obtained by fusion of mtDNA-less (p⁰) cells with cytoplasts derived from fibroblasts of human individuals spanning a broad range of ages (20 weeks fetal and 103 years) (Laderman et al., 1996). A statistically significant donor age-dependent decrease in oxygen consumption rate of the transformants was observed. The cellular mitochondrial DNA (mtDNA) content showed an age-dependent decrease over the entire range of donor ages examined. In the present study, we have focused on the structural analysis of mtDNA from the transformants using a modification of the denaturing gradient gel electrophoresis (DGGE) technique, which can detect even a single point mutation present in 10% of mtDNA molecules (Michikawa et al., 1997).

The D-loop and its vicinity in mtDNA were chosen as the DNA segments to be analyzed because of the presence in this region of sequences involved in regulation of transcription and replication of mtDNA. We chose seven segments which have a single uniform melting domain and range from 161 to 441 bases in size to cover the entire sequence of this region. The 5'-end of one of the PCR primers selected for the amplification of each segment was attached to psoralen for the subsequent UV-induced crosslinking of the complementary strands. The optimum MgCl₂ concentration for PCR and the optimum running time for DGGE were determined for each segment. The mtDNA sequences in the transformants with the highest and the lowest mtDNA content among the cell lines derived from each fibroblast population were compared to each other by making heteroduplexes between the PCR products of each segment before loading them onto the denaturant gradient gel. After analysis of all mtDNA segments in the pairs of transformants derived from 23 differently-aged individuals, we found twelve mtDNA segments in seven age sets which exhibited a different nucleotide sequence in the transformants with the highest and the lowest mtDNA content. Ten of the twelve segments were present in the transformants derived from fibroblasts of individuals over 48 years in age. Surprisingly, ten of those twelve segments exhibited intracellular heteroplasy, and eight of the ten intracellular heteroplasmic states were associated with transformants exhibiting the highest mtDNA content.

We have thus observed a donor age-dependent appearance of intracellular heteroplasmy in the D-loop and its vicinity in mtDNA from transmitochondrial cell lines. For further analysis, we are planning to identify the heteroplasmic mtDNA sequences and try to determine the possible relationship of the intracellular heteroplasmy with the mtDNA content.

References
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229. Further characterization of the recombinant mitochondrial transcription termination factor (mTERF) and analysis of the in vivo expression of the native protein
Patricio Fernandez-Silva, Francisco Martinez-Azorin

We have recently cloned the cDNA for the human mitochondrial transcription termination factor (mTERF) and performed a detailed structure-function analysis of the recombinant mature protein by site-directed mutagenesis and DNA-binding assays. In this way, we have identified a modular organization of mTERF, with two widely separated basic regions and three leucine zipper motifs, which probably form a three-stranded coiled-coil structure. The DNA-binding properties of the in vitro synthesized and natural mTERF are very similar, both binding the target sequence as a monomer in a highly specific way and producing a comparable DNase I footprinting pattern.

We have further characterized the functional activity of mTERF and found that, while the purified in vitro synthesized protein strongly stimulates transcription termination when added to an in vitro transcription system, the recombinant protein is not able to support transcription termination. This result indicates that either a secondary modification of the in vitro synthesized protein or another component, present in the affinity-purified natural product as a minor contaminant, is necessary to confer upon the in vitro product the termination promoting activity. This conclusion is supported by previous results obtained with the mitochondrial in vitro transcription system, which show that KCl concentrations which do not affect transcription initiation nor mTERF binding to DNA, strongly reduce transcription termination. These results suggest that the termination process is more complex than previously thought, requiring at least another component besides mTERF.

In other studies, we have analyzed the expression of the mTERF mRNA in two cell lines and in different human tissues. The mRNA, of about 2.1 kb in size, is
expressed in all tissues and cell lines, being more abundant in the tissues with higher respiratory demand, as muscle and heart, and in the pancreas. An interesting result is the presence in the RNA transfer hybridization blot of a secondary band corresponding to an RNA about 1.4 kb in size, the nature of which is presently under investigation.

Reference

230. Large-scale purification of the human Mitochondrial Transcription Termination Factor (mTERF)
Jordi Asin, Francisco Martinez-Azorin

Transcription of the rRNA coding region from the heavy strand of mitochondrial DNA occurs at a 20- to 50-fold higher rate relative to the downstream genes. In this phenomenon, an essential role is played by the mitochondrial transcription termination factor (mTERF), a DNA-binding protein that binds to a 28 base pair region within the tRNA<sub>Glu</sub> gene.

The human mTERF cDNA has been cloned, sequenced and expressed in an <i>in vitro</i> transcription-translation system, as well as in <i>E. coli</i> and insect cells transfected with recombinant baculovirus. The mature recombinant protein, while exhibiting the expected specific DNA-binding capacity, is unable to promote transcription termination in the <i>in vitro</i> system, suggesting that a posttranslational modification and/or an additional component is necessary to activate the transcription termination promoting capacity of the protein. Furthermore, a detailed structure-function analysis has provided evidence for a novel DNA-binding motif, in which three leucine zippers form an intramolecular three-stranded coiled-coil that brings two widely separated basic domains in close register with the mTERF target DNA sequence.

Present work is being focused on large scale purification of histidine-tagged mTERF expressed in <i>E. coli</i> or in baculovirus-transfected insect cells in order to carry out an X-ray crystallographic analysis of the protein, as well as to raise antibodies against the protein. A third goal is the study, by structural analysis, of the role of phosphorylation in the transcription termination activity of the protein.

References

231. Molecular cloning of the cDNA and gene for the mouse mitochondrial transcription termination factor (mTERF)
Xiaojun Zhao

In mammalian mitochondrial DNA (mtDNA), the only non-coding sequence is found in the D-(displacement) loop region, which is involved in initiation of mtDNA replication and transcription of the (G+T)-rich heavy (H) strand and (A+C)-rich light (L) strand. Another regulatory region of mtDNA is the short termination sequence, located in the segment immediately adjacent and downstream of the 16S rRNA/leucyl-tRNA boundary (Kruse et al., 1989). This region plays a key role in an H-strand transcription termination event at the 3' end of the mtDNA, resulting in the 20- to 50-fold higher expression of the rRNA gene region relative to the downstream genes. A human mitochondrial transcription termination factor (mTERF) has been identified and characterized from our laboratory, which plays a central role in this termination event (Daga et al., 1993). Recently, an A->G transition in the middle of the mTERF-protected mtDNA segment has been demonstrated to be associated with the human MELAS encephalomyopathy and with maternally inherited diabetes mellitus (MIDM). This mutation dramatically reduces the binding affinity of mTERF for its target sequence (Fernandez-Silva et al., 1997). In order to study the functional role of mTERF in controlling transcription termination, as well as the pathogenesis of MELAS and MIDM, I have initiated experiments aimed at knocking the mouse mTERF gene out. I have so far cloned both cDNA and genomic DNA of the mouse mTERF. The DNA sequences of three cloned cDNAs were determined and the putative mouse mTERF coding region was found to exhibit about 72% identity relative to the human mTERF. The putative mouse mTERF precursor contains a mitochondrial targeting sequence and the mature mTERF, three leucine zippers, of which one is bipartite, and two widely spaced basic domains, which appear to highly conserved with the human mTERF. Northern blot analysis has shown that the mouse mTERF is widely expressed in mouse tissues, including heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis. The 12 kb genomic DNA fragment containing the majority of the mouse mTERF gene is currently being analyzed using restriction mapping and DNA sequencing.

References

232. Functional analysis of <i>In vivo</i> and <i>In organello</i> footprinting of HeLa cell mitochondrial DNA
Vicente Micol, Patricio Fernandez-Silva

<i>In vivo</i> and <i>In organello</i> footprinting techniques based on methylation interference have been utilized to investigate protein-DNA interactions in the transcription initiation and rDNA transcription termination regions of human mitochondrial DNA (mtDNA) in functionally active mitochondria. In particular, the changes in methylation reactivity of these regions in response to treatment of the organelles with ATP or ethidium bromide, which affects differentially the rates of mitochondrial rRNA and mRNA synthesis, have been analyzed. Two major sites of protein-DNA interactions have been identified in the main control regions of mtDNA, both <i>In vivo</i> and <i>In organello</i>, which correspond to the regions of the
light-strand promoter and heavy-strand rRNA-specific promoter. The *in organello* footprinting of the latter showed ATP- and ethidium bromide-dependent modifications, that could be correlated with changes in the rate of rRNA but not of mRNA synthesis. By contrast, no ATP effects were observed on the *in organello* footprinting pattern of the termination region and on *in vitro* transcription termination, strongly suggesting that ATP control of rRNA synthesis occurs at the initiation level. Several methylation interference sites were found upstream of the whole H-strand transcription unit, pointing to possible protein-DNA interactions related to the activity of this unit. *In vivo* footprinting of the rRNA transcription termination region of human mtDNA has revealed a very strong protection pattern, indicating a high degree of occupancy of the termination site by the mitochondrial transcription termination factor (~80%).

233. Pathogenetic mechanism of the MELAS mutation

Anne Chomyn, José Antonio Enriquez, Vicente Micol, Patricio Fernández-Silva

A mutation at position 3243 in the human mitochondrial tRNA<sub>Leu(UUR)</sub> gene causes the MELAS syndrome [mitochondrial encephalopathy and lactic acidosis, with stroke-like episodes (Goto et al., 1991)], and in other families, maternally inherited diabetes mellitus. This mutation occurs in the binding site of mTERF, the mitochondrial transcription termination factor (Kruse et al., 1988), but does not appear to affect the steady state levels of transcripts of the DNA upstream and downstream of the binding site (Chomyn et al., 1992).

In a further investigation of the pathogenetic mechanism of this mutation, we have carried out an *in vivo* footprinting analysis of the tRNA<sub>Leu(UUR)</sub> gene, and found that occupation of the binding site is not significantly altered quantitatively or qualitatively in mutant cells. This finding is consistent with our earlier finding that termination of transcription *in vitro* is not affected in mutant cells. Looking at another function of the tRNA<sub>Leu(UUR)</sub> gene, we have investigated the steady state level of the tRNA<sub>Leu(UUR)</sub> and its degree of aminoacylation and have found both to be reduced. Thus, it appears that a reduction in the amount of the charged tRNA contributes to the pathogenetic effect of the mutation. However, unlike the situation found in cells carrying the MERRF mutation in the tRNA<sub>Leu(UUR)</sub> gene (Enriquez et al., 1995), there appears to be no correlation of reduction in synthesis rate of a given polypeptide with the number of UUR codons in its reading frame. Furthermore, we find no evidence of prematurely terminated translation products. Our investigations are leading us to the hypothesis that the 3243 mutation affects protein synthesis at the level of initiation of translation. Experiments are being done to test this hypothesis.

234. An ND6 frameshift mutation leads to a failure of assembly of mtDNA-encoded subunits of NADH dehydrogenase and a non-functional enzyme

Yidong Bai

The mammalian mitochondrial NADH dehydrogenase (Complex I) is composed of more than 40 subunits encoded by either the nuclear or the mitochondrial genome. An increasing number of mutations in mtDNA-encoded subunits of this enzyme have been shown to be involved in Leber's hereditary optic neuropathy, but the functions of these subunits are largely unknown. We report here the isolation of a mouse cell line which has a frameshift mutation in the ND6 gene resulting in the lack of that subunit. The absence of the ND6 subunit leads to the failure of assembly of other mtDNA-encoded subunits of Complex I, while at least some of the nuclear-encoded subunits involved in the redox reactions are assembled normally. In this mutant cell line, the oxygen consumption dependent on Complex I function is strongly reduced, and the NADH:Q1 oxidoreductase activity is absent. The ND6 mutant cell line also fails to grow in medium containing galactose instead of glucose, indicating an impairment in the oxidative phosphorylation function. All these results point to a essential role that the ND6 subunit plays in Complex I activity.

235. Isolation and characterization of a mouse ND5 mutant cell line

Yidong Bai

We have continued our efforts in screening for mouse cells which are respiration deficient due to mutations in the mitochondrial genome among rotenone-resistant A9 derivatives (Annual report 203, 1996). One of the cell lines (3A) showed a significant decrease in NADH dehydrogenase (Complex I) activity. However, mitochondrial protein synthesis analysis failed to provide evidence pointing to an abnormal subunit.

The mitochondria from 3A cells were transferred into mouse mtDNA-less cells, and 27 transformants were isolated. Surprisingly, a wide spectrum of Complex I activities were detected among the transformants, from wild-type level to severe deficiency. We isolated two of the transformants with the highest (3A19) and the lowest (3A20) Complex I activity for further characterization. The mitochondrial protein synthesis patterns revealed that the ND5 subunit of Complex I was almost absent in 3A20, while 3A19 had a normal product. Subsequent sequencing analysis showed a C to A mutation in 3A20 cells, which changed an arginine codon to a mitochondrial stop codon. Consequently, the ND5

References


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product, normally 608 amino acids long, was truncated at the 106th amino acid.

The mitochondrial genome is very dynamic as compared to the nuclear one, and heteroplasmy is quite common. Our results have indicated that mitochondria-mediated transformation of mtDNA-less cells can serve as a purifying method to isolate cells carrying a homoplasmic mutation for further investigation. Furthermore, the transformants with different Complex I activities provide us with an opportunity to study the threshold level of the ND5 product in Complex I functions.

236. Allotopic expression of mitochondrial genes in human cells

Yehua Weng, Susan S.-A. T. Lai, Anne Chomyn

We are investigating the possibility of complementing mutations in mitochondrial protein-coding genes by expressing a wild-type version of the gene in the nuclear-cytosolic compartment and importing the gene product into mitochondria. Our test gene is the one for ND3, which encodes a subunit of NADH dehydrogenase. We have made several bipartite constructs, consisting of a mitochondria targeting sequence joined to the universal code equivalent of the ND3 gene. We have used the mitochondria targeting sequence of the human cytochrome c oxidase subunit 4, a nucleus-encoded mitochondrial protein, in monomer or dimer form, with two or eight amino acids following the signal cleavage site, or alternatively, the targeting sequence from the bovine ATP synthetase subunit 9, a very hydrophobic nucleus-encoded mitochondrial protein.

We are able to synthesize all of these hybrid proteins in vitro using a coupled transcription-translation system. We are using these hybrid proteins in vitro mitochondrial import assays. As a positive control, we are using the precursor of mTERF, the mitochondrial transcription termination factor (Fernandez-Silva et al., 1997). The constructs encoding those proteins that are imported with the greatest efficiency will be used to transfect HeLa cells, which make an electrophoretic variant of ND3.

Publications


Fernandez-Silva, Martinez-Azorin, F., Micol, V. and Attardi, G. (1997). The human mitochondrial transcription termination factor (mTERF) is a multizipper protein but binds to DNA as a monomer, with evidence pointing to intramolecular leucine zipper interactions. The EMBO J. 16, 1066-1079.


molecules to the fetus or infant is a mechanism by which mammalian neonates acquire humoral immunity to antigens encountered by the mother. The protein responsible for the transfer of IgG is called FcRn, for Fc receptor neonatal. Unlike other known Fc receptors which are composed of tandemly repeated immunoglobulin-like domains, FcRn is a heterodimer that shows an intriguing structural similarity to class I major histocompatibility complex (MHC) molecules. The FcRn light chain is β2-microglobulin, the same light chain that is associated with class I MHC molecules, and the heavy chains of FcRn and class I MHC molecules are related by low but significant sequence similarity. MHC class I molecules have no reported function as immunoglobulin receptors; instead they bind and present short peptides to T cells, and stable cell surface expression depends on peptide binding. The similarity at the primary, tertiary, and quaternary structure levels between FcRn and class I MHC molecules suggests that they share a common ancestor, in spite of their apparently unrelated immunological functions.

We solved the crystal structures of a soluble version of rat FcRn both alone and as a complex with Fc (Burmeister et al., 1994a,b). The structural studies suggest that FcRn dimerizes in response to IgG binding, which may serve as a component of a signal to initiate internalization and subsequent transcytosis of IgG/FcRn complexes. We hypothesize that formation of an oligomeric ribbon of FcRn dimers on adjacent membranes bridged by IgG molecules is also required for FcRn function (see Figure 1, following page). The FcRn interaction with IgG represents a well defined receptor/ligand system in which to address questions of broad biological significance related to receptor-ligand interactions, receptor function, signaling induced by ligand binding, and protein trafficking. Specifically, our hypothesis about IgG-mediated bridging of FcRn dimers on adjacent membranes has implications for signaling events as well as for intracellular trafficking of vesicles. Because FcRn is now well characterized biochemically and structurally, and we can assay its function in transcytosis, this hypothesis is testable. Using information from the three dimensional structures of FcRn and its complex with Fc, we can design mutants that disrupt the dimerization capability of FcRn or the bridging capability of Fc and quantitate the effects of these mutations upon FcRn binding (using a surface plasmon resonance assay) and function (using a transcytosis assay). Thus the FcRn/IgG interaction represents an ideal system to study questions of the effects of receptor dimerization upon signaling and function, and the evolutionary origin of the MHC fold in immune recognition.

We have recently become interested in other MHC homologs, including proteins encoded by viruses. Both human and murine cytomegalovirus (HCMV, MCMV) express a β2-microglobulin binding relative of MHC class I heavy chains, probably as part of the viral defense mechanism against the mammalian immune system. Our biochemical studies show that the HCMV homolog associates with endogenous peptides resembling those that bind to class I MHC molecules. Current efforts focus upon defining the structure and function of these homologs in order to understand why viruses make them and how they interfere with the host immune system.

We've also begun work on Zn-α2-glycoprotein (ZAG), a soluble MHC class I homolog present in low concentrations in most bodily fluids. ZAG is found in much higher concentrations in some mammary carcinomas, as well as in cysts from breast gross cystic disease suggesting that ZAG participates in the development of breast diseases. In order to identify the function of ZAG and its relationship with breast cancer, the protein was purified from breast cyst fluid and serum and crystallized. A three dimensional structure determination is in progress. The comparison of its structure with the structure of MHC class I proteins will clarify the intriguing relationship between those molecules and will provide the basis for the search for ligands that bind to ZAG. A combination of structural studies, computational analysis, and ligand binding experiments will be used for analyzing the function and potential roles of ZAG, both in physiological conditions and in the evolution of breast cancer.

References

237. Identification of critical IgG binding epitopes on the neonatal Fc receptor
Daniel E. Vaughn, Christina M. Milburn, David M. Penny, W. Lance Martin, Jennifer L. Johnson
The neonatal Fc receptor (FcRn) binds maternal immunoglobulin G (IgG) during the acquisition of passive immunity by the fetus or newborn. FcRn also binds IgG and returns it to the bloodstream, thus protecting IgG from a default degradative pathway. Biosensor assays have been used to characterize the interaction of a soluble form of rat FcRn with IgG, and demonstrate that FcRn dimerization and immobilization are necessary to reproduce in vivo binding behavior. We have identified several FcRn amino acid substitutions that disrupt its affinity for IgG and examined the effect of alteration of residues at the FcRn dimer interface. The role of these amino acids can be interpreted in the context of the previously reported structures of FcRn and a complex of FcRn with the Fc portion of rat IgG.

238. High affinity binding of the neonatal Fc receptor to its IgG ligand requires receptor immobilization Dan Vaughn
The neonatal Fc receptor (FcRn) binds maternal immunoglobulin G (IgG) during the acquisition of passive immunity by the fetus or newborn. In adult mammals, FcRn also binds IgG and returns it to the bloodstream, thus protecting IgG from a default degradative pathway. Biosensor assays have been
Figure 1 (see Bjorkman Summary)
used to characterize the interaction of a soluble form of FcRn with IgG. We used the statistical method of cross-validation to show that there are two classes of non-interacting binding sites, and these are sufficient to account for previously observed non-linear Scatchard plots of FcRn/IgG binding data. We demonstrated that immobilization of FcRn on the biosensor surface reproduces the high affinity IgG binding observed for membrane bound FcRn, whereas immobilization of IgG results in lower affinity binding similar to that of the FcRn/IgG interaction in solution. The dependence of FcRn/IgG binding affinity on the coupled molecule provides further evidence in support of the previously hypothesized model that an FcRn dimer forms the high affinity IgG binding site.

239. Structural basis of pH dependent antibody binding by the neonatal Fc receptor

Dan Vaughn

We are interested in the molecular mechanism of pH dependent immunoglobulin G (IgG) binding by the neonatal Fc receptor (FcRn). FcRn binds IgG with nM affinity at pH 6, but does not bind at µM concentrations at pH 7.5. In addition to the pH dependence of IgG binding, previous work in our laboratory revealed two additional pH dependent properties: FcRn is more stable at pH 6.0-6.5 compared to pH 7.5-8.0 as assayed by thermal denaturation profiles, and the dissociation rate of FcRn heavy chain is an order of magnitude slower at pH 6 than at pH 8. Taken together, these results suggest that the interaction between the two chains of the FcRn heterodimer is altered over this narrow pH range. The structure of FcRn was previously determined in our laboratory at an acidic pH. We have now solved the structure of FcRn at an alkaline pH. This structure determination reveals an extensive carbohydrate mediated interaction facilitating the dimerization of FcRn molecules to form the high affinity binding site for IgG at pH 6. We have also further refined the structure of FcRn at acidic pH to include this additional carbohydrate structure. These structures were compared with specific attention to the pH dependence of FcRn stability and IgG affinity. We can now propose a mechanism for pH dependent antibody binding to FcRn based on these structures and the body of structure/function literature concerning this interaction.

240. Evidence for IgG stimulated dimerization FcRn at the cell surface

Tirunelveli S. Ramalingam, Yang Liu

The neonatal Fc receptor (FcRn) transports maternal IgG from ingested milk to the bloodstream of the newborn. Binding of IgG is pH dependent, with high affinity binding occurring at pH 6 - 6.5 (the pH of ingested milk in the gut) and no detectable binding at pH 7.5 (the pH of blood). Crystal structures of the receptor alone and of an FcRn/Fc complex revealed a dimer of FcRn heterodimers which was hypothesized to be involved in binding of IgG. Two FcRn molecules bind to a single Fc both in solution and in the FcRn/Fc co-crystals, but the packing in the crystal creates two distinct 2:1 FcRn/Fc complexes. One of the 2:1 complexes utilizes a dimer of FcRn heterodimers observed in crystals of FcRn alone. We are interested in learning if the crystallographically observed FcRn dimer is relevant for IgG binding, and whether or not dimers of FcRn pre-exist on a cell surface or are induced by binding of IgG. Pleiotropic effects produced by many cytokines and growth factors are communicated into the cell by ligand induced dimerization of their respective receptors on the cell surface. Hence it is tempting to hypothesize that dimerization of FcRn in response to IgG binding transmits an endocytosis signal which initiates transcytosis of the receptor/IgG complex. In order to assess if membrane bound FcRn dimerizes in response to IgG binding, we constructed a chimeric cDNA encoding the extracellular domain of FcRn and the transmembrane and intracellular domains of the Trk tyrosine kinase. Upon dimerization of the chimeric gene product in transfected cells, the extracellular tyrosine kinase domains should be brought into spatial proximity such that cross phosphorylation will occur. Phosphorylation experiments at pH 6.0 in transfected CHO expressing the chimera showed that the basal level phosphorylation of the receptor was enhanced in the presence of IgG or Fc, while no appreciable change in phosphorylation was detected at pH 8.0. This suggests that the FcRn dimerizes at the cell surface in response to IgG binding. Further experiments using this dimerization readout system will involve use of a chimeric Fc, in which only one side of the Fc homodimer contains an FcRn binding site (see Abstract #5 by Lance Martin) and FcRn mutants with alterations at the dimer interface.

241. Neonatal Fc receptor and IgG: ligand-induced receptor oligomerization

W. Lance Martin

The neonatal Fc receptor (FcRn) mediates the passive acquisition of humoral immunity by neonatal and prenatal mammals from their mothers. In adult mammals FcRn is also responsible for rescuing IgG from a default catabolic pathway. The receptor performs both of these functions by binding to the IgG ligand at pH 6 - 6.5 and releasing IgG at pH 7.5. The cocystal structure of the FcRn and the Fc fragment of its IgG ligand at pH 6 contains the receptor and the ligand in an oligomeric array of repeating 2FcRn:1Fc complexes (Figure 1). Determining whether the formation of this oligomeric array is important for the receptor's function in vivo is the focus of this research. The oligomeric array contains two possible arrangements of 2FcRn:1Fc complexes, and these two different complexes contain different sets of interactions between the receptor FcRn and the ligand Fc. Biochemical and physiological experiments indicate interactions important to each of the two different complexes are also important to receptor function. I will attempt to confirm these
studies by examining the receptor’s interactions with a mutant heterodimer Fc molecule capable of forming the interactions with the receptor in only one of the two complexes. In addition to continuing biochemical studies which indicate the necessity of all the interactions of the oligomeric ribbon, I will also attempt to visualize the ribbon in situ in polarized epithelial cells in which transfected FcRn can undergo transcytosis. Using the FcRn/Fc cocystal structure as a guide, we have constructed various FcRn and Fc mutants which are predicted to disrupt formation of the oligomeric ribbon by either disrupting FcRn dimer formation or by disrupting the ability of Fc to bridge between receptor dimers on adjacent membranes. The dimensions of transport vesicles in polarized cells transfected with wildtype FcRn and mutant FcRn, or treated with wildtype or mutant Fc, will be compared. Vesicles will be visualized using electron microscopy or confocal microscopy, which offers the possibility of visualization in live cells.

242. Direct visualization of the FcRn-IgG oligomeric network formation between membranes under physiological flow conditions.

Tirunelveli S. Ramalingam

The neonatal Fc receptor (FcRn) presents a paradigm for studies of transcytosis. Some clues about the possible structure(s) mediating transcytosis were obtained from the X-ray crystal structures of FcRn and its complex with Fc fragment of IgG. It reveals an oligomeric array of 2:1 complex of FcRn and Fc in a network (Figure 1). It had been postulated that these networks can be formed between adjacent or opposing membranes and may be present in vesicles or tubules during transcytosis. To test this, we have designed a parallel plate flow chamber to directly visualize the bond formation between opposing membranes using real-time Epi fluorescence microscopy. A glass plate coated with an FcRn embedded lipid bilayer forms the lower plate and a deck made of polycarbonate forms the upper plate of the parallel-plate flow cell. The upper and lower plates are separated by 260 μm thick silicone rubber which provides a seal for the plates under vacuum. The whole set-up will be mounted on a Nikon Epi-fluorescence microscope stage equipped with a video-camera for real-time on-line recording. Glass plates coated with full-length FcRn embedded in lipid bilayers will be saturated with BSA (Bovine Serum Albumin) in the presence and absence of IgG. This will be followed by a flow of cells expressing membrane-bound FcRn. If IgG can bridge FcRn dimers on adjacent membranes, the cells should stick to the FcRn containing bilayer in the presence of IgG. By varying the shear flow rates near the wall of the glass plate, we can estimate the strength of IgG binding to FcRn and the kinetics of association and dissociation with IgG under physiological flow conditions.

243. Role of the cytoplasmic tail of FcRn in transcytosis and phosphorylation based signal transduction in epithelial cells

Tirunelveli S. Ramalingam

FcRn is an Fc receptor expressed in the fetal yolk sac of rat, mouse and human placenta which mediates the transport of maternal IgG from ingested milk (pH 6-6.5) to blood (pH 7.5). Though the extracellular portion of FcRn has been well studied biochemically and structurally, very little information is available about the role of its 44 amino acid cytoplasmic tail. The intracellular portion of many membrane bound receptors including other Fc receptors contain information which help sorting signals in a temporal and hierarchical fashion. In addition, the tail has access to proteins in the cytoplasm which constitute cellular sorting and phosphorylation based signal transducing machinery (Mellman, 1995). Using cell biological techniques, we will study the role of cytoplasmic tail in transcytosis of FcRn/IgG complexes and in phosphorylation based signal transduction in polarized MDCK cells or hepatocytes. Finally, we will try to solve the three dimensional structure of the cytoplasmic tail by X-ray crystallography to gain insight into the structural requirements of its signal transducing machinery.

Reference


244. Design and characterization of mutations in FcRn at the counterpart of the MHC class I peptide binding groove

José Lebrón

The three dimensional structure of FcRn is similar to the structures of class I MHC molecules. However, although the two α-helices that form the sides of the MHC peptide binding groove are present in FcRn, they are closer together than their MHC counterparts and the FcRn groove does not bind peptides. Two amino acids contribute to closure of the FcRn cleft and its inability to bind peptides (an arginine within the FcRn counterpart of the MHC class I pocket A used for the peptide N-terminus, and a proline within the α2 domain helix located at a kink closing one side of the cleft). Since FcRn does not use its counterpart of the MHC peptide binding groove for binding Fc, the functional significance of the closed cleft is not clear. By introducing class I MHC residues at these positions in FcRn, we can test if FcRn can bind peptide, and how that influences its ability to bind Fc. In addition, by introducing these changes into a class I MHC molecule (H-2K<sup>d</sup>), we can test to see if these changes alone are sufficient to prevent peptide binding. We have expressed two different FcRn mutants in which Pro 162 and Arg 164 were replace by their class I MHC counterpart residues. Neither mutant binds IgG with detectable affinity. Since the binding site for IgG is distant from the mutated residues, this result suggests a conformational change in the receptor which affects
ligand binding. Preliminary CD studies support the idea of a conformational change in the FcRn mutants. The Kd mutant has also been expressed, and will be analyzed for its ability to bind endogenous peptides. These studies will increase our understanding of how FcRn interacts with IgG, as well as how evolution has exploited the MHC structural motif for different immunological recognition processes.

245. Structural studies of a human IgA Fc receptor

Clinton L. White, Peter M. Snow

The human IgA Fc Receptor (FcαR) has only recently been cloned. Some sequence similarity has been noted between FcαR and the human p58 NKIR family. We are interested in determining the crystal structure of FcαR alone and when bound to IgA Fc. In addition, we would like to characterize the FcαR-IgA Fc binding interaction using the BIAcore technology. We have expressed the two extracellular domains of FcαR in a baculovirus system and will soon initiate preliminary crystallization and BIAcore experiments.

246. Structural studies of a Herpes Simplex Virus Immunoglobulin G Fc Receptor

Tara Chapman, Malini Raghavan

Herpes Simples Virus (HSV) encodes an immunoglobulin G (IgG) receptor that may aid virally infected cells in evading the host immune response by acting to coat virus-infected cells with IgG so as to reduce exposure of viral antigens to immune effector cells. The HSV-encoded Fc receptor is a heterodimer of proteins gB and gL. Soluble versions of these proteins have been expressed in Chinese hamster ovary cells. The soluble proteins are secreted from transfected cells as a heterodimer and have been shown to bind human, but not rat, IgG. Large scale production of these proteins is in progress to produce protein for crystallization trials.

247. Structural studies of murine Ly49 C-type lectin NK cell inhibitory receptors

Yang Liu, Peter Snow

NK cells recognize and destroy cells that have lost expression of MHC class I molecules. Under normal circumstances, NK cells are turned off by recognition between MHC class I molecules on the potential target cell and a new family of inhibitory receptors on murine NK cells; the Ly49 molecules. When the target cell does not express class I MHC molecules, as can occur during a viral infection or in tumors, the activating function of the NK cell is not turned off by Ly49, and the target cell is lysed. In the case of a viral infection that downregulated MHC class I expression, this recognition of "missing self" by NK cells is complementary to the cytotoxic T cell surveillance which is regulated by T cell receptor and MHC class I engagement. To further understand the molecular recognition between the Ly49 and MHC class I molecules, my project focuses on studying the interactions between Ly49A and its MHC class I ligand, H-2Dd. To achieve this goal, I will use both of the following methods: a) Determination of the crystal structure of the extracellular domain and the carbohydrate recognition domain (CRD) of Ly49A, both alone and complexed with its ligand H-2Dd; b) Qualitative and quantitative analysis of the interactions between Ly49A and H-2Dd using the BIAcore biosensor system. At present, two forms of Ly49A and Ly49I have been successfully expressed using the baculovirus system. Crystallization trials using purified Ly49A are underway and microcrystals have been obtained. We have also expressed the ligand of Ly49A, H-2Dd, in Chinese hamster ovary cells, which will be used for biosensor and crystallization experiments.

248. Structural studies of human natural killer receptors

Clinton L. White, Carol Wu1, Tara L. Chapman, Watson Shen, Jongsun Kim2, Marco Colonna2

Human natural killer inhibition receptor (NKIR) molecules regulate natural killer cytotoxicity by interacting with major histocompatibility (MHC) ligands on a target cell. We would like to determine the crystal structure of the p58 and p70 forms of human NKIRs, both individually and when bound to their MHC ligands, and characterize the binding affinity of the NKIR-MHC complexes. Small single crystals of a p58 NKIR have been grown, frozen, and shown to diffract to ~4Å using synchrotron radiation. Currently, we are trying to express the extracellular domain of a human p70 NKIR in both mammalian and insect cell systems. In addition, several p58 NKIR-MHC ligand pairs have been identified from gel shift analysis and BIAcore biosensor studies have been initiated to quantitate the observed binding interactions.

249. Characterization of two virally encoded MHC class I homologs: UL18 of human cytomegalovirus and m144 of murine cytomegalovirus

Tara L. Chapman, Amy Zheng1, Peter M. Snow2, Watson Shen, Yang Liu

Both human and murine cytomegaloviruses (HCMV, MCMV) downregulate expression of conventional class I MHC molecules at the surface of infected cells. This allows the infected cells to evade recognition by cytotoxic T cells, but leaves them susceptible to natural killer (NK) cells which lyse cells that lack class I molecules. Both HCMV and MCMV encode β2-microglobulin (β2m)-binding class I MHC heavy chain homologues within their genomes that might serve as decoys for NK inhibitory receptors. Sequence comparisons suggest that the HCMV class I homologue (UL18) contains the well characterized groove that serves as the binding site in MHC molecules for peptides derived from endogenous and foreign proteins. By contrast, the MCMV homologue...
(m144) contains a substantial deletion within the counterpart of its C2 domain and might not be expected to contain a groove capable of binding peptides. We previously showed a soluble form of UL18 expressed in CHO cells binds a mixture of endogenous peptides with characteristics similar to those eluted from class I molecules, that is, "anchor" residues, and a predominance of short peptides derived from cytoplasmic proteins (Fahnestock et al., 1995). We have now expressed a soluble version of m144, verified that it is a heavy chain/ß2m complex, and showed that it does not associate with endogenous peptides yet is thermally stable. We are currently assaying for potential interactions between UL18 and soluble forms of human p58 Ig-like NK cell receptors and a heterodimeric c-lectin-like NK cell receptor using biosensor, gel shift and immunoaffinity assays. Similar experiments are ongoing to study potential interactions between m144 and the Ly49 family of murine NK receptors. Using underglycosylated forms of UL18 and m144, we have begun crystallization trials for eventual three dimensional structure determinations.

Reference

250. Structural and functional characterization of M080R, an MHC Class I Homolog from Mollicusm Contagiosum Virus
Zsuzsa A. Hamburger, Tara Chapman
The recent sequencing of the genome of the human poxvirus, Mollicusm contagiosum, revealed that the virus encodes an MHC class I homolog, M080R (Senkevich et al., 1996). From viral DNA (generous gift of R. Mark and L. Buller, St. Louis University School of Medicine), we have used PCR to generate full length M080R and a version encoding a truncated, soluble form of M080R, and subcloned these into eukaryotic expression vectors. Currently, we are trying to express the truncated M080R in Chinese hamster ovary cells as well as in a baculovirus expression system. We are interested in performing crystallographic along with functional studies of M080R in order to determine the role this MHC class I homolog may play in evasion of the host immune system by the virus.

Reference

251. Structural and functional studies of HLA-G, its receptors on NK cells, and further implication in maternal-fetal tolerance
Huazhang (Watson) Shen, Peter M. Snow, Jennifer L. Johnson
Maternal-fetal tolerance is a puzzle that has bothered immunologists for over 40 years. Simply stated, the fetus should be rejected by the mother's immune response because it has half the paternal genetic material, which is "non-self." One way of evading the maternal immune attack is by the downregulation of polymorphic MHC class I molecule at the placenta, but this makes the placenta susceptible to an attack mediated by natural killer (NK) cells which lyse cells that lack class I MHC molecules. HLA-G is a non-classical human class I MHC molecule specifically expressed at the placenta where classical MHC-I and II molecules are not expressed. Because of its low level of sequence polymorphism, HLA-G is proposed to be a key molecule for conferring maternal-fetal tolerance at the placenta. In order to elucidate potential interactions between HLA-G and NK cell inhibitory receptors, we expressed a soluble form of an HLA-G/ß2m heterodimer using the baculovirus system. Soluble forms of human NK receptors CD94 and NKG2A were also expressed using recombinant baculoviruses and shown to form a covalent heterodimer. Crystallization and BLACore experiments will be initiated to try to understand the potential interaction between these proteins.

Reference

252. Downregulation of class I MHC molecules is a strategy to evade the cellular immune response
Melanie J. Bennett, Peter M. Snow
Downregulation of class I MHC molecules is a means to evade the cellular immune response that occurs in virally infected cells and some types of human cancer cells. Human cytomegalovirus encodes a family of proteins, US2-US11, that prevent cell surface expression of class I molecules by a variety of mechanisms. The US proteins may provide a paradigm for the class I downregulation mechanisms that result in viral escape from T cell-mediated destruction and perhaps also contribute to immune evasion by tumor cells. In contrast, murine cytomegalovirus encodes a protein, gp34, that binds to class I molecules and travels with them to the cell surface. Its function may be to prevent class I molecules from stimulating killer cell inhibitory receptors to prevent cell lysis through that pathway. Based on sequence analysis, the US proteins and gp34 are predicted to be type I transmembrane proteins with N-terminal domains that interact with class I molecules and short C-terminal cytoplasmic tails. Our goal is to express soluble forms of US2, US3, US6, US11 and gp34 and to study their interactions with class I molecules using surface plasmon resonance techniques and x-ray crystallography. Expression of soluble US2, US3, US6 and US11 proteins in a baculovirus system has thus far been unsuccessful, as has been expression of soluble US3 as a maltose binding protein fusion or as a thioredoxin fusion in E. coli. However, we have expressed a soluble form of gp34 in a baculovirus expression system which yields ~3 mg/L. Soluble gp34 can be purified by a single passage over a nickel column (based on an added His6 tag in the construct). The ability to obtain large quantities of pure gp34 will permit us to characterize it biochemically and eventually generate full length structures.
and to analyze its interactions with class I molecules and as well as to screen for crystallization conditions.

1 Caltech Protein Expression Center

253. Crystallographic and biochemical characterization of a secreted form of HFE

José Lebrón, Melanie Bennett, John Feder

Hereditary hemochromatosis (HH) is a disease characterized by the deposition of iron in different organs of the body. The gene associated with HH was recently identified (Feder et al., 1996) and named HFE. 83-100% of HH patients are homozygous for a mutation in HFE that replaces a cysteine by a tyrosine (C257Y).

HFE is an heterodimer consisting of a heavy chain related by sequence and domain organization to class I MHC molecules, and β2m, the class I light chain. MHC class I molecules present peptides to cytotoxic T cells during immune surveillance, whereas HFE functions in an as yet incompletely characterized way in the control of iron absorption. In order to compare the three dimensional structure of HFE to MHC molecules and to elucidate its role in iron metabolism, we expressed and purified a soluble version of an HFE/β2m heterodimer, starting with cDNA encoding HFE provided by Mercator Genetics. Biochemical analyses of purified HFE showed that, unlike conventional class I MHC molecules, HFE does not associate with endogenous peptide, ruling out an autoimmune component to HH whereby autoreactive T-cells respond to HFE presenting a self peptide, which leads to a problem in iron metabolism. We recently solved the crystal structure of the HFE/β2m heterodimer. The structure reveals that HFE indeed shares a common three-dimensional fold with MHC class I molecules. Further structural and biochemical studies are in progress to characterize ligands of HFE and to elucidate the role of HFE in iron metabolism and in the immune system.

Reference
Feder et al. (1996). Nature Genetics 13, 399-408.

1 Mercator Genetics, Menlo Park, CA

254. Biochemical characterization and crystallization of Zn-α₂-glycoprotein

Luis M. Sánchez, Carlos López-Otín

Zn-α₂-glycoprotein (ZAG) is a 41 kDa soluble protein that is present in most bodily fluids. In addition, ZAG accumulates in fluids from breast cysts as well as in 40% of breast carcinomas, which suggests that ZAG plays a role in the development of breast diseases. However, the function of ZAG under physiological and cancerous conditions remains unknown. Because ZAG shares 30-40% sequence identity with the heavy chains of class I major histocompatibility complex (MHC) proteins, we compared the biochemical properties of ZAG with those of classical class I MHC molecules. We purified human ZAG from breast cyst fluid and serum and produced a panel of anti-ZAG monoclonal antibodies. Binding assays and acid elution experiments revealed that, in contrast to class I MHC proteins, ZAG does not bind peptides or the class I light chain, β2-microglobulin (β2m). Nevertheless, CD studies indicated that ZAG is thermally stable in the absence of bound peptide or associated β2m, as opposed to class I MHC molecules which require the presence of both β2m and peptides for stability. These data indicate that the function of ZAG has diverged from the peptide presentation and T cell interaction functions of class I molecules. To gain insight into the function of ZAG and to compare the three dimensional structures of ZAG and class I MHC molecules, we produced ZAG crystals that diffract beyond 2.7 Å and have initiated an x-ray structure determination.

Reference
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255. Crystallographic study of Hemolin, an insect immune response protein related to immunoglobulins

Xiao-Dong Su

Insects have an efficient defense system against infections. Numerous antibacterial immune proteins induced by bacterial infection have been identified in the giant silk moth, Hyalophora cecropia. One such protein is hemolin, a 48 kDa protein present in low but significant amounts in the hemolymph of cecropia pupae. The concentration of hemolin is increased by a factor of approximately 20 after injection of dead or live bacteria into the pupae. Hemolin binds both to insect hemocytes and to bacteria, suggesting a possible function in mediating phagocytosis of bacteria by hemocytes and a functional analogy to lipopolysaccharide binding proteins involved in the innate immune response in vertebrates. A cDNA clone of hemolin was isolated (Sun et al., 1990), and analysis of the 413 residue sequence revealed that the protein contains four internal repeats characteristic of immunoglobulin-like domains. We are interested in the structural characterization of this protein in order to investigate its immune properties and its relationship to vertebrate members of the immunoglobulin gene superfamily. A soluble form of hemolin was expressed using a recombinant baculovirus system (Gastinel, Vaughan, Faye, and Bjorkman, unpublished results), and we have purified and crystallized soluble hemolin.

Data were collected from cryopreserved crystals to 2.3 Å resolution using synchrotron radiation. The crystal structure was solved by the MIR method using platinum and pressurized xenon derivatives. The structure revealed that each of the four domains has an immunoglobulin V-like folding topology. The domains are arranged so that the first two are anti-parallel to the last two in an arrangement resembling a closed horseshoe. To our knowledge, the hemolin structure represents the first Immunoglobulin superfamily member in which Ig-like domains are arranged in an anti-parallel fashion. We are currently investigating the hypothesis that concentrated patches of positive charge at the center of the horseshoe and the two ends
of the protein may be involved in interactions with negatively charged lipopolysaccharide moieties of bacterial cell walls.

Reference

256. Crystal structure determination of Invasin
Zsuzsa A. Hamburger, Ralph R. Isberg

The ability to bind to and enter normally non-phagocytic cells is an important property required of many bacterial pathogens in order to establish infection. Yersinia pseudotuberculosis is an enteropathogenic bacterium capable of penetrating such cells. During the course of an infection, food born Yersinia must cross the intestinal epithelium before entering the lymphatic system and finally colonizing the liver and spleen. Previous work has shown that the protein responsible for mediating uptake of the bacterium is a 986 amino acid outer membrane protein called invasin (Isberget et al., 1987). Invasin binds to several members of the β1 integrin family, including α3-α6β1 and αvβ1. It is thought that by binding to an integrin receptor, invasin is able to activate host signaling that results in reorganization of the actin cytoskeleton to form pseudopods that envelop the bacterium. Interestingly, invasin binds to integrins at a mutually exclusive site with fibronectin, another integrin ligand, although the two proteins are not homologous based on sequence, and invasin lacks the RGD sequence important for fibronectin-integrin binding. Also, while invasin-integrin binding results in bacterial uptake, fibronectin-integrin binding does not result in fibronectin uptake. We are interested in determining the three dimensional structure of invasin and invasin bound to integrin receptors in order to gain further insight into the mechanism of bacterial uptake. The C-terminal 492 amino acid region of invasin, capable of mediating binding and uptake, has been expressed and purified in the laboratory of Ralph R. Isberg. We have grown crystals of invasin and collected a complete native data set to 2.5 Å resolution from two crystals at the HHMI X4A beamline at Brookhaven. We are currently working on determining conditions for producing isomorphous heavy metal derivatives of the invasin crystals. Further studies will include growing crystals of selenomethionyl substituted invasin (expressed and purified in Dr. Isberg’s laboratory) and co-crystallization of invasin with β1 integrins for structure determinations.

References
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Publications

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Summary: Although we still know much more about prokaryotic DNA replication than about eukaryotic DNA replication, the last few years have seen tremendous strides in the eukaryotic field. The structure of chromosomal replication origins has been defined, at least in yeast chromosomes. A functional replication machine has been reconstituted from purified mammalian enzymes and proteins, using the SV40 genome as a model replicon. The proteins that interact with chromosomal origins have been identified in yeast and have been shown to be conserved in both structure and function in vertebrates.

Despite these achievements, not all important eukaryotic DNA replication proteins have been identified. A missing link has been the helicase that
moves the chromosomal replication fork. During the past year we have made significant progress in studying a good candidate for such a helicase that was discovered using yeast genetics and molecular biology two years ago in our laboratory. Continuing from previous studies that established a role for this helicase in DNA replication, we have now shown that the helicase interacts with DNA polymerase α, the polymerase responsible for initiation both at origins of replication and at each Okazaki fragment on the lagging strand. In addition, we have found genetic and biochemical evidence for an interaction of the helicase with a nuclease involved in maturation of Okazaki fragments. Since yeast, thus far, lacks an in vitro replication system, to determine the role of Dna2 in replication directly, we have identified the Dna2 gene of *Xenopus* and have set up the *Xenopus* in vitro replication system, in which we will test the effect of removing or mutating Dna2 and the proteins with which it interacts.

Our laboratory has been studying the DNA polymerases involved in replication for many years. In fact, we were the first to show that there was more than one essential DNA polymerase at chromosomal DNA replication forks. What we still don't know, is how the three polymerases divide up the labor during DNA replication. During the past year, our work has focused on the least well understood DNA polymerase, pol ε. There is some dispute as to how pol ε participates in DNA replication, since the SV40 system can dispense with pol ε. We have discovered that pol ε is a dimer and that a C-terminal zinc finger is involved in essential protein/protein interactions. This is significant because the *E. coli* replicase is also a dimeric enzyme, with one monomer dedicated to the copying of each strand, suggesting that pol ε may be organized at the eukaryotic fork in the same way. This work has involved site-directed mutagenesis, yeast two hybrid analysis and overexpression of mutants in baculovirus infected insect cells.

Perhaps our most exciting advance is the discovery over the past year of one of the first important substrates of yeast cyclin-dependent kinase, Clb5,6/Cdk1, at the G1/S phase transition of the cell cycle. Three proteins essential for the initiation of DNA replication associate with origins in late mitosis or early G1 - Orc, Cdc6 and the Mcm proteins. These so-called pre-replication complexes, however, do not initiate replication until Clb5,6/Cdk1 becomes active late in G1. We have shown that Cdc6 is a substrate of Cdk1 in late G1. Our results further suggest that phosphorylation of Cdc6 leads to its degradation by the ubiquitin dependent pathway, and that this is an important element of cell cycle regulation. By degrading Cdc6 after an origin fires, re-initiation is prevented until Cdc6 can be resynthesized. Expression of Cdc6 does not occur until late mitosis, and only then can new protein associate with Cdc6-free origins for use in the next cell cycle. Further studies have shown that Cdc6 interacts directly with the sequence specific origin binding protein, Orc. Perhaps the biggest surprise is that Cdc6 interacts directly with the Mcm proteins. Degradation of Cdc6 may be required to release the Mcms, which appear to move away from origins with the replication fork.

257. A yeast replicative helicase, Dna2, interacts with yeast FEN-1 nuclease

*Martin Budd, Judith L. Campbell*

We have recently described a new helicase, the DNA helicase, that is essential for yeast DNA replication (1). We have shown that the yeast FEN-1(FEN-1) nuclease interacts genetically and biochemically with Dna2 helicase. FEN-1 is implicated in DNA replication and repair, and the mammalian homolog of yFEN-1 (DNaseIV, FEN-1, or MF1) participates in Okazaki fragment maturation. Overproduction of yFEN-1 encoded by the RAD27/RTH1 gene suppresses the temperature-sensitive growth of *dna2-1* strains. Overproduction of DNA2 helicase suppresses the temperature sensitive growth defect of *rad27/rth1D* strains. *dna2-1 rad27/rth1D* double mutations are inviable demonstrating the two mutations are synthetically lethal. The genetic interactions are likely due to direct physical interaction since epitope tagged yFEN-1 and endogenous yFEN-1 coimmunopurify with tagged Dna2. A likely interpretation is that one role of Dna2 helicase is processing of Okazaki fragments.

Reference


258. Genetic and physical interaction between the yeast DNA2 helicase and Ctf4, a DNA polymerase a binding protein

*Tim Formosa*, Judith Campbell, Martin Budd

Ctf4 is a protein that binds to the catalytic subunit of DNA polymerase α. (1) Genetic experiments show that Ctf4 is important for normal DNA replication but is not essential. To further investigate the nature of the complexes containing Pol1p and Ctf4p, mutations which were synthetically lethal with Ctf4 deletion strains were identified. One such mutation maps to the DNA2 gene. A deletion of the Ctf4 gene is synthetically lethal with *dna2-1, dna2-2*, and *Δrad27/rth1* mutant strains. These genetic interactions suggest that Ctf4 may stabilize a complex containing Dna2p and yFEN-1. Dna2 mutations caused chromosomal instability, but the effect was small compared to Dcf4 strains. Immuno-affinity purified Dna2p is found to contain Ctf4p. In addition mutations of Dna2 caused a small reproducible elongation of telomeres suggesting a role in suppression of telomere elongation analogous to the role of DNA polymerase α in suppressing telomere elongation. The data suggests that Dna2, yFEN-1, and Ctf4 function coordinately in DNA replication.

References


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259. The role of DNA2 protein in the repair of damaged DNA

Martin Budd, Tim Formosa, Judith Campbell

The dna2-1 and dna2-2 mutations have been tested for sensitivity to DNA damaging agents to ask the question whether dna2 protein is involved in repair. Both the dna2-1 and dna2-2 strains are more sensitive to x-rays than DNA2+ strains. However both strains are significantly more resistant to x-rays than rad52 or rad6 strains. dna2-1 and dna2-2 strains are sensitive to methyl methane sulfonate. Neither strain is sensitive to UV. The x-ray sensitivity of both dna2-1 and dna2-2 strains is suppressed by overproduction of yFEN-1, although rad27/rad1D strains are not sensitive to x-rays. We previously observed that the growth defect of dna2-1 strains at 37°C is suppressed by overproduction of yFEN-1. Neither the dna2-1 nor the dna2-2 strains are significantly defective in the ability to maintain the stability of dinucleotide repeats. The result differs from a rad27/rad1D strain in which dinucleotide repeat length is unstable.

To analyze the mechanism of dna2 protein in repair dna2 rad multiple mutants were constructed and analyzed for epistatic or synergistic effects on survival after x-rays and UV. Yeast has 5 pathways of repair, the excision repair pathway (rad1), the post-replication repair pathway (rad6), the recombinational repair pathway (rad52), the blunt ended ligation repair pathway (Ku), and the mismatch repair pathway. dna2-2 rad52 and dna2-1 rad52 strains are more sensitive to UV than either rad52 or dna2-2 strains. dna2-1 rad52 strains are more sensitive to x-rays than either dna2-1 or rad52 strains. dna2-2 rad50 mutants are more sensitive to x-rays than either dna2-2 or rad50 strains. rad50 protein is presumed to be part of a 5' to 3' exonuclease complex involved in processing double strand breaks for repair and evidence from the Weaver laboratory suggests it is involved in the KU pathway of repair. Dna2 protein interacts with FEN1p (rad27p) and Ct4p protein in DNA replication. DCf4p rad52 strains were found to be no more sensitive to UV radiation than rad52 strains suggesting that dna2 protein is involved in repair independent of one of its partners in DNA replication, the Ct4p protein. rad52 rad27 double mutants are inviable.

dna2-1 rad6 and dna2-2 rad6 strains are no more sensitive to UV or x-rays than rad6 strains. Interactions with the rad1 pathways are in progress. The data suggests that dna2 protein is a part of the rad6 post-replication repair pathway to repair UV and x-ray damage. Further work will analyze whether interaction is part of the error-free or error-prone pathway. In addition the dna2-1 rad52 double mutant analysis suggests that dna2 protein may be involved in the KU pathway of repair since these strains are more sensitive to x-rays than rad52 strain or dna2-1 strains. The KU pathway is the repair pathway involved in gene rearrangements at the T cell receptor and B cell receptor loci, and a major pathway of repair in mammalian cell. Possible roles for dna2 protein in repair involve a 5' to 3' exonuclease step in x-ray repair. In addition dna2 may be involved in translesion DNA synthesis during post-replication repair. However the mechanism of the rad6 post-replication repair pathway is largely unknown in yeast.

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260. A Dna2 structure-function study and identification of interacting proteins

Martin Budd, Judith Campbell

To further understand the role of the Dna2 protein in DNA replication and repair a structure function study of the dna2 protein is being done. The dna2-1 protein is being expressed in yeast under the gal10 promoter. The dna2-1 mutation is a Proline to Serine change in AA 504 in a region of the protein that is conserved in human, worm, and Xenopus dna2 (Budd et al., 1995). The dna2-1 mutation maps in a domain distinct from the helicase domain. Probable defects of the dna2-1p involve protein-protein interaction and candidate proteins are dna2p itself, yFEN-1, Ct4p and additional dna2 interacting proteins. In addition Dna2 protein has been observed to form multimers when assayed by gel filtration. The dna2-1p will be tested for a defect in self interaction.

Previous work has identified two proteins which interact with Dna2, yFEN-1 and Ct4p protein. To identify additional interacting proteins, Dna2 associated proteins will be sequenced by mass spectroscopy at the Resource Technology Center at the University of Washington as part of a collaboration headed by Lee Hartwell. Hemaglutinin tagged Dna2 protein has been purified through an 12CA5 immunoaffinity step. A 220 kd band is observed in the immunoaffinity purified protein which does not react with the 12CA5 antibody. Since the 220 kd protein is larger than the immunoaffinity tagged dna2 (160 kd) it is probably not dna2 and is being sequenced by mass spectroscopy.

Reference

261. Identification of yFEN1 homologs and determination of their relationship to DNA2, a helicase involved in DNA replication

Wonchae Choe, Martin, Budd, Judith L. Campbell

A structure-specific endonuclease recognizes a specific 5' flap DNA structure at the junction where the two strands of duplex DNA adjoin a single-stranded region. Structure-specific endonucleases that have been identified are human, mouse and yeast flap endonuclease. Our laboratory has shown that DNA2 and yFEN1 interact physically and genetically. Since FEN1 is involved in the processing of the Okazaki pieces, we suggest that DNA2 protein is also maturation of 5' end of Okazaki fragments. So, we are trying to determine if yFEN1 deletion strains and dna2 deletion strain are defective in Okazaki processing.
in vivo. Interestingly, the yfen1 deletion mutant is a temperature-sensitive mutant. Thus, yFEN1 is not essential, though yFEN1 is performing an important function. So, we postulate that there would be at least another yfen1 homolog in S. cerevisiae. Using a computer search for yFEN1 homolog, we found three types of yFEN1 homologs. We are trying to elucidate the relationship between the DNA2 and yFEN1 homologs by biochemical and genetic methods.

262. Cloning of Xenopus DNA2 gene
Qingquan Liu, Judith L. Campbell
In Saccharomyces cerevisiae, a gene called DNA2 encodes an essential DNA helicase which is required for DNA replication (Budd & Campbell, 1995). Furthermore, Dna2 interacts with Rad27, suggesting that Dna2 may be involved in processing Okazaki fragments (Budd & Campbell, 1997). Dna2 can also bind to POB1, a DNA polymerase a binding protein. The function of this interaction is yet to be answered (Formosa et al., submitted). The yeast DNA2 homologues from human and C. elegans have also been cloned, but their biological role is completely unknown.

In order to study the function of Dna2 in higher eukaryotes, we designed degenerate primers according to a conserved amino acid sequence motif among yeast, human and C. elegans Dna2 homologues, did PCR using Xenopus eDNA as the probe, and thus cloned the DNA2 gene from Xenopus. The Xenopus Dna2 has 1053 amino acids and is highly homologous to yeast, human and C. elegans Dna2. Currently, we are trying to express Xenopus DNA2 in E. coli and insect cells. We have already made the polyclonal antibodies against Xenopus DNA2 C-terminus. After we get the purified DNA2 protein, we will use the in vitro DNA replication system from Xenopus eggs to do the immunodepletion and addback experiments to find whether Xenopus Dna2 is involved in DNA replication.

References
Formosa, Campbell, and Budd, submitted.

263. Role of DNA polymerase epsilon in eukaryotic DNA replication, transcription, cell-cycle transitions
Rajiv Dua, Judith L. Campbell
The focus of the research is to understand the role of DNA polymerase epsilon in eukaryotic replication, transcription and cell-cycle regulation using Saccharomyces cerevisiae as the model system. DNA polymerases are nuclear enzymes that have the responsibility of copying the genome with high accuracy. The yeast pol ε holoenzyme consists of five different subunits having molecular masses of 256kDa, 80kDa, 34kDa, 31kDa, and 29kDa. The 256kDa subunit is the catalytic subunit. The POL2 gene encoding the catalytic subunit has been cloned and sequenced. Previously, the Campbell laboratory has shown that POL2 gene is essential for replication in yeast and also participates in DNA repair. Recently, pol ε has also been indicated in transcription and cell-cycle checkpoint. Navas and coworkers have shown that pol ε is involved in linking the DNA replication and cell-cycle apparatus. They showed that pol2-11 and pol2-12 mutants fail to arrest the cell-cycle in response to blocks to DNA replication caused by hydroxyurea. Genetic analysis suggested that the extreme carboxyl terminus of pol ε which contains putative zinc fingers plays a crucial role in the signaling pathway from replication apparatus to cell-cycle machinery. However, the mechanism by which the C-terminus of pol ε senses and relays the signal is not known. It has been proposed that zinc fingers might act as a sensor of single-stranded DNA that forms when pol ε progression during replication is blocked. At the same time one can not ignore the fact that a protein-protein interaction site important to relay the signal may be defective in pol2 mutants. To further understand the role of C-terminus, various site-directed deletion mutants in the C-terminus of pol ε has been prepared. Each mutant contains a sequential deletion of 10 amino acid residues from the C-terminus of pol ε. Genetic analysis of these mutants has indicated that a cluster of 20 amino acid residues near the zinc finger region in the C-terminus is important for POL2 function. Secondly, we have used yeast two hybrid system to demonstrate that the C-terminus of pol ε interacts with one other known DNA replication protein. Internal deletion analysis of C-terminus of pol ε was performed to identify the region required for the interaction. The expression of coexpressed proteins was followed by Western analysis. The identified mutation in the C-terminus of pol ε blocks the interaction. In addition, yeast two hybrid analysis indicated that DPB2 protein which copurifies during purification of pol ε also interacts directly with pol ε. The results also suggests that the C-terminus of pol ε folds independently of its amino-terminus. In order to show protein-protein interactions in vitro, coimmunoprecipitation experiments are in progress.

To understand the roles of C-terminus of POL2 in DNA repair, the deletion mutants of POL2 were also examined for sensitivity toward methyl methane sulfonate (MMS) and inducibility of RNR3 in the presence of MMS. The results indicated that first zinc finger in the C-terminus of POL2 perform a function in DNA repair.

The zinc fingers of POL1 and POL3 differ from each other and from POL2. Does this mean that they have different functions in each protein? Do they have different targets or do they differ in DNA binding. To address this, I swapped the zinc finger of POL2 with zinc finger of POL3. The heterologous finger did not complement pol2Δ for growth. This suggests that zinc finger of POL2 and POL3 might have unique functions. Biochemical analysis of the mutant proteins is in progress.

For purification and biochemical analysis of pol ε and mutant proteins we are using baculovirus expression
in Sf9 insect cells. The C-terminus POL2 gene is cloned in baculovirus transfer vector and proteins will be purified by using nickel column.

In addition to our study on DNA polymerase epsilon, we are also working on structure based site directed mutagenesis of Taq DNA polymerase. This work is collaborated with Prof. Richard’s group at Caltech and Applied Biosystems Inc. (ABI). The work is focused on understanding the mechanism of catalysis by Taq polymerase with an eventual goal of improving Taq polymerase for DNA sequencing. Based on molecular modeling of the active site, we have constructed various mutants of Taq polymerase. The mutant proteins have been overproduced and purified. The study on determining various kinetic and binding parameters of mutant Taq polymerases is in progress at ABI.

264. S. cerevisiae Cdc6 and control of origin firing

Suzanne Elsasser

The initiation of chromosomal DNA replication is thought to proceed as follows: the origin of replication is bound by ORC and provides a foundation for the subsequent assembly of other initiation factors. Cdc6 associates with the origin, followed by the MCMs. The origin bound complex of ORC, Cdc6, possibly the MCMs and probably other factors constitutes the so called prereplicative complex, the assembly of which is thought to be required for subsequent origin firing. It has been shown that the preRC may only form in the absence of active Clb/Cdc28, corresponding to late M and G1. When Clb/Cdc28 is activated at the G1/S transition, origins are both fired and prevented from firing again. Since Clb/Cdc28 prevents repeated origin firing, phosphorylation of preRC proteins by this kinase is likely to be a key step in maintaining the arrow of the cell cycle.

We have previously shown that Cdc6 binds tightly to and is phosphorylated by Cdc28 (Elsasser et al., 1996). In addition, we have shown that Cdc6 interacts only with the Clb form of Cdc28 and only in the absence of Sic1, an inhibitor which regulates the G1/S transition. Taken together, these observations suggest that the interaction of Cdc28 with Cdc6 may prevent repeated initiation from a single origin.

Cdc6 is known to be an unstable protein, and this instability depends on Cdc4, a protein that mediates the G1/S transition by degrading Sic1. We have found that a 14 residue deletion within the Cdc28 binding region confers stability on Cdc6, indicating that phosphorylation by Cdc28 may be a requisite step in degradation. This raises the question of whether the suppression of repeated origin firing is due to modification or subsequent degradation of Cdc6. We have used charge to alanine mutagenesis across the Cdc28 binding region to generate a stabilized protein that is presumably still phosphorylated. Using this mutant, we will study the effect of Cdc6 modification in the absence of degradation. Since we do not observe wholesale increase in DNA content in strains carrying alleles of Cdc6 that do not bind Cdc28, we suspect that there is at least one other protein sensitive to Clb/Cdc28 surveillance.

Reference

265. Roles of B-type cyclin/Cdc28 kinase in the formation of Cdc6/ORC complexes during DNA replication

Kathy Luo, Judith L. Campbell

Regulation of the G1/S transition is necessary for orderly cell cycle progression, so failure of control leads to unrestricted proliferation and tumorgenesis. Blocks to re-replication in a single cell cycle maintain the integrity of the genome. In yeast, the same B-cyclin/cyclin-dependent kinase (CDK) complex regulates both the G1/S transition and the block to re-replication in S, G2 and M. Potential Cdk targets are the Origin Recognition Complex (ORC), which is bound to origins of replication throughout the cell cycle, and Cdc6, which has been proposed to bind to ORC in late M or early G1. I am interested in whether Clb5/Cdc28 regulates the association of Cdc6 with the origins (ORC) during the yeast cell cycle.

The specific aims are: First, to demonstrate that Cdc6 interacts with ORC in yeast which has been shown by others using ORC and Cdc6 produced in insect cells (Liang et al., 1995). Second, to map which part of the Cdc6 protein interacts with the ORC. Third, to determine if the interaction of Cdc6 with ORC affects the interaction of Cdc6 with Clb5/Cdc28. The outcome of the proposed studies will shed light on how the ORC/Cdc6 interaction controls initiation of replication both in yeast and in higher eukaryotic organisms, which have structural and functional homologs of Cdc6 and ORC.

Currently, I am investigating the interaction of ORC and Cdc6 in an in vitro system. To detect the ORC complex, the endogenous ORC2 gene has been tagged with a triple epitope (3XHA) at the N-terminus, which can complement a temperature-sensitive orc2-1 mutant at non-permissive temperature (37°C). Since the ORC complex consists of six subunits which can form a complex stable to 1 M salt, tagging one subunit of ORC will allow detection of the whole complex. Cdc6 has been purified from E. coli as a GST-Cdc6 fusion protein and immobilized on glutathione beads. The beads were incubated with yeast extracts carrying the 3XHA-ORC2 construct in the presence of ATP. After extensive washing, the bound proteins were eluted with 5 mM glutathione and analyzed by Western blotting with monoclonal antibody to the HA epitope. 3XHA-OrC2 present in the yeast extracts was clearly retained on the bGST-Cdc6 beads. Furthermore, bGST-Cdc6/K114E, a protein with a point mutation in the consensus nucleotide binding domain also interacts with 3XHA-OrC2 at the same level as the wild type of Cdc6. There is no binding of 3XHA-OrC2 either with
bGST bound to the glutathione beads nor with just beads alone can be detected.

Now I am in the process of mapping the ORC interaction domain in the Cdc6 protein. Five GST-Cdc6 deletion constructs have been generated by Campbell’s lab and our collaborator Ambrose Jong’s lab. GST-Cdc6 fusion proteins containing different lengths of Cdc6, such as amino acids 1-48, 1-191, 1-232, 1-392, and 191-513, will be purified on glutathione beads and used to test whether they can bind to yeast 3xHA-Orc2 in our in vitro system.

Meanwhile, bacterially expressed His-Mcm5 fusion protein has been purified by a combination of nickel NTA-resin column and preparative electrophoresis. The gel band containing the His-Mcm5 fusion protein was cut off and used to generate antibody against Mcm5, one of the six subunits of Mcm protein complex which has been shown to interact with ORC through Cdc6 in Xenopus (Coleman et al., 1996). To detect whether Mcm5 is also presented in the Cdc6/ORC complex in yeast, all the protein blots from in vitro fusion protein has been purified by a combination of stripping off and re-probed with the anti-Mcm5 antibody.

References

266. Role of architectural elements in combinatorial regulation of initiation of DNA replication in Escherichia coli
Piotr Polaczek, Kelvin Kwan, David A. Liberles
In Escherichia coli containing multiple origins of replication, all origins fire at precisely the same time in the cell cycle and each origin fires only once per cell cycle. According to the classic replicon model, replication is controlled by two elements, a replicator and an initiator (Jacob et al., 1963). The former is a cis acting sequence that governs replication of adjacent sequences, an origin of replication (oriC). The latter is a protein that acts on the replicator to trigger replication. However, extensive analysis of regulation of oriC activation has indicated that the timing of activation is regulated by a number of proteins that interact at oriC in a specific temporal program to create a complex with a precise geometry that facilitates the conversion of the origin into a fork. We attempted to evaluate the contributions of different elements to the overall architecture of the oriC template.

Bending of DNA is a prerequisite of site-specific recombination and gene expression in many regulatory systems involving assembly of specific nucleoprotein complexes. We have investigated how the uniquely clustered Dam methylase sites, GATCs, in the E. coli origin and their methylation status modulate the geometry of oriC and its interaction with architectural proteins such as integration host factor (IHF), Factor for inversion stimulation (Fis) and DnaA initiator protein. IHF binds to a specific site and induces DNA curvature in oriC (Polaczek, 1990). Three GATCs, strategically positioned within the IHF protected region, are unique to the oriC site. Methylation of the GATC sites enhances IHF binding and alters the IHF-induced bend at oriC. GATC motifs also contribute to intrinsic DNA curvature at oriC and the degree of bending is modulated by methylation. The IHF-induced bend at oriC is further modified by Fis protein and IHF affinity for its binding site may be impaired by protein(s) binding to GATCs within the IHF site. The GATC sites at oriC affect the DNA conformation and GATCs, in conjunction with the IHF and Fis-induced bends, are critical cis-acting elements in specifying proper juxtapositioning of initiation factors in the early steps of DNA replication. Thus, the unique oriC DNA stretch, the replicator, is not merely a scaffold for the assembly of initiation proteins but is a dynamic partner in the regulation of its own function.

References

267. Evidence for two types of subunits in the bacterioferritin of M. magnetotacticum
Elizabeth Bertani, Jerry Huang1, Barbara Weir1, Joseph Kirschvink2

The most remarkable property of the bacterium, Magnetospirillum magnetotacticum (MS-1), is its ability to convert iron into single-domain crystals of the mineral magnetite (Fe3O4). Magnetite is valuable not only for its magnetic characteristics and hardness as a material, but also for its potential as a geobiological tracer.

Both eukaryotes and prokaryotes store large amounts of iron in specialized structures composed of the proteins ferritin or bacterioferritin (Bfr), respectively. Since ferritin had been implicated in the deposition of magnetite in chitons, we considered the possibility that Bfr might play a similar role in the conversion of iron to magnetite in bacteria.

Typically, the ferritin structure consists of 24 subunits of 17-19 kDa, assembled into a hollow shell with up to 4000 iron atoms incorporated into the cavity. In the case of mammalian ferritin, there are two types of subunits, designated the H- and L-chains. The H-chain has a ferroxidase activity which is lacking in the L-chain. In contrast, E. coli Bfr consists of 24 identical subunits which not only have ferroxidase activity, but also also bind 12 heme groups to well-conserved Met52 residues.

Using PCR and DNA hybridization, we have now identified two overlapping bfr genes in MS-1 with the potential of producing two distinguishable types of subunits. One, Bfr1, has strong sequence similarity to the single Bfr subunit found in E. coli and retains the heme-binding Met52 residue, but has diminished ferroxidase activity. The other, Bfr2, which has weaker similarity to E. coli Bfr, has the Met residue shifted to position 49, but retains full ferroxidase activity. In addition, the Bfr2 type of subunit is completely different in the region of the molecule that
is known to be involved in metal binding. Which, if any, of these properties is important for magnetite deposition remains to be elucidated.

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268. A search for magnetotactic archaebacteria in Mono Lake
Megan Andrews¹, Joseph Kirschwink²,
Elizabeth Bertani

It has been suggested that all magnetotactic systems are descended from a single monophyletic origin. Examples of organisms that either produce the mineral magnetite (Fe₃O₄) and/or are magnetotactic have been described for two of the three primary phylogenetic domains, the eubacteria and the eukaryotes, but not for the third, the archaebacteria. In order to fill this gap, we have begun a search for magnetotactic archaebacteria.

Since archaebacteria tend to inhabit extreme environments, we began our search at Mono Lake, which is known to be pH 10-11 and to have about three times the salinity of sea water. Samples were obtained from several parts of the lake and subjected to a one-hour magnetic extraction. Material that collected at the magnet was frozen for future examination. In addition, samples of mud and water were also collected. PCR was then performed on DNA extracted from the samples, using primers specific for archaebacterial 16S ribosomal RNA, and the products were cloned and sequenced. Samples from mud and water yielded sequences with extremely high similarity to archaebacteria in Mono Lake. PCR products were also obtained from the magnetotactic material and are currently being processed.

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Summary: The focus of the lab has been the coupling of theoretical, computational and experimental approaches for the study of structural biology. In particular, we have placed an emphasize on developing quantitative methods for protein design with the goal of developing a fully systematic design strategy that we call "protein design automation." Our initial work in this area has focused on the selection of amino acids for the buried hydrophobic core of proteins. More recent work has focused on defining the limits of specificity in protein design, catalytic design, the design of peptides that are targeted to specific alleles of class I MHC's, and the coupling of backbone variation and core amino acid selection.

In addition to our design efforts, the lab has been interested in issues related to protein folding and stability. In particular, we have a strong interest in developing instrumentation for studying fast folding kinetics. To this end, we have been engaged in a collaborative project with Professor Yu-Chong Tai, an electrical engineer at Caltech, to use silicon microfabrication technology to develop a practical multistage quench-flow device capable of achieving dead times well below one millisecond.

269. De Novo protein design: Fully automated sequence selection
Bassil I. Dahiyat¹, Stephen L. Mayo

We report the first fully automated design and experimental validation of a novel sequence for an entire protein. Using our computational design algorithm based on the physical chemical potential functions developed in our previous work (Dahiyat and Mayo, in press; Dahiyat and Mayo, 1996; Dahiyat, et al., 1997) we screened a virtual combinatorial library of 1.9 x 10²⁷ possible amino acid sequences for compatibility with the design target, a ββα protein motif. To compare to experimental screening techniques, a corresponding peptide library consisting of only a single molecule for each 28 residue sequence would have a mass of 11.6 metric tons. A BLAST search shows that the optimally designed sequence, Full Sequence Design 1 (FSD-1), has very low identity to any known protein sequence. The solution structure of FSD-1 was solved using nuclear magnetic resonance spectroscopy and indicates that FSD-1 forms a compact, well ordered structure that is in excellent agreement with the design target structure.
270. Automated design of the surface positions of protein helices  
Bassil I. Dahiyat, David Ben Gordon, Stephen L. Mayo

Using a protein design algorithm that quantitatively considers side-chain interactions, the design of surface residues of α-helices was examined. Three scoring functions were tested: a hydrogen-bond potential, a hydrogen-bond potential in conjunction with a penalty for uncompensated burial of polar hydrogens, and a hydrogen-bond potential in combination with helix propensity. The solvent exposed residues of a homodimeric coiled coil based on GCN4-p1 were designed by using the Dead-End Elimination Theorem to find the optimal amino acid sequence for each scoring function. The corresponding peptides were synthesized and characterized by circular dichroism spectroscopy and size exclusion chromatography. The designed peptides were dimeric and nearly 100% helical at 1 °C, with melting temperatures from 69-72 °C, over 12 °C higher than GCN4-p1, while a random hydrophilic sequence at the surface positions produced a peptide that melted at 15 °C. Analysis of the designed sequences suggests that helix propensity is the key factor in sequence design for surface helical positions.

271. Automated design of protein β-sheets  
David Ben Gordon, Stephen L. Mayo

We examine the application of intrinsic secondary-structure propensities to the design of β-sheets. Statistically-derived propensity energies were incorporated into a sequence selection algorithm based on the Dead-End Elimination Theorem. Twelve surface-exposed β-sheet positions of the immunoglobulin-binding domain of streptococcal protein G were redesigned using energy potentials in which the scale of the β-sheet propensity was varied. To evaluate the designed sequences, inverse PCR mutagenesis was used to assemble genes for bacterial expression, and the resulting proteins were characterized by circular dichroism spectroscopy. Designed proteins all folded reversibly, with melting temperatures ranging from 29 °C to 67 °C. Proteins designed with highly scaled propensity had significantly higher melting temperatures than those designed with lower propensity, though they had fewer inter-residue electrostatic interactions. We conclude that propensity is a more important contributor to β-sheet surface stability than side-chain pair interactions.

272. Penalizing exposure of hydrophobic area on protein β-sheet surfaces  
Arthur G. Street, Stephen L. Mayo

In our effort to design entire proteins, the best surface amino acids (residues) of β-sheets remain difficult to determine. Unlike in the core of the protein (Dahiyat and Mayo, in press), van der Waals restraints are not very constraining, and unlike the surface of α-helices (Dahiyat et al., 1997), a hydrogen bonding potential is not sufficient either. One potential energy term that may help is to directly benefit residues according to their “β-sheet propensity” (Minor and Kim, 1994; Smith et al., 1994). These are energies derived from studies of highly solvent-exposed sites, which are chosen to minimize complicating context effects. Propensities thus measure how much each amino acid inherently “likes” to be in a β-sheet.

An alternative energy term, which we are examining, is one that penalizes the exposure of hydrophobic area. This agrees with notions of a “reverse hydrophobic effect” (Fukula and Sauer, 1990) that says hydrophobic residues should be disadvantaged on the surface of proteins.

Penalizing exposed hydrophobic surface area is in fact partly an issue of “negative design,” of making alternative folds less stable so that a sequence will fold to the given structure (Sun et al., 1995).

The system we use is the βl immunoglobulin-binding domain of streptococcal protein G (Gβl) (Gallagher et al., 1994). We use a protein design algorithm based on the Dead-End Elimination Theorem (Desmet et al., 1992), which analyzes side-chain packing, to find the sequence leading to the minimum energy structure. The wildtype sequence of Gβl incorporates Ile at position 6, on the surface in the middle of a central β-strand, in a position that is 64% buried.

A total of six point mutants were made at position 6, mostly to amino acids of similar β-sheet propensity, using molecular biology methods. Their stabilities were measured as melting temperatures using circular dichroism.

Using these results, we scaled the hydrophobic exposure penalty and found a strong correlation between the experimental stabilities and the calculated energies. The mutations with residues of worse β-sheet propensity had stabilities generally lower than predicted. Thus it may be necessary to include both β-sheet propensity and a hydrophobic exposure penalty in order to properly design protein β-sheet surfaces.

References

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273. Coupling backbone flexibility and amino acid sequence selection in protein design
Alyce Su1, Scott Ross, Catherine Sarisky1, Stephen L. Mayo

Using a protein design algorithm that quantitatively considers side-chain packing, the effect of explicit backbone motion on the selection of amino acids in protein design was assessed in the core of the β1 domain of streptococcal protein G (Gβ1). Concerted backbone motion was introduced by varying Gβ1's secondary structure parameter values. The stability and structural integrity of seven of the redesigned proteins were experimentally determined and showed that core variants containing as many as six out of ten possible mutations retain native-like properties. The relaxation properties of the backbone amide 15N were measured and the dynamic order parameter (S2) was mapped for those mutants with significantly altered core compositions. The difference between the S2 distribution for these mutants and the wildtype protein reflects regions responsible for backbone flexibility. NMR structural analysis of the most overpacked core mutant reveals the accessible backbone modes for regions responsible for backbone flexibility. This result demonstrates that backbone flexibility can be explicitly combined with amino acid side-chain selection and that the selection algorithm is sufficiently robust to tolerate perturbations as large as 15% of Gβ1's native super-secondary structure. The approach presented here can be further viewed as the starting point for rational design of novel backbone folds.

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274. Design of a hyperthermophile in the optimization of boundary residues
Sandra M. Malakauskas, Stephen L. Mayo

Our approach to protein design is two-fold: backbone design and sequence selection. With respect to the latter, our computer algorithm, based on the Dead-End Elimination Theorem (Desmet et al., 1992), historically calculated a benefit energy for those residue and template interactions that resulted in a burial of hydrophobic surface area. However, experimental (Dahiyat and Mayo, in press) and theoretical data (Sun et al., 1995) suggest that it is also necessary to apply a penalty for exposure of hydrophobic surface area. Though there does not appear to be a thermodynamic reason for the necessity of the penalty, we suspect it reduces the structural heterogeneity of our modeled structures. As an additional consequence, we expect the modeled structures to be inherently more stable as they bury increased hydrophobic surface area (Chan et al., 1995). Initial experimental evidence supports this expectation.

The penalty for exposure of hydrophobic surface area, κ, was first optimized in the context of the boundary positions of our model system, protein G. Boundary residues are those that can be either exposed to solvent or buried within the core of the protein depending on changes in their χ torsion angles. W43 was chosen as a test position and was mutated to various amino acid identities. The experimentally determined melting temperatures of the corresponding recombinant molecules were compared to the calculated energies. The correlation was greatest when κ was increased from 0 to 1. This value of κ was then used to do an initial optimization of four boundary positions in protein G.

The four residues chosen for optimization were T16, T18, T25 and V29. The result was two nearly identical ground states: WIEI and IIEI. The latter was chosen for experimental analysis. Initial temperature melts revealed that IIEI is a hyperthermophile, as it failed to unfold even at 99 °C. Chemical denaturation done at 50 °C yielded a free energy of unfolding equal to 5.4 kcal/mol. Wildtype controls remain to be done for comparison. Finally, the preliminary NMR solution structure confirms our expectation that IIEI should retain the wildtype fold. Next, we will attempt to optimize all 13 boundary residues, with our ultimate goal being to redesign the entire protein.

References

275. De novo protein design: Fully automated sequence selection of a new motif
Chantal S. Morgan1, Stephen L. Mayo

The protein design algorithm developed in the group (Dahiyat and Mayo, 1996) has been used to design a protein with a different motif from those used to develop the algorithm. Since the computational algorithm was developed using physical chemical principles rather than protein-specific considerations, extension of the design algorithm to an alpha helical motif of 51 residues will demonstrate the generality of the design methodology. Initially, twelve core positions of the protein were redesigned; the resultant protein was synthesized and experimental evidence demonstrated that it is a folded, stable protein. The surface positions have been redesigned, both in sections and all together. Next, the boundary positions will be redesigned and the resultant protein will be experimentally analyzed. With improved computational power, it may be possible to design all 51 residues in one calculation.

Reference
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276. Design and synthesis of proteins with novel binding sites for small organic ligands
Frederick S. Lee, Stephen L. Mayo

A computational methodology was developed to add novel ligand binding activities to protein templates. Small hydrophobic pockets of proteins
created by site-directed mutagenesis have been shown to bind small organic ligands such as benzene and indole (Eriksson et al., 1992; Feher et al., 1996). The B1 domain of streptococcal protein G (GPl) (Gronenborn et al., 1991) that lacks natural affinities towards small ligands was used as our initial template. Potential binding sites for benzofuran were sought for by mutating one and every residue of GPl into a set of pseudo residues, which are conceptually similar to transition state analogs of a chemical reaction. Side chains surrounding the mutation were allowed to repack as well. The side-chain conformations of both the natural and pseudo residues were described by discrete states. The side-chain rotamers. The combinatorial search for the optimal sequence of rotamers was solved using the Dead-End Elimination Theorem (Desmet et al., 1992), which was implemented in our protein design automation modules (Dahiyat & Mayo, 1996). As a result, five GPl variants were discovered. Each contains one or more mutations to form the putative binding site for benzofuran. The genes for all five mutants were constructed and the corresponding proteins were expressed in E. coli. Binding activities of these mutants were assayed by thermal upshift (Eriksson et al., 1992) and fluorometric titration (Hands  

References
Hands  

277. Microfabricated silicon mixers for submillisecond quench-flow analysis
Dirk Bökenkamp, Stephen L. Mayo
A primary technique used to determine the rate constant of liquid phase chemical reactions is the rapid mixing of two solutions and the measurement of the time dependent concentrations of the products formed. Several mixers have been developed in order to achieve efficient mixing in a few milliseconds. The Ball mixer described by Berger and coworkers (Berger et al., 1968) utilizes the turbulent wake of a spherical surface and is currently used in commercially available stop-flow and quench-flow instruments. Free jet (Takahashi et al., 1997) and multicapillary (Moskowitz and Bowman, 1966) mixers have been shown to mix in the microsecond time scale but are limited to only a single mixing stage and continuous-flow operation, and are therefore currently not applicable to stop-flow or quench-flow techniques. All of these mixers operate on the principle that high turbulence generates vorticity which increases the contact area of the reactants and reduces the mixing distance. Since diffusion over small distances is fast, rapid mixing is achieved.

Stop-flow and quench-flow methods are the most common techniques used to investigate protein folding. In a stop-flow instrument, refolding occurs after rapidly diluting a chemically unfolded protein (Jackson and Fersht, 1991) and the process is studied by a variety of spectroscopic techniques (Evans and Radford, 1994). In contrast, quench-flow instruments contain a series of mixers that start and stop a reaction over a defined period of time. The product is analyzed afterwards, independent of the instrument. The minimum observation time is determined primarily by the dead time of these instruments, which is dependent on the shortest delay lines, the mixing efficiency and the fluid flow rates. To date, observation times for stop-flow and quench-flow instruments less than a few milliseconds have not been attainable. Initial events such as collapse of a polypeptide chain and secondary structure formation in proteins often cannot be observed. Revealing these processes has been the major driving force in developing new experimental methods (Eaton et al., 1996). Eaton and coworkers (Chan et al., 1997) recently described a study of cytochrome c folding in the submillisecond time scale using a continuous-flow method. Despite the valuable insights into fast protein folding kinetics provided by these techniques, they provide only limited structural information. Detailed structural data in proteins can be obtained by examining the exchange of hydrogens and deuterons, followed by structure analysis using NMR (Mayo and Baldwin, 1993) or mass spectrometry (Miranker et al., 1996). However, this approach typically requires more than one mixing stage and cannot be performed in a continuous-flow apparatus.

Recent advances in silicon micromachining make it feasible to miniaturize mixer assemblies onto a single silicon chip. In collaboration with Yu-Chong Tai, Electrical Engineering and Applied Physics, Caltech, we designed several prototype silicon devices. Decreasing mixer volume and distances between the mixing chambers combined with high flow velocities enabled us to achieve greatly reduced dead times (Desai et al., 1997). The 1 cm x 1 cm x 1 mm silicon chips are comprised of two simple "T" mixers connected by delay lines of different length. Interfacing to the commercially available fluid delivery system (Biol  

Get more insights on the submillisecond time scale, because it proceeds with
the same chemical mechanism but has a higher rate constant. The silicon mixers feature a minimum observation time of 110 µs and a dead time of 100 µs, which is more than ten times faster than any currently available quench-flow system. The data not only show good agreement between different flow velocities in one chip but also between different delay lines in different chips.

We also confirmed mixing events visually, using chemical indicators as mixing probes (Li and Toor, 1986).

References

Publications

Professor: James H. Strauss
Senior Research Associate: Ellen G. Strauss
Research Fellows: Soichi Nukuzuma,
Robbert van der Most, Kyongmin Hwang Kim
Volunteer: Chiyoko Nukuzuma
Research and Laboratory Staff: Edith M. Lenches
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Summary: We are interested in two groups of animal viruses, the alphaviruses and the flaviviruses. These viruses contain an RNA genome of about 11,000 nucleotides and are enveloped, having a lipid envelope that surrounds an icosahedral nucleocapsid. Both alphaviruses and flaviviruses are vectored by mosquitoes and infect both mosquitoes and a wide range of vertebrates. We wish to understand the replication of these two groups of viruses at the molecular level and to study their evolution, and how it relates to the broader question of the evolution of all RNA viruses.

There are about 26 recognized alphaviruses, many of which cause important human diseases including encephalitis, polyarthritis, and febrile illnesses accompanied by rash and joint pain. The genomic RNA of 11,700 nucleotides is an mRNA encoding four nonstructural proteins that together make up the RNA replicase. The structural polypeptides that are used to assemble progeny viruses are translated from a subgenomic 26S mRNA. Both the nonstructural and structural proteins are translated as polyprotein precursors, which are proteolytically processed to the final products.

The flaviviruses contain about 70 members, most of which are important human pathogens causing hundreds of millions of cases of human disease annually such as yellow fever, dengue fever, and encephalitis. The genomic RNA of about 10,700 nucleotides is the only viral message RNA and is translated into a long polyprotein that is cleaved to give both the structural proteins required for virus assembly and nonstructural proteins required for RNA replication.

Our studies have been greatly aided by the development of cDNA clones that allow us to express all or parts of the viral genomes. In the case of the alphaviruses, we have constructed complete cDNA clones of four viruses: Sindbis, Ross River, Semliki Forest, and Aura virus from which infectious virus can be recovered after transcription of RNA in vitro with T7 or SP6 RNA polymerase. These clones allow us to map mutations in viruses, to site-specifically construct new mutants of the virus, and to construct chimeric viruses in which the proteins from one virus must interact with proteins of a second virus. Such studies allow us to study the functions of different proteins and their interactions with one another, and to construct viruses of altered virulence, including potential vaccine strains. Full length clones of several flaviviruses have also been constructed by investigators in several laboratories.

The abstracts below describe efforts to understand in detail the mechanisms involved in control of RNA synthesis, viral assembly, and receptor mediated endocytosis to initiate infection. In the past year our studies have been concentrated on the following topics: (1) Isolation and characterization of receptors for alphaviruses using anti-idiotypic antibodies or using a filter binding assay; (2) Isolation and characterization of a receptor for flaviviruses, and mutation of a putative receptor binding domain of flaviviruses; (3) Studies on the evolution and geographic spread of alphaviruses by comparison of sequences of various members of the genus; (4) Construction of chimeric viruses to study virus
assembly and the use of reverse genetics to characterize "passage-adapted" chimeric viruses; (5) Studies on superinfection exclusion of alphaviruses in mosquito cell lines.

278. Characterization of soluble recombinant envelope protein from yellow fever virus as a probe to identify cellular receptors

Robbert van der Most

Flaviviruses are enveloped viruses that enter their target cells by receptor-mediated endocytosis. However, no host cell receptor has been identified for any flavivirus so far. Since the viral envelope protein is the viral moiety most likely to interact with the receptor, this protein would therefore be the best probe to identify the receptor. The crystal structure of the E protein of one flavivirus has been solved and revealed a three-domain structure in which an immunoglobulin (Ig) -like domain is probably responsible for cell binding.

To obtain sufficient quantities of E protein, we chose to express the yellow fever virus (YF) E protein using recombinant baculoviruses, in collaboration with Peter Snow from the protein expression facility. Initially, the viral E protein was co-expressed with a second viral protein, prM, since co-expression was thought to be important for proper folding of the E protein. However, the resulting baculovirus recombinant produced very small amounts of protein. By expressing the E gene alone and replacing the viral secretion signal by a baculovirus secretion signal, we obtained a baculovirus recombinant that produced secreted E protein at approximately 1 µg/ml. The recombinant E protein has a C-terminal His6-tag, allowing rapid purification on Ni-columns.

To assay biological activity of the recombinant protein, we first measured its binding to susceptible cells (SW13 cells), by immunofluorescence and flow cytometry. Specific binding was readily detectable, using antibodies against YF or a monoclonal antibody against the His6-tag. To determine whether the recombinant protein could compete with YF for cell binding, plaque reduction assays were performed. Preliminary results indicate that incubation of SW13 cells with soluble E protein prior to infection results in a 4-fold decrease in the number of plaques. Since these results establish that the soluble protein indeed binds the YF receptor, we can now proceed with biochemical studies aimed at isolating the receptor. In a first series of experiments, radiolabeled membrane preparations from SW13 cells are incubated with soluble E protein, followed by purification on a Ni-column. If this approach leads to the identification of a cellular protein that specifically interacts with the E protein, the procedure will be scaled up, which would allow purification of sufficient protein for microsequencing.

279. Identification of a putative receptor binding site on the yellow fever virus envelope protein

Robbert van der Most

Attachment of flaviviruses to target cells is presumably mediated by the viral envelope (E) protein. Interestingly, the Ig-like domains (see above) of the E proteins of many mosquito-borne flaviviruses contain an Arg-Gly-Asp (RGD) motif, or a closely related sequence. Since the RGD sequence plays an important role in the binding of several cell adhesion molecules (e.g. fibronectin, vitronectin) to their integrin receptors, it is tempting to speculate that the RGD motif in flavivirus E proteins is also involved in cell attachment. To address this question, an infectious cDNA clone of yellow fever virus (YF) (strain 17D) was used to mutate the RGD motif. Mutated RNA transcripts were transfected into human cells (SW13) and mosquito (C6/36) cells, and viability of the mutant viruses was assayed by immunofluorescence and titration of progeny virus.

The results show that the Gly residue is critical for propagation of YF in both human and mosquito cells: substitution of Gly by Ala (RAD) severely impairs spread of the infection and decreases the viral titer >100-fold. In contrast, replacement of the Asp residue by Glu (RGE) does not impair viral spread or infectivity. The third position does play a role however, because preliminary evidence suggests that a non-conservative Asp to Ser substitution (yielding RGS) partially inhibits viral spread. The double mutant RAE displays the same phenotype as RAD. These results correlate well with the different sequences found in flaviviruses: the Gly residue is conserved among all mosquito-borne flaviviruses (with the exception of dengue 2 virus) and two flaviviruses (West Nile virus and Kunjin virus) carry the sequence RGE. Because the YF strain Asibi, the parent of the 17D vaccine strain, has the sequence TGD, we also created the mutants TGD, TGE, TAD and TAE. TGD and TGE both yield viral titers comparable to wild type (RGD). However, YF-TAD and YF-TAE appear to be impaired in spread, and TAE grows to >100 fold lower titers than wild type. Again, these data implicate the Gly residue as essential for viral infectivity in both human and mosquito cells.

Our data pose two new questions. First, do these mutations indeed impair cell binding? Second, assuming that the RGD motif is involved in cell binding, is the receptor an integrin? The observation that the Gly to Ala substitution has a strong effect would be consistent with this notion, but the lack of effect of the Asp to Glu mutation and the observation that the Arg to Thr mutation does not affect infectivity seem inconsistent with it. One explanation could be that RGD-containing E proteins indeed bind integrins, but that the replacement of Arg by Thr changes receptor specificity. We are currently developing approaches to address both of these questions.
280. Superinfection exclusion of alphaviruses in three mosquito cell lines persistently infected with Sindbis virus

Ellen G. Strauss, Edith Lenches, Adam Karp

Three Aedes albopictus (mosquito) cell lines persistently infected with Sindbis virus excluded the replication of both homologous (various strains of Sindbis) and heterologous (Aura, Semliki Forest and Ross River) flavivirus, yellow fever virus, replicated equally well in uninfected and persistently-infected cells of each line. Sindbis virus and Semliki Forest virus are among the most distantly related alphaviruses, and our results thus indicate that mosquito cells persistently infected with Sindbis virus are broadly able to exclude other alphaviruses, but exclusion is restricted to members of the alphavirus genus. Superinfection exclusion occurred to the same extent in the mosquito cell line C7-10, in which acute primary infection with Sindbis results in strong cytopathic effects, and in the C6/36 line, where acute infection does not cause visible cellular alterations. However, superinfection of persistently infected C7-10 cells by the homologous virus does not produce cytopathology, consistent with the idea that cytopathology requires significant levels of viral replication.

One of the first steps in alphavirus replication is the synthesis of minus strand copies of the plus-strand viral genome. We have shown that the minus strand replicase must contain uncleaved nonstructural polyproteins, and that subsequent buildup of the virally encoded nonstructural proteinase during the infection cycle results in fully cleaved proteins that can no longer synthesize minus strand templates. We suggest that sufficient proteinase is present in the persistently infected cells that the polyproteins of the superinfecting viruses are cleaved while nascent and no minus strands can be made to start replication, and that this is the molecular basis for superinfection exclusion among alphaviruses.

281. Mayaro and the evolution of alphaviruses

Ellen G. Strauss, Scott Weaver, James H. Strauss

All alphaviruses are arthropod-borne, primarily by various species of mosquitoes, and all in nature undergo an obligate alternation between replication in the vector and replication in a vertebrate host. For some alphaviruses, such as Sindbis and the New World encephalitic viruses, the major natural vertebrate reservoir is birds, including migratory birds, while for others, the vertebrate reservoir is small mammals, including primates. The particular vertebrate host affects the global distribution of these viruses, and viruses whose primary vertebrate hosts are birds are more widely distributed and more uniform in sequence than viruses whose primary vertebrate host is mammals, where local endemic strains of virus often occur. As one example of such spread, very similar strains of Sindbis virus have been isolated in Northern Europe and in South Africa, while strains circulating in the intervening latitudes are quite different. Birds migrate predominantly in a north-south pattern, and thus there are many examples of spread of bird-associated alphaviruses within the New World or within the Old World, but natural mechanisms for transfer between the hemispheres are much rarer. We have been able to detect only a few incidences of transfers of this kind, although the activities of man, in particular jet airplane travel, may significantly alter these distribution patterns in the future.

Although in general the New World viruses can be clearly distinguished from the Old World alphaviruses by sequence, a few anomalies have been observed, and these have given us clues as to the evolutionary history of these viruses. One such anomaly is Mayaro virus, a New World isolate closely related to the Semliki Forest group. A phylogenetic tree based upon the alphavirus sequences is shown in Figure 1, on which a time scale has been introduced using the rates of divergence which have been determined for a few members of the group. It should be noted that this scale is only a best approximation and could be in error by a factor of 2 or more, but almost certainly not as much as a factor of 10. From this analysis, the following conclusions can be drawn:

1. The current lineage of alphaviruses originated in the New World about 4000 years ago; (2) Alphaviruses have been introduced into the Old World two times—once about 3500 years ago to found the Semliki Forest virus group, and once 1600 years ago to found the Sindbis virus group; (3) About 1800 years ago, a recombination event occurred between Eastern equine encephalitis virus and a Sindbis-like progenitor to found the

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Figure 1. Phylogenetic tree of the alphaviruses derived from sequences in nonstructural protein nsP4 and glycoprotein E1.
Western equine encephalitis group in the New World; and (4) Mayaro, an Old World virus related to Semliki Forest virus, was reintroduced into the New World about 1500 years ago.

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282. Detection and isolation of cellular receptors for Sindbis virus

Soichi Nukuzuma, Chiyoko Nukuzuma

The interaction between viral attachment proteins and cellular receptors is one of the factors determining tissue tropism and virulence of a virus. Using an anti-idiotypic antibody as a probe Wang et al. (1991) found that the major receptor used by Sindbis virus (SIN) to enter chicken embryo fibroblast cells (CEF) appeared to be a 63-kDa protein which is not identical to the Sindbis receptor on BHK cells (Wang et al., 1992). To isolate protein(s) that interact with Sindbis virus and that might therefore be involved in virus entry, we first devised a slot blot assay for virus binding and used it to screen CEF cell membrane preparations. Increasing amounts of CEF proteins were spotted onto a PVDF membrane with a slot blot apparatus, incubated with biotinylated SIN virus, followed by anti-biotin antibody conjugated with peroxidase. This assay gave positive results for virus binding, and we have now developed this assay further, encouraged by the results of Ludwig and Smith (1996) who used a virus overlay binding assay to identify mosquito proteins that bind Venezuelan equine encephalitis (VEE) virus. CEF membrane proteins were separated by SDS-PAGE, blotted onto PVDF membranes, and incubated with 35S-labeled SIN virus or biotinylated SIN. Bound virus was detected either by direct autoradiography (using labeled virus) or by enhanced chemiluminescence using peroxidase. Using the enhanced chemo-luminescent reaction which is more sensitive than direct autoradiography, we identified a 97-kDa protein as a presumptive receptor on CEF. However, this putative SIN receptor appears to be present in extremely low concentration in these preparations. We are currently developing a procedure for isolating proteins bound to biotinylated Sindbis from large volumes of CEF membrane preparations using magnetic beads with streptavidin coupled on their surface, but the procedure needs to be optimized for pH, temperature, ionic strength and solubilizing agents for the membranes.

References

283. Mouse monoclonal antiidiotypic antibodies as probes for the Sindbis virus receptor

Chiyoko Nukuzuma, Soichi Nukuzuma

Animal viruses initiate infection of susceptible cells by binding to receptors expressed on the cell surface, followed by entry into the cell. The distribution of these receptors determines the host range and tissue tropism of viruses. The alphaviruses possess a very wide host range that encompasses insects and many species of vertebrates, and it is an intriguing question as to whether they use the same receptor in all these divergent hosts, or whether the alphaviruses have evolved the ability to use different receptors in different organisms.

A series of rabbit polyclonal antiidiotypic antibodies (α-Ids) made to mouse monoclonal antibodies (mAbs) that neutralized the infectivity of Sindbis virus (SIN) were previously studied by K.-S. Wang in our laboratory (Wang et al., 1991). One of the α-Ids (anti-mAb 49) bound to the surface of chicken embryo fibroblasts (CEF), partially blocked virus binding, and immunoprecipitated a chicken protein with a molecular weight of 63kDa.

In the present study, we are attempting to generate mouse monoclonal α-Ids (which should immunoprecipitate the receptor for SIN), by screening hybridoma supernatants for reactivity with surface proteins of CEF cells. Balb/C mice were immunized with protein G-purified mAb 49 conjugated to keyhole limpet hemocyanin. Fusions were performed by the Monoclonal Antibody Facility. Hybridoma supernatants were screened for binding to CEF membrane proteins by a LAB-ELISA system. Microtiter plates were coated with purified CEF membrane proteins, incubated with hybridoma supernatants, followed by biotinylated anti-mouse IgG, and then HRP avidin, and detected by a colorimetric peroxidase reaction. Using this screen 850 hybridoma supernatants have been examined, of which 100 hybridoma supernatants were positive, i.e. they bound to the immobilized CEF membrane proteins. These 100 supernatants are currently being assayed for their ability to block Sindbis virus binding to CEF by a plaque reduction assay. A monoclonal which binds to CEF and can block Sindbis virus infection can then be used to isolated the CEF receptor for Sindbis virus in sufficient quantities for biochemical characterization.

Reference

284. Characterization of adapted virus from the SIN(RREl) chimera

Kyongmin Huang Kim, Edith Lenches

During the assembly of alphaviruses, a pre-assembled nucleocapsid buds throught the cell plasma membrane to acquire an envelope containing two virally-encoded glycoproteins, E2 and E1. Last year, our group described a chimeric Sindbis virus, in which the 6K and E1 genes and 3'NTR were replaced with those of Ross River(RR) virus. The virus yield of this chimera was only 107 that of wild type SIN. The conformation of the SIN E2/RR E1 heterodimer was different from that of SIN E2/E1 heterodimers and no interaction between intracellular nucleocapsids and viral glycoproteins at the cell plasma membrane could be detected in the electron microscope.
When this chimeric virus was passaged 10 times in BHK cells, "adapted viruses" arose that grew 100 fold the better than the original chimera. Examination of multiple independent adapted viruses showed that most contained several mutations in the SIN E2 and RR E1 region, and some mutations occurred more than once, indicating possible important residues. To test whether these mutations were responsible for the adaptation phenotype, some of these mutations were introduced back into the original chimera, either singly or in various combinations, and the resultant viruses tested for replication efficiency. Some of the tested mutants are the following: in SIN E2, 131 Lys to Glu, 237 Val to Phe, 248 Asp to Tyr and 380 Ile to several amino acid residues; and in RR E1, 310 Ser to Phe, 411 Gln to Leu, and 433 Cys to Arg.

With single mutations, the chimera with SIN E2 237 Val to Phe mutation gave a 100-fold better yield than the original chimera and the chimeras with 310 Ser to Phe or 433 Cys to Arg each gave 10 fold better yields. When the latter two mutations were combined, the double mutant gave 100-fold more virus. Chimeras with triple mutations (237 Phe/310 Phe/433 Arg or 131 Lys/310 Phe/433 Arg) gave 1000-fold higher yield of virus. Interestingly, the SIN E2 131 Lys to Glu mutation did not improve virus production when it was introduced alone. However, when this mutation is combined with two other mutations, as described above, the triple mutant chimera produced 10-fold more virus than the double mutant without the change at 131. Taken together, it seems probable that these mutations were selected because they adapt RRE1 and SIN E2 to one another and allow SIN E2/RR E1 heterodimers to assume a conformation which facilitates virus budding.

285. Regulation of Alphavirus RNA synthesis

Kyongmin Hwang Kim

The four nonstructural proteins of Sindbis virus (SIN), nsP1, nsP2, nsP3 and nsP4, are translated in that order as a polyprotein from the genomic RNA, are processed by the nonstructural proteinase located in the carboxy-terminal half of nsP2, and together form the viral RNA replicase. The roles of the nonstructural polyproteins, cleavage intermediates, and mature proteins in Sindbis(SIN) virus RNA synthesis have been studied using site directed mutants. Some of these mutants have defects in processing at the 1/2 and/or the 2/3 cleavage sites, others have a serine sense codon in place of the normal opal termination codon between nsP3 and nsP4. From these studies it has been proposed that Sindbis RNA replication is tightly regulated by differential proteolysis of P123. Early in infection, nsP4 and unprocessed P123 form transient minus-strand RNA synthesis complexes. Later in the infection cycle, the high level of viral protease activity (due to the presence of nonstructural proteinase nsP2 and intermediates containing nsP2) eliminates de novo synthesis of P123 and minus-strand RNA synthesis ceases; nsP4 and the P123 cleavage products then form stable plus-strand RNA replication complexes.

In contrast, Semiliki Forest (SF) virus has an arginine sense codon instead of the opal termination codon between nsP3 and nsP4. In SF infected cells, P123 and nsP4 should be synthesized in 1:1 ratio and no excess P123 is available to serve as a source of transacting proteinase. It has been postulated that RNA replication complexes containing unprocessed P123 might be able to synthesize plus-strand RNA, or that SF might be using a slightly different mechanism for RNA replication. Also, nsP4 of SF has been reported to be produced by autoproteolysis by a protease activity found in nsP4 rather than the nsP2 protease.

To test how SF regulates RNA synthesis, we constructed a full length cDNA clone of SF virus and SF viruses were recovered after transfection of RNA transcripts. From this full length SF cDNA clone, several mutants were constructed similar to SIN mutants: the 1/2 and/or the 2/3 cleavage sites were blocked, and/or the arginine sense codon between nsP3 and nsP4 was changed to an opal termination codon. With this series of mutants, we can now examine the effects of these changes on the ability of viral replicase complexes to make plus- and minus-strand RNAs. We can also ask whether the nsP2 protease is responsible for cleavage at the 3/4 site in SF and the effect of proteolytic processing throughout the SF infection cycle.

Publications


neural crest. This migratory precursor population is more accessible than the central nervous system.

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Summary: The mechanisms that control the development of neuroepithelial precursor cells into the neurons and glia of the mammalian nervous system are not understood. We have focused our analysis of mammalian neural development on the neural crest. This migratory precursor population gives rise to the neurons and glia of the peripheral nervous system, which is simpler and experimentally more accessible than the central nervous system. However, we anticipate that many of the rules governing the development of the neural crest will apply to central neuronal and glial progenitors as well. We seek to understand the 'logic' underlying the segregation of different lineages (is lineage restriction stochastic, or accomplished by progressive restriction of developmental potentials, and if the latter is there an order to the restrictions?); we also seek to identify key regulatory molecules and cell-cell interactions that control this process.

In vivo and in vitro studies in other laboratories using chick and quail embryos have revealed that the earliest crest cells are multipotential. The development of a culture system for rodent neural crest permits a combination of the power of mouse molecular genetics with in vitro cell biological approaches. We have succeeded in isolating and culturing early mouse and rat neural crest cells, using the low-affinity NGF receptor as a cell surface marker. Using serial subcloning experiments, we have demonstrated that these cells are not only multipotent, but exhibit at least limited self-renewal, suggesting they have stem cell-like properties. This finding has allowed us to define the factors and mechanisms governing self-renewal and lineage commitment of neural crest stem cells. For example, we have identified three distinct growth factors that promote differentiation of each of three crest-derived lineages: BMP2, TGFβ and GGF promote development of neuronal, smooth muscle and glial lineages, respectively.

To identify transcription factors that control the lineage restriction of neural crest stem cells, we are taking two complementary approaches. One involves isolating mammalian homologues of Drosophila genes involved in early neuronal development. For example, we have isolated homologues of the proneural achaete-scute genes, called Mash genes. Gene knockout experiments in the mouse have revealed that the early expression of Mash1 in neural crest cells reflects an essential function for this gene in the generation of several classes of autonomic neurons. More recently, we have identified a novel family of proteins distantly related to MASH1, called the neurogenins, which are expressed in populations of developing neurons, such as sensory neurons, that do not express MASH1. Another approach is to clone genes encoding regulatory proteins that control the transcription of structural genes specifically expressed in neuronal lineages, such as SCG10. A negative regulatory element that restricts SCG10 expression to neurons has been delineated, and a novel "silencer" protein that binds to this element, called NRSF, has been cloned. Functional studies of NRSF are in progress.

The ability to manipulate the expression of wild-type or dominant negative forms of these transcription factors in cultured neural crest stem cells, using retroviral vectors, permits us to understand how these intracellular molecules control and execute the programs of differentiation triggered by growth factors, and the extent to which they commit the cells to particular fates. In parallel with these in vitro studies, analogous experiments are being carried out in chick embryos to determine how these molecules control crest cell fate in vivo. Complementing these gain-of-function manipulations are gene knockout experiments in mice, that determine the necessity of these molecules for crest development. Together, these approaches allow an analysis of the intercellular and intracellular regulatory circuitry that controls the lineage diversification of a multipotential stem cell population.

286. Integration of multiple instructive cues by neural crest stem cells

Nirao M. Shah

Growth factors can influence lineage determination of neural crest stem cells (NCSCs) in an instructive manner, in vitro. Since NCSCs are likely exposed to multiple signals in vivo, these findings raise the question of how stem cells would integrate such combined influences. BMP2 promotes neuronal differentiation while GGF2 promotes glial differentiation; if NCSCs are exposed to saturating concentrations of both factors, BMP2 appears dominant. By contrast, if the cells are exposed to saturating concentrations of both BMP2 and TGFβ1 (which promotes smooth muscle differentiation), the two
factors appear co-dominant. Sequential addition experiments indicate that NCSCs require 48-96 hrs in GGF2 before they commit to a glial fate, whereas the cells commit to a smooth muscle fate within 24 hrs in TGFβ1. The delayed response to GGF2 does not reflect a lack of functional receptors, however, since the growth factor induces rapid MAP kinase phosphorylation in naive cells. Furthermore, GGF2 can attenuate induction of the neurogenic transcription factor MASH1, by low doses of BMP2. This short-term anti-neurogenic influence of GGF2 is not sufficient for glial lineage commitment, however. These data imply that NCSCs exhibit cell-intrinsic biases in the timing and relative dosage sensitivity of their responses to instructive factors, which influence the outcome of lineage decisions in the presence of multiple factors. The relative delay in glial lineage commitment, moreover, apparently reflects successive short-term and longer-term actions of GGF2. Such a delay may help to explain why glia normally differentiate after neurons, in vivo.

287. Isolation and functional characterization of mitogenic factor for mammalian neural crest stem cells

Jaesang Kim

Neural crest cells represent an ideal system to study the biological characteristics of stem cells and the mechanism of lineage diversification. In particular, a successful culturing of rat neural crest stem cells (NCSC) in this laboratory allowed an in vitro analyses of the growth and differentiation of mammalian NCSC. Conditions for growth of NCSC and commitment to specific lineages have been defined from these analyses.

The growth of NCSC in vitro requires the addition of chicken embryonic extract (CEE) to an otherwise chemically defined medium. I am currently attempting to purify and identify the mitogenic factor or factors from CEE. Conventional chromatographic techniques are used for preliminary characterization of the CEE derived mitogen. The mitogenic activity is assayed for by substitution of CEE with chromatographic fractions in a growth assay of NCSC. The activity is recovered in a single fraction over two ion exchange columns (DEAE and SP Sepharose) and can be safely dialyzed in a membrane with the molecular weight cutoff of 12500 daltons suggesting that the mitogen is a single protein factor. Moreover, approximately 50% of the cells grown in the presence of the second column fraction became MASH1+ within 24 hours upon being challenged with BMP2. A similar response is seen among cells grown in the presence of CEE thus indicating that a similar degree of neurogenic potential is retained by cells and that the purified mitogen is not inducing a commitment to a particular lineage. A more rigorous test of multipotency, in the form of serial subcloning experiments as well as further purification, will also be attempted.

288. Mechanisms of neural crest differentiation

Sebastian S. Gerety

Previous work from our lab has identified three factors, GGF2, BMP2, and TGFβ2, that act instructively on neural crest lineage choice, generating primarily glia, neurons, and smooth muscle, respectively. BMP2 was shown to be expressed in vivo at a location consistent with its potential role as a neural inducing signal, near sites of differentiation of crest derived sympathetic neurons. I am interested in confirming the role of BMP2 in vivo through the use of retroviral expression vectors. By overexpressing a dominant negative form of the BMP2/4 Type I receptor, I will block signaling through the BMP2 pathway in migratory neural crest cells in the chick embryo. If BMP2 plays the role suggested by our previous data, we would expect to see a reduction in sympathetic neuronal differentiation. A second aspect of neural crest fate determination I am examining is the specificity of fates generated by BMP2 and TGFβ in vitro: While TGFβ generates exclusively smooth muscle cells, BMP2 generates both neurons and smooth muscle cells. The fact that BMP2 and TGFβ are both members of the TGFβ Superfamily suggests an interesting possibility that there is some crosstalk, either at the level of receptors, or downstream signaling pathways. I am interested in dissecting this issue of specificity again through the use of retroviral vectors. By infecting primary neural crest in culture with constructs overexpressing down-stream effectors, constitutively active or dominant-negative forms, I hope to better understand the mechanistic aspects of these cell fate decisions.

289. Functional studies of NRSF during mouse neurogenesis

Zhoufeng Chen

NRSF (neuron-restrictive silencer factor) is a zinc finger transcription factor that binds the neuron-restrictive silencer element, a consensus motif identified in many neuron-specific genes. During embryogenesis, NRSF is expressed in many non-neuronal tissues including the somites, limb buds, and head mesenchyme. In the nervous system, NRSF is expressed in undifferentiated neuronal progenitors but not in differentiated neurons. Therefore, we hypothesized that NRSF may negatively regulate the expression of neuronal genes, either by repressing the expression of neuronal genes in non-neuronal tissues, or by preventing premature neurogenesis in neuronal tissues or both.

To understand the function of NRSF in vivo, we generated NRSF-null mutant mice. The NRSF-null mutants become growth retarded from E9.5 and die between E10.5-11. Histological examination revealed a disorganization of myotomal cells and head mesenchyme cells. To further characterize the phenotype of NRSF-null embryos, the expression patterns of a battery of molecular markers were
examined. The expression of one early neuronal marker TuJ1, a neuronal tubulin cytoskeleton protein, has been found to be derepressed in several non-neuronal tissues including myotome and limb ectoderm. Ectopically expression of TuJ1 is also found in a thin layer of cells surrounding the heart, the cranial neural crest cells and the ectoderm adjacent to the otic placode. In the nervous tissues, the expression of TuJ1 is highly upregulated. These data demonstrate that NRSF is a master control gene, and is required for repressing the expression of some neuronal genes in both neuronal and non-neuronal tissues.

290. NRSF function in development

Alice J. Paquette

During neuronal differentiation genes characteristic of the differentiated state come to be expressed. There are two basic mechanisms for this transcriptional up-regulation: Transcription of these genes can be enhanced by a neuron-specific, positive transcription factor, or, conversely, these genes can be de-repressed after having been silenced by a neuronal-progenitor-specific, negative transcription factor. Surprisingly, it appears as though the latter mechanism may play an important role in neurogenesis.

To identify proteins that regulate neuron-specific gene expression, the 5' regulatory region of a gene specifically expressed in all differentiating neurons, SCG-10, was characterized. It was found to contain a sequence element that conferred transcriptional repression, but only in non-neuronal cell lines. This suggested that a factor might be present in these cell lines keeping at least this neuron-specific gene silent. Mobility shift assays confirmed the presence of a factor that bound to this element in non-neuronal cell lines and indicated that the activity was absent from cell lines that expressed neuronal markers. The gene encoding this Neuron-Restrictive Silencing Factor (NRSF) was cloned according to its ability to bind the element and was determined to encode a zinc-finger DNA binding protein. NRSF is expressed both in most non-neuronal tissues and in the area of the neural tube where neuronal precursor cells, but not terminally-differentiated neurons, reside. Database searches showed that sequences similar to the binding element were found within the regulatory regions of many neuron-specific genes, besides the original, SCG-10. After testing a sample of these sequences for their ability to bind NRSF and to mediate silencing, a functional consensus sequence was determined and used to compile a list of tens of genes likely to be regulated by NRSF.

Given this large set of target genes and the widespread expression of NRSF outside the nervous system during development, a major role for NRSF in addition to regulating neurogenesis is proposed. NRSF may be necessary in a wide array of developing tissues to prevent them from expressing genes inappropriate for that tissue. (Although the majority of potential NRSF targets are neuron-specific genes, many are not.) Using retrovirus-mediated gene transfer a dominant-negative version of this transcription factor, composed of the DNA-binding domain alone, has been used in chick to interfere with the endogenous function of NRSF during development. Currently Type III Beta Tubulin and Ng-CAM, two previously identified direct targets of NRSF, have already been found to be de-repressed specifically in dominant-negative retrovirus-infected regions of the developing embryo. Further studies using this system will certainly provide additional valuable insight as to the role of NRSF during development.

291. MASH1 maintains competence for BMP2-induced neuronal differentiation in post-migratory neural crest cells

Liching Lo, Lukas Sommer

The interplay between growth factors and transcription factors in vertebrate neurogenesis is poorly understood. MASH1 is a basic helix-loop-helix (bHLH) transcription factor that is essential for autonomic neurogenesis. Bone morphogenetic protein (BMP) 2, and its relative BMP4, have been shown to induce expression of MASH1 and to promote autonomic neuronal differentiation in neural crest stem cells. The relationship between expression of MASH1 and the neurogenic competence of neural crest cells has not been investigated, however. We have examined the function of MASH1 in neurogenic competence using a population of immuno-isolated neural-crest-derived progenitor cells. Post-migratory neural crest cells isolated from fetal rat gut expressed MASH1, yet comprised a mixture of committed neuronal precursors and non-neuronal cells. The non-neuronal cells remained competent to differentiate to neurons, however, if challenged with BMP2. Such competence declines with time and is paralleled by a decline in MASH1 expression in the cells. Expression of endogenous MASH1 can be maintained by BMP2; in turn, constitutive expression of MASH1 from a retroviral vector maintains competence for neuronal differentiation in response to late addition of BMP2. These data suggest that MASH1 promotes competence for neurogenesis, in a manner similar to its homologs, the proneural genes achaete-scute in Drosophila. They also reveal an unexpected feedback interaction between BMP2 and MASH1 during neuronal differentiation. MASH1 may play multiple roles at successive stages of development within a neurogenic lineage, only one of which is revealed by a loss-of-function mutation.

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292. Essential role of neurogenin in murine sensory neurogenesis

Qiufu Ma, Zhoufeng Chen, Lukas Sommer

We are interested in how mammalian peripheral neurons are specified from neural crest stem cells. Interesting questions include lineage segregation
between sensory and autonomic, and the further segregation of neuronal sublineages such as nociceptive versus proprioceptive sensory neurons. Earlier studies from several groups have identified Mash1, a basic helix-loop-helix (bHLH) transcriptional regulator, as a key determinant for autonomic neurons. Most recently, we and others have identified two highly related bHLH genes, ngn1/Math4C/neuroD3 and ngn2/math4A, which are exclusively expressed in early sensory precursor cells. ngn's are structurally related to atonal, a Drosophila proneural gene, and to NeuroD, a putative vertebrate neuronal differentiation gene. Functional studies of a Xenopus ngn related gene, x-ngnr-1, have suggested that ngn's function as neuronal determination genes.

To further study the role of ngn1 during development of the murine nervous system, a null mutation of ngn1 has been generated by homologous recombination. We found that NGN1 is essential for a subset of cranial sensory ganglia including trigeminal, vestibulo-cochlear, and Superior/Jugular complex. NGN1 is also required for development of the nociceptive neurons in the dorsal root ganglia (DRG). In these affected ganglia, neither differentiated neuronal markers (TuJ1 and SCG10) nor putative neuronal differentiation factors (NeuroD and Math3) are expressed. These data are consistent with the idea that NGN1 may function as a neuronal determination factor during mammalian sensory neurogenesis, as does X-NGNR-1 in Xenopus.

293. Functional analysis of bHLH genes involved in the development of sensory and autonomic lineages in ovo
Sharon E. Perez

One of the central questions in developmental neurobiology is how distinct classes of neurons are specified. Two different bHLH genes, MASH1 and neurogenin-1, are required for the development of two neural crest derivatives, the autonomic and sensory neurons of the trunk. Loss of function analyses have indicated that MASH1 is required for the development of the autonomic lineage (Guillemot et al., 1993; Sommer et al., 1995), while neurogenin-1 plays a role in the development of the sensory lineage (Ma, Chen and Anderson, in preparation). Ectopic expression studies of these genes are likely to reveal additional functional information; we are particularly interested in whether expression of either bHLH gene restricts neural crest cells to a particular fate, and whether maintained expression of either gene causes the developmental program for that lineage to "stall".

The MASH1 and neurogenin-1 ORFs have been tagged with myc epitopes, and cloned into an avian retroviral vector, RCAS-B. To label the pre-migratory neural crest, either one of the experimental viruses, or a control reporter virus, is injected into the lumen of the neural tube in chick embryos at HH stages 10-13. Animals are harvested a minimum of 36 hours later, and frozen sections are processed to detect either reporter expression or the bHLH bearing virus. In addition to a comparison of the positions of infected cells, the samples will be examined for perturbations in cell type specific marker expression via immunohistochemistry or non-radioactive in situ hybridization. Finally, the consequences of ectopic expression on cell proliferation and survival will be examined via BrdU and Tunel labeling.

294. The development of sensory and autonomic neurons in an in vitro explant culture system
Amy L. Greenwood

The trunk neural crest gives rise to two main classes of neurons in vivo: autonomic neurons of both the sympathetic and enteric ganglia and sensory neurons found in dorsal root ganglia near the spinal cord. Sensory neurons are a heterogeneous population in terms of their peripheral targets, morphology and expression of molecules such as neuropeptides, ion channels and growth factor receptors. The sensory and autonomic lineages segregate early in development thus raising questions about the mechanism, timing and logic of this lineage segregation and the production of the sensory sublineages. In vitro studies have contributed much to our knowledge of the cellular and molecular mechanisms that control differentiation of crest into the autonomic fate. The study of sensory neurogenesis, however, has proven more difficult because this type of neuron does not arise under standard conditions of culturing neural crest.

To begin to address the relationship between the sensory and autonomic lineages I have been studying neurogenesis in explant cultures of the neural tube. Both types of neurons develop in these cultures; sensory neurons and precursors can be identified by expression of Brn3.0, a POU-domain transcription factor, while autonomic neurons are identified by Phox2, a homeodomain transcription factor. These two populations develop in the same relative locations as they are found in vivo. Sensory neurons develop in close proximity to the neural tube whereas autonomic neurons are often found more distally in clusters in the periphery of the explant. Additionally, the number of sensory neurons that develop is dependent upon the presence of the neural tube; that is, the longer the neural tube is left in culture the more sensory neurons are produced. Sensory neurons may rely on the neural tube as a source of necessary signals, as a source of precursor cells, or both. I am interested in testing the competence of early and late migrating crest to give rise to both kinds of neurons and in the effect of factors known to be present in the neural tube on cell fate choices in the crest.

295. Rodent - chick chimeras: A novel approach to study mammalian neural crest differentiation
Patricia M. White

My project has been to develop an in vivo system to assay the developmental potential of rodent neural
crest stem cells populations. Such a system can be used to test hypotheses of progressive restriction of cell fates: I can isolate populations believed to represent partially restricted precursors and show what such cells have the capacity to become when presented with the embryonic microenvironment. This complements in vitro studies of the neural crest already done in this laboratory, where others have analyzed the cell fates of the progeny of single cells in vitro, and demonstrated instructive effects of individual growth factors present in the embryo at the right time and place. My analysis provides the most rigorous test of restrictions deduced from their studies.

By transplanting rat precursors into chicks at early stages of development, I can show rat crest cells isolated in vitro can migrate to the chick aorta and turn on the general neuronal marker SCG-10, as well as more specific autonomic markers like GATA-2, Phox-2, and tyrosine hydroxylase. This is consistent with sympathetic neuron differentiation. Crest cells also migrate to the spinal nerve, consistent with Schwann cell development, and some express myelin protein P0. Finally, in my transplantation assay, I do not see rat crest cells colonizing the sensory ganglion and expressing neuronal markers, even though I can demonstrate quail contributions to the sensory ganglion using the same transplantation technique.

By varying the cell types injected, I have shown rat cells with the potential to associate with the sensory ganglion and express the rat specific neuronal markers SCG-10 and Brn-3a are present in the neural tube after 3 days of culture, but not in the emigrated crest. This implies rodent crest cells that have emigrated from the tube in vitro are restricted from the full choice of fates shown to be possible of emigrated crest cells in vivo.

296. Eph receptor-ligand interactions in the peripheral nervous system development

Hai Wang

I am currently studying the role of Eph family receptor tyrosine kinases and their transmembrane ligands in the PNS development. Two such Eph family transmembrane ligands - Htk-L in rat and Lerk2 in chick, are expressed in caudal somite halves. Trunk neural crest cells and early motor axons avoid caudal somite halves during their migration/projection. Previous studies have suggested the existence of inhibitory/repulsive candidate molecules in the caudal somite halves. To test the hypothesis that Htk-L and Lerk2 are inhibitory/repulsive molecules for crest cells and motor axons, we used a series of in vitro assays such as a transfilter chemotaxis assay, protein stripe assay, and growth cone collapse assay. Such explant and culture studies revealed that both Lerk2 and Htk-L repulsive guidance activities toward migratory neural crest cells as well as early motor axons explanted from rat neural tubes (see reference).

In collaboration with Zhoufeng Chen, I am in the process of making Htk-L knockout mice in order to study Htk-L’s role in vivo. A tau-lacZ tracer gene was inserted into the Htk-L locus by homologous recombination. Htk-L is expressed in early caudal somite halves, and hindbrain segments (r1, r2, r4, r6). Later it is expressed in forebrain cortical neurons, and transiently in motorneurons. Thus, the Htk-L mutant mice may have one or more developmental defects, such as somite polarity disruption, dorsal root ganglia malformation, and motor/cortical axon mis-guidance. The tau-lacZ marker could be used to dissect these potential defects.

Reference

297. The purification of central nervous system stem cells

Sean J. Morrison

Stem cells proliferate and differentiate during fetal and neonatal life to form the central nervous system (CNS). CNS stem cells have been proven to exist by retrospective analyses such as the retroviral marking of clones in vivo and the generation of multilineage colonies in vitro (reviewed in Morrison et al., 1997). Rare stem cells persist in the adult CNS, where they have been implicated in the turnover of neurons in the olfactory bulb, and the hippocampus. In addition to yielding insights into the regulation of self-renewal and differentiation, stem cell biology is thought to be central to improving the understanding of tumorigenesis in the CNS, and to developing the ability to transplant or regenerate nervous system tissue. The purification of hematopoietic stem cells opened doors that dramatically advanced analogous scientific and clinical goals. We are attempting to purify stem cells from the rat telencephalon, which gives rise to the cerebral cortex, and from the rat olfactory epithelium, which gives rise to olfactory sensory neurons throughout life. We have developed an assay in which primitive progenitors from each tissue efficiently form colonies in vitro. Under clonal culture conditions most such progenitors proliferate as nestin+ cells with neuroepithelial morphology and then differentiate into neurons and astrocytes. Nestin is an intermediate filament protein expressed by primitive CNS progenitors. We have identified a panel of cell surface antigens by fluorescence activated cell sorting (FACS) that are expressed heterogeneously by cells from each tissue. Differences in antigen expression correlate with the ability to form multilineage colonies in vitro. Currently we are testing whether a combination of surface markers uniquely identifies a population of cells from each tissue that is multipotent and self-renewing.

Reference
Summary: Development of the nervous system involves several stages, including the proliferation of precursor cells, their determination to become neurons, their differentiation into specific neuronal and glial types, the formation of precise networks, and the maintenance of the functional integrity of the system. All these events underlie the behavior of the organism. Our research group on *Drosophila* addresses these questions, using mutants to dissect the processes of development, physiology, and behavior.

Many genes and proteins show homology between *Drosophila* and vertebrates, and both *Drosophila* and humans suffer from many genetic developmental abnormalities. Memory loss in humans is associated with degeneration of specific neurons, as in Alzheimer's disease and other neurological disorders. We are studying similar degenerative phenomena in *Drosophila*. In the *Drosophila* mutant *drop-dead*, lack of expression of the normal gene leads to the onset of brain degeneration and early death. In addition to a detailed study of this gene and its function, we have isolated a series of other brain degeneration mutants that display varied modes of cellular degeneration with age. Genetic analysis of such mutants provides a powerful tool for evaluating neuronal and glial cell interactions, and the isolation of molecular factors that are important in maintaining the integrity of the brain.

The development of the compound eye of the fly has provided a fertile field for investigation of cell-cell interaction and the determination of cell fate. We are approaching the question of the evolution of these mechanisms by a comparative molecular analysis of the development of the eye of the grasshopper, which is much lower on the insect evolutionary tree and which, unlike the fly, does not undergo complete metamorphosis.

**298. Transgenic studies of the *drop-dead* gene**

Laurent Seroude, Seymour Benzer

The *drop-dead* (*drd*) mutants have shortened adult lifespan; after a few days, the flies have locomotor difficulties, become paralysed, and die (Hotta and Benzer, 1970). Electron microscopy shows early onset of brain degeneration (Buchanan & Benzer, 1993). The *drop-dead* gene spans roughly 15kb, contains eight exons and encodes a novel, multiple transmembrane protein. We isolated two genomic cosmid DNAs which contain the same sequences upstream of *drd*, but only one also carries coding regions. These were used to generate transgenic flies carrying duplications of the *drd* region and tested for their capability of rescuing the early death and female sterility of several *drd* alleles. Only the duplication containing the *drd* coding region was able to fully rescue, demonstrating that we have cloned the correct gene.

To investigate the function of the *drd* gene product, we used transgenic flies to study the *drd* function's temporal and spatial requirement. To control the time when *drd* is expressed, we used the heat-inducible promoter *hsp70*. We found that over-expression of *drd* in wild-type flies did not induce any dominant phenotype. By applying heat-shock at various times during development in an *drd* mutant background, we showed that to prevent early death of the adult fly and produce a normal trachea (see abstract by C. Kim), *drd* function is required during a short time window at the end of metamorphosis. We also have generated transgenic lines to express, under the control of the *drd* promoter, DRD protein carrying immuno-tags. Most of the lines did not rescue *drd* mutants indicating that the constructs do not carry a fully functional *drd* promoter. However, we found so far two lines that were able to rescue. It is probable that, in those two cases, the construct has been inserted in the genome close to an enhancer driving expression in the correct place and time. We are currently investigating the DRD
expression driven by these lines. We are also currently investigating the \textit{drd} function's spatial requirement using the GAL4-UAS system (Brand and Perrimon, 1993).

References


299. Expression and Function of the \textit{Drosophila} DRD Protein

Changsoo Kim, Seymour Benzer

Using fusion protein expressed by a drop-dead (\textit{drd}) cDNA construct, a monoclonal antibody was generated against the N-terminal region of the DRD protein. Staining of tissues with the antibody showed expression in a variety of cell types, including tracheal, hypodermal, hindgut, cone, and follicle cells. The common characteristic of these cells is that they secrete components of cuticle, which provides rigidity and flexibility to the various structures.

By fluorescence microscopy, we observed that the inner linings of tracheae show a blue fluorescence visible upon UV excitation. In \textit{drd} mutant flies, although the chitinous wall of the trachea is present, the blue fluorescence is absent. An elastic material, resilin, which produces blue fluorescence, is a protein cross-linked by di-tyrosine bonds that has been found in tendons, ligaments, foot pads, and other flexible structures of various insects (Anderson and Weis-Fogh, 1964). We identified di-tyrosine in acid hydrolysates of detergent-insoluble material from flies. In \textit{drd} mutants, the tracheae are fragile, suggesting easy collapse upon physical stress, consistent with a lack of the flexible component. Since \textit{drd} brain cells undergo DNA fragmentation, characteristic of apoptosis, anoxia may underlie the early death of the mutant flies.

The \textit{drd} gene encodes a novel, multimembrane protein without known motifs and functional domains. In homogenates of fly heads, a ~95 kDa molecule is found in membrane fractions by Western blot analysis, using anti-DRD antibody. Immunohistochemistry shows that the staining is punctate inside cells, suggesting that the DRD protein may be present in intracellular vesicles. In developing tracheae, DRD staining is found both inside tracheal cells and in the inner linings of the tracheae, indicating that the DRD vesicles synthesized in cells migrate to the cuticular layer to discharge the contents of vesicles. Based on the amino acid of the conceptual DRD protein, it is unlikely that DRD itself is resilin. Therefore, we propose that the DRD protein is involved in vesicular transport of a soluble precursor to the tracheal inner wall, where cross-linking occurs to produce the blue fluorescent protein.

Reference


300. \textit{Drosophila} drop-dead mutations accelerate the time course of age-related markers.

Blanka Rogina\textsuperscript{1}, Seymour Benzer, Stephen L. Helfand\textsuperscript{1}

Mutations of the \textit{drop-dead (drd)} gene in \textit{Drosophila melanogaster} lead to striking early death of the adult animal. At different times after emergence from the pupa, individual flies begin to stagger and, shortly thereafter, die. Anatomical examination reveals gross neuropathological lesions in the brain. The life span of flies mutant for the \textit{drop-dead} gene is 4 to 5 times shorter than for normal adults. That raises the question whether loss of the normal gene product might set into motion a series of events typical of the normal aging process. We used molecular markers, the expression patterns of which, in normal flies, change with age in a manner that correlates with life span. In the \textit{drop-dead} mutant, there is an acceleration in the temporal pattern of expression of these age-related markers.

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301. Mosaic analysis of drop-dead

Archana Hayaran, Seymour Benzer

The \textit{drop-dead (drd)} mutation has a shortened adult lifespan (Hotta and Benzer, 1970). The mutant flies appear normal after eclosion, but after a few days have locomotor difficulties, then become paralyzed and die. We also find that, beginning on day 1 after eclosion, \textit{drd} flies are sluggish compared to wild type flies when tested in the countercurrent distribution apparatus (Benzer, 1967). While a significant reduction in the tracheal blue fluorescent protein has been observed (see abstract by Kim and Benzer), the focus of the phenotype remains obscure. Hence, we generated gynandromorphs by paternal loss of the X chromosome (Szabad \textit{et al}, 1995) carrying the internal marker \textit{armadillo-lacZ} (Vincent \textit{et al}, 1994). Twenty two gynandromorphs were analysed for lifespan, sluggish behaviour, presence of tracheal blue fluorescence protein, and absence of lacZ expression to identify mutant tissues. Out of these, 17 gynandromorphs showed a reduced lifespan (3 days) and sluggish behavior, while the remaining five stayed alive for at least 15-18 days, with no sluggishness. Among the group of 17, although degeneration was observed in most tissues, 10 flies did not show local correlation with respect to either lacZ staining or blue fluorescent protein. 7 flies showed degeneration in the abdomen along with explosion of the crop. The five flies which remained alive for 15-18 days also did not show local autonomy; large areas of various tissues were mutant, yet the flies survived. Two flies had tracheal blue fluorescence significantly lower in one half of the brain, and in one of those it was low in one half of thoracic ganglion and muscles as well. These preliminary data suggest that there may not be a unique autonomous focus for the \textit{dropdead} phenotype.
References

302. A partial suppressor of the Drosophila drop-dead mutation displays extended longevity
Yi-Jyun Lin, Seymour Benzer

drop-dead (drd) mutant Drosophila die within two weeks after eclosion, whereas normal flies can live more than 12 weeks at ambient temperature (Hotta & Benzer, 1972; Buchanan & Benzer, 1993). To study the molecular function of drd, we performed genetic enhancer/suppressor screening of the drd flies. Male Canton-S flies were mutagenized with ethylmethane sulfonate (EMS), and crossed to FM7-balanced females. The survival of approximately 12,000 F1 males carrying drd1 and mutagenized autosomes were scored, and the ones that lived more than two weeks were established as lines. One such line, tentatively termed Longlife (Lf), partially suppressed the drd1 phenotype, extending its lifespan to 22 days. This effect appears to be drd specific; it does not extend the life of three other drd alleles tested.

In the absence of the drd mutation, heterozygous Lf flies themselves displayed a 40 % increase in longevity compared to cohorts without the Lf mutation. We are currently mapping the location of Lf, and cloning and characterization of the gene will be pursued.

References

303. Screening of lifespan extension mutants in Drosophila
Yi-Jyun Lin, Seymour Benzer

Aging is a general biological process that involves complex physiological and biochemical events. Toward a genetic dissection of the processes involved, we are conducting a screen for extended lifespan in Drosophila melanogaster by single gene mutations. The survival curves of 300 lines of flies with single P-element insertions at various locations in the genome were measured to identify ones that outlive normal controls. One such mutant line, methuselah (mth), has been studied in detail. Heterozygous flies have normal lifespan, but ones homozygous for the P-element consistently show a greater than 50% increase in lifespan. This is true both at 25 and 29 °C. When exposed to paraquat, a free radical generator, they are much more resistant than control flies, consistent with previous observations on fly populations of extended longevity derived by selective breeding (Arking et al., 1991). Cloning of the gene and expression studies are in process.

Reference

304. Identification of reduced-lifespan mutants in Drosophila by P-element screening
Kyung-Ta Min, Seymour Benzer

We screened over 600 P-element inserted autosomes for reduced lifespan. Thirteen mutants were found which show a variety of lifespans. These are being analyzed by plasmid rescue and sequencing of DNA adjacent to the P element. Some of these identify previously known genes. Two are alleles of the Drosophila mutants shortsighted (Treisman et al., 1995) and headcase (Weaver & White, 1995). A third P-element mutant disrupts the 42A actin gene (Fyrberg et al., 1981), for which no mutant phenotype has previously been known. The mutant shows severe preadult lethality; escapers die early in adulthood. The remaining genes are novel, showing no significant sequence homology with known genes. These mutants are being characterized by genetic, histological, and molecular techniques.

References

305. Degenerative diseases in Drosophila mutants resembling mammalian brain syndromes
Kyung-Ta Min, Seymour Benzer

Various brain degeneration diseases are characterized by late onset, gradual progression, and finally death, but have still unknown etiology and mechanisms. To investigate the use of the fly as a model system to identify genes related to such diseases, we screened for mutants showing reduced life associated with brain degeneration.

Two such mutants, produced by EMS mutagenesis, have different patterns of brain degeneration as characterized by histology and transmission electron microscopy. The brain of the spongecake mutant exhibits regionally specific membrane-bound vacuoles mimicking those seen in human Creutzfeldt-Jakob disease. In one day old spongecake flies, maintained at 29°C, various axon terminals in the optic lobe neuropil become inflated. These occur both singly and in groups. At early stages, these can be identified as axon terminals by their content of abundant synaptic vesicles and mitochondria. With ages, the vacuoles develop in the optic lobes, beginning in the medulla, then spreading also to the lamina and the lobula, but not to the central brain.

In a second mutant, eggroll, both neural and glial cells contain cytoplasmic inclusions that are rare in normal brain. These are already visible in the neuropil and the cortex region of one day old flies. As aging progresses, various types of multilamellated structures arise in the neuropil. By 4 or 5 days, there is widespread degeneration, including cortex, lamina, medulla, lobula, and central brain. By 12 days, there is also retinal degeneration. Dense, multilamellated inclusions resembling those in eggroll have been seen in...
many human disorders, including Tay-Sachs disease, Niemann-Pick disease, and Fabry disease.

In the fly, the pathological mechanism and progression of disease can be investigated from the earliest stages. The genes can readily be mapped and cloned, thus identifying a constellation of genes, the normal functions of which are needed to maintain the integrity of the aging brain. spongectae, eggroll, and others, including chocolate chip and meringue, also found in the screen, may provide insight into mechanisms and possible candidates genes for similar human syndromes.

Reference


306. A maternally-transmitted, virulent form of Wolbachia causes degeneration and death in Drosophila

Kyung-Tai Min, Seymour Benzer

Wolbachia, a microorganism of the Rickettsial family, is found in the ovaries of various arthropods and is maternally transmitted. It is known to cause cytoplasmic incompatibility, parthenogenesis, or feminization in various insect species. The relationship between bacterium and host is usually regarded as symbiotic; cytoplasmic incompatibility between infected males and uninfected females produces reproductive isolation that can enhance evolution; the other mechanisms enhance production of progeny. We have found a new strain of Wolbachia and given it the name popcorrnia. It is maternally transmitted in Drosophila melanogaster. While quiescent during the fly’s development, it begins massive proliferation in the adult, causing widespread degeneration of tissues, including brain, retina, and muscle. This leads to early death, as occurs in human diseases such as typhus, caused by the insect-transmitted, pathogenic Rickettsia prowazekii.

Electron microscopy reveals cells packed with the bacteria resembling bags of filled with popcorn, hence the name. The bacteria, detectable by PCR, antibody, and direct microscopic observation, are maternally transmitted to previously uninfected male and female progeny, which in turn acquire the syndrome. However, there is no cytoplasmic incompatibility; infected males crossed with uninfected females, and vice versa, result in normal numbers of progeny. Tetracycline treatment of carrier flies eliminates both the bacteria and the degeneration, and restores normal lifespan.

Comparison of the 16s rDNA sequence with Wolbachia known in other insects shows over 98% identity. These bacteria may be useful for studying the disease-causing mechanisms of pathogenic rickettsia. Tests on various strains of Drosophila melanogaster commonly used in genetic experiments reveals that a surprising number are infected with less virulent forms of Wolbachia, which might affect their longevity, behavior, and other phenotypes.

References


307. Sluggish behavior mutants of Drosophila

Archana Hayaran, Seymour Benzer

Although there has been extensive work in vertebrates on the physiology and biochemistry of neurotransmitters, one would like to learn more about their genetic control. Drosophila, with its powerful genetics, can serve as a model organism to address this issue. Most efforts in neurotransmitter research have focused on immunohistochemical, biochemical, retrograde uptake, and in vitro experiments; there are relatively few genetic studies (Greenspan et al., 1980). A preliminary screen by Ghysen (1975), using the countercurrent distribution method (Benzer, 1967) yielded a dozen different mutations, on the X chromosome alone, that exhibited sluggish behavior. Only one of these has so far been cloned. The mutant sluggish A (slgA) was found to be defective in the mitochondrial enzyme proline oxidase, which is involved in biosynthesis of glutamate from proline (Hayward et al., 1993). Proline and glutamate serve as neurotransmitters in mammals; in the fly, glutamate is a neurotransmitter at the neuromuscular junction (Restifo & White, 1990). It is intriguing that a mouse mutant with a defect in proline oxidase likewise exhibits sluggish behaviour (Kanwar et al., 1975).

In a resumption of this project we are carrying out mosaic analysis of slgA by generating gynandromorphs via loss of the paternal X chromosome (Szabad et al., 1995) carrying the internal marker armadillo-lacZ (Vincent et al., 1994). Preliminary analysis indicates that the cortex of the central nervous system, which contains the neuronal and glial cell bodies, is the focus.

Since all but slgA and another (SB37) of the original sluggish mutants have since been lost, we undertook a new mutagenesis using ethylmethanesulfonate, and obtained four new sluggish mutants among 200 lines, using countercurrent distribution.

Functional and molecular characterization of these is in progress.

References


308. Compound eye development in the grasshopper versus the fly

Markus Friedrich, Seymour Benzer

We analyzed compound eye development in the embryo of the grasshopper (Schistocerca gregaria). Comparison with the fruitfly Drosophila allows one to identify evolutionarily conserved and divergent pattern formation processes in insect compound eye development.
Unlike the fly, the grasshopper does not undergo complete metamorphosis: the eye is not formed from an imaginal disc. An individual hatched from the egg already has some ommatidia. At each molt, more facets are added anteriorly. Nevertheless, as in the fly, there is a morphogenetic front (MF), equivalent to the Drosophila morphogenetic furrow, which reappears at each molt. BrdU incorporation experiments show that cell proliferation is similarly tightly controlled at the grasshopper MF, with a first mitotic wave anterior to it and a second mitotic wave posterior, but absence of cell proliferation within.

The early stages of ommatidial assembly are highly conserved between both species. A mature precluster with a central R8 cell appears first and is later surrounded by cell pairs R2/R5 and R1/R6 and the posterior-most R7. The R3/R4 cell pair, located anterior to R2/R5, does not contact R8. Confocal microscopy of tissue labeled with MAb 1C10, which detects grasshopper Lachesin (Karlstrom et al., 1993), reveals that the sequence of neuronal maturation of the future photoreceptor cells is also highly conserved. Thus, the early steps in the developing retina of the grasshopper embryo share many features with the Drosophila eye disc, as is the case for a previously studied beetle species (Friedrich et al., 1996).

On the other hand, the course of later ommatidial differentiation in the grasshopper looks much different from Drosophila. The formation of a centrally fused rhabdome in the adult grasshopper ommatidium requires the central R8 to move peripherally and to take up a position either dorsal or ventral to the adjacent R7-cell. This event is preceded by the formation of a so-called cone cell thread either dorsal or ventral to R7. R8 invariably moves towards the position that the posteriorly located cone cell thread had taken. This results in the formation of two ommatidial subtypes which are randomly distributed over the eye field. The "sloppiness" of the pattern is in contrast to the much greater precision in arrangement in the evolutionarily more advanced Drosophila eye.

References

309. Development and evolution of the insect ocellus

Markus Friedrich, Seymour Benzer

In Drosophila, as in many other insects, in addition to the compound eyes there are ocelli on top of the head. The fly has three, a lateral pair and a third on the midline that is ontogenetically derived by fusion of two halves. Morphologically, the ocelli contain three cell types, photoreceptors, lens-secreting cells and tapetal cells, the latter corresponding functionally to the pigment cells of the compound eye (Yoon et al., 1996). Like compound eyes, ocelli are also present in other arthropod phyla indicating that both sense organs diverged from an ancestral form at least 500 million years ago (Brusca & Brusca, 1990). It is therefore interesting to ask what developmental mechanisms are shared in the formation of compound eyes and ocelli.

In screening a set of monoclonal antibodies (Fujita et al., 1982) for cross-reaction between fruit fly and grasshopper, we found that MAb 36F7 labels the grasshopper tapetal cells, both during embryogenesis and in the adult. We used this and markers of neurogenesis to study the early differentiation of the ocelli in the grasshopper embryo. The earliest expression of neuronal and tapetal cell markers was seen at about 40% of embryonic development, around the same time as development of the grasshopper compound eyes starts. Double labeling experiments suggested very little time difference, if any, between determination of the earliest tapetal and photoreceptor cells. This appears to be different from development of the grasshopper compound eye, where the photoreceptor cells clearly differentiate earlier than the accessory pigment cells. MAB 36F7 does not label the Drosophila tapetal cells. Further studies will compare subsequent steps in differentiation of the ocellus and the compound eye in grasshopper, and the corresponding events in the fly.

References

310. Evolutionary comparison of the photoreceptor cell specific transcription factor Glass

Markus Friedrich, Seymour Benzer

The Drosophila glass gene encodes a zinc finger transcription factor which is required for the development of all of the fly's photoreceptive organs, including the compound eyes, the ocelli and the larval Bolwig's organ (Moses & Rubin, 1991). Glass protein is specifically expressed in tissues which give rise to these organs, and also in a few poorly characterized cells in the brain (Ellis et al., 1993). The latter might also be photoreceptors (Voshall & Young, 1995).

There is also evidence for brain photoreceptor cells in other insect species (Fleissner et al., 1993), but nothing is known about the evolutionary origin and conservation of such cells. Attempts to clone the glass gene in vertebrates have so far failed (Heather et al., 1997).

To address these issues, we will use the zinc-finger region of the Glass protein, which shows strong conservation at the amino acid level between Drosophila melanogaster and Drosophila virilis (O'Neill et al., 1995), to screen for corresponding monoclonal antibodies. Some of these may serve to detect the Glass protein across species and thus provide early markers for brain photoreceptor cells in insects.

References
311. Glia in the chiasms and medulla of the 
*Drosophila melanogaster* optic lobes 
Simone Tix, Eckhart Eule, Karl-
Friedrich Fischbach, Seymour Benzer

Different classes of glia cells in the optic lobes of *Drosophila melanogaster* were defined by the enhancer trap technique, using expression of the *lacZ* reporter gene. At both the outer and inner optic chiasms, there are dual stacks of glia, arrayed from dorsal to ventral, interspersed between the crossings of axonal fiber bundles. The giant glial cells of both the outer and inner chiasms are similar with respect to nuclear shape and position, suggesting similar functions of these cell types.

Another class of glia is found in the medulla neuropil. Their cell bodies anchor in the most distal region of the neuropil, and their processes extend into the deeper axonal layers.

Birth dating using BrDU shows that both groups of chiasm glia are born early in larval life; they may participate in the development of the optic lobe. The medulla glia are born later and may be involved primarily in adult functions.

In wild type, and in mutants with structurally altered optic lobes, the numbers of tract-associated glial cells in the outer and inner optic chiasms depend on the number of visual columns, whereas the complement of medulla neuropil glia correlates with the volume of the optic lobe.

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312. Generation of cDNA expression libraries enriched for in-frame sequences 
Clayton A. Davis, Seymour Benzer

Bacterial cDNA expression libraries are made to reproduce protein sequences present in the mRNA source tissue. However, there is no control over which frame of the cDNA is translated, because translation of the cDNA must be initiated on vector sequence. In a library of non-directionally cloned cDNAs, only some 8 percent of the protein sequences produced are expected to be correct. Directional cloning can increase this by a factor of two, but does not solve the frame problem. We have therefore developed and tested a library construction methodology using a novel vector, pKE-1, with which translation in the correct reading frame confers kanamycin resistance on the host. Following kanamycin selection, the cDNA libraries contained 60-80% open, in-frame clones. These, compared to unselected libraries, showed a 10-fold increase in the number of matches between the cDNA-encoded proteins made by the bacteria and database protein sequences. cDNA sequencing programs will benefit from the enrichment for correct coding sequences, and screening methods requiring protein expression will benefit from the enrichment for authentic translation products.

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**Publications**


**Professor:** William J. Dreyer  
**Senior Research Fellow:** Jost Vielmetter  
**Graduate Students:** Anita Gould, Robert P. Lane, Frank Miskevich  
**Research and Laboratory Staff:** John Page, David Wang, Jian Zhang

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**Support:** The work described in the following research reports has been supported by:  
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National Institutes of Health, USPHS  
**Summary:** One of the most important and exciting topics in biology today is the question of how the DNA in the chromosomes of a fertilized egg choreographs the sculpturing of an entire organism, including the most complex structure of all, the brain. For many years it has been clear to me that this embryological process must involve heritable DNA changes. In other words, that most cell lineages make use of a DNA editing mechanism much like that used by the immune system.

During the process of embryogenesis, genetic programs generate a myriad of different cell types, and in the process, the newly committed cells somehow acquire new memory. The fate of each cell type and its future options become altered as specific lineages unfold and new cell types are generated. The heritable changes that occur program the expression of cell-surface molecules that provide very specific addresses for each of the new cells as they assemble the embryo.

I have been developing collaborative projects to explore the possible role of DNA changes in the heritable memory of cell lineages as well as to study the cell-surface molecules responsible for the address codes that are specifically generated in developing cell types.

Evolving out of these collaborative efforts are studies in the area of genetic control of brain structure and behavior. New genome databases and powerful new methods offer research options that have not been available previously and make these new projects feasible. Many aspects of the project started about ten years ago and reported in the abstracts below have been
completed or phased out since they are no longer appropriate in the post-genomic era.

In that project we developed methods permitting us to isolate and greatly purify cell surface molecules that play a role in neural development. The approach has been very productive and has lead to the identification of a number of interesting proteins that have been studied in this laboratory and others. Other novel and intriguing antibodies produced as a part of that project remain to be studied and arrangements are being made to share them with other laboratories.

References

313. A screen for new cell surface proteins involved in an embryonic address code
Jost G. Vielmetter, Jian Zhang, David Wang, Robert P. Lane, Frank Miskevich

One of the projects in our laboratory was aimed at the discovery of novel cell surface proteins with a potential addressing role in embryonic development. Although biological studies make it clear that many cell types must express address molecules, we have focused our studies on the development of the sophisticated cellular network in the nervous system. The nervous system is believed to be assembled with the aid of cell surface molecules that provide an address system for growing neurites and direct them to their correct targets. We anticipate that address molecules must be expressed in very distinct patterns perhaps, for example, reminiscent of the elaborate and compartmentalized expression of homeobox genes in the insect and vertebrate nervous systems. We have initiated a second-generation screen for such molecules by using an approach that we have already applied successfully to one fraction of cell surface molecules. That first screen produced monoclonal antibodies that identified most of the neural cell adhesion molecules that have since been studied in this (see abstracts below re Bravo and neogenin) and in other laboratories. Other new and interesting proteins detected in that first screen remain to be studied but we have nevertheless moved on to identify and study new families of receptors.

Our experimental paradigm includes techniques that allow us to specifically isolate cell surface molecules from a given tissue and to raise monoclonal antibodies against defined size fractions of those molecules. The tissue we chose for this project was the optic tectum of 8 day old chicken embryos, because it is well known that at this stage millions of retinal ganglion cell axons are in the process of growing into the tectum and searching their appropriate targets, presumably by using cell surface addressing systems.

Tecta from about 5,000 embryos were used in order to obtain adequate quantities of the purified proteins derived from axonal and cell surfaces. We are now in the process of screening tissue sections for restricted patterns of expression. We have found numerous different and restricted patterns resulting from staining sections with monoclonal antibodies prepared against a single 44kD protein fraction. These monoclonal antibodies have detected an intriguing new family of neural cell surface molecules. It remains to be determined if these molecules play a role in cellular addressing during embryogenesis or in other functions that can explain the restricted pattern of expression of each of these different 44 kD proteins.

314. Neogenin is believed to play a functional role in cell differentiation and axonal growth like the related tumor suppressor molecule DCC
Jost Vielmetter

We have discovered a molecule, neogenin, which is presumed to be involved in neuronal differentiation (Vielmetter et al., 1994) and we have cloned and sequenced both the chicken and the human homologs. These show a surprisingly high similarity of over 90% at the amino acid level (See Abstract 313 ). Our studies of expression of neogenin during early retinal development in the chicken embryo are compatible with the notion that it plays a role in terminal differentiation. (Vielmetter et al., 1994).

Many receptor molecules play an important role during embryogenesis as cells undergo the transition from proliferation to differentiation. The molecular mechanisms regulating cell growth and differentiation also play a key role in aberrant and uncontrolled growth leading to tumors. Of possible relevance in this regard is the fact that the only molecule found in the databases to be closely related to neogenin is DCC (Deleted in Colorectal Carcinoma). Typically, tumor suppressor genes are essential for normal control of growth, cessation of proliferation, and signaling the onset of differentiation. In many cases they have been shown to be involved in the control of cell cycle. Therefore chromosomal deletion or inactivation by mutation of both alleles of tumor suppressor genes is associated with abnormal growth and tumor development. Most tumor suppressor genes so far identified function intracellularly; DCC is the only known cell surface receptor molecule in this group, and it is believed to play a role in cell interactions and the signaling of terminal differentiation.

Both neogenin and DCC contain conserved sequences in their intracellular domains which share significant homology to known src homology type 3 (SH3) binding motifs and serine/threonine phosphorylation sites, which are of particular relevance for a possible linkage of these proteins to cell signaling pathways (see below).
315. Src homology type 3 (SH3) domain binding motifs in the intracellular domain of neogenin, a possible link to signaling pathways

Jost Vielmetter, Frank Miskevitch

A key aspect that needs to be understood in a receptor molecule that signals cellular changes is its linkage to the signal transduction pathways inside the cell. Cellular changes as a response to interaction with specific ligands are presumed to be triggered by signaling pathways that are linked to intracellular domains.

The SH3 domain is a type of protein binding motif which is found in an increasingly large number of proteins, including non-receptor tyrosine kinases, phospholipase C, cytoskeletal proteins, and adapter proteins such as Grb2 and Crk (Mayer and Eck, 1995). One function of these domains is to link cell surface receptors lacking kinase or phosphatase activity to signaling pathways within the cell. Deletions of the SH3 domain in the non-receptor tyrosine kinase src cause loss of cell cycle control and failure of differentiation, as do deletions in the c-Abl cytoplasmic tyrosine kinase. It is widely accepted that abnormal tyrosine phosphorylation caused by these mutations leads to malignant transformation. The signaling pathways of the src family of kinases leading to transformation are not fully understood. It is interesting to note that the pattern of expression of c-src, c-yes and fyn kinases in developing neurons parallels the regulation of neogenin expression.

The intracellular domain of neogenin contains seven SH3 domain-binding motifs. These putative binding sites provide a critical clue as to how neogenin may be able to transduce extracellular signals. The intracellular domain of neogenin has no homology to any protein other than DCC, and thus does not appear to be a kinase or phosphatase. However, due to the presence of the SH3 domain binding sites, numerous different proteins could interact with the intracellular domain. Of the seven core SH3 binding motifs of Pro-X-Pro-X-Pro, six are conserved not only between human and chicken neogenin, but also between chicken neogenin and Xenopus and human DCC (See Abstract 314), suggesting that they are vital to the proper functioning of these molecules. For neogenin, one site is a class I SH3 binding site (Arg-X-Pro-X-Pro) and another is a modified class II binding site (Pro-X-Pro). These conserved motifs strongly suggest that neogenin will interact with at least one and possibly several SH3 domain containing proteins. Other novel Pro-X-Pro sites may interact with new types of SH3 domains. Future experiments involving the use of recombinant neogenin in affinity chromatography, immunocoprecipitation and expression library screening are aimed at the identification of proteins that interact with and bind to the intracellular domain of neogenin.

316. Searching for proteins which interact with the chick cell surface protein neogenin

Frank Miskevitch, Jost Vielmetter

N2. n found on early chick retinal ganglion cells at the time when those cells first begin to form. This receptor is expressed at high levels as the axons extend towards their targets and is dramatically down regulated thereafter. The molecule has been cloned and sequenced in our laboratory. It was found to be a member of the immunoglobulin (Ig) superfamily molecule and is most closely related to the human gene DCC, deleted in colorectal cancer. In order to begin to understand the biological function of this molecule, we are looking for proteins that interact with both the extra- and intracellular (See Abstract 315) regions of the molecule. We are currently using several approaches to find binding partners for neogenin. We have expressed the Ig domains in the yeast Pichia pastoris at high levels, and are currently using the recombinant protein for affinity chromatography, immunoprecipitation, and expression library screening experiments. The intracellular domain of neogenin has been expressed in a T7 based bacterial expression system, although the plasmid appears to be unstable due to generating a toxic gene product. We are currently attempting to address this problem using a more stringently controlled vector, different host strains, and/or different induction methods. We have also used a monoclonal antibody (10-22A8) to immunopurify neogenin and associated proteins from chicken brain extract. Using this antibody, we have preliminary evidence that suggests a 60 kD protein co-purifies with neogenin.

317. Expression of neogenin Ig domains and monoclonal antibody production

Frank Miskevitch, Jost Vielmetter

We have used the Pichia Pastoris expression system to produce a 4 Ig domain recombinant construct from the chicken neogenin molecule. The protein is secreted at approximately 80 mg/L of yeast culture and is purified in a single step from the culture media using immobilized metal affinity chromatography. The protein was then injected into Balb-C mice and monoclonal antibodies were raised. Many neogenin antibodies were identified on chicken embryonic tissue sections of the retina and tectum and the corresponding hybridoma lines were subcloned. These monoclonal antibodies are useful tools for studies of the sites of expression and function of neogenin in vivo and in vitro.

318. Recombinant expression of chick Bravo domains used in functional studies

Robert P. Lane , Jost Vielmetter

Both yeast and bacterial expression systems have been used to generate gram quantities of recombinant Bravo protein. To date, the entire extracellular domain, intracellular domain and the alternatively spliced 5th fibronectin domain have been expressed and purified protein identified by N-terminal protein
sequencing. Two kinds of functional studies have been performed using these domains: in vivo perturbation experiments, and in vitro ligand binding experiments.

Both expressed domains and monoclonal antibodies to chick Bravo have been injected into E3 chick embryonic eyes. This is the stage of development when the retinal ganglion cells of the eye differentiate and extend axons, and the growth cones of these axons are begin to use presumptive axon guidance mechanisms to target these cells to the visual centers of the brain. Lipophylic dyes (Dye-I, Dye-O) are used to subsequently visualize the axons and whole mounts are examined by confocal microscopy.

The second class of functional experiments performed using recombinant Bravo protein is affinity chromatography to identify presumptive ligands. Expressed protein is coupled to affi-gel support and brain extracts are chromatographed over the domain to identify retarded protein fractions. To date, a presumptive 70kD ligand has been identified as having a high binding constant to the expressed alternatively spliced fibronectin domain of Bravo, although its identity is not yet known. This is work in progress.

319. Expression studies of the alternatively spliced isoforms of chick Bravo
Robert P. Lane, Jost Vielmetter

The 5th fibronectin repeat of Bravo is alternatively spliced in both the chicken and human homologs of the Bravo molecule (see Abstract 318), suggesting important functional constraints of these isoforms. We have raised a monoclonal antibody specifically against the alternatively spliced 5th fibronectin repeat of Bravo. To date, it appears that in the brain, spinal cord, peripheral nervous system and all tissues examined, wherever Bravo is expressed, so is the alternative exon. That is, it is unlikely that the isoforms are tissue specific, neither both isoforms may be expressed on the same cell or on different cells in the same tissues. We are currently exploring this question at higher resolution by double staining techniques and confocal microscopy to further address the functional significance of this alternative splicing event.

320. Cloning and chromosome mapping of the human Bravo gene
Robert P. Lane, X-N Chen, K. Yamakawa, Jost Vielmetter, J.R. Korenberg

Using primers from the chick Bravo sequence, low stringency PCR was performed on a human embryonic library. An appropriately sized band was cloned, sequenced, and identified as a cDNA with very high sequence conservation to the chick Bravo gene. This PCR product was subsequently used to screen this library for cDNA clones in order to do more definitive and extensive sequencing of the presumptive Bravo homolog. The cDNA shares its most significant sequence similarity with chick Bravo (Genbank database) and is 82% identical at the amino acid level, including 100% identity in the transmembrane and intracellular domain. Also conserved is the presumptive alternative splicing at the 5th fibronectin repeat, suggesting high evolutionary constraint on functional modulation of this molecule. Human Bravo clones were used in a collaborative effort with the Korenberg laboratory at Mount Sinai to map the gene to a human chromosomal locus using fluorescent in situ hybridization (FISH). The human Bravo gene has been localized to a chromosomal band 7q31.1-2 that has been shown elsewhere to contain a tumor suppressor gene but no other human genetic diseases have as yet been localized to this region. We are currently exploring the possibility that Bravo may be involved in early differentiation and cell cycle control.

321. Evolutionary relationships of neural immuno­globulin superfamily members: functional interpretation using molecular phylogeny
Robert P. Lane, Jost Vielmetter, Michael Frolich
The immunoglobulin supergene family now contains an enormously diverse set of proteins all of that have the signature immunoglobulin domain, a beta-sheet structure of about 100 amino acids. It was first identified because of its role in antigen binding and other intermolecular interactions in the immune system. In the nervous system, an ever-growing subfamily of proteins has been identified that, in addition to multiple Ig domains, also typically contain multiple fibronectin type III repeats. These, like Ig domains, are beta-sheet structures (topologically nearly identical to Ig domains) that are also involved in various ligand-binding interactions. We have analyzed the molecular phylogeny, or evolutionary history, of a specific group of these proteins all of which contain 6 Ig domains and 4-5 fibronectin type III repeats. Each of the species homologs for Bravo/NrCAM, LI/NgCAM, neuroglial, neurofascin, axonin-1/TAG-1, Contactin/F11, Big-1/Tag-like, and Big-2 has been examined in this study. Using cladistic/phylogenetic software (Paup 3.1.1 and MacClade) and with alignments derived from structural modeling, we have recapitulated the probable evolutionary history of this protein family. We have also examined the phylogenetic relationship of individual domains and specific structural units (specific beta strands and loops) in order to attempt to capture a functional history based on patterns of conservation and divergence. From these studies we propose, in addition to a particular evolutionary history for individual proteins, a model whereby domain order has been selected for in order to maintain important ligand interactions between domains that involve the bottom loops of one domain interacting with the top loops of the C-terminal adjacent domain, as well as important intervening sequences between pairs of domains. In addition, a cooperative interaction of multiple domains with ligands is suggested.
Biochemical modulation may also complement prior use. Regulation of synaptic efficacy by mechanisms of synaptic regulation in the central nervous system. It has long been postulated that memories are encoded in the brain through long-lasting changes in the strength of synapses triggered by their prior use. Regulation of synaptic efficacy by biochemical modulation may also underlie mood changes and is important for minute by minute information processing. CNS neurons communicate primarily through synapses where presynaptic terminals release neurotransmitters that open ion channels and alter the electrical potential across the membranes of their postsynaptic target neurons. Many of these transmitters can also initiate relatively long-lasting biochemical changes in the signaling machinery of the synapse that change the strength of future transmission at the altered synapse. We are studying the molecular organization of the postsynaptic signaling machinery at glutamatergic synapses, the principal excitatory synapses in the mammalian brain.

In one major project, we are using cell biological, microchemical and molecular genetic mechanisms to systematically identify the protein components of a prominent synaptic organelle called the postsynaptic density or PSD (Kennedy, 1997). The PSD is a dense thickening of the submembranous cytoskeleton that is especially prominent beneath the postsynaptic membrane of glutamatergic synapses, opposite the presynaptic terminals. A number of functions have been proposed for the PSD, including mediation of adhesion of pre- and postsynaptic membranes, regulation or aggregation of postsynaptic receptors, physical stabilization of the synaptic junction, signal transduction in response to activated postsynaptic membrane receptors, and information storage. However, its actual functions are still being determined. An organelle that resembles the PSD can be isolated by subcellular fractionation of brain homogenates. In previous years, we have found that this "PSD" fraction contains signal transduction machinery that responds to activation of receptors, as well as proteins that may help to regulate adhesion between the presynaptic and postsynaptic membranes. Many of the proteins that we have identified as major components of the PSD fraction, including CaM kinase II, PSD-95, the NR2B subunit of the NMDA receptor, and densin-180 are co-localized at glutamatergic synapses in CNS neurons. We have begun to study the functions of some of these newly identified PSD components. In this past year, we have identified two new components of the PSD, both of which are regulatory proteins that interact with members of the GTP-binding protein family. One of these is a new Ras GTPase activating protein (ras GAP) that we have termed synaptic p130 ras GAP. The other protein, previously named citron, is an apparent effector protein that associates with the GTP bound form of Rho. We are continuing to unravel some of the interactions among these proteins that lead to formation and regulation of postsynaptic signal transduction complexes.

In a second major project, we are studying the dynamic regulation of Ca2+/calmodulin -dependent protein kinase II (CaMKII), an enzyme that has been shown to be a crucial component of several important neural protein phosphorylation pathways. Studies in our lab and many others have demonstrated that this protein kinase plays a critical role in permitting changes in synaptic strength that are believed to underlie memory formation in the mammalian brain (Kennedy, 1994). CaMKII is autophosphorylated when it is activated by an increase in Ca2+ concentration. The activity of the kinase ceases only when it is dephosphorylated by cellular phosphatases. We have used a new immuno-cytochemical method, developed in our lab, that permits the visualization of specific phosphorylated sites on known proteins in fixed tissue sections to study highly localized activation of type II CaM kinase in situ during physiological stimulation that leads to long-lasting changes in synaptic strength. This year, we have published a study in which we visualized such changes at synaptic endings following tetanic stimulation of a neural pathway in slices from the hippocampus, a brain area intimately involved in memory formation. Our findings challenge several
major models for the actions of CaM kinase II during generation of long term potentiation. We are continuing
to establish the pattern and time course of activation of CaM kinase II during changes in synaptic strength
in intact neural circuits.

References

322. A novel Ras GTPase-activating protein from
the rat forebrain PSD fraction
Hong-Jung Chen

We have identified a novel protein that contains a Ras GTPase-activating (Ras-GAP) domain as a major
protein component of apparent MW 130 kD in the rat forebrain postsynaptic density (PSD) fraction. The protein was partially purified by SDS-PAGE
fractionation of the PSD fraction and trypsinized in the
gel piece. Peptides were released from the gel and separated by HPLC. Peptide fractions were analyzed by MALDI-TOF mass spectrometry, and sequenced by
gas phase Edman degradation in the Protein and Peptide Microanalysis Laboratory. We designed sense and antisense primers for the polymerase chain
reaction (PCR) based on the peptide sequences. Using combinations of sense and antisense primers for different peptides, we performed RT-PCR with
dNA+ RNA from rat forebrain as template and obtained a specific ~270 bp PCR product. This PCR product was used as a probe to screen a rat brain
cDNA library. Twenty-four positive clones were purified and sequenced. We assembled a nearly complete sequence from 7 clones with overlapping
sequences. The ~3.2 kb cDNA contains an open reading frame and encodes a protein of ~113 kD that contains 12 of the original peptide sequences. Blast search
revealed that this protein has significant homology (25-33% identity) to the carboxyl halves of p120Ras-GAPs from mammals and other organisms and contains the ras-GAP fingerprint motif. The sequence also reveals a putative PH domain, a proline-rich region and several potential CaMKII phosphorylation sites. This synaptic p130 ras GAP appears to represent a new class of mammalian ras GAPs.

323. Citron, a Rho-interacting protein, is a component
of the rat forebrain PSD fraction
Wandong Zhang, Michelle L. Apperson

Proteins migrating at apparent MW 180 kD were
purified from the rat postsynaptic density fraction by
SDS-PAGE. Proteins within excised gel bands were
digested with trypsin. Tryptic peptides were purified by HPLC and sequenced by gas phase sequencing in the Protein and Peptide Microanalysis Laboratory. A
BLAST search of the Genbank database revealed that
4 of 7 peptide sequences are present in a protein named citron previously identified in mouse by the yeast two-
hybrid method as a GTP-Rho binding partner proposed to be a GTP-Rho/Rac effector (Madaule et al., 1995). We have cloned the rat citron gene and the encoded
protein is ~98% identical to mouse citron. The sequence reveals a coiled-coil domain, a rho-binding region, a
PH domain, a Zinc-finger like motif, multiple possible CaMKII phosphorylation sites, and a TSXV motif that
may bind to PDZ domains. We have raised an antibody against citron and shown by western blot that citron is
enriched in the forebrain PSD fraction. Immuno-
cytochemistry is being carried out to identify the cellular localization of citron in neuronal cultures and brain sections. We propose that citron participates
in control of the cytoskeleton within spines under the
regulation of the rho family of small GTPases.

Reference

324. Identification of proteins in the PSD fraction by
mass spectrometry
Randall Walikonis, Ole N. Jensen3, Matthias
Mann1, Mary B. Kennedy

Mass spectrometry offers a rapid and sensitive
method for the detection of the molecular masses of individual peptides in a mixture. The laboratory of Matthias Mann has developed chemical methods and
computer algorithms to analyze the spectrum of tryptic peptides produced by digestion of individual protein
bands from polyacrylamide gels (Mortz, et al. 1996;
Lamond and Mann, 1997). This mass spectrum can then
be used to scan a non-overlapping database of known proteins in order to determine the identity of the
protein if its sequence exists in the database. Under
optimal conditions, this technique permits the
identification of sub-picomolar amounts of proteins in
SDS-gels. It can be used routinely to identify picomolar
amounts of proteins from gels. It can often detect
individual proteins in gel bands that contain mixtures of two or three proteins.

We have initiated a project to identify proteins in the
PSD fraction by this method. The postsynaptic
density fraction was prepared by conventional methods
(Cho et al., 1992), and divided into three samples that
were extracted with different detergents. One sample
contained the PSD pellet obtained after one extraction of a synaptosome fraction with 0.5% triton-X100, the second contained the pellet obtained after two
extractions with 0.5% triton-X100, the third contained
the pellet obtained after extraction once with 0.5%
triton-X100 and then once with 3% sarcosyl. Twenty
µgs of each of these fractions was prepared for SDS-
PAGE fractionation and loaded into the wells of mini-
gels of varying percentages of acrylamide. After
electrophoresis of the proteins, the gels were stained
with Coomassie blue and individual protein bands were
Experimental Laser Desorption Ionization (MALDI) Time of Flight (TOF) mass spectrometry. Several molecules that were
previously identified as components of the PSD fraction
were identified, as well as at least seven proteins that
had not been previously identified. Two of these new
proteins are clearly contaminants from the outer mitochondrial membrane. The others may be newly-identified PSD constituents. We are presently preparing antibodies with which to determine the enrichment of the new proteins in the PSD fraction, and their subcellular distribution in cultured neurons.

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1European Molecular Biology Laboratory (EMBL), Heidelberg, Germany

325. Quantitation of staining for phospho- and nonphospho-CaMKII in hippocampal slices
Yannan Ouyang, David B. Kantor, Erin. M. Schuman, Mary B. Kennedy

CaM kinase II has been recognized to play a critical role in synaptic plasticity in the hippocampal CA1 region. It comprises 2% of the total protein in the hippocampus and about 1% in the forebrain. About half of the kinase is present in the cytosol of cell bodies and dendrites. The other half is particulate and some of the particulate kinase is concentrated in the post synaptic density. CaM kinase II is autophosphorylated when it is activated, and then it remains active and becomes independent of calcium, until it is dephosphorylated by protein phosphatases. In our laboratory, we have raised antibodies that recognize either the phosphorylated or the nonphosphorylated form of CaM kinase II (Patton et al., 1993).

We have developed a method to examine the distribution of phosphorylated and nonphosphorylated CaMKII in hippocampal slices by immunocytochemistry and confocal microscopy (Kindler and Kennedy, 1996). Slices treated in various ways are fixed and 50 µm sections are cut from the 500 µm slices and double-stained for phospho- and nonphospho- kinase. Digital images of staining with each antibody are acquired with a Zeiss confocal microscope. We wish to apply statistical analyses to data acquired in this way, in order to determine changes in distribution of phosphorylated CaMKII produced by various physiological and pharmacological manipulations. Therefore, we have developed a procedure for quantifying differences in brightness of staining between “control” tissue and “experimental” tissue. One example is the “two pathway” experiment in which the control pathway is stimulated from one side of a hippocampal slice and the experimental pathway is stimulated (eg. tetanized) from the other side. We use macro programs written with the aid of the scientific data visualization and analysis program MacPhase (Otter Solution, Whitesboro, NY) to collect the average brightness values from rectangular “regions of interest” (ROIs) of fixed size positioned on corresponding P-CaMKII and NP-CaMKII images between each of the stimulating electrodes and the recording electrode. Brightness averages from the control side and from the tetanized side of the three sections are summed for each of the slices. Finally, we compute the ratios of the summed brightnesses from the tetanized side to those from the control side. The data are transferred to Excel (Microsoft, Redmond, WA) for statistical analysis by ANOVA and t-test.

References

326. Tetanic stimulation produces an increase in total staining for CaMKII in dendrites of hippocampal neurons
Yannan Ouyang, Gabriel Kreiman, David B. Kantor, Alan J. Rosenstein, Erin M. Schuman, Mary B. Kennedy

Calcium/calmodulin dependent protein kinase II (CaMKII) is believed to play a crucial role in synaptic plasticity in area CA1 of the hippocampus. Both LTP and LTD are disrupted in hippocampal slices from mutant mice from which the alpha subunit of CaMKII is deleted (Silva et al., 1992; Stevens et al., 1994); however, the molecular mechanisms underlying each form of plasticity are still unknown. We used double-label immunocytochemistry of fixed hippocampal slices with antibodies against activated, autophosphorylated CaMKII (P-CaMKII) and non-phosphorylated CaMKII (NP-CaMKII) to show that both forms of CaMKII are significantly elevated in dendrites in area CA1 30 min after induction of LTP by tetanic stimulation of Schaffer collaterals (Ouyang et al., 1997). In contrast, in cell bodies only P-CaMKII is elevated by the same stimulus. Our results suggest that new CaMKII can be synthesized locally in neuronal dendrites in response to tetanic stimulation and that long-lasting changes in dendritic CaMKII activity produced by tetanus may be caused by new synthesis rather than by long-lasting changes in the proportion of P-CaMKII. This finding may provide a function for the relatively large quantity of alpha-CaMKII message found in hippocampal dendrites.

We are now testing this hypothesis by investigating changes in P-CaMKII and NP-CaMKII at earlier times after tetanus, and after blockade of protein synthesis with anisomycin.

References
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327. Optimization of antibody concentration for visualization of phospho-CaMKII in neural tissue
Kathryn A. Stofler
The Kennedy Lab has developed an immunohistochemical method for imaging the relative distribution of the active (autophosphorylated) and inactive (nonphosphorylated) forms of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) in sections of rat hippocampus (see abstracts 4 and 5). The technique makes use of immunohistochemical labeling with fluorescent antibodies specific for either phospho-CaMKII or nonphospho-CaMKII. The brightness of staining for the two different forms of CaMKII in hippocampal slices is determined with the use of a laser-scanning confocal microscope. Sections to be examined are first incubated with a primary antibody that recognizes only the form of CaMKII that is autophosphorylated at threonine-286 (a mouse monoclonal antibody, 22B1) and a primary antibody that recognizes only the form of CaMKII that is not phosphorylated at this site (an affinity-purified rabbit polyclonal antibody). Then the sections are incubated with a mixture of fluorescently-labeled secondary antibodies, fluorescein-Goat anti-mouse IgG and Cy3-Goat anti-rabbit IgG. The secondary antibodies bind to their respective primary antibodies and label the location of the two forms of CaMKII. The two fluorophores can be detected separately by the microscope with the use of different sets of filters. To ensure reliability of the visualization method, I have determined the optimum concentrations of primary antibodies to obtain the brightest staining for each form of kinase and the best signal to noise ratio in the images. The results indicate a linear increase in image brightness for increases in 22B1 concentration between 0.2 mg/ml (1/70 dilution of ascites fluid) and 0.5 mg/ml (1/30 dilution of ascites fluid). The optimum concentrations of affinity purified rabbit serum against nonphospho CaMKII are similar.

328. Linearity of visualization of phospho- and nonphospho-CaMKII in hippocampal slices
Mary Elizabeth Mosier
The Kennedy Lab has developed an immunohistochemical method for imaging the relative distribution of the active (autophosphorylated) and inactive (nonphosphorylated) forms of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) in sections of rat hippocampus (see Abstracts 325 and 326). To establish the quantitative validity of this method, I have examined the relationship between the user-defined contrast setting on the laser-scanning confocal microscope and the brightness of the fluorescent images obtained after staining tissue as described in abstract 6. The confocal microscope records images by detecting the light that passes through a small pinhole as the laser scans the specimen to activate fluorescence at each point in the specimen. The photodiode that detects the light is linear in its response over a broad range, but the image is laid down by the computer in eight bit mode. That is, the image brightnesses are binned into 256 brightness values and recorded as brightnesses from 0 to 255. The upper and lower limits of the computer sampling and the width of each bin are determined by user-defined settings (the "brightness" and "contrast," respectively). The setting that can most critically effect the linearity of the data is the contrast setting. Several images were taken of a given optical section, each at a fixed brightness setting and different contrast settings on the microscope. (The images were taken at low magnification so that there was no bleaching of the fluorophores.) Average brightness values of the images were obtained with the MacPhase program and plotted against the contrast setting at which the image was taken. I found that the increase in brightness was linear over a very broad range of contrast settings, verifying the validity of the assumption of a linear change in brightness with changes in the contrast setting.

329. A phospho-specific antiserum against NR2B phosphorylated at Serine-1303
Alan Rosenstein, Mary Kennedy, R.V. Omkumar
We are interested in identifying postsynaptic proteins that are regulated by CaM kinase II, which is highly concentrated in the glutamatergic postsynaptic density. We have shown that the 2B subunit of the NMDA receptor is a target for CaM kinase II in the postsynaptic density, and have identified the site of phosphorylation as serine 1303 (Omkumar et al., 1996). To establish that serine 1303 is phosphorylated in vivo, and to study the regulation of its phosphorylation in intact tissue, we have raised a rabbit antiserum that specifically recognizes NR2B only when it is phosphorylated on serine 1303. A synthetic 12-mer peptide with the sequence surrounding serine-1303 was obtained from the Peptide Sequencing Facility at Caltech. Ten mgs of the peptide was phosphorylated enzymatically with CaMKII, purified by HPLC, and conjugated to keyhole limpet hemocyanin (KLH). Rabbit antisera were raised and tested on immunoblots against 0.2 µg phosphorylated and 0.2 µg nonphosphorylated fusion protein containing serine 1303. The serum of one rabbit showed good specificity for phospho-GST-tNR2B. To neutralize antibodies against nonphosphorylated GST-tNR2B, the serum was preabsorbed with 6 mole equivalents of the nonphosphorylated antigen peptide at 4°C overnight before dilution for use on immunoblots. Nonphosphorylated and phosphorylated rat brain homogenates were prepared by incubation for 1 hour at 30°C to permit dephosphorylation of proteins by endogenous phosphatases. One ml was then removed and prepared for SDS-PAGE. The rest of the dephosphorylated homogenate was added to an equal volume of prewarmed phosphorylation mixture containing 1 µM okadaic acid to inhibit protein phosphatases and incubated for 5 minutes at 30°C,
then prepared for SDS-PAGE. The rabbit serum preabsorbed with the antigen peptide was tested on Western blots of phospho- and non-phospho homogenate to determine its specificity for intact phosphorylated NR2B. The serum bound to a band at 180 kDal at the position of NR2B in the phospho-homogenate, but not in the dephospho-homogenate, confirming its specificity for intact phosphorylated NR2B.

Reference

330. Phosphorylation of NR2B by CaM kinase II regulated in vivo in hippocampal slices
Mary B. Kennedy, Alan L. Rosenstein, R. V. Ornkurnar

We have found that serine 1303 on the NMDA receptor subunit NR2B is a good substrate for CaMKII in brain homogenates and in the PSD fraction. We have previously shown that in acutely prepared slices of rat hippocampus, the basal level of active, autophosphorylated CaM kinase II can be manipulated by incubating the slices in buffer containing EGTA, which lowers the level of active kinase from an average of 9% to about 4%; or in okadaic acid, which raises the level of active kinase to about 27%. To determine if serine 1303 is phosphorylated in intact hippocampal neurons under any of these conditions, hippocampal slices (500 µm thick) were prepared and placed on filter paper soaked with Ringer buffer in an oxygenated environment. After one hour, groups of twelve slices were immersed in oxygenated Ringer buffer, or in the same buffer containing either 5 µM okadaic acid, or 0 CaCl₂/0.25 mM EGTA, for an additional hour at room temperature in an oxygenated atmosphere. The slices were then immediately transferred to a teflon/glass homogenizer containing 600 µl SDS-PAGE sample buffer plus 2 units DNase and homogenized. Each homogenate was boiled for 5 minutes and applied to SDS-PAGE gels (25 µg of homogenate protein per lane). Fractionated proteins were transferred to nitrocellulose and processed for immunoblots with anti-phosphosite-specific rabbit serum preabsorbed with nonphospho-antigen as described in abstract 8. Bands reacting with the antibody were visualized with NBT/BCIP phosphatase color reagent after incubation with alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies. We clearly detected the presence of the phosphorylated site in a protein band at the position of NR2B in homogenates of slices incubated under each condition. The band detected by the antisera was substantially darker in homogenates of slices incubated in okadaic acid, consistent with phosphorylation by CaM kinase II which is more highly active in slices under that condition.

Reference

331. Determination of phosphorylation sites for CaM kinase II in densin-180
Frank Asuncion

Densin-180 is an adhesion molecule-like protein that is localized at synapses and enriched in the postsynaptic density fraction (Apperson et al., 1996). It has a pattern of sequence-homology domains similar to that found in the family of leucine-rich repeat containing glycoproteins. In addition, it contains a PDZ domain at its carboxyl terminus which we presume to be located in the cytosol. Densin-180 is phosphorylated by CaM kinase II in the postsynaptic density fraction (Apperson et al., 1996). To study the role of this phosphorylation in regulating densin-180, we decided to identify the site or sites of phosphorylation by CaM kinase II. Because the amount of densin-180 present in the postsynaptic density fraction is not sufficient for practical determination of the sequence surrounding the site(s) of phosphorylation, I have used recombinant fusion proteins containing portions of the C-terminal domain of densin-180 fused to the C-terminus of glutathione-S-transferase. I have shown that CaM kinase II phosphorylates these fusion proteins at two sites, one within the PDZ domain and the other outside the PDZ domain. The site within the PDZ domain is phosphorylated at a rapid rate with a K₉ₐ of approximately 1 µM. I am presently generating large quantities of the phosphorylated fusion proteins for sequencing of the phosphorylation sites.

Reference

Publications


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Summary: The Lester-Davidson group continues its interest in molecular aspects of neuronal function.

(a) Knowledge about the structure-function relations among ion channels has reached the point where channels can be constructed with varying and, in some cases, predictable departures from normal activity. In particular, we have designed ligand-gated channels, such as nicotinic and serotoninergic receptors, that are constitutively active or hyperexcitable. Potassium channels too can be designed along the lines of the weaver mutation to act as nonspecific cation channels.

(b) At the same time, mouse genetics has reached the point where "knock-in" point mutants can be constructed. These mice substitute the mutant channels for the native channels in the very cells that normally express these channels—and only in these cells.

(c) Furthermore, problems in synaptic plasticity are being studied by methods depending on recombinant adenovirus-mediated gene transfer.

We are therefore constructing knock-in mice that express constitutively active cation channels, on the assumption that such mice will express robust phenotypes and that these phenotypes will enlighten us about the natural expression, functional oligomeric state, and distribution of the channels in question. We also suspect that these mice will become models for several types of neurological (and perhaps psychiatric) diseases. We have arranged collaborations to study behavior of these mice.

We continue our collaboration with the Dougherty group on basic aspects of ion channel structure-function, studied with unnatural amino-acid incorporation. We have concentrated on residues expected to be generally useful for investigations on membrane proteins. Over the past year, we have learned how to incorporate photosensitive residues. One such residue can be activated to cleave the main chain; another can be "uncaged" to reveal a fully functional native tyrosine side chain. Another generally useful residue contains tyrosine. Although the experiments now take place in frog eggs, we would like to extend them to somatic cells.

One class of potassium channels, the GIRKs, are effectors for G proteins. The proteins known as "Regulators of G protein Signalling" play important roles in shaping the kinetics of these responses. The channels also appear to interact with other cells signalling molecules.
Neurotransmitter transporters, another important class of neuronal molecule, are beginning to yield their mysteries. Our experiments on transporters combine flux, electrophysiology, and site-directed mutagenesis. Recombinant adenovirus is an efficient vector for introducing genes into post-mitotic neurons with a high per-cell efficiency and fairly high level of expression. Using these methods, in collaboration with Professor Erin Schuman’s group (and as described in her section of the Annual Report - see Kantor et al., 1996 reference), we have demonstrated that endothelial nitric oxide synthase plays the principal role for the generation of NO, in long term potentiation.

In addition, adenovirus-mediated gene transfer has been used to study the role of G protein-gated inward rectifiers in down-modulating neuronal excitability.

Other collaborations with the Schuman group involve studies of the effects of the neurotrophin, BDNF, interacting with its receptor (Trk B) on synaptic efficacy.

Jerome Pine Subgroup. The subgroup led by Pine continued work using advanced technologies to study neural systems. The neuron probe is a new mode of communication with the CNS; the multielectrode array will be used to study learning in cultured networks; and the neurochip will be used for detailed studies of synaptic plasticity in small cultured networks. In addition, for use with voltage sensitive dyes, a new high speed CCD camera has been developed. The two-photon laser scanning microscope built by Potter in the Imaging Center will also be used to study morphological changes of stained neurons.

332. Examining the physiological role of ion channels: Generation of a “knock-in” mouse with point mutations in neuronal nicotinic acetylcholine receptors

Mark W. Nowak, Melihat Fidan-Nowak, Purnima Deshpande, Cesar Labarca, Hong Dang, Paulo Kofuji

In an effort to understand the physiological role of the neuronal nicotinic acetylcholine receptors (nAChRs) we will generate “knock-in” mice containing well-characterized point mutations in the various nAChRs. We have chosen as our first target the α4 subunit. Frontal lobe autosomal dominant nocturnal epilepsy has, in part, been shown to result from a point mutation in the α4 subunit such that the receptor becomes less active. In addition, the α4 subunit in association with the β2 subunit forms the high affinity nicotinic binding site in the brain, and most likely is involved in nicotine addiction.

The type of point mutation that we have generated will render the neuron expressing the mutant α4 subunit “hyperexcitable.” That is, the mutant receptor has an increased open time upon activation leading to prolonged depolarization of the neuron. Mutations of this type are found in the muscle nACHR in the degenerative disease Slow Myasthenia Channel Syndrome. In this disease mutations are primarily found in the M2 transmembrane domain which forms the ion channel pore. We have extensively examined the effects of ion channel pore mutations in the mouse muscle nACHR in the Xenopus oocyte expression system.

Using the information from our studies on the mouse nACHR we have generated the following mutants in the ion channel pore of the rat α4 subunit: Leu9'Ser, Leu9'Ala, Thr12'Pro, Val13'Met, Leu17'Ser, and Leu17'Ala. Oocytes were coinjected with the mutant α4 subunit along the β2 subunit, and the effects of the mutations examined. Mutant α4:β2 receptors displayed increases in acetylcholine (ACh) sensitivity 3-100-fold.

Summary: AChR α4:β2 mutants

We focused on the Leu9'Ala and Leu9'Ser mutations which increased ACh sensitivity 10- and 100-fold, respectively. From our studies on the mouse nACHR we know that the increased ACh sensitivity results, in part, from an increase in the open time of the receptor. Voltage-jump relaxation measurements for these two mutant receptors indeed showed pronounced increases in the relaxation time constants. In addition, these mutations increased the oocyte leak current which was blocked by open-channel blockers. This observation is consistent with spontaneous receptor activity. Based on these findings the α4 Leu9'Ala and Leu9'Ser mutations should render a neuron “hyperexcitable.”

The M2 transmembrane domain is located in exon 5 of mouse α4. Following the sequencing of a genomic clone containing exon 5 the Leu9'Ala and Leu9'Ser mutations were made. We are currently inserting into this construct a cassette containing a neomycin.
resistance gene and the Herpes thymidine kinase gene for selection purposes.

333. Hyper-active 5HT3 receptor knock-in mice
Hong Dang, Cesar Labarca, Purnima Deshpande, Nicolas Guy1, David Julius1

To study how ion channels function in the brains of intact animals, we are introducing a hyper-active 5HT3 channel into the native genetic environment of mice by gene-targeting (a "knock-in" strategy, in contrast to a "knock-out" strategy where a native gene is inactivated). The choice of this particular ligand-gated ion channel was based on (1) the likelihood that this receptor is encoded by a single gene (there is no genetic evidence for other subunits, thus little chance for compensation from other subunits) and (2) the low calcium selectivity in vitro of the 5HT3 receptor, which indicates that it is unlikely to be cytotoxic. Heterologous expression in Xenopus oocytes was used to examine shifts of the receptor’s sensitivity to serotonin. Two point mutations (a valine to either serine or alanine) in the M2 channel-lining domain were found to decrease the EC₅₀ values by both 100- and 60-fold.

The same mutations were made in a piece of genomic DNA containing the exon coding for the M2 region. A Neo-TK (neomycin resistant gene-thymidine kinase gene) cassette flanked by two loxP sequences was inserted in the nearby intron to provide both positive (Neo) and negative (TK) selection markers. This DNA will then be introduced into mouse embryonic stem (ES) cells. One copy of the wild-type exon replaced by the mutated DNA through homologous recombination, will be selected by G418 resistance and screened by PCR and Southern blotting. The removal of the selection cassette from the genomic DNA will be achieved through negative selection (FIAU) against the TK gene after transient transfection with the Cre gene, whose product, CRE recombinase, deletes DNA sequences between two similarly oriented loxP units, leaving a footprint of 34 bps in the intron.

Clones of ES cells containing the intended mutations will be used to generate mice carrying the above mutations. These animals may then be analyzed to identify specific alterations associated with a mutation.

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334. Couplings between distant regions in the nicotinic acetylcholine receptor: Acidic residues near the agonist binding site and hydrophobic residues in the pore
Wenge Zhong1, Haiyun Zhang2, Dennis A. Dougherty1

Conventional and nonsense suppression mutagenesis methods and double mutant cycles were employed to study functional interactions within the muscle nicotinic acetylcholine receptor expressed in Xenopus oocytes. We measured macroscopic dose-response relations for receptors with natural and unnatural mutations at tyrosine 93 in the α subunit (αY93) and/or D (aspartic acid) to N (asparagine) mutations at 174 in the γ and 180 in the δ subunits. Comparison among the EC₅₀ values for these receptors leads to the conclusion that there is no hydrogen bond between αY93 and γD174 or δD180. However, these D-to-N mutations decrease the receptor's sensitivity to acetylcholine (ACh). Single-channel analysis indicates that the D-to-N mutations affect gating of the channel primarily by a decrease in the opening rate, in addition to a moderate increase in the channel closing rate. The D-to-N mutations were also studied in combination with mutations from wild-type leucine to serine at the 9' position of the M2 pore-lining region. The opposing effects on EC₅₀ values are not all multiplicative, suggesting remote energetic couplings between the acidic residues and the 9' leucine residues, particularly in the β subunit. These acidic residues participate in the intramolecular transduction pathway that couples agonist binding to channel gating.

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335. Leucine mutations at the 9' position of the acetylcholine receptor M2 domain: Kinetic bases of the changes in channel gating
Haiyun Zhang1, Purnima Deshpande, Cesar Labarca

In nicotinic acetylcholine receptors (nAChRs), a highly conserved leucine residue occurs in the pore-lining M2 region at the 9' position and is thought to comprise at least part of the gating mechanism. Previous results show that mutation of this residue to serine produces a leftward shift of ~ tenfold in the ACh dose-response relation for each subunit mutated. We expressed nAChRs with varying numbers (m*) of Leu9'Ser mutant subunits in Xenopus oocytes and studied both macroscopic voltage-jump relaxations and single-channel properties in outside-out patches. Mutated AChRs have (1) slower voltage-jump relaxations (~ 200-fold greater time constant than wild type), (2) longer ACh-induced openings and bursts, (3) briefer closed times, and (4) more frequent spontaneous openings. These effects increase with m*. For example, average channel open times for wild-type α βγδ (m* = 4) receptors are 1.8 ± 0.1 ms and 28 ± 3 ms, respectively. The spontaneous openings, however, have an average open time of 0.3 ± 0.1 ms, which remains constant for all m*.' In addition, the spontaneous opening frequency increases ~ 10⁴-fold from α βγδ (m* = 1) to α βγδ (m* = 4). In order to study the consequence of the Leu9'Ser mutations for synaptic function, synthesized postsynaptic currents were produced with brief ACh pulses delivered to outside-out patches. The currents decay as expected from channel burst duration. For instance, the α βγδ (m* = 4) receptor displays a time constant of 1340 ± 200 ms (~ 600-fold greater than for the wild-type receptor). Thus, (1) both longer and more frequent openings contribute to the ~ 10⁴-fold shift in EC₅₀ values.
between the wild-type receptor and the receptor with
m9 = 4, and (2) the highly conserved 9' leucine is
crucial for appropriately brief synaptic events.
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336. The role of hydrophobic residues in the M2
domain of the AChR in channel gating
Cesar G. Labarca, Haiyun Zhang, Purnima
Deshpande
The M2 transmembrane domain of the AChR has
numerous hydrophobic residues, mainly Leu and Val.
We previously showed that mutation of Leu 9' to Ser
altered the gating of the channel. The presence of each
mutated subunit decreased the EC 50 value by roughly a
factor of 10, and the effect was nearly independent of
the subunit mutated. The frequency of spontaneous
openings increased with the number of mutated
subunits. We have now examined the effect of mutating
hydrophobic residues to Ser in M2 at positions other
than 9'. In receptors with a subunit mutations at
positions 7, 11', 13', 15', 16', and 17', the dose response
relation for ACh shifts to the left; the largest shift
occurs at position 13', with an EC 50 value over three
orders of magnitude lower than that for the wild type.
In a helical wheel representation, most of these
residues cluster on two opposing faces and orthogonal
to the polar 6' and 10' residues that face the open
lumen, as though these hydrophobic positions face
the flanking M2s of neighboring subunits. In contrast,
receptors with 8' mutations show a rightward dose-
response shift; these residues would face away from
the open pore. Thus, gating is influenced by multiple
hydrophobic interactions (a) between the M2
transmembrane helices surrounding the ion pore and
(b) between the M2 helices and the distal surrounding
domains of the receptor. We propose a model for AChR
gating.

337. Site-specific, nitrobenzyl-induced, photochemical
proteolysis applied to ion channels in vivo
Pamela M. England, Dennis A. Dougherty
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A method for site-specific, nitrobenzyl-induced
photochemical proteolysis (SNIPP) of diverse proteins
expressed in living cells has been developed based on
the chemistry of the unnatural amino acid (2-
nitrophenyl)glycine (Npg). Using the in vivo
nonsense codon suppression method for incorporating
unnatural amino acids into proteins expressed in
Xenopus oocytes, Npg has been incorporated into
two ion channels: the Drosophila Shaker B (ShB)
K' channel, and the nicotinic acetylcholine receptor
(nAChR). Functional studies in vivo show that
photolysis of a protein containing an Npg residue
does lead to peptide backbone cleavage at the site of
the novel residue. Using this method, evidence is
obtained for an essential functional role of the
"signature" Cys128-Cys142 disulfide loop of the
nAChR α subunit.
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338. Unnatural amino acid mutagenesis: incorporation
of a caged tyrosine in the α subunit of the mouse
nicotinic acetylcholine receptor
Jeffrey C. Miller, Scott K. Silverman
The ability to incorporate unnatural amino acids
in proteins expressed in Xenopus oocytes provides a
unique opportunity to specifically place sidechains
with novel behaviors within proteins in living cells.
We have placed a photolabile nitrobenzyl tyrosine
derivative, tyr(ONB), in the mouse nicotinic
acetylcholine receptor α subunit. Wild-type tyrosine
was replaced with this residue at α subunit positions
93, 127, 190, and 198. With the aid of modified
electrophysiologial recording equipment, channel
currents from oocytes expressing the mutated receptors
were recorded in real time as the tyrosines were
uncaged by 1 ms pulses from a 250 J per pulse flash lamp.
Before the tyr(ONB) is uncaged the oocytes show only
small responses to acetylcholine; they show much
larger responses after light-induced uncaging of
tyr(ONB). When flashes are delivered to the oocytes
in the presence of acetylcholine, the time constant for
the current increases may be measured. The time
constant varies both with the site at which the caged
tyrosine was incorporated and with the pH of the
recording solution. Time constants ranging from 20 ms to
several seconds have been observed. The underlying
explanation for this variation is under investigation.
The possible uses of the tyr(ONB) residue and
similar residues such as caged serine and caged
phosphoserine include probing the time course of
signaling events in neuroreceptors with millisecond
time resolution. In favorable cases, caged sidechains
might be used to probe the pKa of residues within some
membrane proteins.
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339. Unnatural amino acids for in vivo site-specific
labeling of membrane proteins
Justin P. Gallivan, Dennis A. Dougherty
A key structural issue for all membrane proteins is
the transmembrane topology. Defining which regions
of a protein are intracellular, extracellular or
embedded in the membrane is not only essential for
determining how a protein folds, but also how it
functions. There are relatively few methods to
determine the transmembrane topology of functional
proteins expressed in living cells, and most involve the
introduction of major structural perturbations. To
address this issue, we have developed a new
experimental method to determine the surface
accessibility, and ultimately the transmembrane
topology, of membrane proteins expressed in Xenopus
oocytes.

We have used the in vivo nonsense suppression
technique to incorporate biotinylated unnatural amino
acids into functional ion channels expressed in
Xenopus oocytes. Binding of 125I-streptavidin to biotinylated
receptors was used to determine the surface
accessibility and thus the topology of individual amino
acids. We have incorporated biotinylated amino acids into sites in the main immunogenic region (MIR) of the nicotinic acetylcholine receptor and assayed their accessibility to streptavidin. Biocytin is efficiently incorporated into five sites in the MIR, and extracellular streptavidin binds to one residue in particular, α70, thus establishing its position on the receptor surface.

We believe that the incorporation of biotinylated amino acids represents a potentially general method for determining the surface accessibility and topology of membrane proteins. In work over the next year, we hope to use the method to experimentally determine the transmembrane topology of other ion channels and neurotransmitter transporters.

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340. Role of conserved proline in transmembrane domain M1 of ligand-gated ion channels
Hong Dang, Justin Gallivan

A conserved proline residue is found in the first transmembrane domain of every subunit of the ligand-gated ion channel super-family. The location of this conserved proline residue, between the extracellular ligand-binding domain and the main channel-lining domain (M2) in the primary sequence, hints at its involvement in the transduction of ligand-binding signals to the gating of the ion channel. Preliminary study--by conventional mutagenesis of the α7 homomeric nicotinic receptor expressed in Xenopus oocytes--indicates that replacing the conserved proline residue with either glycine, threonine, or phenylalanine results in a reduced number of surface antagonist (α-bungarotoxin) binding sites and a complete inactivation of nicotine currents. Proline is the only imino acid naturally incorporated de novo into proteins. Its α-nitrogen is part of a pyrrolidine ring, which places unique constraints on the polypeptide backbone around it. The unique nature of proline and the degree of conservation of this residue in ligand-gated ion channels suggest that replacement with any of the other 19 amino acids by conventional mutagenesis would represent too drastic a change from the native conformations that would render the receptor inactive.

The stop codon suppression method is well-suited for introducing subtle changes into this conserved proline residue. This approach allows the incorporation of synthetic amino acid analogs at a specific site on a given protein. The main problem we encountered in using the stop codon suppression approach was that the injected aminoacyl-tRNA was inefficient in suppressing the normal termination process at a particular site. To evaluate the feasibility of such an approach for the homomeric receptors expressed from oocytes, a stop codon (TAG) (replacing the one for M1 proline) was introduced into cDNA clones of the α7 nicotinic and the 5HT3 receptors. Co-injection of the TAGed mRNA and prolyl-tRNA into oocytes produced no detectable nicotinic current for the α7 receptor, but yielded a whole cell current of about 200-400 nA for the 5HT3 receptor at a saturating serotonin concentration. By introducing proline analogs and characterizing the resultant receptor physiology in oocytes, the role of this conserved residue in the function of the ligand-gated receptors may be elucidated.

341. Studying modulation of the voltage-gated potassium channel Kv1.3 with unnatural amino acid probes
Yanhe Tong

Potassium channels play a significant role in setting the membrane potential as well as in setting the frequency and duration of action potentials in living cells. Voltage-gated potassium channels are a subfamily of potassium channels which open following membrane depolarization. Recent work has demonstrated that the voltage-gated potassium channel Kvl.3 can be phosphorylated at multiple sites not only by serine/threonine protein kinases, but also by tyrosine kinases. Using the newly developed nonsense codon suppression method, “caged” tyrosine, serine and threonine can be incorporated into putative phosphorylation sites of the channels expressed in Xenopus oocytes. In short, the specific phosphorylation sites will be protected from kinases by a photolabile protecting group. The phosphorylation of the channel will begin only after photolytic deprotection. The rate of deprotection can be controlled by the light intensity, and it is possible to achieve deprotection in ~2 s. Therefore, modulation of the channel kinetics can be measured during the phosphorylation process. Coexpression of Kvl.3 containing the caged side chain, together with various protein kinases or lipases or activating endogenous kinases in oocytes expressing unnatural amino acid-incorporated Kvl.3, will provide information about the phosphorylation profile of the channel. This study not only will help us to understand the mechanisms of channel regulation by neurotransmitters, hormones, and the second messenger system, but also will establish a new method for studying phosphorylation mechanisms of ion channels, receptors, and other cytosolic proteins. The system generated in this study will also serve as a set of valuable tools for studying various signaling pathways.

342. Expression and circular dichroism studies of the extracellular domain of the α subunit of the nicotinic acetylcholine receptor
Anthony P. West, Jr.1, Pamela J. Bjorkman, Dennis A. Dougherty

To provide material suitable for structural studies of the nicotinic acetylcholine receptor, we have expressed and purified the N-terminal extracellular domain of the mouse muscle α subunit. Several constructs were initially investigated using Xenopus oocytes as a convenient small-scale expression system. A fusion protein (α210GPI) consisting of the
210 N-terminal amino acids of the α subunit and a glycosyl-phosphatidylinositol anchorage sequence conferred surface α-bungarotoxin binding in oocytes. Coexpression of α210GPI with an analogous construct made from the δ subunit showed no evidence of heterodimer formation. The α210GPI protein was chosen for large scale expression in transfected Chinese hamster ovary cells. The α210GPI protein was cleaved from these cells and purified on an immunoaffinity column. Gel and column chromatography show that the purified protein is processed as expected and exists as a monomer. The purified protein also retains the two distinct, conformation-specific binding sites expected for the correctly folded α subunit. Circular dichroism studies of α210GPI suggest that this region of the receptor includes considerable β-sheet secondary structure, with a small proportion of α-helix.

343. Deactivation kinetics of GIRK currents are accelerated by RGS proteins

P. Kofuji, C. A. Douplnik

One well-described aspect of native G protein-activated inwardly rectifying K⁺ channels (GIRK) is their fast activation and deactivation kinetics with step applications of agonists for G protein-coupled seven helix receptors. In atrial cells, acetylcholine (ACh)-evoked GIRK currents deactivate in < 1 s with rapid removal of ACh, presumably due to the rapid disassociation or removal of Gβγ dimers from the channel. The off rate of the muscarinic response is much faster than the intrinsic GTP hydrolysis rate of Gαi or Gαo subunits measured in vitro (2-5/min), suggesting GIRK channels may possess an intrinsic GTPase activating protein (GAP) activity similar to other G protein effector molecules. We investigated the molecular determinants of GIRK deactivation by coexpression of various components of this signalling pathway in oocytes and CHO cells. In CHO cells transfected with plasmids encoding GIRK1, GIRK2, and the m2 type muscarinic receptor, application of 1 µM ACh induced GIRK currents that activated rapidly (τ_{act} ~ 500 ms), but deactivated slowly (τ_{dec} = 5-13 s) compared to atrial cells (though this rate is similar to the GTPase rate of Ga subunits). The ACh-evoked currents were abolished by pertussis toxin pretreatment, indicating the involvement of Gi or Go proteins. Similar slow deactivation of GIRK currents is seen in oocytes. We investigated the effect of the newly described class of proteins, “regulators of G protein signalling” (RGS), which accelerate the in vitro GTPase activity of Gαi and Gαo proteins via a direct interaction with the Ga proteins (Berman et al, Cell 86: 445, 1996). Coexpression of the RGS4 isoform with GIRKs and the m2R in CHO cells, led to expression of GIRK currents that now deactivated with a time constant (τ_{dec} = 0.4 to 1.5 s) comparable to that measured for atrial cells (τ_{dec} = 0.5 to 1 s). We conclude that GIRK channels do not act as GAPs as previously suggested; instead, RGS proteins exert this role in vivo.

344. A C-terminal peptide of the GIRK1 subunit directly blocks the G protein-activated K⁺ channel (GIRK) expressed in Xenopus oocytes

Tudor Luchian, Tudor Luchian, Nathan Dascal, Carmen Dessauer, Dieter Platter, Alfred Gilman, Wolfgang Schreibmayer

The cytosolic C-terminal region of the G protein-activated, inwardly rectifying potassium channel subunit GIRK1 has been proposed to form a blocking gate that may keep the channel closed in the absence of activation by the GTPγS subunits. As an initial test of this hypothesis, we expressed GIRK channels (of GIRK1/GIRK5 and GIRK1/GIRK4 subunit composition) in Xenopus laevis oocytes, and we studied effects of two synthetic peptides in isolated inside-out membrane patches. The GIRK channel was activated by bath application of a non-hydrolysable analogue of GTP, GTPγS, or of the purified recombinant Gα12 subunits. A peptide (DS6) derived from a sequence very near the end of the C terminus of GIRK1 reversibly blocked GIRK activity by 83.4% ± 3.1% (n=10) at 100 µg/ml. IC₁₀ of the DS6 block was 7.9 ± 2.0 or 3.5 ± 0.5 µg/ml, and the Hill coefficient was 1.2 ± 0.3 or 0.75 ± 0.08, for GIRK channels of GIRK1/GIRK5 or GIRK1/GIRK4 composition, respectively. Application of a control peptide derived from the cytoplasmic N-terminal part of GIRK1 (DS4) at a concentration of 100 µg/ml was without effect. Dose dependency of GIRK activation by purified Gα12 showed that DS6 significantly reduced the maximal achievable open probability but did not significantly alter the EC₅₀ value for activation by Gα12. Thus, a DS6 block of GIRK channels does not result from competition between the peptide and functional GIRK channels for the available Gα12. Single-channel analysis of the DS6 block revealed that burst duration was reduced whereas long closed times between bursts were markedly increased, accounting for the observed inhibition, while other gating parameters remained virtually unchanged. Analysis of voltage dependence of a GIRK block by the DS6 peptide revealed that inhibition depends slightly on voltage, increasing at more negative potentials. Our data support the hypothesis that the distal part of the carboxy-terminus of GIRK1 is a part of the intrinsic gate that keeps GIRK channels closed in the absence of Gα12.

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345. A regenerative link in the ionic fluxes through the weaver GIRK2 and GIRK4 channels underlies the pathophysiology of the mutation

Scott K. Silverman, Paulo Kofuji, Dennis A. Dougherty

The homozygous weaver mouse displays neuronal degeneration in several brain regions. Previous experiments in heterologous expression systems showed...
that the GIRK2 (G protein-gated inward rectifier K⁺) channel bearing the weaver pore-region GYG to SYG mutation (1) is not activated by G₂ₐ subunits, but instead shows constitutive activation; and (2) is no longer a K⁺-selective channel, but conducts Na⁺ as well. The present experiments on weaverGIRK2 (wvGIRK2) expressed in Xenopus oocytes show that the level of constitutive activation depends on intracellular Na⁺ concentration; in particular, manipulations that decrease intracellular Na⁺ produce a component of Na⁺-permeable current activated via a G protein pathway. Therefore, constitutive activation may not arise because the weaver mutation directly alters the gating transitions of the channel protein; instead, there may be a regenerative cycle of Na⁺ influx through the wvGIRK2 channel, leading to additional Na⁺ activation. We also show that the wvGIRK2 channel is permeable to Ca²⁺, providing an additional mechanism for the degeneration that characterizes the weaver phenotype. We also demonstrate that the GIRK4 channel bearing the analogous weaver mutation has properties similar to those of the wvGIRK2 channel, providing a glimpse of the selective pressures that have maintained the GYG sequence in nearly all known K⁺ channels.

1Division of Chemistry and Chemical Engineering

346. Integrin interactions with GIRK channels
Jancy C. McPhee, Yan Dang

A three amino acid motif, arginine-glycine-aspartate (RGD), just extracellular to the first transmembrane domain, is well-conserved on all known G protein-gated inward rectifier channel (GIRK) subunits. This sequence is the same as the ligand frequently recognized by another family of transmembrane proteins called integrins. Integrins often recognize an RGD sequence found on extracellular matrix molecules; accordingly, they have been associated with functions such as cell-substrate adhesion or cell-cell adhesion. Interestingly, they have also been implicated in neuronal excitability in the hippocampus, neurite outgrowth, and stretch-induced neurotransmitter release. It is conceivable, therefore, that integrins may regulate ion channel activity. Because of the presence of the classic integrin ligand recognition site, RGD, on all GIRK subunits, we decided to examine whether GIRK activity, localization, or expression is affected directly by interaction with integrin.

For our first approach, we injected RNA into Xenopus laevis oocytes to express the muscarinic acetylcholine receptor and a combination of GIRK1 and GIRK4 subunits. We compared the expression of WT and mutant subunits with the extracellular GIRK RGD site mutated to RGE. This type of mutation should disrupt GIRK channel-integrin interactions, if they exist. The GIRK1-RGE/GIRK4-RGE double mutant complex produced no detectable current. The GIRK1-RGE/GIRK4 complex consistently expressed at a level 3-4 times lower than that produced from the same amount of WT GIRK1/GIRK4 RNA. The GIRK1/GIRK4-RGE complex expressed at about 10-fold lower levels than the double WT GIRK subunit complex. Voltage-jump relaxation steps clarify that, in the one mutant/one WT subunit combinations described above, the mutant subunits were actually being expressed and that observed current was not merely due to the expression of the WT subunit. We were intrigued that a direct interaction of GIRK and integrin may occur and that this interaction might regulate the amount or timing of GIRK expression or localization in oocytes. Comparison of the total protein level of the WT and mutant channel constructs using Western blot analysis revealed that the mutant constructs were expressed at similar levels to the WT. Preliminary experiments isolating plasma membrane fractions from oocytes expressing these channels suggested that the mutant channels were located in the plasma membrane to the same extent as WT. Therefore, the mutation of the RGD sight seems to decrease GIRK current without eliminating channel protein synthesis or localization to the plasma membrane in oocytes. To directly show that the channel interacts with integrin, we are currently performing co-immunoprecipitation experiments to show that the expressed WT GIRK channel and the endogenous Xenopus integrin form a complex.

In the near future, we plan to shift our analysis to native cells that contain GIRK channels, such as atrial cells. Co-immunoprecipitation experiments can be performed with these cells, and whether application of an RGD-containing peptide can compete for integrin-GIRK channel interactions and alter GIRK activity will also be explored. Expressing WT and mutant channels in mammalian cells by transfection of CHO cells is also in our future plans. Using this system, we hope to get a more detailed analysis of GIRK function and localization than we could using oocytes and, hopefully, elucidate the role of integrin in the regulation of GIRK activity.

347. Cyclic nucleotide-gated channels: Structural basis of ligand efficacy
Jun Li, William N. Zagotta

Most proteins exhibit allosteric coupling, which can be defined as indirect interactions between physically remote sites. Hemoglobin and nicotinic acetylcholine receptors are the exemplar proteins traditionally used in the study of allosteric mechanisms. Recently, however, the cyclic nucleotide-gated (CNG) channels have emerged as another excellent model system. This is because CNG channels do not desensitize and the atomic structures of some of their homologous proteins, such as E.coli CAP, are readily available. In a re-examination of structure-function studies concerning binding and gating in CNG channels, we draw attention to some mechanistic implications not yet seriously considered before.

We showed that each of the three parameters in the Monod-Wyman-Changeux (MWC) model, K, f,
and L₀ specifies one of the three basic processes that comprise the ubiquitous, ligand-mediated conformational changes in proteins: the initial binding (K), the spontaneous conformational changes (L₀), and the amount of free energy transferred from additional binding to the stabilization of conformational changes (f). f thus represents ligand efficacy. It is a measure, on the one hand, of the increase in binding strength due to the conformational changes and, on the other hand, of how much of the free energy released by the additional binding contributes to the conformational changes. We then reviewed studies which have revealed structural motifs responsible for these three functional aspects. Finally, we proposed that most of the documented functional modifications can be classified into the K types, f types, or L₀ types.

Using CNG channels as an example, we applied this classification scheme to natural and recombinant mutations, as well as to many modes of channel modulation.

The MWC model is a basic model for CNG channels: it captures key features of allosteric transitions. In applying this model, however, one almost always has to elaborate on it in order to accommodate additional properties, much in the same way that the ideal gas laws must be modified to better characterize real-life gaseous matters.

The MWC model is a basic model for CNG channels: it captures key features of allosteric transitions. In applying this model, however, one almost always has to elaborate on it in order to accommodate additional properties, much in the same way that the ideal gas laws must be modified to better characterize real-life gaseous matters.  

4. Single-channel analysis of the activation mechanism of CNG channels

Jun Li

According to one hypothesized mechanism for the activation of cyclic nucleotide-gated (CNG) channels, based on the Monod-Wyman-Changeux model, in the absence of ligand there is a small but finite opening probability (L₀); each additional binding stabilizes the open state by an additional factor (f), such that a fully liganded channel is favored to open (L₀f₀+1). This simple model assumes complete symmetry among binding sites. But an actual CNG channel may not display such ideal symmetry: the binding events may have a range of affinities or contribute a range of stabilization factors. We examined the openings of a single, heterologously expressed, homomeric CNG channel under steady-state conditions, with the hope that the statistical properties of the channel's behavior may help to distinguish among these possibilities.

We used the cloned rat olfactory CNG channel expressed in Xenopus oocytes. The single-channel openings show neither subconductance nor severely flickery openings. Most of the openings last between 1 and 500 ms. At higher ligand concentrations longer openings occur more frequently and briefer openings decrease in frequency. These properties simplified the analysis, especially in comparison with other CNG channels which show fast flicker (<1 ms) or more than one conductance level. However, several technical/analytical difficulties did arise: (1) endogenous stretch-activated channels are very similar to the expressed CNG channels and were present in most patches, (2) the openings do not show obvious bursting patterns, which are predicted by some common models and widely observed among ligand-gated channels, and (3) even at a steady level of ligand the opening probabilities often change with time (i.e., 20%-80%), suggesting spontaneous shifts in binding affinity, gating mode, or both. Preliminary analysis indicates that some asymmetries among binding events definitely exist. Thus, the original, three-parameter MWC model must be extended to describe the activation of the rat olfactory CNG channel.

4. Second messengers, trafficking-related proteins, and amino acid residues that contribute to the functional regulation of the rat brain GABA transporter GAT1

Michael W. Quick¹, Janis L. Corey²

Recent evidence indicates that several members of the Na⁺-coupled transporter family are regulated and this regulation in part occurs by redistribution of transporters between intracellular locations and the plasma membrane. We elucidate components of this process for both wild-type and mutant GABA transporters (GAT1) expressed in Xenopus oocytes using a combination of uptake assays, immunoblots, and electrophysiological measurements of membrane capacitance, transport-associated currents, and GAT1-specific charge movements. At low GAT1 expression levels, activators of PKC induce redistribution of GAT1 from intracellular vesicles to the plasma membrane; at higher GAT1 expression levels, activators of PKC fail to induce this redistribution. However, co-injection of total rat brain mRNA with GAT1 permits PKC-mediated modulation at high transporter expression levels. This effect of brain mRNA on modulation is mimicked by co-injection of syntaxin 1a mRNA, and is eliminated by injecting synaptophysin or syntaxin antisense oligonucleotides. Additionally, botulinum toxins, which inactivate proteins involved in vesicle release and recycling, reduce basal GAT1 expression and prevent PKC-induced translocation. Mutant GAT1 proteins, in which most or all of a leucine heptad repeat sequence was removed, display altered basal distribution and lack susceptibility to modulation by PKC, delineating one region of GAT1 necessary for its targeting. Thus, functional regulation of GAT1 in oocytes occurs via components common to transporters and to trafficking in both neural and non-neural cells, and suggests a relationship between factors that control neurotransmitter secretion and the components necessary for neurotransmitter uptake.

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350. Amino acid residues responsible for low-pH potentiation of the transport-associated current in two mammalian serotonin transporters
Y-W. Cao, S. Mager

Mammalian serotonin transporters (SERTs) expressed in Xenopus oocytes display at least two currents uncoupled from substrate flux: the leakage current in the absence of 5-HT and the transport-associated current in the presence of 5-HT. Both currents are due to “channel-like” behaviors in serotonin transporters. We previously reported that acidic pH strongly potentiates the transport-associated current in rat SERT (rSERT) but not in human SERT (hSERT). To understand the mechanism of this low pH potentiation and its possible relevance to channel gating, we performed domain-swap analysis between rSERT and hSERT, followed by site-directed mutagenesis. We found that a single amino acid difference at position 490 (threonine in rSERT and lysine in hSERT, in the extracellular loop between TM9 and TM10) is responsible for the difference in response to low pH. However, since threonine is predominantly protonated already at a neutral pH, we propose that the low-pH potentiation of the transport-associated current is due to the protonation of the two neighboring glutamate residues (at position 493 and 494). The positively charged lysine in hSERT would prevent the protonation of these two glutamate residues and thus abolish the low-pH potentiation in hSERT. Consistent with this model, mutations at these two glutamate residues in rSERT also abolished the low-pH potentiation. These results indicate that one or both of the negatively charged glutamate residues may play an important role in a gating process at the serotonin transporter.

351. Rethinking neurotransmitter transporter topology
N. Yu, Y-W. Cao, S. Mager

Covalent cysteine-modification was used along with site-directed mutagenesis to study the membrane topology of the GABA transporter GAT1. Treatment of Xenopus oocytes expressing GAT1 with either (2-sulfonatoethyl)methane-thiosulfonate (MTSES) or [2-(trimethylammonium)ethyl]methanethio-sulfonate (MTSET) (3.75 mM, 30 min) significantly decreased the GABA transport current (to 22% and 10% of the controls, respectively). Substitution of cysteine at position 74 to alanine (C74A) eliminated the sensitivity to these membrane-impermeant compounds, indicating that C74 is exposed to the extracellular side. Furthermore, wild-type (WT) GAT1 treated with MTSET generated large Na+ leakage currents that were undetectable with unmodified WT transporters; Li+ leakage currents also increased several-fold. MTSET had no effect on either Na+ or Li+ leakage currents in the C74A mutant. Interestingly, substitution of tryptophan with leucine at position 68 (W68L), a mutation that affects ion binding and permeation, decreased the transporter’s sensitivity to both reagents. Effects of C74 modification on ion conductance and altered reactivity observed with the W68L mutant suggest that C74 lies in or near the permeation pathway. These findings, together with recent evidence placing the TM2-TM3 loop on the extracellular side, suggest that the TM2 region does not traverse the membrane. We speculate that TM2 may form a loop structure reminiscent of the cation channel P-loop.

352. Use of and precautions with a codon-optimized green fluorescent protein as a reporter gene in co-transfection experiments
B. M. Sullivan, M. Lanzrein, N. Davidson, M. C. Jasek

We describe a method of co-transfecting a reporter gene for efficient recognition of cells for single cell electrophysiology. In cultured mammalian cells, a codon-optimized form of GFP, denoted plasmid Green Lantern-1 by the supplier (for which we use the abbreviation GL or pGL), was co-transfected with three additional plasmids, two coding for G protein-gated inwardly rectifying potassium (GIRK) channel subunits and one coding for the muscarinic m2 receptor. Electrophysiological recordings demonstrated a 98% correlation between GL fluorescence and the presence of GIRK currents. The highest expression levels of the gene(s) of interest were obtained when small amounts of GL plasmid were used. The inhibitory effect of coexpression of large amounts of GL plasmid was observed for each of several co-transfected test genes, including two isoforms of nitric oxide synthase and lac-Z. Furthermore, the inhibitory effect was not observed when an equimolar amount of the lac-Z plasmid was substituted for the GL plasmid. Our data show that GL provides most effective, non-interfering recognition of single cells for physiological experiments if GL is used in the lowest amount that gives a signal in a sufficient fraction of cells.

353. Functionally significant differences between the two isoforms of the nitric oxide synthase that are constitutively expressed in the brain
Brian M. Sullivan, Yong-Xin Li

Nitric oxide (NO) is an important signaling molecule in multiple brain regions. The “neuronal” and “endothelial” isoforms of nitric oxide synthase, which produce nitric oxide in response to calcium/calmodulin binding, are each constitutively expressed in multiple partially-overlapping cell types and regions within the brain. Using adenoviral vectors
to induce high-level expression of the neuronal and endothelial isoforms (nNOS and eNOS, respectively) of the nitric oxide synthase in cell lines and primary neuronal cultures, we address the question "Why have two enzymes in the brain that catalyze the same reaction?". Previous work by others has shown that the subcellular localizations of the enzymes are determined by different mechanisms; the eNOS is fatty acylated and the nNOS contains a PDZ domain. We are using immunocytochemistry combined with fluorescence tagging to determine if there are differences in the subcellular localizations of the enzymes in infected cultured hippocampal neurons. We are also using a nitric oxide-sensitive electrode, which is able to discern concentrations of a few nM NO, to monitor nitric oxide production in infected Chinese Hamster Ovary (CHO) cells. This assay will allow us to determine whether the enzymes differ in their ability to release nitric oxide into the extracellular space and whether they have different calcium sensitivities. We have measured NO production in both intact and homogenized infected CHO cells using this electrochemical device. We detect ~100 nM NO produced by a 35-mm dish of infected CHO cells in the presence of a calcium ionophore. NO production, as assayed by the electrode, is sensitive to NO-synthase inhibitors. We are using the system to analyze differences between the nNOS and the eNOS. Early results suggest that the enzymes release NO to the extracellular space differently; they may also have different calcium sensitivities.

354. Activation of GIRKs overexpressed by recombinant adenovirus blocks hippocampal neuron excitability
Markus U. Ehrengruber, Craig A. Doupnik, Youfeng Xu, Justine Garvey, Mark C. Jasek, Norman Davidson

G protein-gated inward rectifier K⁺ channel subunits (GIRK1-4) have been cloned from neuronal and atrial tissue and function as heterotetramers. To examine the inhibition of neuronal excitation by GIRKs, we overexpressed GIRKs in cultured hippocampal neurons from 18 d rat embryos which normally lack or show low amounts of GIRK protein and currents. Adenoviral recombinants containing the cDNAs for GIRK1, GIRK2, GIRK4, and the serotonin 5-HT₁A receptor were constructed. Typical GIRK currents could be activated by endogenous GABA₉, serotonin 5-HT₁A, and adenosine A₁ receptors in neurons coinfected with GIRK1+2 or GIRK1+4. Under a current clamp, GIRK activation increased the cell membrane conductance by 1-2-fold, hyperpolarized the cell by 11-14 mV, and inhibited action potential firing by increasing the threshold current for firing by 2-3-fold. These effects were not found in non- and mock-infected neurons, and were similar to the effects of muscarinic stimulation of native GIRK currents in atrial myocytes. Two inhibitory effects of GIRK activation, hyperpolarization and diminution of depolarizing pulses, were simulated from the experimental data. These inhibitory effects are physiologically important in the voltage range between the resting membrane potential and the potential where voltage-gated Na⁺ and K⁺ currents are activated, that is where GIRK currents are outward. Adenoviral GIRK overexpression can now be tested for its effect on (hippocampal) synaptic transmission and in model systems of certain biological malfunctions, including epilepsy.

355. Analysis of gene expression induced by BDNF in the hippocampus
Markus Lanzrein, W. Bryan Smith, Hyejin Kang, Erin M. Schuman, Norman Davidson

Long-lasting synaptic potentiation (LTP) in the hippocampus can be divided into an initial phase (1-3 hrs) that relies upon modification of existing proteins and a late phase (>3 hrs) that is dependent on transcription and translation. Thus, new genes are induced during the late phase of LTP which presumably function in the maintenance of the synaptic potentiation. The neurotrophins BDNF and NT3 can induce a long-lasting enhancement in synaptic transmission in the Schaffer collateral - CA1 synapses in the rat hippocampus. This enhancement is also sensitive to inhibitors of protein synthesis and therefore requires de novo synthesis of proteins. For a better understanding of neurotrophin action and synaptic plasticity, it would be interesting to know which genes are induced in the hippocampus upon neurotrophin treatment. We are approaching this problem by constructing cDNA libraries from acute hippocampal slices that have been potentiated by BDNF treatment and from unpotentiated control slices. Due to the limited amount of tissue available, we are employing PCR-based methods to generate library cDNA. Subtractive hybridization is then used to obtain cDNA libraries that are specifically enriched in genes that are induced by BDNF. As a second approach, we are utilizing serial analysis of gene expression (SAGE). This novel technique is based on the analysis of small (9bp) sequence tags that are derived from a given mRNA population. SAGE allows the quantitation of the expression level of each individual mRNA and differences in expression induced by BDNF treatment can be determined.

356. Presynaptic modulation of neurotransmitter release by BDNF
Yong-Xin Li, Yinong Zhang, Sheri McKinney, Erin Schuman, Norman Davidson

Brain-derived neurotrophic factor (BDNF) enhances neurotransmitter release at central synapses. We investigated the effects of BDNF on E18 hippocampal neurons after ten days in culture. BDNF (100 ng/ml) increased the frequency of excitatory postsynaptic current bursts (EPSCs) to 205 ± 20.8% of the levels found for the control samples and increased the amplitudes of evoked synaptic currents by 45 ± 10%
in four out of seven pairs of neurons. However, there were no detectable effects of BDNF on the resting membrane potential (Vm), input resistance (Rin), action potential threshold (AD), action potential amplitude (AP), or amplitude of the current induced by an application of glutamate (I). BDNF also increased the frequency of miniature EPSCs (mEPSCs) (268.0 ± 46.8 % of the levels found for the control samples) in the presence of 50 nM TTX without affecting the mEPSC amplitude. The effect of BDNF on mEPSC frequency was blocked by the tyrosine kinase inhibitor K$_{252}$ and also by removal of [Ca$^{2+}$]$_{OUT}$. In addition, Fura-2 recordings showed that BDNF elicited an increase in [Ca$^{2+}$]$_{IN}$; this effect was also blocked by K$_{252}$ and dependent on [Ca$^{2+}$]$_{OUT}$. The results demonstrate that BDNF affects synaptic transmission at a presynaptic locus and that this effect is accompanied by a rise in [Ca$^{2+}$]$_{IN}$ in the signal transduction pathway.

357. Use of organotypic hippocampal cultures and antisense suppression methods to examine synaptic physiology
Mark C. Jasek

Examinations of receptor physiology often rely heavily on the use of agonist, antagonist, and other pharmacological agents. In this day of molecular characterization of various classes of receptors and their numerous subtypes, not to mention subunits, it is often the case that selective pharmacological tools are unavailable. This presents a large hurdle in understanding the roles that select receptors and/or their subunits play in synaptic physiology. In order to circumvent this problem, we are developing methods to delete functional proteins from functional synapses. Currently, we are testing two basic antisense methodologies in mammalian cell line cultures. The first method uses mammalian expression vectors which generate antisense RNA. This antisense RNA binds to the select RNA of interest and prevents translation of the protein. Once a suitable antisense RNA-expressing vector has been developed, this will then be transferred into a viral expression system (Adenovirus) allowing efficient infection of various cells or tissue preparations. The second approach utilizes antisense oligodeoxynucleotides, containing phosphorothioate bonds between bases for increased stability, to hybridize with the native DNA and prevent the transcription of RNA of the protein of interest.

The model of synaptic transmission we will be using is the CA3 projection to CA1 pyramidal neurons in organotypic hippocampal slice cultures. This method, originally developed by B. Gähwiler, involves the mounting of hippocampal slices onto coverslips which are placed into tubes containing culture media and subsequently arranged in a rotating roller bin. These cultures survive for several weeks, providing us with the time to reduce protein levels of interest using our antisense methods. Molecules we plan to examine initially include Trk B, metabotropic glutamate, and GIRQ receptors.

358. Cultured neural networks to watch learning happen
Steve M. Potter, Jerry Pine

The accessibility of neural cell cultures makes them ideally suited for the study of emergent, network properties of living neural circuits. These cultures exhibit many of the properties of neural circuits in vivo, including the formation of rich synaptic connectivity, the development of complex patterns of spontaneous activity, and synaptic plasticity in response to electrical or pharmacological stimulation. Traditionally, such cultures are studied using one or two microelectrodes. We believe that such approaches are unable to capture the spatio-temporal distribution of activity and may therefore miss many interesting collective properties of biological neural networks.

Therefore, we have developed three key technologies aimed at manipulating and observing many neurons at a time (more details can be found at http://www.caltech.edu/~pinelab/pinelab.html):

- A 61-electrode Petri dish (multi-electrode array, MEA) for non-invasive (extracellular) recording and stimulation of cultured rat hippocampal neurons (see Figure 1). We plan to use the MEA to characterize the spontaneous patterns of neural activity that develop in these cultures over days or weeks. As a model for learning, we will attempt to influence the activity patterns using similarly patterned electrical stimulation.
- A custom 100-frame/sec CCD camera to observe the electrical activity in cultures stained with voltage-sensitive dyes. This allows us to see patterns of electrical activity in cultured networks with unprecedented temporal and spatial resolution.
- A 2-photon laser-scanning microscope, developed in collaboration with Professor Fraser, which provides submicron resolution in living cells with far less photodamage than confocal microscopy. This allows us to study fine morphological features that subserve single-neuron and network activity, and long-term plasticity. It allows us to image, in a single living specimen, the minute structural changes that accompany learning, across minutes or hours (time lapse imaging) or across days (time sequence imaging). By observing and manipulating the interplay between neuronal activity and morphology, we hope not only to get at how learning and memory works, but also to provide useful parameters for computer models of neural networks. By using multi-neuron techniques, we will establish the importance of network properties and dynamics that may not be apparent using traditional electrophysiological approaches.
Figure 1: Multielectrode array dish and associated preamplifiers and patch panels. The arrays consist of leads and electrodes made of the transparent conductor indium-tin-oxide. The leads are insulated with silicon nitride, and the electrodes are plated with platinum to enhance the signal-to-noise ratio.

359. The Neurochip: A living neural network
Michael P. Maher, Jerome Pine, John Wright, Yu-Chong Tai

Over the past years, we have designed and built a working system in which dissociated embryonic rat hippocampal neurons can be cultured on a micromachined silicon device called a 'neurochip'. The neurochip allows non-invasive, specific, and chronic recording and stimulation of individual neurons in a network.

Immature, freshly dissociated fetal rat hippocampal neurons are placed into an array of 25x25x15 µm³ wells etched into the surface of the chip; each well has an integrated extracellular metal electrode. A silicon nitride canopy over the well holds the soma next to the electrode while allowing neurites to grow normally. Eight 2 µm² cross section, 15 µm long tunnels connect the well interior to the main culture area, permitting the neural processes to meet and form synapses. The morphology and survival of cells in neurochip wells are unaltered relative to controls.

The close proximity of the soma to the electrode permits detection of action potentials (AP's) with high signal-to-noise ratio. Typically, AP recordings are triphasic and 200-300 µV high with 5 µV RMS noise. AP's have stereotyped shape and size for individual cells, with some variation between cells. Electrical crosstalk between channels is negligible.

Passing current pulses through the well electrodes can elicit AP's in the individual cells, as determined by simultaneous recordings of the fluorescence signal of the voltage-sensitive dye RH237. The AP response shows a sharp threshold as the stimulus is increased, and the response is independent of stimulus strength above threshold. Cells can reliably be fired by applying a biphasic 3 µA pulse, 3 msec per phase.

Hippocampal neurons cultured in the neurochip form synapses on each other within days of being
loaded into the wells. Subthreshold synaptic potentials in these cells were detected by firing the neuron in one well while monitoring the RH237 fluorescence signal of another cell. Preliminary experiments indicate that sub-threshold signals can be detected electrically by the well electrodes, allowing us to avoid photodamage associated with fluorescent dye recording.

The neurochip technology allows us to generate a living neuronal network, all of whose elements are under direct experimental control. In the coming year, we plan to use this new system to investigate the pharmacology and activity-dependence of synaptic plasticity of hippocampal neurons. We will also examine the development of the network with and without externally-applied activity patterns. In the long term, we see the neurochip as an extremely powerful tool with which to explore the behavior and dynamics of biological neural networks.

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**Publications**


Gallivan, J., Lester, H.A., Dougherty, D.A. Site specific incorporation of biotinylated amino acids as a means to identify surface exposed residues in integral membrane proteins. Submitted.


Lyford, L.K., Lee, J.W., Desai, S., Labarca, C., Rosenberg, R.L. "Revertant" pharmacology of a non-desensitizing α7 nicotinic acetylcholine receptor mutant expressed in Xenopus oocytes. Labarca, C., in collaboration with Dept. of Pharmacology, University of North Carolina, Chapel Hill and Dept. of Medicine, Duke University Medical Center. *Biophys J.* 72, 2, A149.


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**Summary:** Much of the research in this laboratory involves the overlap and interaction between the nervous and immune systems. The role of neuroepoietic cytokines in PNS and CNS injury and repair, neurodegenerative disease, learning and memory, pain and inflammation is being examined primarily through the use of knockout mice and over-expression in vivo using adenoviral vectors. In addition, two cell surface molecules found in neural and hematopoietic cells, CD9 and Lewis X, are being studied in culture as well as in the embryonic and mature nervous systems.

Cytokines are diffusible intercellular messengers that were originally studied in the immune system. Our group has contributed to the discovery of a new protein family that we have termed the neuroepoietic cytokines, because of their action in both the nervous and hematopoietic/immune systems. In fact, one of these cytokines, leukemia inhibitory factor (LIF), can coordinate the neural and immune reactions to injury. Using both delivery of recombinant LIF protein in vivo and examination of the consequences of knocking out the LIF gene in mice, we find that this cytokine has a powerful regulatory effect on inflammation, both within and outside the nervous system. We are exploring the implications of these findings in the study of animal models of nerve injury and regeneration, pain and autoimmune diseases. We are also examining the role of these cytokines in Alzheimer's disease brain and in an animal model of this disease.

Cytokines may also be involved in the modulation of connections during synaptic plasticity that is an integral part of learning and memory. We are examining the role of LIF and other cytokines in several models of synaptic plasticity in the hippocampus and amygdala of adult rats and mice. This work also involves knockout mice, electrophysiology and delivery of recombinant proteins or the genes that code for them.

A second area of investigation is also concerned with an overlap between the neural and immune systems. In searching for molecules with distinctive expression patterns in the developing mammalian nervous system, we found the protein CD9, a signaling...
molecule on the surfaces of platelets and lymphocytes. Our perturbation experiments with antibodies to this protein suggest a role for CD9 in neurite outgrowth and glial cell adhesion, motility and proliferation. In addition, biochemical studies indicate that CD9 is part of a signaling complex of several very interesting proteins on the neural cell surface. Such complexes are likely to be key players in guiding neurite growth and recognition events during development and regeneration.

Another molecule with a highly restricted regional expression is found in the rostral embryonic forebrain, on the surfaces of dividing and newly postmitotic cells. We find sharp boundaries of expression, which delineate heretofore unknown subdivisions in this region. This molecule is the oligosaccharide Lewis X, a carbohydrate known to be important in inflammation. Current work is aimed at discovering the function of Lewis X in the embryonic brain through the use of cell culture and the analysis of mutant mice in which genes that code for the enzymes that synthesize this molecule have been knocked out.

361. Leukemia inhibitory factor can act as an anti-inflammatory cytokine
Lisa R. Banner, Clifford J. Woolf

The neuroepoietic cytokine leukemia inhibitory factor (LIF) plays a role in the response to injury in both the peripheral and central nervous systems. To test whether LIF regulates neurogenic inflammation, we have studied its role in peripheral inflammation and inflammatory pain hypersensitivity. Intraplantar injection of complete Freund's adjuvant (CFA) elevates LIF mRNA. Knockout mice with a null mutation for LIF have a basal mechanical sensitivity similar to wild type mice and both groups of mice respond to intraplantar CFA with mechanical hyperalgesia. In the knockout mice, however, the degree of swelling produced by the inflammation is substantially greater in spatial extent and amplitude, and is associated with a greater inflammatory cell infiltrate. In addition, LIF knockout animals display a greater up-regulation of both interleukin (IL)-1β and NGF in response to CFA. Conversely, intraplantar injection of LIF if administered 10 min prior to CFA injection, delays the development of mechanical and thermal hypersensitivity, as well as the associated up-regulation of IL-1β and NGF. These data imply that up-regulation of LIF during inflammation serves an endogenous anti-inflammatory role.

362. LIF regulates the inflammatory response to peripheral nerve injury
Ronit Lahav, Shi-Ying Kou, Paul H. Patterson

Transaction of nerve leads to axonal degeneration and demyelination. In addition, the local inflammatory response includes cell infiltration (including macrophages, neutrophils and mast cells) out of blood vessels into the nerve. Although inflammation is tightly associated with the degeneration and regeneration processes, little is known of how it is controlled and how it influences axonal regrowth and restoration of neural function. One candidate regulatory agent is the cytokine leukemia inhibitory factor (LIF). LIF expression is strongly up-regulated following injury to the PNS and CNS, and experiments with LIF knockout mice have shown that this cytokine is required for the normal neuronal response to damage. Moreover, exogenous LIF can enhance the survival of degenerating motor and sensory neurons. To determine if LIF is involved in the inflammatory response to peripheral nerve injury, we compared the number of macrophages, neutrophils and mast cells in crushed adult sciatic nerves from LIF null mutant mice and wild type littermates. We find that the early cell infiltration response is greatly diminished in the absence of LIF, indicating that LIF is required for both the neuronal and inflammatory responses to this type of injury. We are currently asking if the absence of LIF also influences axonal regeneration.

363. LIF can induce chemotaxis in mouse peritoneal macrophages
Shigeki Sugiuira
The cytokine leukemia inhibitory factor (LIF) is strongly induced at sites of injury in the adult PNS and CNS. Moreover, LIF is required for appropriate peripheral neuron responses to injury. Our recent work has shown that LIF knockout mice display a markedly deficient initial inflammatory response to sciatic nerve crush. Unlike wild type mice, LIF knockout mice exhibit a relatively sparse inflammatory cellular infiltrate at the site of injury 24 hr following crush (see R. Lahav abstract). This suggests that LIF could affect immune cells directly. To investigate whether LIF has a chemotactic activity, this and other proteins were tested on mouse peritoneal macrophages in a microchamber assay. LIF does induce migration, and this migration depends on a positive gradient of LIF. Therefore, LIF is chemotactic rather than merely chemokinetic. Moreover, IL-6 and oncostatin M, which belong to the same family as LIF, do not display this chemotactic activity toward such macrophages. Therefore, at least part of LIF's role as a critical positive mediator of the early inflammatory response in nerve may be directly on the immune cells.

364. Analysis of neuronal and glial phenotypes in brains of mice deficient in LIF
Lakshmi Bugga, Reto A. Gadient, Karen Kwan1, Paul H. Patterson

Leukemia inhibitory factor (LIF) can regulate the survival and differentiation of certain neurons and glial cells in culture. To determine the role of this cytokine in vivo, we have examined the brains of adult mice in which the LIF gene was disrupted.
Immunohistochemical staining of neurons for choline acetyltransferase, tyrosine hydroxylase, serotonin, parvalbumin, calbindin, neuropeptide Y, vasoactive intestinal polypeptide and calcitonin gene related peptide revealed no significant differences between mutant and wild type (WT) brains. In contrast, analysis of glial phenotypes demonstrated striking deficits in the LIF-knockout brain. Staining with several anti-glia fibrillary acidic protein (GFAP) antibodies showed that the number of GFAP-positive cells in various regions of the hippocampus in the female mutant is much lower than in the WT. The null male hippocampus also displays a significant, though less marked, deficit. The number of astrocytes in the mutant hippocampus, as determined by S-100 staining, is not, however, significantly different from WT. In addition, the level of GFAP in the mutant hippocampus as determined by immunoblotting does not differ from WT. We hypothesize that the striking, sexually dimorphic deficit in GFAP staining of astrocytes is due to altered accessibility of the antibody in sections of mutant animals. Histochemical staining of female, but not male, mutants reveals a significant deficit in myelin basic protein content in three brain regions, suggesting alterations in oligodendrocytes as well. Thus, while overall brain histology appears normal, the absence of LIF in vivo leads to specific, sexually dimorphic alterations in glial phenotype.

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365. Expression of LIF in normal and Alzheimer's disease brain
Reto A. Gadient, Rainer Lemke

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive memory loss and cognitive impairment. There is increasing evidence that inflammation may be crucial for the development and progression of AD. Inflammatory cytokines, such as interleukin (IL)-6 and IL-1α have been detected in the amyloid plaques found in the cortex and hippocampus of AD brain, as are acute-phase proteins, which are known to be regulated by these cytokines in the liver. Moreover, anti-inflammatory drugs have shown efficacy in ameliorating AD symptoms and time of onset. The neuroinflammatory cytokine LIF plays a crucial role in many inflammatory processes, such as in the acute-phase reaction of the liver and following peripheral nerve injury. We are therefore asking whether LIF is involved in the inflammatory process in AD brains. Using in situ hybridization we studied the expression of human LIF mRNA transcripts in normal and AD brain. In all areas investigated including hippocampus, frontal (A9) and temporal (A22) cortex, we find that LIF mRNA is predominantly expressed by neurons. In contrast, astrocytes and microglia do not display detectable levels of LIF mRNA. The localization of LIF transcripts is similar in AD and control brains, but the overall staining intensity is diminished in the former. This reduction correlates with the significant loss of neurons in AD.

We are currently analyzing LIF protein expression in these brains.

366. The role of cytokines in long-term potentiation
Joanna Jankowsky, Erin Schuman, Brian Derrick

Long-term potentiation (LTP) in the mammalian hippocampus is currently one of the best characterized physiological paradigms for studying learning and memory. We are interested in understanding how local environmental factors, cytokines in particular, can exogenously affect, or are themselves endogenously regulated by the induction of LTP. Cytokines expressed by neurons could be involved in synaptic plasticity directly, while cytokines produced by reactive astrocytes and microglia during the electrophysiological recording process used in typical experiments could also influence these events. We are currently examining 19 cytokines and receptors in hippocampal LTP in vitro and in vivo. We and others have shown that exogenous cytokines can influence LTP in hippocampal slices. Moreover, using semi-quantitative RNAse protection and RT-PCR assays to examine relative mRNA levels, we have preliminary evidence of LTP-induced changes in mRNA expression of several cytokines in vivo. Several other cytokine mRNA levels increase in vivo simply with electrode insertion, as might be expected with an inflammatory response to injury. The same questions are being asked in acute hippocampal slice cultures. Future work will center on LTP in vivo using chronically implanted electrodes. This will obviate acute inflammatory effects and focus more directly on cytokine mRNA changes with LTP induction.

367. Adenovirus-mediated expression of neuroinflammatory cytokines in brain
Kazuhiko Oishi, Youfeng Xu, Donghong Ju

Adenovirus vectors are particularly attractive vehicles for gene transfer in brain because they can induce gene expression in postmitotic neurons. To examine the role of leukemia inhibitory factor (LIF) in neurogenic inflammation and nerve regeneration, we are expressing the LIF cDNA, as well as other neuroinflammatory cytokines, in wild type and null mutant brains using adenoviruses. We have inserted cDNAs for LIF, IL-6 or LacZ under the control of a CMV promoter into first generation adenoviruses. To assess these recombinants, we infected primary cultures of rat hippocampal neurons and fibroblasts as well as the rat hippocampus in vivo. Histological staining reveals efficient gene transfer to the cultured cells. LacZ expression was also observed 2 days after injection of LacZ adenovirus into adult rat hippocampus. Moreover, RT-PCR analysis demonstrated an increase in LIF and IL-6 mRNAs after infection with LIF or IL-6 adenoviruses. Since it has been reported that adenoviruses can evoke an immune response, particularly at high titer, we are
368. LIF is an autocrine/paracrine factor for sympathetic neurons

Jr-Gang Cheng

Leukemia inhibitory factor (LIF) alters neuronal phenotype both in vitro and in vivo. Since it can be produced by glia and other non-neural cells, LIF is a candidate target-derived differentiation factor as well as an injury-response factor. We now have evidence that LIF can be produced by neurons and can act as an autocrine/paracrine factor. A reverse transcriptase-polymerase chain reaction assay detects LIF mRNA in rat sympathetic neuron cultures, and in situ hybridization combined with MAP2 immunocytochemistry indicates that most of the cells expressing LIF mRNA are, in fact, neurons. The neuronal lysate as well as the conditioned medium contain proteins that are specifically recognized by anti-LIF antibodies, and these antibodies also specifically stain the cultured neurons. In addition, incubation of high density sympathetic neuron cultures with anti-LIF antibodies reduces basal expression levels of LIF target genes, indicating that the endogenously produced cytokine is acting on the neurons under these conditions. We also find that the LIF transcript is expressed in sympathetic and sensory neurons in vivo as well.

369. Bone morphogenetic proteins modulate STAT signaling by neuropoietic cytokines

Jr-Gang Cheng

Cytokines are thought to play important roles in the developing and mature nervous system. Bone morphogenetic proteins (BMPs) and neuropoietic cytokines can regulate the neurotransmitter, neuropeptide and morphological phenotypes of neurons in culture. We find that these two cytokine families act antagonistically on cultured sympathetic neurons: expression of the neuropeptide genes induced by neuropoietic cytokines is modulated by BMPs. Moreover, we find that the actions of neuropoietic and BMP cytokines converge downstream on the tyrosine-phosphorylation and nuclear transport of STAT3 (signal transducer and activator of transcription 3). Thus, by modulating STAT-3 activation, BMPs could have much wider effects than previously thought.

370. Cardiotrophin-1 induces the same neuropeptides in sympathetic neurons as do neuropoietic cytokines

Jr-Gang Cheng

Cardiotrophin-1 (CT-1) was cloned from mouse embryoid body for its ability to induce growth of heart cells. Predictions of its secondary structure indicate that CT-1 belongs to a family of cytokines with a four helical bundle structure, and sequence comparisons reveal a weak homology to leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF). Using a RT-PCR assay with rat sympathetic neuron cultures, we find that CT-1 induces and suppresses the expression of the same set of neuropeptide and neurotransmitter synthetic enzyme mRNAs as do LIF and CNTF. In addition, the effects of CT-1 and LIF are not additive, and CT-1 does not require a GPl-linked component to mediate its actions. Our functional data confirm that CT-1 is a member of the neuropoietic cytokine family, and suggest that the CT-1 receptor complex contains the gp130 signal transducing component.

371. A novel fucosyltransferase is involved in the synthesis of the FORSE-1/LEX carbohydrate recognition molecule in embryonic cerebral cortex

Karen L. Alleendorfer, J. B. Lowe

The LeX carbohydrate recognition molecule is present on the surface of proliferating neuroepithelial cells in the embryonic cerebral cortex, and is specifically recognized by the FORSE-1 monoclonal antibody. In the embryonic brain, this carbohydrate epitope appears to be carried primarily by neutral glycolipids. In order to determine the function of LeX, we sought to study the brains of embryos in which the synthesis of the LeX carbohydrate has been disrupted. The final step in LeX biosynthesis is the addition of an alpha (1,3) fucose residue by an alpha (1,3) fucosyltransferase (FucT). There are five known human alpha (1,3) FucT genes (FucT 3-7), and biochemical analysis of FucT activity in developing brain indicates that it is of the FucT4 type. Mouse homologues of FucT4 and FucT7 have been cloned, and knockout mice have been generated. These animals exhibit loss of LeX-containing selectin ligands in the immune system, as well as defects in lymphocyte rolling and homing. Northern blots of wild-type E13 mouse brain indicate low levels of FucT4 mRNA and undetectable levels of FucT7 mRNA. We immunostained the brains of embryos lacking FucT4, FucT7 or both enzymes at E11.5 and E12.5, and find that the brains of all of these embryos exhibit normal FORSE-1 staining in the proliferative neuroepithelium of the telencephalon. Moreover, these embryos appear to have normal brain morphology and lack neurological symptoms during development and in adulthood. Thus, it appears that FucT4 and FucT7 are not required for LeX biosynthesis in the embryonic brain.

372. Association of the tetraspan protein CD9 with integrins on the surface of S-16 Schwann cells

Michael Hadjiargyrou, Zaven Kaprielian, Nobuo Kato

The cell surface glycoprotein CD9 is a member of a group of proteins known as the "tetraspan" or "transmembrane 4 superfamily". Previous work with non-neural cells has shown that CD9 associates in cis with integrins and small GTP-binding proteins on the cell surface. To extend our recent findings showing that perturbation of CD9 alters Schwann cell adhesion,
proliferation and migration, as well as neurite outgrowth in sympathetic neurons, we have searched for CD9-associated proteins in S-16 Schwann cells. We find that CD9 is specifically co-precipitated from S-16 cell extracts by antibodies against integrins α3, α6 and β1. In addition, double immunofluorescence labeling as well as co-capping experiments indicate that CD9 is specifically co-localized with these integrins on the cell membrane of S-16 Schwann cells.

373. Perturbation of the tetraspan membrane protein CD9 promotes neurite formation in a partially α3β1 integrin-dependent manner.

Shilpi A. Banerjee, Michael Hadjiargyrou

The tetraspan cell surface glycoprotein, CD9, has been implicated in cellular signaling during growth and differentiation in the haematopoietic and nervous systems. Since CD9 expression is induced early in development in sensory and sympathetic neuroblasts, we investigated the role of CD9 in neurite outgrowth. We plated dissociated cells from neonatal sympathetic ganglia on immobilized anti-CD9 antibodies or on antibodies against other cell-surface molecules. We find that B2C11, an anti-CD9 antibody that we have previously shown to activate Schwann cells in vitro, promotes robust neurite outgrowth from sympathetic neurons that is greater than that on other antibody surfaces and is comparable to neurite outgrowth on a collagen substrate. In addition, B2C11 causes dramatic morphological changes in neurons and glia from dissociated ganglia, including a flattening of these cells. Since CD9 interacts with integrins in many cell types including Schwann cells, and specifically with the α3β1 integrin in some cells, we tested whether the effect of B2C11 on neurite outgrowth is mediated by this integrin. An anti-α3β1 antibody, Ralph 3-1, attenuates the extent of neurite outgrowth on B2C11 and collagen, but not on laminin. Since the α3β1 integrin has been shown to mediate neurite outgrowth on different substrates, these results provide a functional significance for the CD9-α3β1 interaction; downstream signaling may be activated by this cis interaction on the cell-surface in response to external cues that promote neurite outgrowth.

Publications


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Summary: The apparently simple electrophysiological output of a single synapse is the result of a vast and complicated biochemical and molecular machinery. The exquisite control of synaptic strength is achieved by the use of myriad molecular signals which vary in their production, diffusion constants, signal transduction mechanisms, and targets of action. A major goal of the Schuman lab is to understand how different types of molecular signals contribute to synaptic transmission and plasticity. We examine synaptic transmission and plasticity in the hippocampus, a brain structure implicated in memory formation in humans and animals. We study short- and long-term synaptic plasticity induced by electrical stimulation (long-term potentiation-LTP) as well as plasticity induced by different signaling molecules. We are examining different kinds of signals, from highly diffusible gases to adhesion molecules, whose mechanism of action range from direct chemical interactions with proteins to stimulation of protein kinase cascades to potential mechanical perturbations of synaptic structures. We are interested in the mechanisms by which these signals modulate synaptic transmission themselves or interact with other signals during physiologically-induced synaptic plasticity. We combine electrophysiological, biochemical and molecular biological techniques to address fundamental issues related to the formation and maintenance of different types of plasticity.

Our previous work and that of many other labs, suggested that NO may act as a retrograde messenger...
to deliver postsynaptically-derived information to the presynaptic neuron. There are three isoforms of the enzyme responsible for generation NO: the neuronal, endothelial and inducible NO synthases (NOS). Our lab has addressed the role of eNOS by combining pharmacological tools with recombinant adenovirus (Ad) vectors designed to specifically manipulate eNOS function. In collaboration with Norman Davidson, we constructed an Ad vector containing a truncated eNOS (TeNOS) which lacks catalytic activity and may function as a dominant negative inhibitor of eNOS function. Expression of the TeNOS selectively blocked LTP at synapses which usually exhibit NO-dependent LTP. Why is eNOS used in LTP? The eNOS differs from the other isoforms in that it is directly associated with membranes, owing to its myristoylation. In another set of experiments we found that an inhibitor of myristoylation can block LTP. Moreover, Ad-medicated expression of an eNOS targeted to the plasma membrane by a myristoylation-independent mechanism can rescue this block of LTP. The membrane localization of eNOS likely serves two critical functions: it optimally positions the enzyme both to respond to Ca\textsuperscript{2+} influx across the membrane and to release NO into the extracellular space.

Neurotrophins, proteins which promote the survival and differentiation of neurons during development, are expressed in the CA1 region of the hippocampus throughout postnatal development and into adulthood. We have examined the role that they play in synaptic plasticity and found that both BDNF and NT-3 can enhance synaptic strength in the adult hippocampus. These are three broad areas that we are pursuing related to this observation: the signal transduction mechanisms employed by BDNF and NT-3, including their potential role in stimulating local protein synthesis; their role in activity-induced plasticity; and their potential to produce structural changes in mature synaptic structures. Our approach to the latter issue involves an on-line analysis of structural changes promoted by the neurotrophins utilizing Caltech’s two-photon microscope (collaboration with the Scott Fraser group). We have recently obtained time-lapse images of well-resolved synaptic structures in hippocampal slices for up to 20 hours. We are now examining the effects of acute exposure to neurotrophins on the synaptic structures. Another project involves time-lapse imaging of mossy fiber sprouting in hippocampi from rats which have experienced pilocarpine-induced seizures.

We are also examining the role of cadherins family ofcell adhesion molecules in synaptic plasticity. Several labs have shown that cadherins are localized to synapses to the hippocampus. We have recently shown that function-blocking cadherin antibodies or peptides can prevent LTP, without interfering with basal synaptic transmission. We are now examining the molecular mechanisms by which cadherins influences synaptic strength and the involvement of cadherins in the formation and maintenance of synapses.

A relatively new focus of the lab concerns the more global issue of how information is processed in the brain. We are interested in how the coordinated activities of ensembles of neurons represent information and how the circuitry of the hippocampus gates or influences information flow. Information reaches the CA1 region of the hippocampus via the conventional trisynaptic loop as well as via a direct input from entorhinal cortex. We are examining how these two inputs interact and influence the output of CA1 pyramidal neurons. In collaboration with Gilles Laurent, we are conducting simultaneous intracellular recordings from multiple neurons in the slice and investigating how the ensembles of cells respond to noisy inputs and patterned activity. Eventually, this multi-electrode technology will be married with molecular intervention (e.g. adenovirus and transgenic approaches) in order to record form individual neurons with altered molecular identifies and observe the effects on the network properties and information processing.

374. Nitric oxide synthase subcellular localization and long-term potentiation

David Kantor, Mariela Zirlinger, Norman Davidson

Recent studies have demonstrated that eNOS may be the NOS isoform which produces NO during the induction of LTP. Specifically, work from this lab has shown that eNOS must be localized to the plasma membrane by virtue of N-terminal myristoylation in order to participate in the signal transduction events leading to LTP. In COS-7 cells, the membrane localization of eNOS has been shown by others to facilitate the extracellular release of NO, raising the possibility that myristoylation of eNOS is necessary for NO to fulfill its proposed role of retrograde messenger. This study addresses how the subcellular localization of eNOS affects the extracellular release of NO in cultured neurons, and in turn how the characteristics of NO release affect LTP in hippocampal slices. Initially, hippocampal neurons were infected with adenovirus expressing eNOS, challenged 48 hrs later with glutamate, then assayed colorimetrically for NO production using the dye ABTS. Preliminary results showed that application of 50 \mu M glutamate resulted in a 4.3 fold increase in absorbance over baseline, while co-application of 50 \mu M glutamate with 50 \mu M AP-5 resulted in a doubling of the absorbance over baseline.

In addition, we are currently experimenting with a fluorescent NO-sensitive dye which can insert into plasma membranes by virtue of its lipophilic moiety, and thus potentially reveal both temporal and spatial information about NO production. Initial experiments demonstrated that this dye is sensitive to NO, as treatment with either SIN-1 or saturated NO solutions...
converts the dye from a non-fluorescent to a fluorescent state with an emission peak at 595 nm. Further confocal imaging of neurons treated with this dye revealed uniform dye loading and easy visualization of fine processes. Ultimately this dye could be used to correlate the site of NO production with the location of both glutamate receptors and NOS. Both of these detection methods will also be used to ass whether different NOS isoforms give rise to NO with different spatial and temporal release characteristics.

375. Two-photon time-lapse imaging of living hippocampal spines
David Kantor, Steve Potter, Adam Mamelak
Numerous studies have established the role of several neurotrophins in promoting morphological changes in synaptic structures during development of the nervous system. Recent evidence from this lab has demonstrated that the neurotrophins BDNF and NT-3 dramatically enhance synaptic transmission in area CA1 of adult rat hippocampal slices. Further, this enhancement is dependent upon new protein synthesis, and most likely employs the protein synthesis machinery and mRNAs located at or near dendritic spines. Given the fact that many of the mRNAs detected in dendrites encode cytoskeletal proteins, such as MAP-2, the intriguing possibility exists that neurotrophins many also induce remodeling of synaptic structures in the adult animal.

To test this idea directly we have begun to examine the effect of BDNF application on synaptic spine morphology in living adult hippocampal slices using two-photon laser scanning microscopy (TPLSM). Pyramidal neurons in area CA1 were labeled either extracellularly by microinjection of the lipophilic dye DiO, or intracellularly using a micro electrode loaded with fluorescein-conjugated dextran. Following labeling, slices were maintained in modified Krebs Ringer solution which permits slice survival for 12 hours or more. Because of the substantially reduced light dose afforded by TPLSM over standard confocal microscopy, prolonged 3-D time-lapse movies of well resolved dendritic spines are possible without extensive phototoxicity or photobleaching. In preliminary experiments, dendritic spines were imaged for up to 12 hours, with images collected every 15 to 30 minutes. At 63X magnification, densely clustered spines were evident along large extents of dendritic arbors, and displayed morphological changes (such as elongation or retraction) over the course of several hours, even under baseline conditions. In several cases following prolonged time-lapse imaging, slices demonstrated healthy field potentials, and exhibited long-term potentiation.

376. Time-lapse imaging of mossy fiber sprouting in hippocampal slices using two-photon laser scanning microscopy
Adam N. Mamelak, Steve Potter, David Kantor, Daniel Lowenstein, Scott Fraser
While hippocampal mossy fiber sprouting (MFS) is routinely observed in fixed specimens form epileptic patients and following status epilepticus in animals, MFS has never been observed in vitro or in vivo. Direct observation of MFS in living hippocampal slices should reveal many dynamic aspects of axonal reorganization and provide insight into its molecular basis. We have developed a technique to perform time-lapse imaging of MFS in hippocampal slices using TPLSM. Pilocarpine (320 mg/kg I.P.) was used to induce MFS in 32-day-old rats. Animals were sacrificed 7-21 days later, and hippocampal slices were prepared. Slices were perfused in oxygenated ACSF supplemented with serum-free media at 32C. Moss fibers were labeled by extracellular pressure injection of DiO, and then imaged as z-series stacks on a TPSLM at intervals of 15-20 min, for periods of 8-24 hrs. Slice viability at the completion of the imaging session was determined by measurement of field potentials in stratum radiatum. Individual time point images were processed (ImageSpace and NIH Image software) and assembled into time lapse movies.

Physiologically viable slices were maintained for periods of up to 24 hrs and time lapse imaging was performed. Photobleaching of dye tended to degrade the image over time, although it did not generally affect slice viability. Preliminary data suggest that MF growth can be visualized from day 14 and beyond, and that fibers extend and retract in a dynamic fashion during this time. Neurite extension towards both the hilus and IML was evident, and collateral sprouting was seen. The cells of origin for these MFS were not well visualized. Thus, MFS appears to be dynamic, involving both neurite retraction and extension. Further studies are in progress to better characterize these events, and provide more specific labeling of MFS. In addition, this technique may provide a system for investigating the role of biological modifiers on MFS.

377. Different role for TrkB signaling in long-term potentiation in the adult rat hippocampus
Hyejin Kang
Several recent studies have demonstrated the importance of neurotrophic factors in synaptic plasticity in the hippocampus. We examined the role of TrkB ligands in hippocampal long-term potentiation (LTP) using function-blocking TrkB antiserum and Trk-IgG fusion proteins. Incubation of hippocampal slices with TrkB antiserum did not have any effect on basal synaptic transmission, short-term synaptic plasticity or LTP induced by several trains of tetanic stimulation.
The anti-TrkB treated-slices, however, showed significant deficits in the expression of LTP induced by either theta-burst stimulation or pairing postsynaptic depolarization with low frequency stimulation. Consistent with these results, prior enhancement of synaptic transmission by BDNF did occlude the subsequent induction of LTP by theta-burst stimulation although it has previously been shown to spare tetanus-induced LTP (Kang & Schuman, 1995).

To address the mechanism for the differential roles of TrkB function in different forms of synaptically-induced LTP, we tested two kinds of LTP induction protocols with provide the same total number of pulses but with distinct temporal patterns. Treatment with TrkB antisera significantly attenuated LTP induced by three trains of theta-bursts (spaced; 120 pulses), without affecting LTP induced by a continuous delivery of 120 pulses (100 Hz), which suggests that the pattern of stimulation might be more important than the absolute number of pulses in determining the involvement of TrkB function. The late-phase of LTP (L-LTP) was also significantly impaired in slices pretreated with TrkB antisera or TrkB-IgG, or previously potentiated with BDNF. This blockade had a specific time window. When TrkB-IgG was applied 30 min after LTP induction, it caused previously potentiated synapses to return to baseline. In contrast, when TrkB-IgG application was delayed 70 min after the first tetanus there was no blockade of L-LTP. Taken together, these results suggest that the activation of TrkB by its endogenous ligands such as BDNF and NT-4/5 within an hour of LTP induction, plays a critical role in maintaining L-LTP.

Reference

378. Localization of neurotrophins BDNF and NT-3 and their receptor tyrosine kinases TrkB and TrkC in the rat hippocampus
Lin Z. Jia

Neurotrophins are proteins essential for regulation of neuronal survival and differentiation during development. Recently, it has been shown that brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) may also play a role in adult synaptic plasticity. In order to begin a detailed molecular study of the role of BDNF and NT-3 in modulating plasticity, it is important to first determine their mechanism of action. Although it has been known that the two neurotrophins each bind to a specific tyrosine kinase receptor (Trk)—BDNF to TrkB and NT-3 to TrkC—the localization of the neurotrophins and their respective receptors have not yet been studied thoroughly in the adult rat brain.

Localization of endogenous BDNF, NT-3, TrkB, and TrkC was carried out by immunocytochemical experiments using confocal microscopy. Hippocampal slices were prepared from young (6-8 weeks) adult male Sprague-Dawley rats. Slices were fixed with 4.0% paraformaldehyde and 0.2% glutaraldehyde in 0.1M phosphate buffer for 4 hours. Individual slices were then cut into 50 mm sections, permeabilized with 0.7% Triton X-100 for 1 hour, and incubated with an optimal dilution of primary antibody in preblock solution overnight at 4°C. Sections were washed in preblock solution, incubated with an optimal dilution of the secondary antibody for 1 hour at room temperature, and then rinsed and mounted on glass slides. Following immunostaining, confocal images were obtained with a Zeiss LSM 310 laser-scan confocal microscope. Fluorescein-conjugated secondary antibodies were viewed at 488nm and Cy3-conjugated secondary antibodies were viewed at 543nm. Images were visualized with either a 10X objective or a 63 X oil immersion objective. Brightness and contrast settings were kept constant for all images taken within a given set of experiments.

BDNF and NT-3 were present in abundant quantities in the hippocampus. The highest concentration of the two neurotrophins was found in the CA1 region of the hippocampus. However, localization of the two proteins could also be detected in the CA2 and CA3 regions. In the CA1 region, BDNF and NT-3 localized to the apical dendritic processes. None of the neurotrophin-positive cells showed cytoplasmic or nuclear localization. Staining of BDNF and NT-3 were clear and punctate.

TrkB and TrkC could also be detected in the hippocampus. However, due to the poor quality of the antibodies, localization was not very clear. From preliminary experiments, it can be determined that both Trk receptors also localize to the CA1, CA2, and CA3 regions of the hippocampus. In the CA1 region, TrkB and TrkC antibodies can be visualized in the apical dendritic processes. Like BDNF and NT-3, there was no cytoplasmic or nuclear localization of the two receptors.

Further experiments will be conducted to see if BDNF colocalize with TrkB and NT-3 with TrkC in the hippocampus. Together, these experiments help provide a detailed molecular look a the role of BDNF and NT-3 in modulating synaptic plasticity.

379. Involvement of cadherin molecules in synaptic plasticity in the adult hippocampus
Lixin Tang

We have been investigating the potential involvement of classic cadherins, in particular N-cadherin (Ncad) and E-cadherin (Ecad), in synaptic transmission and LTP in the CA1 region of the adult hippocampus. Our previous studies have shown that cadherins and catenins are expressed in synaptic sites in the adult hippocampus, and suggested that N-cadherins are involved in hippocampal LTP by using an Ncad-derived "HAV" containing synthetic peptide (AHAVD) and an adhesion-blocking antibody.

To further the study, we applied Ecad-derived "HAV" peptide (SHAVS, 0.2 mM) and found it also
Reduced LTP whereas the scrambled sequence was without effect. In an attempt to achieve a more potent effect, we preincubated slices with Ncad or Ecad-derived “HAV” 17-mer peptides (mM) and found these peptides significantly inhibited LTP without affecting I/O, PTP or PPF when compared to adjacent slices preincubated with a scrambled HAV sequence. Moreover, slices preincubated in an adhesion blocking Ecad antibody also showed less LTP than adjacent control slices, whereas slices preincubated in Ncad or Ecad cytoplasmic antibodies showed normal LTP when compared to ACSF treated adjacent slices.

To ensure these different reagents affect cadherin-mediated adhesion, we performed direct centrifugal force-based cell adhesion assay on mouse L-cells expressing exogenous Ecad and found that both the Ecad-derived “HAV” peptides and the Ecad antibody inhibit Ecad-mediated adhesion to a greater degree than the Ncad-derived peptides or the Ncad antibody. Longer HAV peptides were more effective than shorter peptides, whereas scrambled peptides and preimmune IgG were without effect.

We are currently making a recombinant adenovirus vector containing a mutant Ncad cDNA which lacks most of the extracellular domain and has been shown to disrupt endogenous cadherin adhesion when expressed in target cells (Fujimori et al., 1993). We are going to infect hippocampal slices with this recombinant adenovirus to knock-out endogenous cadherin function and ask whether LTP is subsequently affected.

Reference

380. High-frequency stimulation in stratum lacunosum-moleculare gates Schaffer collateral-evoked spiking in hippocampal CA1 pyramidal cells
Hannah Dvorak-Carbone, Brian Tabas

The temporoammonic projection from layer III of entorhinal cortex to area CA1 of the hippocampus terminates in stratum lacunosum-moleculare (SLM). Activation of the temporoammonic pathway by stimulation in SLM generally results in a small glutamatergic EPSP followed by fast (GABA	extsubscript{A}) and slow (GABA	extsubscript{B}) IPSPs in pyramidal neurons.

Because of the distal location of the synapses, the response recorded at the soma of a CA1 neuron after SLM stimulation is generally very small. However, both the excitatory and the slow inhibitory components of the response to stimulation in SLM summate a higher stimulation frequencies. A cell which shows virtually no response to a single stimulus may be hyperpolarized by as much as 5 mV in response to a train of ten stimuli at various frequencies; this summation does not increase at frequencies greater than 50 Hz.

Similarly, a single stimulus in SLM applied before a single stimulus in stratum radiatum (SR; activating the Schaffer collaterals), at interstimulus intervals ranging from five to 800 ms, has little or no effect on the SR-evoked EPSP or spike. However, when a train of ten stimuli at 100 Hz is applied to SLM prior to an SR stimulus that would cause the cell to fire when applied in isolation, the SR-evoked spike is inhibited by an SLM burst-evoked IPSP. We are currently investigating whether this effect is due to interneuron activation by temporoammonic axons, or direct activation of interneurons by the stimulating electrode. These results support the idea that the temporoammonic projection may serve as an inhibitory “gate” regulating the output of CA1 pyramidal cells.

Publications
The segmental ganglia of the embryonic insect central nervous system (CNS) contain a relatively small number of neurons that make genetically defined connections. The basic ganglion architecture and many of the specific pathways are conserved between a species with large neurons suitable for cell biology studies (the grasshopper Schistocerca) and a genetic organism (Drosophila). In Drosophila, we have focused primarily on the development of the embryonic neuromuscular system, which is a simpler subset of the CNS. In each abdominal hemisegment, about 40 motor neurons send axons out within 5 motor nerve pathways, innervating 30 body wall muscle fibers in a highly stereotyped pattern. In Schistocerca, we have developed reagents that allow us to investigate motor axon development, and have also studied development of serotonergic interneurons.

We identified a set of surface proteins that control tyrosine phosphorylation and are involved in selective cell recognition during axonogenesis in the embryonic CNS. These proteins are receptor-linked protein tyrosine phosphatases (RPTPs) that have extracellular domains related to those of neural cell adhesion molecules such as fasciclin II and N-CAM. Four such adhesion molecule-like RPTPs are selectively expressed on CNS axons (including motor axons) in the embryo.

We studied the phenotypes of single and multiply mutant embryos lacking one or more of the four axonal RPTPs. Three of the RPTPs (DPTP69D, DPTP99A, DLAR) control growth cone guidance decisions along the ISN and SNb motor nerve pathways. These RPTPs function in a hierarchy to control ISN guidance past intermediate targets ("guideposts") that they encounter on their way to their target dorsal muscles. They also display partially redundant activities in controlling defasciculation of SNb axons from the common ISN pathway. At a later SNb choice point, however, DLAR acts to suppress a DPTP99A signal that favors an incorrect (bypass) axon trajectory. Our results show that the relationships among the tyrosine phosphatases are complex and dependent on cellular context. At certain growth cone choice points, two phosphatases cooperate, while at another choice point these same phosphatases can act in opposition to one another (abstract 1; Desai et al., 1996, 1997).

Mutations eliminating DPTP10D expression do not affect motor axon guidance on their own, and do not strongly synergize with the other Rptp mutations to produce such defects. Double mutant embryos lacking both DPTP10D and DPTP69D, however, display a unique CNS phenotype in which many interneuronal axons that should navigate within the longitudinal tracts instead grow across the CNS midline (abstract 2). Larry Zipursky’s group (UCLA), in collaborative studies with our group, has shown that Ptp69D single mutants display a specific defect in optic lobe innervation during larval/pupal development. In these mutants, the axons of the R1-6 photoreceptors fail to terminate in their normal target zone (the lamina), and instead continue on to the medulla, which is normally innervated only by R7 and R8 axons. In summary, our results show that specific RPTPs or combinations of RPTPs control many different pathway decisions made by motor neuron and interneuron growth cones.

To define the signaling pathways in which the RPTPs act, it will be necessary to identify their intracellular substrates and extracellular ligands. We purified a transmembrane protein, gp150, that is a probable substrate for DPTP10D, and used protein sequence information to obtain cDNA clones encoding it. The short cytoplasmic domain of gp150 contains four tyrosine-containing motifs related to SH2 domain-binding sequences found in the signaling chains of the mammalian T cell antigen receptor. These tyrosine residues can be phosphorylated by Drosophila Src kinase in transfected Schneider 2 (S2) cells, and DPTP10D reverses this phosphorylation. gp150 also associates with the downstream SH2-domain signaling molecules Drk and Dshc in S2 cells (abstract 3). We have isolated mutations in the gp150 gene, and are analyzing their phenotypes (abstract 4). In collaboration with Larry Zipursky’s group, we also analyzed the embryonic phenotypes of embryos that lack expression of the dock SH2-SH3 adaptor protein, which is a potential downstream effector for tyrosine kinases and RPTPs (abstract 5). We are continuing to analyze signal transduction by RPTPs in S2 cells, and are studying the formation of RPTP homodimers and heterodimers (abstract 6). We are also examining the roles of the protein kinase A signaling system in controlling CNS development in Drosophila (abstract 7). We had shown earlier that this system determines midline cell fates in the grasshopper.

In our current studies in the grasshopper, we have shown that the motor axon system can develop in culture, and have used drug perturbation to study the roles played by tyrosine phosphorylation in controlling guidance of motor neuron growth cones (abstract 8). We have also analyzed the roles played by tension mechanisms in controlling arborization by local serotonergic (s1) neurons (abstract 9).

Our studies on olfactory signal transduction have focused primarily on defining the ligands for odorant receptors. In collaboration with Cori Bargmann’s group at UCSF, we have shown that a nematode 7-helix receptor, Odr-10, which is required for the behavioral response of the worm to the volatile odorant diacetyl, functions as a diacetyl-specific odorant receptor in human cells (abstract 10). Finally, we have shown that the olfactory cyclic nucleotide-gated cation channel, which is involved in odorant signal transduction, is also expressed in the brain, where it may participate in the regulation of synaptic function (abstract 11; Bradley et al., 1997).

References
381. Competition and cooperation among receptor tyrosine phosphatases control motoneuron growth cone guidance in Drosophila.

Chand J. Desai, Neil X. Krueger1 Haruo Saito1, Kai Zinn

The neural receptor tyrosine phosphatases DPTP69D, DPTP99A, and DLAR are involved in motor axon guidance in the Drosophila embryo. Here we analyze the requirements for these three phosphatases in controlling growth cone guidance decisions along the ISN and SNb motor pathways. Any one of the three suffices for the progression of ISN pioneer growth cones beyond their first intermediate target in the dorsal muscle field. DLAR or DPTP69D can facilitate outgrowth beyond a second intermediate target, and DLAR is uniquely required for formation of a normal terminal arbor. A different pattern of partial redundancy among the three phosphatases is observed for the SNb pathway. Any one of the three suffices to allow SNb axons to leave the common ISN pathway at the exit junction. When DLAR is not expressed, however, SNb axons sometimes bypass their ventrolateral muscle targets after leaving the common pathway, instead growing out as a separate bundle adjacent to the ISN. This abnormal guidance decision can be completely suppressed by also removing DPTP99A, suggesting that DLAR turns off or counteracts a DPTP99A signal that favors the bypass axon trajectory. Our results show that the relationships among the tyrosine phosphatases are complex and dependent on cellular context. At growth cone choice points along one nerve, two phosphatases cooperate, while along another nerve these same phosphatases can act in opposition to one another. Current studies are directed toward the identification of the tyrosine kinase(s) regulated by these three RPTPs.

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382. Genetic analysis of the functions of DPTP10D and DPTP69D in Drosophila embryonic CNS development

Qi Sun, Sami Bahri1, William Chia1, Kai Zinn

The DPTP10D gene is adjacent (5') to a gene encoding a large protein of unknown function, Bifocal. Deletions were generated from a P element located just 3' to bifocal. One of these deletions, 59, removed all of the Bifocal and DPTP10D coding sequences. Homozygous 59 (Ptp10D) mutants are viable and fertile. The Ptp10D; Ptp69D double mutant combination produces a unique and highly penetrant CNS phenotype not seen in any other single, double or triple RPTP mutant combination. Within the CNS, three interneuronal pathways labeled by antibodies against Fasciclin II (FasII) develop within the longitudinal tracts. The medial Fas II pathway (MP1 pathway) develops normally in double mutant embryos, but the later-developing FN3 and lateral FasII pathways do not form. By stage 16, only a single fused pathway is observed in place of the normal three longitudinal bundles. Abnormal FasII-positive commissural axons are also observed in these embryos, suggesting that axons in the two outer FasII longitudinal pathways have been diverted across the midline. Mutations in another Drosophila gene, roundabout (robo), cause axons in the medial FasII bundles to grow across the midline, but do not affect axons in the more lateral pathways. robo and the Ptp10D; Ptp69D mutant combination produce an additive triple mutant phenotype in which all FasII-expressing axons appear to be diverted to the midline. robo has been shown to be downstream of commissureless (comm), a mutation that completely eliminates commissure formation. In comm mutants, all commissural axons instead follow longitudinal pathways, and no axons cross the midline. When comm is combined with the Ptp10D and Ptp69D mutations, its phenotype is partially suppressed, and axons are observed to cross the midline. These results suggest that the DPTP10D and DPTP69D phosphatases are involved in midline crossing decisions. These two RPTPs may participate in some of the same signaling processes as the Comm and Robo proteins.

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383. The transmembrane glycoprotein gp150 is a substrate for the receptor tyrosine phosphatase DPTP10D in Drosophila cells

Sarah J. Fashena1

We have begun to explore the downstream signaling pathways of receptor protein tyrosine phosphatases (RPTPs) that control axon guidance decisions in the Drosophila central nervous system. We have focused our studies on the adhesion molecule-like gp150 protein which binds directly to and is an in vitro substrate for the RPTP DPTP10D. Here, we show that gp150 and DPTP10D form stable complexes in Drosophila Schneider 2 (S2) cells and in wild-type larval tissue. The DPTP10D cytoplasmic domain is sufficient to confer binding to gp150. gp150 has a short cytoplasmic domain containing four tyrosines, all found within sequences similar to immunoreceptor family tyrosine-based activation motifs (ITAMs). We demonstrate that gp150 is tyrosine phosphorylated in wild-type larvae, underscoring the possible significance of the ITAM-like elements. Moreover, gp150 becomes tyrosine phosphorylated in S2 cells following incubation with PTP inhibitors or upon coexpression of the Dsrc tyrosine kinase. Furthermore, phosphorylated Dsrc and the signaling molecules Dshc and Drk form stable complexes with gp150, thereby implicating them in a putative gp150 signaling pathway. When co-expressed, either full-length DPTP10D or its cytoplasmic domain mediates gp150 dephosphorylation, whereas a catalytically inactive DPTP10D cytoplasmic domain does not. The neural RPTP DPTP99A can also induce gp150 dephosphorylation, but does not coimmunoprecipitate...
with gp150. Taken together, the results suggest that gp150 might transduce signals via phosphorylation of the ITAM-like elements. Phosphorylation of these elements could be modulated in vivo by RPTPs such as DPTP10D.

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384. Isolation of gp150 mutations by P-element mutagenesis
Qi Sun

Genetic studies of four Drosophila R-PTP genes have revealed their roles in axon guidance. But we still know very little about the signal transduction pathways of these RPTP genes. A former postdoc in our group, Shin-Shay Tian, isolated a new membrane protein, denoted gp150. Biochemistry studies done by Tian and Sarah Fashena (see abstract 3) showed that gp150 can specifically bind to DPTP10D, and can be dephosphorylated by DPTP10D and DPTP99A, which indicated that gp150 might be a substrate of DPTP10D and DPTP99A. No known mutants have been mapped to the gp150 locus. As we could not predict the phenotype of the gp150 mutant, we chose to do P-element mutagenesis, and screened for lines with mutated gp150 sequences. We have isolated a new P-element line, P46, with an insertion about 5 kb upstream of the gp150 open reading frame. By doing imprecise excision, we generated several deficiency lines. One of them, D24, has a deletion that extends through most of the gp150 3'-UTR. D24 is homozygous lethal, with no obvious nervous system defects detectable by staining with anti-Fasciclin II antibody. We still do not know whether gp150 expression is disrupted in P24, and whether the lethality is caused by gp150. We plan to generate more excision lines from P46, and examine mutant embryos by in situ hybridization to determine whether they affect gp150 mRNA expression.

385. Mutations affecting expression of the SH2-SH3 adaptor protein Dock block formation of a synapse on muscles 6 and 7
Chand Desai, Paul Garrity1, Haig Keshishian2, Larry Zipursky1, Kai Zinn

The Dock adaptor protein is the ortholog of the vertebrate Nck oncogene product. Dock mutations affect photoreceptor axon guidance during optic lobe innervation in larvae. Here we show that these mutations also have a subtle effect on motor axon patterning in the embryo. In embryos lacking zygotic Dock function, formation of one synapse is delayed. This synapse is formed by the RP3 neuron at the cleft between muscles 6 and 7. Dye injection studies show that the RP3 axon grows out normally to the vicinity of the 6/7 cleft, so Dock does not appear to be strongly involved in motor axon guidance. The 6/7 synapse eventually forms in Dock mutants, and muscle innervation in third instar larvae is indistinguishable from the wild-type pattern. This phenotype is identical to that previously described for the late bloomer (lbl) mutation by Corey Goodman's group. lbl Dock double mutants have phenotypes identical to those of lbl or Dock single mutants, so there is no synergy between these mutations. This result may suggest that Dock and Lbl function in the same (partially redundant) pathway for synapse formation. When both maternal and zygotic Dock function are eliminated, an additional axon guidance phenotype within the CNS is observed. In this phenotype, the lateral Fasciclin II-expressing longitudinal bundle (see abstract 2) fails to form normally.

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386. Characterization of dimer formation among fly CNS RPTPs
Bruce Burkemper

Recent crystallographic studies have shown that the first cytoplasmic domain of mammalian RPTPs, can form a dimer in which a region of each domain (denoted as the "wedge") inserts into the active site of the opposite monomer. These results suggest that a dimeric RPTP is likely to be catalytically inactive. As with receptor tyrosine kinases (RTKs), dimerization of the RPTPs may be an important means by which their catalytic activity is regulated. We are investigating whether the four Drosophila axonal RPTPs, which all have sequences related to the wedge region, can form dimers. Sarah Fashena and Anya Varshavsky have obtained preliminary evidence indicating that a heteromultimer including DPTP10D and DPTP69D can exist. To follow up on these results, I have generated expression vectors encoding epitope-tagged versions of the cytoplasmic domains of the RPTPs. These will be transiently transfected into Drosophila S2 cells. IPs followed by Westerns will identify constructs that interact with eachother in S2 cells, and are thus likely to form dimers in vivo. Various constructs with mutations in the wedge domain will then be tested to identify sequences that mediate dimerization. Finally, mutations that prevent dimer formation will serve as the basis for constructs that will be used to make transgenic flies and assay their phenotypes. We are particularly interested in examining whether heterodimers involving Dlar and DPTP99A, since our genetic experiments showed that Dlar suppresses the activity of 99A (abstract 1).

387. PKA in the development of the embryonic Drosophila midline
Te-Yi Kung

cAMP-dependent protein kinase (PKA) is known to have an important role in many signal transduction pathways, including those involved in the insect embryonic development. In grasshopper, Barry Condron earlier demonstrated that PKA activity is required for the determination of midline cell identities. In Drosophila, several labs reported that the proper function of PKA crucial for hedgehog signaling during fly imaginal disc development. To study the role(s) of PKA in Drosophila midline development, we have
take advantage of several recently available PKA transgenic constructs that either increase or decrease PKA activity. Midline specific expressions of these altered PKAs was achieved by crossing UAS-PKA lines to midline enhancer trap-Gal4 lines, and the results were interpreted by staining with different midline specific antibodies. In one cross in which PKA was constitutively turned on in rhomboid-expressing midline cells, the staining pattern obtained with an antibody against the BarH1 homeobox protein was altered. Extra cells and and mislocated cells were observed in stage 16 embryos. These effects were highly penetrant. We are currently working on the identification of these cell groups. We will also attempt to determine if reducing PKA activity using dominant negative regulatory subunit constructs can affect the fates of these cells.

388. Regulated neurite tension as a mechanism for determination of neuronal arbor geometries in vivo.

Barry G. Condron¹, Kai Zinn

Tension in neurites has been hypothesized to be an important determinant of the structures of neuronal arbors. A recent proposal suggests that tension mechanisms also generate the complex folding patterns of the cerebral and cerebellar cortices. Tension has actually been demonstrated, however, only for dissociated neurons or neuronal cell lines cultured on artificial substrates. The complex three-dimensional structures of neuronal arbors make it very difficult to study neurite tension in vivo. Here we describe a system in which tension can be demonstrated and perturbed in an intact insect central nervous system (CNS). The axon of a local serotoninergic interneuron known as s1 forms a stereotypical bifurcation in the embryonic CNS neuropil. The geometry of this bifurcation node is highly conserved between embryos and held constant during development. Current models for the development of such stereotyped geometries usually propose that they are created and maintained by neurite adhesion to localized substrates. Here we show that the structure of the s1 bifurcation node is actually determined by balanced tension between three fixed points. This was revealed by selectively transecting each of the branches that intersect at the node. Transections are followed by a rapid restructuring ('snapping') of the node geometry. The data suggest that tension-driven mechanisms determine the complex geometry of the entire s1 arbor, and might also control the geometries of other arbors in invertebrate and vertebrate nervous systems.

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389. Development of the grasshopper neuromuscular system

Kaushiki Menon

We are interested in identifying signal transduction pathways participating in neuromuscular development of insects. There exists a well-established culture system in grasshopper embryos that allows us to look at a period in development when the motoneurons are going to the target muscles. This allows us to screen different drugs that block specific signalling systems and determine their effects on the developing motoneuron pathways. We have identified a motoneuron marker in grasshopper - a polyclonal antibody made against fly synaptotagmin. Synaptotagmin (Syt) is a synaptic vesicle transmembrane protein involved in calcium-regulated fusion with the presynaptic membrane. We also generated a monoclonal antibody against the grasshopper Syt using the fly protein as the antigen. These antibodies cross-react with grasshopper motoneurons and stain the intersegmental (ISN) and segmental (SN) nerves during development. Towards the end of embryogenesis, the syt antibody stains only the synapses both in the CNS and in the periphery. We have also identified various neurons that contribute to the ISN and SN nerves. Dil labelling of the two nerves halfway during embryogenesis shows a similar pattern of labelling as that seen in the flies. Both ipsilateral and contralateral neurons send their axons into these motoneuron pathways. Currently, we are using inhibitors to block different signalling pathways by culturing the embryo in the presence of drugs, followed by immunohistochemistry with the Syt antibody. We have found that a protein tyrosine kinase inhibitor and a PI3kinase inhibitor have similar effects. They both seem to affect axon guidance rather than inhibit neurite extension as seen in the vertebrate systems. We are also conducting grafting experiments to identify the regions of the bodywall tissue that provide repulsive or attractive cues for the developing motoneuron pathways in the grasshopper embryo.

390. The C. elegans 7-transmembrane protein Odr-10 functions as an odorant receptor in mammalian cells

Yinong Zhang, Joseph H. Chou¹, Jonathan Bradley¹, Cornelia I. Bargmann¹, Kai Zinn

The nematode C. elegans exhibits behavioral responses to many volatile odorants. Chemotaxis toward one such odorant, diacetyl (butanedione) requires the function of a 7-transmembrane receptor protein encoded by the odr-10 gene. To determine whether Odr-10 protein is an odorant receptor, it is necessary to express the protein in a heterologous system and show that it responds to diacetyl by activation of a G protein signaling pathway. Here we demonstrate that human cells expressing Odr-10 on their surfaces exhibit a transient elevation in intracellular Ca²⁺ levels after diacetyl application. Volatile compounds which differ from diacetyl only by the addition of a methyl group (2,3-pentanedione) or the absence of a keto group (butanone) are not Odr-10 agonists. Behavioral responses to these compounds
are not dependent on odr-10 function, so Odr-10
specificity in human cells can explain in vivo
specificity. The apparent affinity of Odr-10 for
diacetyl observed in human cells is consistent with the
diacetyl concentration ranges that allow efficient
nematode chemotaxis. Odr-10 expressed in human cells
also responds to two anionic compounds, pyruvate and
diacetyl production by certain bacterial species. Ca^{2+}
elevation in response to Odr-10 activation is due to
release from intracellular stores.

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391. Functional expression of the heteromeric
"olfactory" cyclic nucleotide-gated channel in the
hippocampus: a potential effector of synaptic
plasticity in brain neurons
Jonathan Bradley¹, Yinong Zhang, Robert Bakin²,
Henry A. Lester, Gabriele V. Ronnett³, Kai Zinn
Cyclic nucleotide-gated (cng) channels are
important components of signaling systems mediating
sensory transduction. In vertebrate photoreceptors,
light activates a signaling cascade that causes a
decrease in intracellular cGMP concentrations, closing
retinal cng channels. Signal transduction in olfactory
receptor neurons is thought to proceed via G protein­
mediated elevation of intracellular cAMP in response
to odorant binding by 7-helix receptors. cAMP opens
the olfactory cng channel, which is highly permeable
to Ca^{2+}. Here we demonstrate by in situ hybridization
and immunohistochemistry with subunit-specific
antibodies that both subunits of the heteromeric rat
olfactory cng channel are also widely expressed in the
brain. Expression of the retinal rod cng channel,
however, can be detected only in the eye. In the adult
hippocampus, the olfactory cng channel is expressed on
cell bodies and processes of CA1 and CA3 neurons.
In cultured embryonic hippocampal neurons, the channel
is localized to a subset of growth cones and processes.
We recorded conductances with the
electrophysiological characteristics of the heteromeric
olfactory cng channel in excised inside-out patches from
these cultured neurons. We also show that Ca^{2+} influx
into hippocampal neurons in response to cyclic
nucleotide elevation can be detected using fura-2
imaging. Cyclic nucleotide elevation has been
implicated in several mechanisms of synaptic
plasticity in the hippocampus, and these mechanisms
also require elevation of intracellular Ca^{2+}. Our results
suggest that the "olfactory" cng channel could regulate
synaptic efficacy in brain neurons by modulating Ca^{2+}
levels in response to changes in cyclic nucleotide
concentrations.

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Summary: Our laboratory is devoted to the study of brain evolution and the neural bases of visual perception. This year, we have found that in species with large brains and slow development, the sex which is the primary caregiver of offspring tends to live longer (see Abstract 392). We have done comparative studies of the brain in lemurs (Abstracts 393, 394, 395) and recombinant inbred strains of mice (Abstract 396). We have also studied the mechanisms of size and distance perception (Abstracts 397, 398, 399).

392. Nurturing and survival in primates: Caretakers live longer
John Allman, Aaron Rosin, Roshan Kumar
In primate species, relative brain mass is scaled with the time required to mature. Thus, the rearing of large-brained offspring requires parental support for commensurably long periods. Moreover, large brained offspring are mostly single births and the interbirth intervals are long. The parents must live a long enough time to sustain the serial production of a sufficient number of young to replace themselves (allowing for the death of offspring before they can reproduce). Therefore, we hypothesize that in large-brained, slow developing species, the sex that bears the greater burden in the nurturing of offspring will tend to survive longer. Our hypothesis runs contrary to the common sense notion that the extra burden of carrying a heavy infant would be associated with an increased risk of dying. We have tested our hypothesis by constructing survival tables for primates and comparing the tables according to sexual division of nurturing.

In Figure 1A, human data from the U.S. population from 2 historical periods indicate a significant female survival advantage throughout life in 1900 and in 1983. The female advantage is evident for both periods in spite of large differences in the mortality rates between these periods. Similar female advantages are evident in the survival tables for most countries in both the contemporary period and throughout the 20th century. What has not changed during this period is that human females have remained the primary caregivers for their offspring. Figure 1B indicates that female gorillas have an even greater survival advantage than contemporary human females. We have also found a similar female advantage in orangutans, Pongo pygmaeus. Figure 1C shows that female white-handed gibbons (Hylobates lar) also enjoy a substantial survival advantage. Gibbons live in monogamous pairs, but the females carry the infants without paternal assistance. Figure 1D indicates that in contrast to the other apes, male siamangs (Hylobates syndactylus) possess a small survival advantage. Like gibbons, siamangs are monogamous; however siamang fathers are the only apes to carry their infants, and their offspring appear to be more closely bonded to them than to their mothers. Figures 1E and F indicate that past sexual maturity, owl monkey (Aotus) and titi monkey (Callicebus) males have a substantial survival advantage. Both owl monkeys and titi monkeys live in monogamous pairs and the fathers carry their offspring from soon after birth except for brief nursing periods on the mother and occasional rides on older siblings. We found that white-faced saki females (Pithecia pithecia) have a small survival advantage. Sakis are members of the same subfamily, the Pithecinae, as owl and titi monkeys, and generally are reported to be monogamous as well, but saki mothers carry their infants without paternal assistance. In golden-lion (Leontopithecus rosalia) and emperor tamarins (Saguinus imperator), we also found a small female advantage. Tamarin fathers carry infants in cooperation with other family members, but the infants are usually born as twins, and thus there is less necessity for long parental survival. In Callimico goeldii we found little difference between male and female survival. In Callimico, births are single, and the father carries the infant in cooperation with other family members; however, maturation occurs at only 1 year of age, far earlier than any other monkey.

Our hypothesis is also supported by data from the mammal with the largest brain, the killer whale, Orcinus Orca. The whale calves are single births with long interbirth intervals, and they remain in close association with the mother throughout their lives. The fathers appear to have little role in parenting. A long-term demographic study of natural populations of Orcinus indicates that the female life expectancy is about 30 years longer than in males (Olesiuk et al., 1988).

Reference
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393. Cytochrome oxidase blobs in primary visual cortex of Eulemur fulvus and Microcebus murinus
Richard Jeo, Martin Sereno, John Allman
We had the unique opportunity to study the brain tissues from two lemuroid primates: the brown lemur (Eulemur fulvus) and the mouse lemur (Microcebus murinus). The Eulemur, a member of a breeding colony at Caltech, presented with a large, fast growing testicular carcinoma that was deemed inoperable and was clearly causing the animal significant discomfort. It was therefore decided by the attending veterinarian...
Figure 1. Sex differences in survival in primates. The human data were obtained from the United Nations Demographic Yearbook. The survival graphs for non-human primates were calculated from raw birth and death records obtained from zoo studbooks.
that the animal should be euthanized. The animal was deeply anesthetized and was perfused transcardially with a buffered saline solution. The Microcebus, obtained from Duke University, was found dead in its cage and was not perfused. In both cases, the brain was quickly removed and photographed, and each hemisphere was physically flattened and fixed. We then cut 50 μm thick frozen sections parallel to the cortical surface and stained for cytochrome oxidase (CO) activity. For both Eulemur and Microcebus, CO staining revealed a system of dark staining CO blobs in primary visual cortex. Area V2 of Eulemur showed some patchy CO staining, but a stereotyped system of prominent, dark staining, thick and thin stripes (present in Old and New World monkeys) was not evident. Microcebus did not show any evidence of V2 stripes; however, the absence of CO stripes in Microcebus V2 could be attributed to the condition of the brain tissue at the time of histology. In Eulemur we were able to identify area MT, primary auditory cortex (A1) and primary somatosensory cortex (SI), based on dark cytochrome oxidase staining patterns. These findings, together with recent data from the dwarf lemur (Cheirogaleus medius), indicate that two characteristic features of the visual cortex in anthropoid primates, namely the cytochrome oxidase blobs in V1 and cortical visual area MT, are also present in lemuroid primates. (Preuss and Kaas, Brain Behavior and Evolution 1996, Paolini et al., Society for Neuroscience Abstracts 1994). On the other hand, the presence of prominent thick and thin CO stripes in V2 may be a specialization of the visual system in anthropoid primates.

References

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394. The structure of the visual pulvinar in nocturnal and diurnal prosimians
Emmanuel P. Gilissen, Evelyn McGuinness, Colin T. McDonald1, John Allman

Some aspects of primate brain structure, such as the distribution of acetylcholinesterase within the lateral geniculate nucleus (LGN), have been related to the daily activity patterns. This thalamic nucleus shows a nocturnal-like pattern of acetylcholinesterase distribution in Tarsius, Aotus and in all strepsirhine primates including the diurnal Propithecus verreauxi and Eulemur fulvus. It is quite possible that these lemurs were nocturnal until recently and that the structure of their LGN still reflects a nocturnal pattern despite of their diurnal habits. This feature is consistent with the stability of the LGN in primate brain evolution. In contrast, the pulvinar nuclei of the thalamus are known to be more progressive than the other thalamic nuclei. Within the thalamic nuclei, both the LGN and the inferior (visual) pulvinar have a visuotopic organization. Identified subdivisions of the inferior pulvinar suggest that a common organization exists in both Old and New World monkeys, including Aotus. In this work, we analyze the structure of the inferior pulvinar in diurnal and nocturnal prosimians including Tarsius. Preliminary results suggest that the subdivisions observed in anthropoid primates are lacking in prosimian primates, reflecting a phylogenetic pattern rather than nocturnal/diurnal lifestyles.

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395. Topographical localization of iron in brains of the aged fat-tailed dwarf lemur (Cheirogaleus medius) and grey lesser mouse lemur (Microcebus murinus).
Emmanuel P. Gilissen, Pratik Ghosh, Russell E. Jacobs, John M. Allman

Iron deposits in the human brain are characteristic of normal aging but have also been implicated in various neurodegenerative diseases. Among non-human primates, strepsirhines are of particular interest because of their high iron absorption observed in captivity. In addition, cheirogaleids have proved to be good models for the study of normal and pathological cerebral aging. This study was therefore undertaken to explore iron localization in the brain of aged cheirogaleids (mouse and dwarf lemurs) with histochemistry and magnetic resonance microscopy. Results obtained with both techniques were consistent. There was no difference between the two species. The young animals (3 years old) showed no iron deposits. In the old animals (8 to 15 years old), iron pigments were mainly localized in the globus pallidus, the substantia nigra, the neocortical and cerebellar white matter, the thalamus, and in anterior forebrain structures, probably including the nucleus basalis of Meynert. These results correspond to previous findings in monkeys and humans. The small size, rapid maturity, success in breeding in captivity, and relatively short life expectancy of the cheirogaleids confer considerable advantages on them as a model system for further studies on the mechanisms and effects of iron absorption in primates. Microscopic NMR images clearly reveal many features seen with the histochemical procedure and appears to be a powerful method for simultaneous in vivo visualization and monitoring of age-related changes in brain iron.

396. A quantitative trait loci (QTL) analysis of the forebrain, hindbrain, olfactory bulbs, and cerebellum in the mouse
Emmanuel P. Gilissen, Robin W. Williams

Normal variation in the size of different parts of the brain can be much greater than that of the whole brain (Gilissen and Zilles, 1996). With this in mind, we have begun to map QTLs responsible for variation in the size of well-defined CNS structures, such as the olfactory bulb and cerebellum, that have unique cell
architecture, connections, and patterns of gene expression. Weight of the cerebellum, olfactory bulbs, forebrain, and hindbrain after fixation were measured in 38 recombinant inbred strains of mice (26 BXD and 12 BXH) and 3 parental strains (n brains = 210). All four regions show significant variation among strains with coefficients of variation between 8% and 11%. Weight of the olfactory bulbs ranges from 15 to 23 mg, and that of the cerebellum from 46 to 64 mg. The correlation between CNS components is moderately high with typical $r^2$ values ranging from 0.4 to 0.5 ($r = 0.7$). However, the size of the olfactory bulb shows surprising independence from the rest of the forebrain ($r^2 = 0.3$). To eliminate variation associated with overall brain weight, we computed linear regression residuals for each region. These residuals were then mapped using MapManagerQT. Candidate QTLs with specific effects on the size of the cerebellum were mapped to Chrs 8 and 12 (p nominal $< 0.001$). We also identified a major effect locus controlling the weight of the forebrain to Chrs 8, and confirmed the presence of a major brain weight QTL on Chr 11. QTLs that modulate the weight of the olfactory bulbs and hindbrain have not yet been mapped.

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397. Where in the what pathway: Object distance modulates responses in areas V1 and V4

Richard Jeo, Allan Dobbins1, Jozsef Fiser2, John Allman

To explore the neural basis of size perception, we measured retinal image size tuning properties of cortical neurons at different viewing distances in awake monkeys. Monkeys were trained to fixate a spot on a movable monitor while viewing stimuli of a variety of sizes presented in the cells' classical receptive field. We measured responses to stimuli scaled such that the retinal image size remained unchanged at 3-5 different viewing distances. We found that response magnitude is often strongly modulated by viewing distance. Approximately half of the cells (in areas V1, V2, and V4) showed significant changes in response with viewing distance. Of these, the most commonly observed cells (45%) had responses that were greatest for the shortest distance of the stimuli from the monkey ("Nearness" cells), but the opposite type, cells that responded best for greater distances ("Farness" cells, 34%), were also common. A small number of cells exhibited non-monotonic response with distance. These results imply that distance may act on size information by modulating the gain of the neural response.

It is generally believed that two distinct processing streams exist within primate visual cortex: a dorsal stream involved with spatial processing and a ventral stream (which includes area V4) involved with object recognition. Distance modulation in V4 demonstrates that visuospatial information is not exclusive to the dorsal pathway. We posit that a three dimensional spatial code is common to both processing streams.

Absolute distance is a crucial parameter governing the responses of many V1 and V4 neurons. The perception of absolute distance probably involves interactions between two opposed populations of neurons: Nearness and Farness cells. The combination of size and spatial information in individual neurons is consistent with these neurons serving as a basis for size constancy.

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398. Dynamic size illusions and what they tell us about the physiology of size contrast and size constancy

David Rosenbluth, John Allman

We have discovered some new size illusions which are notable firstly because they are visually quite striking, secondly because they tell us something about the physiology behind size perception, and thirdly because they make good stimuli for electrophysiological experiments. These illusions are dynamic versions of the more familiar classical size illusions. While the classical illusions involve a comparison of the sizes of two identical objects each of which is embedded in a different visual context which modulates our perception of the size of the embedded object, the new illusion involves the perception of the size of a single object which is embedded in a visual context which is dynamically changing. Since this context modulates our perception of the size of the object, and the context is changing, we perceive the object size to be changing dynamically, though it is in fact constant. The object has the appearance of being elastic and of being either stretched or compressed. Most of the classical size illusions can be easily transformed into dynamic illusions but we have focused our attention on two for which the effect seems strongest. These are the Lyer-Muller Illusion and a modification of the Ebbinghaus effect using concentric circles.

There are several notable psychophysical features of these dynamic illusions which lead to a predictive model. The modulatory effect of the context saturates, producing a change in perceived size change of up to 10% of the actual size (about the same as for the static illusions). Oscillation of the context produces an oscillatory percept of object size, with a cutoff frequency of 1-2 Hz. Oscillation at higher frequencies produces a rapidly diminishing effect. Attempts to null the modulatory effect by dynamically changing the size of the target object, are complicated by the fact that the modulatory effect is non-linearly related to the magnitude of the modulatory context. Our model supposes that this modulatory effect stems from the effect of inputs from neurons responding to context on the firing rates of neurons which are tuned for size; that the strength of the connection between the modulatory neurons and the modulated neurons is relatively weak; that the connection between the modulatory inputs and the size tuned neurons is relatively slow; and that the saturation of the
modulatory effect is due to the saturation of the response of the neurons responding to the context.

From this model one would predict that the relationship between the magnitude of the modulatory signal and the modulatory effect is a sigmoidal one. Using these ideas we have successfully been able to null the effects seen in the dynamic size illusions. We are currently using this nulling procedure in psychophysical tests to quantitatively characterize this phenomena.

There is one other notable qualitative observation. Since the object appears to be elastic, we were interested in where most of the stretching was occurring. Was the object mostly stretching at the edges, or was it expanding and contracting isotropically throughout its entire extent? By putting a texture on the object and observing the transformation of the texture elements with the induced change in size of the object, we ascertained that the size change seemed to be uniform throughout the object. Texture elements near the center of the object appeared to expand as much as the texture elements near the boundaries of the object, when the object appeared to expand. For example, in the Lyer-Muller Illusion, we used a dashed line rather than a solid line in the center, and it was observed that the dashed components expanded as the length of the line appeared to expand, and that the location of the dashed elements on the line did not appear to make any noticeable difference.

399. Size, depth, and proportion
David Rosenbluth, John Allman

Depth cues influence size perception to produce size constancy effects. The Ponzo Illusion is a classical psychophysical demonstration of this effect. The functional role of such effects is thought to be in compensating for the variation of retinal image size with distance of the object from the observer to extract absolute size information. Electrophysiological studies from our lab have shown that depth cues modulate the gain of retinal size tuned neurons and this physiological phenomenon may underlie the perceptual phenomenon.

The size of neighboring objects also has a significant influence on size perception. This influence usually produces size contrast effects, which is well illustrated by the classical illusion called the Ebbinghaus illusion. The functional role of such effects is less clear. We hypothesize that a signal conveying information about the relative size of neighboring objects (which we will refer to as proportion information) would be useful directly for object recognition, and indirectly for depth calculations. Contrast effects produce an exaggeration of size differences. We propose that such an exaggeration might be useful in a task such as face recognition where it has been demonstrated that caricature, which is simply the exaggeration of the proportions of facial features, improves face recognition. This information would also be useful indirectly for the computation of depth information from size gradients. One advantage a proportion signal in this context is that it is independent of object distance and so can be used prior to good depth information. The influence of the relative size of objects on our perception of depth can be illustrated by the fact that depth seem to be compressed in pictures taken with a telephoto lens while depth is exaggerated in pictures taken with a wide angle lens.

Both constancy and contrast effects act and interact simultaneously to produce our perception of size and depth, yet they are always studied independently of each other. We are currently conducting psychophysical experiments to study the interaction of these two effects, to measure our sensitivity to proportion differences and to absolute size differences. These experiments will be conducted in parallel with electrophysiological experiments to measure the tuning of neurons to proportion information.

Publications

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Senior Research Fellows: David Bradley, Yale Cohen, Kenneth Grieve, Larry Snyder
Research Fellows: Jyl Boline, Boris Breznen, Chris Buneo, Jim Crowell, David Dubowitz, Alexander Grunewald, Sohaib Kureshi, Philip Sales, Krishna Shenoy, Xindong Liu, Jing Xing
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Summary: Work in my laboratory has focused on the role of the posterior parietal cortex in visual-motion integration, spatial perception, and visual-motion analysis. The posterior parietal cortex is the end point of one of the two major streams of visual processing in the primate visual cortex. The pathway to the parietal cortex is located in dorsal areas of the extrastriate cortex and is involved in spatial aspects of visual processing. It is functionally distinct from the more ventral pathway, which is concerned with color and form perception.

We are studying the role of this area in the kinematics of visual-motor integration. At issue is the fact that visual information is gathered in retinal coordinates and is represented in many structures retinotopically. However, at some point in the nervous system, this information must be converted to spatial coordinate frames for programming accurate motor activity in the world. We have found that the posterior parietal cortex transforms visual information from retinal to head- and body-centered coordinates, and we are presently studying the mechanisms by which this is accomplished.

Recently we discovered an area in the posterior parietal cortex (the lateral intraparietal area, LIP) which is involved in the planning of eye movements. We are currently studying the role of LIP in the planning of sequences of eye movements, how LIP activity changes with changes in motor plans, and how area LIP can use both visual and auditory information in encoding eye movements.

Several cortical areas within the posterior parietal cortex are involved in processing higher order aspects of motion perception. We have developed novel motion stimuli using high-speed computer graphic techniques to study the role of these areas in motion analysis. We use these stimuli to test the monkey motion processing system psychophysically and directly compare it to that of humans. These experiments have focused on the ability to perceive the structure of objects or the environment based purely on motion cues. We have also begun to use these powerful motion stimuli in conjunction with lesion and single cell recording techniques to examine the processing role of each cortical level in the motion processing pathway that includes primary visual cortex, extrastriate cortex and areas in the posterior parietal cortex.

Finally, we have been examining how motion information is used for navigation. One question we are examining is how extra-retinal signals coding eye and head movements are combined with visual stimulus motion signals for the purpose of path perception during self motion.

400. Primate area MT activities reflect the perception of depth-from-motion

D.C. Bradley, G.C. Chang, R.A. Andersen

Monkeys derive a sense of depth from visual motion cues. For example, the 2D projection (shadow) of a revolving, transparent cylinder with opaque dots on its surface appears 3D, even though the image is flat. Using such displays, we showed (Chang et al., 1996) that depth perception was correlated with MT activities in a rhesus monkey. We have since tested a second animal using dual scleral search coils (to measure depth of fixation) and studied the influence of speed gradients. Monkeys fixated while viewing 2D projections of revolving, random-dot cylinders, then reported the direction of the front surface with a saccade. Without disparity cues, 2D projections do not specify which surface is in front, so the monkeys' answers only reflect the "surface order" they perceive (e.g., front-right, back-left). We also showed cylinders whose surface order was specified with varying amounts of disparity (front-to-back differential, 0.075° - 0.6°). Monkeys consistently performed the task as required, reporting above chance at all non-zero disparities. Depth of fixation was unaffected by cylinder viewing. In concurrent MT recordings, cylinders were oriented to align dot motions with each neuron's preferred-antipreferred direction axis. Each neuron's preferred surface order was judged from (correct) responses to the highest-disparity cylinders, whose surface order was perceptually unambiguous. In contrast, surface order in low- and zero-disparity cylinders was often perceived differently on different trials. In 36/97 (37%) of the neurons, responses differed with perceived surface order in one or more of the stimuli (p<.05, ttest). Of these, 27 responded better when the perceived surface order matched the neuron's preferred surface order (correlated effects) while only 9 behaved oppositely (anticorrelated effects). When dot speeds for each surface were made equal (i.e. two flat surfaces sliding over each other), 7/23 neurons (30%) still showed significant perceptual correlates (correlated/anticorrelated effects = 6/1), indicating that differential MT responses do not strictly depend on speed gradients. In summary, MT activities are correlated with bistable depth-order percepts evoked by transparent stimuli. This suggests that MT has a central role in depth-from-motion perception in primates.

References

401. Frames of reference in primate parietal cortex: Separation between 7A and LIP; physiology and simulation

K.L. Grieve, I.H. Snyder, J. Xing, R.A. Andersen

It has been shown that proprioceptive and vestibular signals can modulate responses of posterior parietal neurons in macaques undertaking visual tasks (Snyder et al., 1994). We have investigated the extent to which these influences are separated in parietal...
cortex, by unit recordings in monkeys undertaking saccadic tasks with either rotation of body/ head (proprioceptive: body centered coordinates) or body and head (vestibular: world centered coordinates) prior to the task. Rotations were carried out in the dark. Between tests the animal was rotated to body/head at zero, and room lights briefly turned on. Cells were assigned to parietal fields 7a or LIP on the basis of the position of the track in the chamber, depth of the electrode within a track and response type elicited in the saccadic tasks. Of 31 7a cells, 22% showed response modulation by vestibular input, with only 2% showing proprioceptive modulation. Of 59 LIP neurons, 34% showed modulation by proprioceptive input, and only 3% by vestibular input. Mixed responses were seen in only 3 and 2% respectively.

A three-layered neural network was trained to modulate "retinotopic" responses by "proprioceptive" or "vestibular" signals to provide separate "body-centered" and world centered" maps. Hidden units of this network, trained with equal inputs from both sources, showed, after training, near complete separation closely matching the grouping of the parietal cells above.

We suggest that such separation of influences provides an economic means by which different inputs modulate visual function, resulting in different coordinate frames. These areas can thus provide distinct inputs to other cortical areas subserving different functional roles; LIP feeding to the FEF, involved in control of ongoing saccadic eye movements; and 7a to parahippocampus, involved in construction of spatial memory.

References

402. Auditory responses in LIP I: Training effects
J. F. Linden, A. Grunewald, R. A. Andersen
During the stimulus period of a memory-saccade task, neurons in the macaque lateral intra-parietal area (LIP) show spatially tuned responses to both visual and auditory cues. However, in monkeys not yet trained to make auditory memory saccades, behaviorally irrelevant stimuli evoke visual responses but no significant number of auditory responses (Linden et al., 1996). Does auditory memory-saccade training increase the responsiveness of LIP neurons to auditory stimuli? We trained two macaques to fixate through presentation of behaviorally irrelevant visual and auditory stimuli at two locations, left and right. We recorded while the monkeys were performing this task, both before and after the monkeys had also been trained to perform visual and auditory memory saccades. Among 78 neurons recorded in one monkey before auditory memory-saccade training, we found 16 (21%) spatially tuned visual responses and 3 (4%) auditory responses (t-test left vs. right response, p<0.05). The number of auditory responses was not significant (Fischer-Irwin test, p<0.05). After training, we recorded 68 cells using the same stimulus conditions but different selection criteria: we favored cells which responded either to visual or auditory stimuli on a search task. We found 33 (49%) visual and 5 (7%) auditory responses. Again, the number of auditory responses was not significant. To control for differences in the selection criteria before and after training, we normalized the number of auditory responses by the number of visual responses in each experiment. We thus obtained auditory/visual response ratios of 19% before training and 15% after training. We conclude that auditory memory-saccade training does not significantly increase the responsiveness of LIP neurons to auditory stimuli, and that behaviorally irrelevant auditory stimuli evoke very little response in either naive or trained animals.

References

403. Auditory responses in LIP II: Behavioral gating
A. Grunewald, J.F. Linden, R.A. Andersen
Neurons in the lateral intra-parietal area (LIP) of macaques respond both to visual and auditory stimuli during the stimulus period in a memory-saccade task. Do auditory signals arise in LIP only when the auditory stimulus is behaviorally relevant? We recorded from LIP neurons while a monkey performed two tasks. In the memory-saccade (M) task the monkey fixated a central LED while visual or auditory targets were briefly presented. The monkey maintained fixation until the central LED was extinguished, and then made a saccade to the remembered target location. In the fixation (F) task the monkey also fixated a central LED while stimuli were briefly presented, but at the offset of the central LED the animal maintained fixation. Trials for M and F tasks were blocked, but in each block visual and auditory trials were pseudorandomly interleaved. We recorded the activity of 68 neurons during both tasks. For visual stimuli, about the same number of cells had spatial tuning in the Mand F tasks (53% and 49% of cells, respectively). However, for auditory stimuli many more cells exhibited spatial tuning in the M task (16%) than in the F task (6%). To study population effects we performed a two-dimensional line fit to the response in the F task vs. the response in the M task. The slope was 1 for visual stimuli, and 0.3 for auditory stimuli (significantly below 1, p<0.05). Thus across the population visual stimulus-period responses were not task-dependent, while auditory stimulus-period responses were. Thus a large component of the auditory signal reaches LIP only when the animal plans an eye movement. LIP appears to be a default visual-saccade area, but it can represent targets for saccades regardless of modality.
404. Self-motion path perception during head and body rotation

J.A. Crowell, M.S. Banks, K.V. Shenoy, R.A. Andersen

Purpose: We examined extra-retinal sources of information (vestibular canals, neck-proprioception, efference copy) contributing to the estimation of self-motion during head rotations. Methods: Subjects adjusted a response probe to lie on their perceived efference copy) contributing to the estimation of self-information (vestibular canals, neck-proprioception, linear translation over a ground plane while making the following kinds of gaze rotations: (1) head-on-body (active and passive); (2) passive full-body; and (3) passive body with head stabilized in space. Judgments of self-translation were compared to a motion during head rotations.

Results: Subjects adjusted a probe to lie on their perceived path of self motion after viewing displays simulating linear translation over a ground plane while making the following kinds of gaze rotations: (1) head-on-body (active and passive); (2) passive full-body; and (3) passive body with head stabilized in space. Adjustments of self-translation were compared to a motion during head rotations. Adjustments of self-motion were as large as in the simulated condition when the display was consistent with the neck rotation; judgments were accurate when it was consistent with the (zero) vestibular stimulus.

Conclusions: (1) Self-translation is perceived accurately during head rotations. (2) A combination of vestibular and neck-proprioceptive stimulation is required; neither source of information is sufficient on its own. (3) Our data are consistent with the idea that extra-retinal signals are used to determine whether a visually-specified gaze rotation is due to an eye or head movement (expansions) or perpendicular to the eye movement (rotations). These findings suggest that while MSTd contributes to heading perception, its particular role may vary with behavioral conditions.

References
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405. Perception and neural representation of heading during gaze-rotation

K.V. Shenoy, J.A. Crowell, D.C. Bradley, R.A. Andersen

We recently reported that roughly half of primate cortical area MSTd neurons use eye-pursuit and VOR-cancellation signals to compensate for the optic flow focus (FOE) shifts induced by smooth gaze shifts (pursuit-eye movements: Bradley, et al., 1996; and full-body rotations: Shenoy et al., 1996.) This and evidence that microstimulation influences heading judgements (Britten, 1996) strongly suggest that MSTd participates in the computation of heading. Several studies have found that humans accurately judge heading during eye pursuit, but heading perception during full-body rotation has not been reported. Subjects adjusted a probe to indicate the FOE after viewing displays simulating linear translation toward a wall during pursuit-eye movement or full-body rotation (as in physiology experiments). Compensation was high during eye pursuit (130 +/- 90%, mean +/- SD, n=4) but nearly absent during full-body rotation (4 +/- 1%, n=3). Similar results were obtained in a path perception task simulating translation over a ground plane: 89.9 +/- 14.1% (n=9) and 4.4 +/- 9.0% (n=8), respectively (Crowell et al., 1997). On the other hand, MSTd neural activity suggests nearly equal compensation in the two tasks. Neurons responded similarly during pursuit-eye movements and full-body rotation (one hemisphere, n=56): 79% had preferred direction differences <= 90 deg and directional indices were not significantly different (P=0.12, t-test); compensation was 9.86 +/- 3.44 deg and 8.14 +/- 3.46 deg (mean +/- SEM), respectively, and was not significantly different (P=0.62, t-test); and compensation in individual cells is significantly correlated (rs = 0.47, P < 0.001, Spearman) with near unity slope (1.01, least-squares). These findings suggest that while MSTd contributes to heading perception, its particular role may vary with behavioral conditions.

Reference

406. Comparison of eye pursuit effects on expanding and rotating motion patterns

M.A. Maxwell, J.A. Crowell, D.C. Bradley, R.A. Andersen

A pursuit eye movement causes rigid motion of the entire retinal image in the opposite direction of the eye movement. When viewing an expanding or rotating stimulus, this laminar motion caused by eye movement shifts the center of the retinal motion pattern (the focus of expansion or rotation) in the direction of the eye movement (expansions) or perpendicular to the eye movement (rotations). Recent results (Bradley et al., 1996) indicate that expansion- and rotation-selective neurons in extrastriate area MSTd of macaque use an extra-retinal signal (efference copy or proprioception) to compensate for the shift in the retinal motion focus caused by pursuit eye movements. Here we compare human abilities to compensate for the effects of eye movements using similar expanding and rotating patterns of random dots. Displays were identical to those used by Bradley et al. (1996). Perceptual errors in localizing motion foci were estimated using two psychophysical procedures, a stepwise method of adjustment (n=5) and a variant of the QUEST maximum-likelihood estimator (n=3). Constant errors were always in the appropriate direction.
perpendicular for rotations). Inter-observer variability was high, independent of procedure: the extent of compensation varied from 51-182% (mean 105%) with expanding patterns and from 22-54% (mean 40%) with rotating patterns. However, every observer demonstrated better performance-on average by a factor of 2.6-with expansions than with rotations. This finding is interesting in light of the finding of Bradley et al. (1996), that rotation-selective MSTd cells demonstrate a similar degree of compensation to expansion-selective cells; we speculate that the perceptual difference may reflect differences in the relative numbers of expansion-and rotation-selective cells. A similar argument has been made to explain the difference in perceived speed between comparable expanding and rotating patterns. However, every observer demonstrated better performance-on average by a factor of 2.6-with expansions than with rotations.

References

407. Lesion of a macaque parietal area produces visual extinction in an egocentric framework

C.-S. R. Li, K. L. Grieve, R. A. Andersen

Purpose: Lesion of the posterior parietal cortex in humans results in visual extinction, in which the patients ignore the contralateral stimulus when an ipsilesional stimulus is present. In this study we demonstrated the same phenomenon in behaving primates by selectively lesioning the lateral intraparietal area (area LIP) in the posterior parietal cortex.

Methods and Results: By interleaving regular saccade trials with "extinction" trials, in which two targets appeared on opposite sides of the fixation point, we showed that, compared to control experiments, the monkeys made more ipsilesional saccades in the extinction trials, after muscimol injection in area LIP. Varying the initial fixation point (while keeping the targets at the same retinal locations) showed that the severity of extinction was modulated by the starting eye position in a manner consistent with an egocentric (head- or body-centered) coordinate frame. In a different paradigm, a cue preceded the saccade target at the same location. Two targets appeared at opposite sides of the fixation after the presentation of the cue in the "extinction" trials. In either the regular saccade or extinction trial, the monkey was required to make a saccade to the cued location to receive a reward. It was found that the monkey made more errors when he was cued to make a contralesional saccade after area LIP was inactivated. Furthermore, the frequency of errors was strongly affected by initial eye position.

Conclusions: These results provide evidence that area LIP plays a role in the coding of locations in an egocentric coordinate frame.

408. A change in motor intention, without a spatial shift in attention, activates posterior parietal cortex in monkey

L.H. Snyder, A.P. Batista, R.A. Andersen

Activity in the posterior parietal cortex of monkey has been posited to reflect specific motor intention. The lateral intraparietal area (LIP) and nearby parietal reach region (PRR) are differentially active when eye or arm movements are planned to previously memorized locations. However, other studies have suggested that parietal cortex may encode the spatial locus of visual attention, or may even be involved in spatial shifts of attention. We studied how a change in motor intention, without a spatial shift in attention, would affect LIP and PRR.

Animals fixated and pointed to a central target. A colored cue (150 ms) on either side of the fovea instructed the type (red=saccade, green=reach) and direction of an upcoming movement. Movement was delayed until fixation point offset (2.5 s). On some trials, a second cue appeared 0.75 s after the first. Critically, the second cue was always at the same location as the first. As a result, the second cue sometimes changed the motor plan, but never shifted spatial attention. LIP responses were greater when a saccade was instructed (n=20), and PRR responses were greater when a reach was instructed (n=17). Second cue responses were also motor specific, despite no spatial shift in attention. Furthermore, an instruction to change motor intention evoked a large response, provided that the change was to a preferred movement (a saccade in LIP or a reach in PRR). In LIP, the largest response to a second cue occurred when red followed green. Red following red elicited a smaller response, and green after either red or green was also less potent. In PRR, the largest response occurred when green followed red. These results confirm that LIP is specifically involved in the intention to saccade, and PRR is specifically involved in the intention to reach. Both areas may play a role in shifting motor intention.

409. Time course of the eye position signal in posterior parietal cortex

B. Breznen, C.-S. R. Li, R. A. Andersen

Approximately 20% of posterior parietal cortical neurons code the position of the eyes in the orbit. In the current study we compared the time course of the eye position cells' activity during saccades to visible targets and during memory saccades, both performed in darkness. For visual saccades, the pooled activity of the eye position cells begins to rise 189 ms before the eye movement. In individual cells, 52% show an activity increase before the beginning of the saccade. In these cells the response often consists of a transient component superimposed on sustained tonic activity associated with the new position of the eyes in orbit. In contrast, for the memory saccades the increase of the eye position cells' activity is aligned with the beginning of the eye movement. The cells show very
little or no transient response to the presentation of the
target cue. They are also not active during the memory
period. Their pooled activity begins to rise 2 ms before
the beginning of the eye movement. Thus eye position
cells in posterior parietal cortex show task-dependent behavior: in visual saccades, a target that cues
saccades towards the preferred orbital eye position
of the cell also primes that cell's response; in memory
saccades the appearance of the visual target has no
effect on cell activity; rather, the increase of the
activity is aligned with the movement. The fact that
the eye position signal does not lag behind the eye
movement for memory saccades suggests that at least
the early component of the eye position signal is
computed using corollary discharge of the saccade
command. The even earlier appearance of the eye
position signal does not lag behind the eye
movement. Thus eye position

The process is optimal in the maximum-likelihood
position, perhaps using a gain field mechanism.

The new technique improves on previous
methods in that it makes only weak assumptions
about the distribution of spikes, runs automatically
in real-time, and resolves overlapped spikes.

410. Extra cellular recording from adjacent neurons: I.
A maximum-likelihood solution to the spike
separation problem
M. Sahani, J. S. Pezaris, R. A. Andersen
We present a two-stage solution to the problem of
spike separation in extracellular recordings and apply
it to data collected using tetrodes in macaque cortex.
The process is optimal in the maximum-likelihood
sense, resolves spike superpositions, and can be
executed on-line.

In the first stage, we identify the probability
densities of spike shapes generated by different
neurons in the recording. Spike waveforms are
extracted from the first few minutes of the recording
using a simple threshold. The set of waveforms is
modeled by a hierarchical mixture of multi-
dimensional Gaussians using an Expectation-
Maximization algorithm (Dempster et al., 1977).
Suitable priors can be introduced into the gating
functions of the hierarchy to reflect the constraints
of refractory period and spike-shape variability.

In the second stage, we detect events on-line and
resolve superposed spikes using a multiple matched
linear filtering scheme based on the density parameters
identified as above. The detection of overlapping
spikes is achieved by adaptive thresholds that model
the interaction between spike waveforms. This method
is optimal for Gaussian mixture densities, does not
depend on orthogonalization of the space of waveforms,
and can be run in real-time.

The new technique improves on previous
algorithms in that it makes only weak assumptions
about the distribution of spikes, runs automatically
in real-time, and resolves overlapped spikes.

411. Extra cellular recording from adjacent neurons: II.
Correlations in macaque parietal cortex
J. S. Pezaris, M. Sahani, R. A. Andersen
Multiple single unit extracellular recordings
were made using tetrodes in macaque posterior parietal
cortex while the animal was performing a visual
memory saccade task. Recordings were made over a
4 by 4 mm area at both superficial and deep locations
in one hemisphere. Signals were analyzed using an
Expectation-Maximization algorithm for spike
separation based on spike peak height or the first
two principle components of spike shape. 25 sites
were selected for analysis based on clarity of
separation, yielding 64 total pairs of neurons with
a mode of 3 cells per site.

Crosscorrelograms of spike times were computed
for each pair of isolated cells at each site, normalized
by subtraction of the shuffle predictor, and classified
according to features discovered. 10 pairs (16%) showed
broad peaks (5 ms or greater) spanning zero
delay consistent with distant common input. 13 pairs
(20%) showed medium peaks (2-5 ms) either spanning
zero delay or at small offsets consistent with nearby,
unrecorded, common input. 10 pairs (16%) showed
narrow peaks (1-2 ms) at non-zero delay consistent with
an intrapair excitatory connection. 3 pairs (5%) showed
troughs (3-5 ms) consistent with an intrapair inhibitory
connection. 28 pairs (44%) showed no reliably detected
feature.

Autocorrelograms were also computed for each of
the 69 neurons used in the crosscorrelation analysis and
examined for oscillatory activity. 2 cells (3%) showed
strong peaks consistent with oscillation, both at 12 Hz.
3 cells (4%) were found with weak peaks, two at 12 Hz
and one at 25 Hz. The balance (93%) showed no
evidence for oscillation.

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Summary: The overall research effort in our laboratory continues to be centered on our interest in understanding how information is processed by the neuronal circuitry found in cortical structures in the mammalian brain. Two specific regions are studied: the piriform (cerebral) cortex that is believed to be involved in the classification of olfactory stimuli and the cerebellar cortex which is involved in some form of sensory/motor control. In both cases, the laboratory's research effort involves the application of a wide range of interrelated experimental techniques (www.bbb.caltech.edu/bowerlab).

The central focus of the laboratory's experimental approach continues to be the interaction between computer models and experimental investigations. Most of the computer modeling is based on the known anatomical and physiological structure of the biological network or neuron of interest. At the network
level, we continue to work with models of the olfactory bulb, and the olfactory cortex, and have begun constructing a model of the cerebellar cortex. At the level of single cells we have developed models of mitral cells, cerebellar granule and Purkinje cells, and cerebral cortical pyramidal cells. We have also constructed a "systems" level model of the somatosensory system and its interaction with the cerebellum.

Experimentally, physiological efforts in the laboratory include in vivo and in vitro intracellular investigations of the effects of synaptic inputs on Purkinje cells and olfactory pyramidal cells; receptive field mapping studies of somatosensory inputs to cerebellar cortex; and extracellular recordings of the responses of cerebellar neurons to peripheral stimulation and multi-single neuron recording experiments in both anesthetized and awake behaving animal preparations. Anatomically, we conduct investigations at both the electron microscopic and light levels of the structure of cerebellar cortex and its afferent and efferent pathways. At the behavioral level, we have begun to explore the ways in which rats seek and discriminate odors as well as the way in which rats use their somatosensory surfaces to explore objects. We also continue our behavioral studies of active sensory sensing in weakly electric fish. With respect to biotechnology, a major focus of our efforts continues to be on the development of silicon electrodes, multichannel amplifiers, multichannel discriminators, and data collection software for large-scale multi-single-neuron recording. We also continue our support for the neural simulation system, GENESIS both as a research and an educational tool (www.bbb.caltech.edu/GENESIS).

Our laboratory continues to explore new avenues for communication and collaboration. Not only is support for GENESIS now worldwide, but our laboratory also supports students and research staff in several different locations around the world. Current collaborators include Dr. Jan Bjalik at the University of Oslo, Dr. Mark Ellisman at the University of California San Diego, Dr. Peter Fox at the University of Texas Health Science Center at San Antonio Texas, and Dr. Nigel Goddard at the Pittsburg Supercomputing Center, and Dr. Erik De Schutter at the University of Antwerp Belgium. This "distributed collaborative mode" has now formed the basis for new instute in Computational Biology at Montana State University in Bozeman Montana with which our laboratory is strongly affiliated.

Finally, our efforts to improve elementary school science education through The Caltech Pre-college Science Initiative (CAPSI) have continued to grow. This project now involves 11 school districts in California, and 5 school districts in 5 other states and extends from elementary through middle to high school (www.caltech.edu/~capsi).

412. Anatomical tracing of the projections from cerebral (S1) to cerebellar tactile areas in rats
John H. Thompson, Kjersi A. Lyngstad1, Jan G. Bjalik1, James M. Bower
Peripheral surfaces project to and are represented by a somatotopic pattern in S1 of somatosensory cortex and a "fractured" somatotopic map in the cerebellar hemispheres. The somatotopic map in S1 projects to the fractured one in the cerebellum via the pontine nuclei where it becomes the greatest source of input to the cerebellar hemisphere. Our lab has previously determined electrophysiologically that the S1 projection through the pontine nuclei to the cerebellar hemispheres reflects the fractured somatotopic pattern of direct trigeminal projections to the cerebellum. Numerous previous anatomical studies of the SI-pontocerebellar pathway have failed to illuminate how this is accomplished.

Using precise microelectrode micromapping techniques, we have mapped the pericoronal tactile areas of S1 and crusII. We then made punctate injections of tracer material into the electrophysiologically defined regions. We used iontophoretic injections to stay within well defined "patch" boundaries in the granule cell layer of cerebellar cortex. We also made iontophoretic injections in the corresponding area in S1, layer 5. Depending on the experiment, we used combinations of all or some of the following tracers: WGA-HRP, Fluoro-Gold, RITC, PHA-L, BDA.

The distributions of anterogradely labeled corticopontine fibers and retrogradely labeled pontocerebellar cell bodies were computer reconstructed on Silicon Graphics workstations as point clouds inside a solid model representing the pontine nuclei. Using anatomical landmarks, the distribution patterns from several experiments were superimposed for comparison. Inspection of these and individual cases has revealed several important findings. Gradually increasing regions of origin in S1 project to gradually increasing tissue volumes in the pontine nuclei. There is little variation among animals in pontine location of labelled fibers. We find no evidence of compartments in the pontine nuclei. Gradually increasing target regions in crus IIa of the cerebellum receive input from gradually larger pontine subspaces. Taken together, the data indicate that branching of the pontocerebellar neurons, and not primarily divergence in the corticopontine link, give rise to fractured maps in the cerebellum.

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413. Spatial correspondence between tactile projection patterns and the distribution of anti-zebrin II in the cerebellar folium Cruss IIA of the rat
J.H. Thompson, J.S. Hallem1, G. Gundappa-Sulur, R. Hawkes2, J.G. Bjalik1, J.M. Bower
Granular cell layer maps of tactile responses in the rat cerebellar hemisphere are fractured into topographically discontinuous regions, termed 'patches'. We have studied the spatial relationship
between this fractured somatotopic map and the parasagittally oriented molecular zones of the cerebellar cortex, represented with the Purkinje cell specific peptide Zebrin II. High resolution electrophysiological micromapping techniques were used to map the spatial distribution of individual patches in the crus IIa folium. The medial and lateral borders of the large central upper lip patch (UL-patch) were labelled with Fast Blue or India ink. After immunocytochemical processing for Zebrin II, sections were digitized and the distribution of Fast Blue and India ink tracks and anti-Zebrin positive Purkinje cells were reconstructed in 3-D. The UL-patch boundaries coincided with the boundaries of the molecular zones. The medial border of the UL-patch corresponded with the medial border of either the P5a- or the P5b+ band. The lateral border corresponded with the medial border of either the P6+, the P6-, or the P7+ band.

Following electrophysiological mapping of several smaller patches, Fast Blue was injected in their centers. The tracks in these cases were observed at a distance from the border of Zebrin II bands. We conclude that there is a spatial correspondence between granular cell layer tactile patches and molecular zonation in the Purkinje cell layer. This correspondence between molecular and physiological features may have significance for the organization of cerebellar circuits during development. Supported by Research Council of Norway, The Nansen Foundation and the Human Frontier Science Program.

414. Cerebellar granule cell responses in the awake rat during a tactile discrimination task

M.J. Hartmann, Carolyn Chan, Angela Poole, J.M. Bower

For the last several years, we have been investigating the possibility that the cerebellum is involved in the optimization of sensory data acquisition. This hypothesis leads to the prediction that the cerebellum should be particularly active during fine manipulation, discrimination, and exploratory tasks.

In these projects we have been recording from the granule cell layer of cerebellar folium crus IIa in awake, freely-moving rats, in an effort to better understand the type of sensorimotor processing occuring there. Using a light-weight microdrive developed in our lab, we have recorded activity of the cerebellar granule cell layer (GCL) from multiple sites in Crus IIa in awake, freely-moving rats. During chronic electrode implantation we determined the receptive field at each recording site, and we later confirmed these fields in the awake rat. Behaviors were videotaped and synchronized with the recorded neural signals.

Our previous work has shown that the granule cell layer seems to respond only to the tactile components of the behavior, regardless of whether the tactile stimulus is generated via active or passive touch. Although we had recorded granule cell activity during natural behaviors, such as eating, drinking and grooming, we were interested in the granule cell responses during more carefully controlled tactile stimulation. We therefore trained rats to perform a tactile discrimination task. Rats were trained to discriminate between the rougher and smoother sides of a textured bar. The rat received a reward if it walked towards the side of the bar which had the rougher texture. Training curves showed that the rat reached a 76% correct level after 2 weeks of training. Preliminary data analysis suggests that the granule cells are especially active during the discrimination task and may in fact synchronize their activity to match the rhythmicity of the exploratory whisking behavior.

415. A realistic cerebellar network: Temporal, spatial, direct and indirect influences of granule cell axons on Purkinje cells

F. Santamaria, J. M. Bower

For the last several years, our laboratory has been investigating computational models of the cerebellum. These studies have included, between other things, the development of a realistic model of a Purkinje cell (PC). The aim of our study is to understand the activity of a PC when placed in its natural environment: The cerebellar cortex.

We have built a realistic cerebellar neural network to study the influence of the granule cell (GC) layer activity on overlying Purkinje cells (PC), and have been able to account for the functional organization of the cerebellar cortex. The model is based on the known physiological and anatomical organization of the GC-PC pathway. It takes into account the different concentration and conduction velocity of parallel fibers (PF) depending on their depth in the molecular layer. Specifically, the PF have a higher concentration and are fastest at the top of the molecular layer, and have lower concentration and are slower at the bottom. The simulation makes use of a previously published compartmental model of a single PC (see references). Inhibition, by molecular interneurons, was implemented as three horizontal stripes of synapses along the dendritic tree of the PC that were activated 1 to 2msec after the arrival of the first PF to that region. The simulation also predicts the spatial and temporal patterns of synaptic activation due to the synapses on the ascending branch (AB) of the GC axons.

The simulations have been used to predict the temporal patterns of molecular layer synaptic activity induced by activation of localized regions of the granule cell layer. Specifically, The GC layer was divided in sagittal stripes which were then synchronously activated at different distances from the PC. In the absence of inhibition, the simulation
results in a "beam-like" pattern of PC activation along the course of the PFs. However, even under these conditions, PC responses are not evident several hundred micrometers beyond the focus of GC layer activation. When inhibition was added, responsive PCs were largely limited to the immediate region of GC layer activation. These responses were primarily due to synapses made by the AB. Depending on the distance from the stripe of GC being activated the PC could be either depressed or unresponsive. The interaction between the concentration and velocity of the PF with the fast activation of the inhibitory interneurons were responsible for this complex behavior.

These simulations provide a network based physiological explanation to the lack of beam-like parallel fiber effects reported previously in numerous studies using natural peripheral stimulation.

416. Functional imaging of the cerebellum: sensory or motor?
Lawrence M. Parsons, Peter T. Fox, J.M. Bower

For nearly a century, the cerebellum has been regarded by most neurologists and neurobiologists as a motor organ. Lesions to the cerebellum, while not causing appreciable weakness, cause a characteristic incoordination of movement. Electrophysiological recordings in animals and functional neuroimaging in humans amply confirmed cerebellar activity during movement. However, the research in our laboratory over the last ten years has increasingly indicated that the role of the cerebellum may be quite different than motor coordination, per se. Instead, we have proposed that the cerebellum could be involved in the active control of sensory data acquisition (Bower and Kassel, 1990; Bower, 1997).

To test these ideas using human subjects we are involved in a collaborative effort with the Research Imaging Center at the University of Texas Health Science Center in San Antonio. This effort began with a magnetic resonance imaging study focused on the lateral cerebellar output nucleus (dentate) acquired while subjects performed specific tasks involving both passive and active sensory discrimination. The results demonstrated that the cerebellar lateral nuclei was most active during the acquisition of tactile sensory data, i.e., when the sensory discrimination involves the active repositioning of tactile sensory surfaces through finger movements (Gao et al., 1996). Over the last year addition investigations have been undertaken to look at the activity of cerebellar cortex during these tasks as well as the activity in cerebellar target nuclei (e.g. the red nucleus). These results are laying the groundwork for a substantial revision in our understanding of the role of the cerebellum in brain function.

References

417. A network model of the somatosensory system to study post-lesion reorganization of cerebellar maps
Brigitte Stricane, Josee Morissette, J.M. Bower

The laboratory has been studying the unusual topography of cerebellar cortex for a number of years, focussing on area crusIIa in the lateral hemispheres of the rat. This area contains somatosensory representations of the perioral tactile surfaces, organized in a patchy, mosaic-like pattern. Experiments showed that following a peripheral lesion of the infra orbital nerve that carries upper lip inputs, the crusIIa map reorganizes in a way that preserves this particular fractured somatotopy.

We are now investigating the origine of this cerebellar reorganization with a topographic model of the somatosensory network leading to cerebellar crusIIa. This model is built in GENESIS. The anatomical areas are made of non-spiking units connected in a feedforward manner, according to their known anatomy and physiology. Once all connections are set, the lesion is simulated. Almost no reorganization is observed.

The previous physiological experiments suggested that cerebellar reorganization relies solely on plasticity in its afferents. To test this hypothesis, we propagate in the network a reorganized trigeminal map (obtained from electrophysiological experiments). This results in a cerebellar map that is similar to physiological data, thus supporting our hypothesis.

With the model, we can also explore what properties the network needs to reorganize by itself. Adding weights to some connections bares almost no consequence. Setting uniform lateral connections in the trigeminal induces a reorganization quite different from the physiology. To achieve physiological resemblance, in the trigeminal as well as in the cerebellar maps, the network needs a combination of uniform lateral connections and connection weights. This suggests that the post-lesion reorganizations observed in the somatosensory system rely on both an anatomical influence of neighbor cells and an activity-dependent reinforcement of input from neighbor sensory surfaces.

418. Noradrenaline and stimulus representation in pyramidal cell spike trains
Alexander Protapapas, J.M. Bower

A wide range of experimental evidence has implicated the release of noradrenaline (NA) during attention. For example, NA-releasing neurons in the locus coeruleus appear to be phasically active during attentional cues (Aston-Jones, et al., 1994). Work by Madison and Nicoll (1982) suggested a mechanism by which NA acts to increase neuronal "signal to noise" ratio when they found that addition of NA increased
the number of spikes elicited by constant current pulses in hippocampal pyramidal cells. At the time it was widely believed that neural information was rate coded, so that more spikes meant more information about the stimulus. However, since then a number of studies have suggested that neural information may be coded in the precise timing of individual spikes (Bialek et al., 1991). We therefore decided to re-examine these issues using a more rigorous approach that could actually quantify how a neuron’s representation of stimuli changes in the presence of noradrenaline. This is done by injecting current noise into a piriform cortex pyramidal cell and then attempting to reconstruct the stimulus waveform from the neural spike train. The quality of the reconstruction indicates how much information the spike train carries about the stimulus. Mathematically, this is done using techniques developed by Bialek and colleagues (1991). Our results show that the presence of noradrenaline acts to increase the amount of information the spike train contains about the stimulus.

References

419. Different modes of intrinsic firing activity and resonance in modeled olfactory bulb neuron
Yoshihisa Kubota, J.M. Bower

The goal of this project is understanding physiological role of oscillations in the olfactory bulb (OB). Previous works in our lab have shown that, in slice preparations, rat olfactory bulb neurons fire rhythmically even when glutamatergic and GABAergic synapses are inhibited. However, this spontaneous firing depends on the cell’s membrane potential and not all cells exhibit continuous action potential generation.

In order to understand this heterogeneity in mitral cell behavior, we have constructed a detailed multicompartment model and its reduction to a single compartment. These two numerical models contain all ion channels known to be present in mitral cells as well as calcium diffusion and other membrane mechanisms. We found a single distinct region of parameter space in which modeled mitral cells not only best fit the experimental data but also possess three states of neuronal activity, namely bursting, continuous spiking and silent, depending on conductances 56 of low-threshold Ca channel, inwardly rectifying K, and Ca-dependent K channel.

To further understand mitral cell biophysics, we employed numerical and geometric singular perturbation analyses of modeled neurons. Stability analysis indicates that different neural activities among different neurons taken from the same slice preparation might correspond to each different stable point and stable limit cycle that exist in the phase space. More precisely, mathematical analysis gives a simple explanation of (i) why seemingly different patterns of neural activity were observed among different vOB neurons in the same slice preparation, (ii) how the stable and unstable limit cycles, (iii) which stable points and stable limit cycles correspond to which neuronal states of real neurons, (iv) how bifurcation diagram of the cell changes for different external currents and for different channel densities and (v) how the mitral cell responds to time-varying synaptic inputs.

In addition, detailed knowledge of the structure of the bifurcation diagram led us to suggest a new role of subthreshold oscillation observed in vivo upon odor presentation. We have noted and shown that olfactory bulb neuron can undergo a bifurcation from nonspecific passive response to voltage-dependent and low-frequency (0- to 10- Hz) resonance upon odor-evoked subthreshold oscillation. This qualitative changes in behavior is due to low-threshold Ca channel and Ca-dependent K channel expressed in mitral cells. Bifurcation analysis suggests that, during the subthreshold oscillation, state variables of modeled mitral cells stay in a region of phase space where, (i) overall voltage-response to a periodic current input increases, (ii) frequency response measured by impedance magnitude has a hump in the 0- to 10- Hz range. We also discuss responses of the model to a periodic input.

In conclusion, we propose a novel hypothesis as to the role of odor-evoked subthreshold oscillation in the OB. Upon odor presentation, all locally synchronized mitral cells that undergo subthreshold oscillation, are brought to a special biophysical status in which neurons become a resonant bandpass amplifier.

420. Olfactory search behavior of rats in a wind tunnel
C. Chee-Rutter, S. Wu, J. M. Bower

Rats were initially trained in a light-shielded T-maze to follow a commercially-available odorant (toluene) to a sugar-water reward. Subsequently, olfactory search behavior was examined in a 4' X 10' wind tunnel in a darkened room. Animals followed a toluene "plume" to one of three possible release sites, where they received a sugar-water reward. Non-odorized air and plain water were used as controls for the other sites. The behavior of the animals was videotaped using infrared cameras. In the initial phase of training, the performance of the rats was found to be dependent on both odorant quality and concentration. In the wind tunnel, four search phases were identified, independent of how often an animal performed the task: (1) a possibly non-olfactory exploration phase, (2) an initial "detection" phase where the animal ran perpendicular to the airflow once, (3) a highly variable "localization" phase characterized by multiple perpendicular runs and high activity levels away from the edges of the tunnel, and (4) a "target approach" phase. Within these four search phases, eight simple olfactory-related behaviors were
from olfactory psychophysics, and on a database of monomolecular stimuli. The analogous perceptual multimodal sensory information is acquired through neurophysiological responses to well-defined behaviors, and non-olfactory behaviors which may aid being tested with human subjects, using an automated perceptual odor model could confer to olfactory research support (a) a representation of odor qualities, (b) a space which is based on three well-known observations and (c) a basis from which to make testable hypotheses about olfactory perception. The advantages which a model of perceptual odor space— that is, a representation of the structure of our olfactory experience. With a model of perceptual odor space, we could better interpret behavioral and neurophysiological responses to well-defined monomolecular stimuli. The analogous perceptual space in color vision is described by the "color wheel"– a graphical model of colors we experience, and the relationships between them. Similarly, a model of odor-quality perception should provide a framework to support (a) a representation of odor qualities, (b) a representation of relationships between odor qualities, and (c) a basis from which to make testable hypotheses about olfactory perception. The advantages which a perceptual odor model could confer to olfactory research are tremendous: a model would provide a means for systematically selecting olfactory stimuli based on odor quality, instead of on chemical structure. A model would also serve as a repository for the growing body of knowledge about olfactory perception. For these reasons, we propose a model of perceptual odor-quality space which is based on three well-known observations from olfactory psychophysics, and on a database of over 1000 monomolecular chemical compounds and the odor qualities they evoke. This model is currently being tested with human subjects, using an automated odor delivery system.

**421. Electrolocation in weakly electric fish: behavior and simulation**
*Christopher Assad, James M. Bower*

Weakly electric fish generate external currents to explore their surroundings. During exploration they exhibit characteristic motor behaviors, presumably using strategies to enhance the information content of the electric field patterns detected by the fish's electroreceptors, found in the skin. The resulting electroreceptor "images" are processed in the greatly enlarged cerebellum and other brain areas of these fish.

We have been studying natural exploratory behaviors in these fish to determine the electroreceptor consequences and neuroethological significance of their actions. For this purpose we had previously developed a boundary element method computer simulation to solve Poisson's equation for the electric potential and current in and about the fish. Electric fish models were constructed and calibrated on the electric organ discharges (EOD) mapped around immobilized specimens. The simulations were then used to reconstruct the EODs during a previously published tail-probing behavior of Eigenmannia, and from our own videotapes of Apteronotus exploring objects under infrared light. Simulations of selected exploratory behaviors revealed the EOD fields, modulations from body orientation and objects, and the resulting electroreceptor patterns. The results (1) imply the fish control their body positions to regulate particular features of the electroreceptor image, (2) predict features of the electroreceptor input reaching the brain, and (3) suggest algorithms needed to extract useful signals from the electroreceptor stream.

**422. Fine tuning silicon-based microelectrode arrays for multisite neuronal recording**
*David T. Keuley, Ulrich G. Hofmann, James M. Bower*

We are using silicon probes that are in collaboration with Professor Gregory Kovacs at Stanford University's Center for Integrated Systems designed to simultaneously record extracellular signals from multiple nearby sites in nervous tissue. The probes are silicon based, fork-shaped, with several microelectrodes on each of their multiple shafts, to allow simultaneous recordings at different depths within several columns. Using the probes, we are trying to obtain a detailed look at a local network of neurons.

In the past year we characterized the probes and were able to obtain neurophysiological data from acute preparations of the rat cerebral cortex of up to 16 single electrode sites. Despite the relatively easy data acquisition with the probes from cerebral areas, the probes turned out to strongly dimple and therefore damage cerebellar tissue. To quantify and later avoid this effect, we are varying the production process of our probes and are evaluating the viscoelastic properties of
brain tissue with a new mechano-electrical setup. Major efforts are now under way to first increase our data acquisition abilities to up to 128 channels and second to apply on- and offline dataprocessing capabilities to this new system.

423. GENESIS: A general neural simulation system for computational neurobiology

David Beeman, David Bülth, Venkat Jagadish, Jenny Forss, Michael C. Vanier, Alexander Protopapas, Rogene Eichler West, James M. Bower

For the last several years we have been involved in the design, construction, distribution and support of GENESIS, a General Neural Simulation System. GENESIS supports the realistic modeling of biological neural systems. Over the past year, GENESIS 2.1 development was completed and is being prepared for release. This version of GENESIS introduces new features including two dimensional tabulated channels allowing calcium dynamics and similar mechanisms to be computed more efficiently, support for calculation of the Goldman Hodgkin Katz equation and support for system independent structured binary dataset formats. The second edition of the "Book of GENESIS," which provides tutorial introductions to neural modeling and writing simulations using GENESIS, will be available to be used in courses in the fall. Included with the new edition will be a CDROM version of the GENESIS 2.1 release. We have also implemented a prototype for a neurobiological database system which will provide readily accessible information about neural systems which have been collected and understood in the context of realistic models. The GENESIS system is made freely available on the Internet and we will continue to provide support to GENESIS users through the BABEL users group.

424. The GENESIS model database and modeler's workspace

Jenny Forss, David Beeman, James Bower

We have continued the work on the GENESIS neuronal model database and Modeler's Workspace. This system is intended to facilitate the sharing of information between modelers, and to support the neuroscience research process. The Modeler's Workspace is a tool for extracting and visualizing information from neuronal databases, and for constructing new neuronal models using parts assembled from the databases. It can also be used to attach additional information, such as notes and references, to the models. The last year has been devoted to the implementation of working prototypes for the system. We designed a conceptal schema for a database of single cell GENESIS models. This schema was implemented in PostgreSQL, a hybrid object-relational database management system, and populated with some 30 models. A graphical user interface was designed for the Workspace, and a working prototype was implemented in Java. The Workspace is platform independent and can be run over the WWW. During the coming year we will extend and improve the GENESIS model database, and reimplement it in a fully object-oriented database system. Development of the Workspace will also continue. More functionality will be added, and we will perform user evaluations to highlight any design errors in the system.

References


425. The development of a complex systems informatics approach to neuroscientific discovery using a distributed heterogeneous computing environment

Rogene M. Eichler West, James M. Bower

This project will demonstrate the use of Evolutionary Algorithms (EA) in a distributed heterogeneous computing (HC) environment to build and simulate a model of a Purkinje neuron (PC) using parameters queried from federated neuroscientific databases (NDB) of experimentally-generated data. Advancements will be synergistically made in four important scientific and technological disciplines: (1) Neuroscience - How do the details of Purkinje cell morphology and ion channel distribution effect the information processing of this cell type, thus ultimately the integration of motor and sensory information in the cerebellum? (2) Complex Systems - What is the most efficient and thorough method for searching, optimizing, and interpreting parameters in high dimensional spaces? (3) High Performance Computing (HPC) - How can heterogeneous HPC resources around the world be simultaneously accessed to solve a single scientific problem? and (4) Informatics - How can experimental data located in multiple distributed databases around the world be transparently datamined to discover new principles about brain function?

References


426. CAPSI: Caltech Pre-college Science Initiative

James M. Bower, Jerome Pine, Brian Rasnow, Chris Assad, John Thompson, Dieter Jaeger, Erika Oller, Sherri Minch

In addition to their scientific interests, various members of the laboratory continue to be involved in our efforts to revitalize and transform pre-college science education in Pasadena, the state, the nation, and the world. This program is build around high quality inquiry based hands-on science activities coupled with extensive teacher training and support. Thirteen years ago this project started as a
collaboration with Dr. Jerry Pine (Professor of Physics) as an effort focused on the elementary school classrooms of the Pasadena Unified School District. Over the last five years the project has expanded to include middle school, high school, teacher preservice instruction (i.e. teachers in training), and educational software development. In addition this collaborative effort in Pasadena involves not only Caltech and the Pasadena Unified School District, but also the Claremont School of Graduate Education, UCLA, USC, UC-Santa Barbara, JPL, Amgen corporation, Pacific Telesis, the Maui Chamber of Commerce, Inverness Associates, the Rockwell Foundation, and, at last count, 125 scientist volunteers from Caltech and other local science and technology institutions. As a result of these efforts all 620 K-6 classrooms in the Pasadena Unified School District include a substantial science education component, 12 middle and 6 high school teachers are piloting inquiry-based teaching materials.

In addition to our efforts in Pasadena, CAPS! has been involved for several years in initiating and supporting similar programs in other school districts. The National Science Foundation has established a center for science outreach at CAPS! which is currently working with 12 school districts around the state of California to establish exemplary elementary pilot schools as the first step in district wide change. CAPS! is also an active partner in school reform efforts on the Hawaiian Island of Maui; in Buffalo, New York; Huntsville, Alabama; Cleveland, Ohio; Las Vegas, Nevada and Fairbanks, Alaska. Over the last year two CAPS! inspired pilot programs have also been initiated in France. In this way CAPS! is becoming an international model for scientist involvement in science education reform.

427. Quantifying change: Assessing how CAPS! has changed teaching styles in the Pasadena Unified School District
Sherri Minch, James M. Bower
The ongoing support of teacher professional development is an important component of the approach CAPS! has taken to science education reform. Our intention is to provide teachers with the opportunity for continuous professional development that has a direct effect on improving how they teach in their classrooms. However, a serious impediment to this objective is the current lack of quantitative means for measuring the effect of teacher training on classroom behavior. As pointed out recently by two noted researchers in this area, three key questions are, "To what extent are the intended curriculum reforms implemented?" "To what extent are there variations in the implementation of a reform across sites?" and "What are the variables that are the most closely related to these variations?" (Shymansky & Kyle, 1992).

To answer these questions, we have undertaken a systematic study of the implementation of one of the twenty-eight curriculums currently being used in PUSD classrooms, "Batteries and Bulbs," which is taught at the fifth grade level. Over the last year we completed the collection of data for this study. For example, we have analyzed an anonymous survey in which teachers reported which of the curriculum's fifteen lessons they taught during the quarter in which they had the kit. Straightforward analysis of this data revealed that 75% of the respondents presented at least half of the fifteen lessons, and 50% of the respondents presented two-thirds or more of the lessons. On average, respondents teaching the curriculum in quarter 1, 3, or 4 taught about the same number of lessons, while respondents teaching the curriculum in quarter 2 taught significantly fewer lessons. On average, respondents teaching the curriculum for the third time or more taught more lessons than respondents teaching the curriculum for the first time.

The second method of data collection was classroom observations of teachers presenting lessons from the curriculum. Preliminary analysis of sixteen teachers has revealed that 75% of those teachers allocated a third of their science instruction time or more for hands-on activities. Three populations emerged; almost all of the teachers presenting the curriculum for the first or second time allocated between 40% and 50% of their science instruction time for hands-on activities, while the more experienced teachers fell into two groups, those who allocated less than 40% of the time for hands-on activities and those who allocated more than 50% of their time for hands-on activities.

The next phase of this research will concentrate on characterizing hands-on activity segments. It was obvious from classroom observations that teachers differed significantly how they framed hands-on activity segments and how much independent exploration students were really able to do. This analysis will greatly add to the understanding of how different teachers enact this curriculum. Taken together, this analysis is intended to provide a more solid and quantifiable basis for determining to what extent the intended curriculum reform has been implemented and how much variation exists in the implementation styles of different teachers. With this information in hand, we will be able to state much more specifically what has been achieved in reforming the science education of elementary students in the Pasadena Unified School District as well as other districts which are part of CAPS!.

Reference
428. Montana Center for Computational Neuroscience

John Miller¹, Gwen Jacobs¹, James M. Bower

Over the last year, our laboratory has been involved in the establishment of a new Center for Computational Biology at Montana State University in Bozeman, Montana. The center focuses on three integrated efforts: basic research, education and technological development. The central theme of the program is the analysis of the structural and functional basis of neural computation and sensory-motor integration. With respect to basic research, the center focuses on the development and application of new and emerging technology to understanding how nervous systems compute. Initially this will include the development of large scale single neuron recording procedures, and advanced technique for quantifying behavior as well as the anatomical organization of nervous systems. These efforts will be tied together through the use of advanced realistic modeling techniques. In the last year a building has been approved to house the center, and design work is underway on that building.

¹Montana Center for Computational Neuroscience

Publications


Professor of Biology and Engineering and Applied Science: Christof Koch

Visiting Professors: Joseph Bogen, Heinz Schuster

Visiting Associate: Raye Wang

Research Fellows: Christof Born, Jochen Braun, Fabrizio Gabbiani, Charles Higgins, Giacomo Indiveri, Petr Marsalek, Pamela Reinagel, Jongsan Shin

Graduate Students: Rainer Deutschmann², Reid Harrison², Gary R. Holt³, Timothy Horiuichi³, Laurent Itti³, Arno Klein³, Gabriel Kreiman³, Fritz Kruger³, Amit Manwani³, Theron Stanford³, Martin Stemmler³, Jiajun Wen³

Research and Laboratory Staff: David C. Flowers, Candace Hochenedel

¹Engineering Department, Harvey Mudd College
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- Rockwell International Corporation
- Honeywell Technology Center
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Summary: Research in the laboratory of Professor Christof Koch continues to focus on three areas:

1. Biophysics of Computation in Single Neurons;
2. Understanding Visual Motion, Attention and Visual Awareness at the Psychophysical and Neuronal Level, and

What are the biophysical mechanisms underlying neuronal computations? How complex are single nerve cells? How much information can they transmit? And what is the code used to transmit this information.

Research carried out in our group as part of a program
called "Biophysics of Computation," studies how the biophysics and microanatomy of neurons subserves information processing. Analytical work, backed up by detailed computer simulations of nerve cells—based on electrophysiological and anatomical data from our experimental collaborators—are used to generate experimentally verifiable predictions. We are also analyzing the variability, reliability and randomness of neuronal firing—from cells in the cat LGN, cortical areas V1 and MT in the behaving monkey, different stages in the electrosensory system of the electric fish as well as in simulated networks—in order to understand the existence and precision of the temporal code used by nerve cells to transmit information.

Understanding complex information-processing tasks, such as optical flow, hue perception, or selective visual attention, requires a firm grasp of how the problems can be solved at the "computational" level, and how the resulting algorithms can be implemented onto the known architecture of the visual cortex and associated subcortical areas. We use analytical methods, coupled with detailed computer simulations of the appropriate circuitry in the cat and primate visual systems, to study how these neuronal networks give rise to motion perception and how they are involved in the control of selective visual (focal) attention.

Researchers in our laboratory study motion perception, spatial visual attention as well as awareness or the lack of awareness in blindsight using psychophysical techniques in normal subjects. We are complementing some of these studies using noninvasive fMRI imaging under the identical biophysics and microanatomy of neurons subserves attention.

Early vision in flies, cats, and monkeys by fabricating experimental collaborators—are used to generate experimentally verifiable predictions. We are also called involved in the control of selective visual (focal) perception, spatial visual attention as well as saccade towards such targets as well as follow targets as well as saccade towards such targets (using either visual or auditory triggered saccades).

429. Shunting inhibition does not have a divisive effect on firing rates

Gary R. Holt, Christof Koch

Shunting inhibition—a conductance change with a reversal potential close to the resting potential of the cell—has been shown to have a divisive effect on subthreshold EPSP amplitudes. It has therefore been assumed to have the same divisive effect on firing rates. However, shunting inhibition actually has a subtractive effect on the firing rate in most circumstances, for three reasons.

1. The spiking mechanism effectively holds the time-average membrane potential approximately constant, independent of the firing rate. As a result, the average current through the shunting conductance is approximately independent of the firing rate. This leads to a subtractive rather than a divisive effect for synapses which are electrophysiologically close to the soma.

2. Distal shunting inhibition can only have a divisive effect if the reversal potential of the inhibitory conductance is at the "resting potential" of the cell. This is usually thought to be around -70 mV, which is close to the GABAa reversal potential. However, when the cell is firing, the somatic voltage is clamped to about -50 mV, which means that there is a 20 mV driving force behind GABAa inhibition. There is therefore a subtractive effect of distal shunting inhibition which turns out to be much larger than any divisive effect.

3. Even if the reversal potential of shunting inhibition is set properly for a divisive interaction, the firing rate of the cell is a saturating function of the excitatory input and as a result of this nonlinearity the inhibition acts subtractively when the excitatory synaptic conductance is not small compared to the inhibitory conductance.

For these reasons, regulating a cell's passive membrane conductance—for instance via inhibitory feedback—is not an adequate mechanism for normalizing or scaling its output.

430. Feature extraction by burst-like spike patterns in multiple sensory maps

Walter Metzner, Christof Koch, Ralf Wessel, Fabrizio Gabbiani

Within the central nervous system, higher order neurons need to extract behaviorally relevant features from the sensory input to process particular stimulus variables that ultimately elicit adaptive behaviors. Recent studies have quantified the time-varying information carried by spike trains of sensory neurons in various systems using stimulus estimation methods. Here, we address the question of how this information is processed from the sensory neuron level to higher order neurons in the central nervous system by using the electrosensory system in weakly electric fish as a model.
We determined how electric field amplitude modulations are temporally encoded and processed at two subsequent stages of the amplitude coding pathway. We recorded the responses of P-type afferents and E- and I-type pyramidal cells in the first-order electrosensory nucleus, the electrosensory lateral line lobe (ELL), to random distortions of a mimic of the fish's own electric field. The ELL consists of three distinct somatotopically organized segments which contain the amplitude-coding pyramidal cells. We tested pyramidal cells in the centromedial and lateral ELL maps. Both stimulus estimation and signal-detection methods were applied to the data sets. In contrast to P-receptor afferents, pyramidal cells did not reliably encode time-varying information about the stimulus. Instead, they selectively extracted the up- and downstrokes of the amplitude modulations, thus performing a non-linear pattern recognition task. These temporal features were best encoded by short bursts of spikes rather than by isolated spikes. This computation appeared to be based upon neural integration in the dendritic tree of pyramidal cells. Furthermore, I-type pyramidal cells in the centromedial segment performed a better pattern-recognition task than those in the lateral segment and than E-type pyramidal cells in either segment.

References

1Department of Biology, University of California, Riverside
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431. Biophysical implementation of a multiplication operation in an interneuron of the locust
Fabrizio Gabbiani, Holger Krapp, Christof Koch, Gilles Laurent

The lobula giant motion detector (LGMD) is a wide-field visual neuron responding preferentially to approaching objects on a collision-course with the animal. During approach, the instantaneous firing rate of LGMD is well fitted by a product of two terms proportional to the angular velocity and to an exponential of the size subtended by the object at the retina, respectively. To investigate the biophysical implementation of this multiplication operation, LGMD was stained with cobalt hexamine and Lucifer Yellow (n=14) and the morphology of the cell was reconstructed from stacks of images obtained by 1- and 2-photon confocal microscopy. The membrane time constant and input resistance at various locations in the dendritic tree were determined from current injections in discontinuous current clamp mode. LGMD shows an anomalous rectification manifest as a sag in the membrane voltage trajectory during hyperpolarizing pulses and subsequent rebound excitation. Depolarizing pulses were used to investigate the dependence of the firing frequency on injected current and time-averaged membrane potential. They revealed a strong firing frequency adaptation which is consistent with the presence of voltage and/or calcium activated K-conductances in the membrane of LGMD.

References

432. Decoding visual information from multi-cell recordings of the retina
Pamela Reinagel, David Warland1, Markus Meister1

This theory project involved comparing an optimal linear filter reconstruction method to a nonlinear, multi-layer neural network decoder for extracting stimulus information from multicellular data. In the past year we repeated the analysis on additional data sets, and pursued control experiments to test the performance of our decoding methods on artificially constructed data sets. We confirmed our preliminary findings that the optimal interpretation of each cell's response depended on the responses of other cells in the population, and yet the information encoded by the population of cells was linearly decodable.

1Harvard University, Cambridge, MA

433. Local image statistics and human viewing of natural scenes
Pamela Reinagel, Anthony Zador

This new experimental and theoretical project was motivated by the question of what coding scheme would be optimal for the natural input signal of the visual system. We compiled an ensemble of natural images, and analyzed their low-level statistical structure by both standard Fourier methods and a novel, wavelet-based information theoretical measure. Both these measures involve only local computations which could be carried out in primary visual cortex. The images were then presented to human subjects, whose eye positions were recorded during viewing. Our data showed that when subjects move their eyes about a scene, the images falling on the fovea have a different low-level statistical structure than the image as a whole. Our first conclusion is that low-level local structure in images plays a role in guiding where people look (in addition to the well known high-level or top-down influences). Our second conclusion is that the optimal coding strategies of early visual neurons depend, not only on the structure of natural images, but also on the pattern of sampling behavior.

434. Role of bursts in visual information coding by the LGN
Pamela Reinagel, Dwayne Godwin1, Murray Sherman1

The goal of this project was to measure the visual information encoded by bursts versus by isolated spikes. The study was motivated by the broader question of
how the thalamus works as an information "filter" or "gate" between the sensory periphery and the cortex. In some bursting states (such as sleep) the thalamus is thought to uncouple the cortex from sensory input. The new data confirm and extend previous work by our collaborators which had indicated that, under conditions of visual information transmission by the thalamus, bursts can adopt a new role in encoding visual information. In the new study we show that action potentials within bursts code for qualitatively the same "message" as isolated spikes, despite some expectation of differences, such as in temporal filtering or in detection thresholds. We went on to show that bursts code this information more efficiently (using more of the spike train's entropy to transmit signal). This advantage of bursts probably reflects the fact that bursts code for large stimulus fluctuations, which are both rare in the stimulus (thus, detecting them is highly informative), and less likely to be due to noise.  

We extend this hypothesis to two types of cortico-thalamic connections (E and R-terminals). Our hypothesis is relatively easy to test experimentally, at least in its neuroanatomical form. It is based on certain neuroanatomical connections that have already been established and predicts the general nature of other connections that have not yet been established definitively.

References
1The Salk Institute of Biological Sciences, La Jolla, CA 92037

437. Psychophysical evidence for contour integration in striate cortex
Katsumi Watamabe, Jochen Braun

Purpose: The spatial interactions between local visual filters that are thought to be the basis of contour integration are known to be strengthened by visual learning. We took advantage of this fact to study the extent to which contour integration is based on the connectivity of striate cortex.

Methods: Three naive observers were trained to discriminate the shape of contours composed of collinear Gabor elements and embedded in a dense background of randomly oriented Gabor elements. Contour shape resembled a rotated C, with the opening pointing either left or right, or else either top or bottom. The observer reported which side of the contour was open (2AFC). The threshold spatial separation of contour elements increased with training, and transfer of training between different conditions was tested.

Results: The training transferred neither between different retinal positions nor between different eyes, but did transfer between different global shapes.

Conclusions: Contour integration is mediated almost exclusively by the eye-specific connectivity of striate cortex.

References

438. Visual attention is undifferentiated: concurrent discrimination of shape, color, and motion
Jochen Braun, Jiajun Wen, Christof Koch

Purpose: Visual performance is usually studied with respect to fully attended stimuli. Visual performance with respect to poorly attended stimuli can be studied with the help of a concurrent task that draws attention away from the stimuli of interest. Here we ask whether the outcome of such experiments depends on the nature of the concurrent task.

Methods: We used three attention-demanding tasks: shape discrimination (rotated T/L shapes), color...
Attentional modulation of contrast masking

Jiajun Wen, Christof Koch, Jochen Braun

Purpose: We have shown previously that attention selectively lowers some visual thresholds but not others. Here we investigate how attention affects contrast detection thresholds in the presence of a background pattern ("contrast masking"). The aim is to discover how attention modulates interactions between spatial visual filters.

Methods: The target pattern is a vertical stripe whose luminance profile is given by the 6th derivative of a Gaussian and whose power spectrum centers on 4 cpd. The mask pattern (1.5° diameter, 40% contrast) is a 2D Gabor function convolved with a random array of ~100 Dirac functions. Target and mask patterns appear at 1 of 8 equally probable peripheral locations (4° eccentricity) while attention is engaged near the center of the display by a concurrent task. Observers report presence or absence of the test pattern (the mask being always present) and contrast threshold is determined with a staircase procedure. In Exp. 1, the spatial frequency of target and mask is 4 cpd and the relative orientation varies from 0° to 90°. In Exp. 2, target and mask have similar orientation but the spatial frequency of the mask varies from 1.4 cpd to 8.0 cpd.

Results: Attention reduces the threshold elevation due to the presence of the mask. This reduction is largest when target and mask exhibit similar orientation and spatial frequency.

Conclusion: The fact that attention lowers thresholds only when target and mask have similar orientation and spatial frequency suggests that attention changes the gain of visual spatial filters at the attended location. These results are borne out by a computational model of the interactions between visual spatial filters and their modulation by attention.

References


439. Attentional modulation of contrast masking

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References


440. A model for attentional modulation of spatial vision

Laurent Itti, Jochen Braun, Jiajun Wen, Christof Koch

We are developing a computational model of interactions between visual spatial filters in humans. It accounts for a range of visual spatial thresholds and their modulation by attention. The model assumes that each visual location is analyzed by a population of spatial filters tuned for different spatial frequencies and orientations. Pairs of filters in quadrature phase produce an energy response, which is normalized by the responses of other filters from the population, which have similar tuning properties. Psychophysical performance is derived by assuming that each filter exhibits constant background noise plus Poisson noise. An information-theoretic decision strategy then allows for the computation of a unique behavioral response, from the responses of the entire population of oriented filters. This behavioral response can directly be used to compare the model's output to human psychophysical thresholds.

The model is similar to models of contrast gain control in cat simple cells and produces, without additional assumptions, the "dip-shaped" non-linearity characteristic of human threshold vision. Our model differs from others in that only a relatively narrow normalization pool (halfwidth of weight function about 1 octave and 15 degrees) accounts for human vision.

The model successfully reproduces human contrast, contrast increment, and orientation discrimination thresholds, as well as threshold elevation by contrast masking. An increase in the gain of the oriented units and sharpening of their tuning accounts for the transition observed from the data in the absence of attention to that in the presence of attention. This is obtained by strengthening normalizing interactions between the oriented filters.

References

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4Computation and Neural Systems Graduate Student, California Institute of Technology

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An fMRI study of cortical activity during visual segmentation

Jochen Braun, Thomas Ernst1, Ruye Wang2, Linda Chang3, Laurent Itti, Patrick Ledden3, Christof Koch

The segmentation of a visual scene into regions corresponding to visual objects is thought to be mediated in part by long-range horizontal connections (LRHCs) in early visual cortical areas. We investigated this hypothesis with displays where visual segmentation results from differences in local image orientation, using image elements whose size and separation were matched to the spatial range of LRHCs. Segmented displays consisted of elements of identical size (2D-Gabor functions, spatial period 0.1°-0.7°) in a dense array (mean separation 3-5 times spatial period) and differing in orientation inside and outside 30° eccentricity (total display size 12° x 12°). Non-segmented control displays were identical except that all elements had the same orientation. Three subjects were imaged (4 segmented and 4 non-segmented epochs per scan, each with 30 presentations of 1 s, surface coil fMRI using BOLD contrast, 1.5T GE scanner with EPI, 3 x 3 x 6 mm voxels, 4-8 slices parallel to the calcarine sulcus) while passively viewing the display (1280 x 1024 pixels RGB, 72 Hz refresh, Barco ICD751 with additional magnetic shielding, viewed from 5 m through modified binoculars, Optolyth Alpin 8x30). Activity correlated with visual segmentation was observed in the posterior folds of the ventral occipital lobe, including the calcarine fissure. Calcarine activity was more pronounced for displays with smaller elements. These results are consistent with a role of LRHCs in visual segmentation.

References
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An analog VLSI model of the fly elementary motion detector

Reid Harrison, Christof Koch

Flies are capable of rapidly detecting and integrating visual motion information in behavior-relevant ways. The first stage of visual motion processing in flies is a retinotopic array of functional units known as elementary motion detectors (EMDs). Although the biophysical details of the neural circuit largely remain a mystery, the input/output characteristics have been extensively studied, and an algorithm describing the operation of the EMD was proposed by Hassenstein and Reichardt in 1957. The model suggested that a photoreceptor's input was correlated with the delayed input from an adjacent photoreceptor. This model of motion detection has been shown to be functionally equivalent to more general correlation-based algorithms. We have designed and built a correlation-based insect EMD in analog VLSI hardware. The result is a low-power, continuous-time analog circuit with integrated photoreceptors that responds to motion in real time.

In our circuit, photoreceptor signals are divided into two channels as proposed by Franceschini: one for ON-responses and one for OFF-responses. All signals are represented as currents, not voltages. This allows us to use translinear circuits that take advantage of the exponential behavior of MOSFETs in the subthreshold region of operation.

The time delay is implemented as a translinear, current-mode low-pass filter that gives us a much wider linear range than conventional voltage-mode circuits. This circuit is also less prone to DC offsets, an important feature. To correlate signals, we use a translinear floating-gate circuit to achieve true, current-mode multiplication with a small number of transistors.

The responses of the circuit to drifting sinusoidal gratings qualitatively resemble the temporal frequency response, spatial frequency response, and direction selectivity of motion-sensitive neurons observed in insects. In addition to its possible engineering applications, the circuit could potentially be used as a building block for constructing hardware models of higher-level insect motion integration.

References

Analog VLSI motion chips

Charles M. Higgins, Rainer A. Deutschmann

Information about visual motion is used very effectively by biological organisms, which process it at high speed by using massive parallelism. In a step towards building a biologically-inspired motion processing system, we have designed two compact integrated CMOS sensors which report the two-dimensional direction of a moving edge over a wide range of velocities and contrasts. Each sensor is composed of four individual direction-selective elements with preferred directions 90 degrees apart. The direction of motion is computed locally, in parallel, and in continuous time. These sensors have been implemented in two-dimensional arrays in a 1.2-micron CMOS process, and provide robust direction of motion over more than an order of magnitude of velocity for contrasts over 40%.

We plan to use these sensors as a real-time testbed for investigating biological motion integration algorithms. Integration of the individual motion vectors over spatial subregions of the array should allow us to solve the aperture problem and detect transparent motion in a biologically plausible way. We are currently implementing such algorithms in software, but plan to migrate successful algorithms to real-time hardware using multi-chip analog systems.

There are also many machine vision applications of real-time optical flow chips. The average direction of motion over the entire chip can produce an overall
direction of translation of the entire image, which can be used as egomotion information. The average direction of motion in the presence of expanding optical flow can be used to estimate the focus of expansion. Both chips are capable of producing these averages in real time.

1Special Graduate Student, Caltech

444. Application of analog VLSI motion sensors for robotics applications
Christof Born

In the last years, various analog VLSI motion sensors have been developed. Due to the used technology, these sensors have a number of interesting properties such as the real-time performance, low weight and size and low power consumption. On the other hand, the currently available sensors do not give precise values of speed and direction of motion of moving objects and have only a quite limited number of pixels. Furthermore the performance of the sensors has only been tested with artificial stimuli such as moving black and white bars, and approaches to use these sensors for real-world applications weren't very convincing when compared to existing computer vision approaches. Based on these constraints the question arises whether these sensors could be successfully used in real-world tasks where commonly rather time consuming algorithms based on video images are used. To answer this question a number of experiments were designed, some of which have already been concluded.

First the ability of the sensor to respond correctly to natural images (as opposed to artificial ones) was investigated by measuring the sensor output when rotating the sensor in natural environment. The objective was to recover the final orientation of the sensor after arbitrary movement. We were able to show that as well the speed and the orientation of the sensor could be determined with only small errors. This was an important result since it shows that although the sensors deliver only quite inaccurate results, the integrated outputs still yield the correct results.

The next step is to investigate the performance of the sensors in a navigation task. The objective here is to combine the outputs of several motion sensors and ultrasonic range finders to recover position and orientation of a vehicle that moves in 2d in a natural environment. Such a vehicle is currently under construction. Results are expected in mid '97.

Publications


Summary: We continued to study the neural mechanisms of sound localization in barn owls and of song learning in zebra finches. In both cases, we are particularly interested to link neurophysiology to behavior. The operation of neural circuits with known functions is also in our agenda. The hind brain of barn owls contain a neural circuit for interaural time differences which owls use for sound localization. One component of the circuit is nucleus laminaris which is notorious for the difficulty in isolating single neurons. Using a "loose patch" method, we succeeded in recording from many laminaris cells. A model of coincidence detection which laminaris cells carries out predicted that intense sounds impair the ability of laminaris cells to detect interaural time differences. Our results show that intense stimuli do not affect the processing of time differences by laminaris neurons. If gain control by inhibition is added to the model, it can explain the results (see Peña et al. Abstract 445). For this reason, we are studying inhibition in laminaris by neuropharmacological methods. The time processing circuit is also insensitive to interaural intensity differences under normal stimulus conditions (see Viete et al. Abstract 446). This makes sense, because time and intensity disparities encode different coordinate axes. We also studied the neural mechanisms of a perceptual phenomenon known as binaural fusion. This is the only human auditory perceptual phenomenon that has been studied at the level of single neurons in an animal (see Konishi & Takahashi Abstract 448). Another behavioral project that has bearing on human sound localization addresses the question of monaural sound localization (see Egnor Abstract 449). Two members of our group study the neural mechanisms of singing and song learning in birds. The latest development in this field is the finding that the left and right forebrain song control pathways are highly synchronized during song without any direct connection between them (Schmidt Abstract 450).

445. Tolerance to sound intensity of binaural coincidence detection in the owl's nucleus laminaris
Jose Luis Peña, Svenja Viete, Yehuda Albeck, Masakazu Konishi

Neurons of the owl's nucleus laminaris serve as coincidence detectors for measurement of interaural time difference. The discharge rate of nucleus laminaris neurons for both monaural and binaural stimulation increased with sound intensity until they reached an asymptote. Intense sounds affected neither the ratio between binaural and monaural responses nor the interaural time difference for which nucleus laminaris neurons were selective. Theoretical analysis showed that high afferent discharge rates cause coincidence detectors with only excitatory input to lose their selectivity for interaural time difference when coincidence of impulses from the same side becomes as likely as that of impulses from the two sides. It is hypothesized that inhibitory input whose strength increases with sound intensity protects nucleus laminaris neurons from losing their sensitivity to interaural time difference with intense sounds.

446. The role of GABA-mediated inhibition in nucleus laminaris
Svenja Viete

In order to study the role of inhibition in nucleus laminaris, I made double barrel electrodes in which one of the barrels served as a patch electrode. The patch electrode allowed a good extracellular single unit recording from the laminaris neurons. Using iontophoresis, I injected a 2mM solution of GABA (PH 5.6) to the vicinity of the recorded cell. The low spontaneous activity of the NL neurons seems to remain unaffected by the application of GABA. Both, the response to favorable ITDs and unfavorable ITDs increased after GABA application; the whole ITD curve shifted upward. The ratio between the response to favorable ITDs (peaks) and unfavorable ITDs (troughs) seemed to stay unchanged. Application of Bicuculline, a GABA antagonist had the opposite effect: The responses to favorable and unfavorable ITDs decreased, the ITD curve shifted downward. Again the peak to trough ratio remained unchanged and the spontaneous level seemed to increase slightly. These findings are quite unexpected. GABA usually has a hyperpolarizing effect and leads to a decreased response. R. Hysan reported a similar depolarizing effect of low concentrations of GABA in the chicken laminaris in vitro. Further studies with varying concentrations will clarify the role of GABA in the nucleus laminaris. So far GABA does not seem to be involved in sharpening the ITD responses but may be involved in the gain control that enables the nucleus laminaris to operate over a wide range of intensities.

447. Effects of interaural intensity difference on the processing of interaural time difference in the owl's nucleus laminaris.
Svenja Viete, José Luis Peña, Masakazu Konishi

Interaural time and intensity differences (ITD and IID) are processed independently in the owl's auditory system. This paper examines if this independence is established in nucleus laminaris (NL), the first site of ITD processing. A plot of discharge rate against time difference (ITD curve) is sinusoidal in NL. The ITDs that produce the peaks are called the most favorable ITDs and those that produce the troughs are called the least favorable ITDs. IID had little effect on the discharge rates of laminaris neurons for the most and least favorable ITDs. The degree of peak-trough modulation changed slightly with variation in IID. In contrast, IID in tonal stimuli affected the temporal aspect of ITD curves depending on the difference
between the stimulus frequency and the neuron's best frequency (BF). For frequencies below best frequency IID caused large and systematic shifts in ITD towards the ear in which the sound was louder, whereas for frequencies above best frequency IID caused small shifts in ITD towards the opposite ear. IID had little effects on ITD curves taken with BF or broadband noise. These results can be largely accounted for by the effects of frequency and intensity on the timing of impulses at the level of the cochlear nuclei. Thus, the processing of ITD by nucleus laminaris neurons is independent of IID for behaviorally relevant stimuli, because the timing of impulses is insensitive to sound level when the signal is broadband.

448. Behavioral and neural responses to variation in binaural correlation in barn owls
Masakazu Konishi, Yoshifumi Takahashi

A low-frequency noise delivered to the two ears sounds as if it originated from a phantom source in the middle of the head. If the signal in one ear is delayed, the source appears to shift away from that ear. The singularity and distinctiveness of the source varies as a function of correlation between the left and right signals. A single discrete phantom source is perceived when the two signals are identical and two separate sources, one in each ear, are heard when the two signals are independent of each other. The degree of correlation can be expressed by regression coefficients which are obtained by plotting one signals against the other. Some experiments show that human beings can perceive phantom sources even when binaural correlation is as low as 0.2-0.3.

Barn owls rapidly turn their heads toward sound sources. This response can be induced by sounds delivered by earphones. Owls turn their heads as if they localized sound sources in real space. The time difference between the left and right signals determines the angle of head turning. The standard deviations of the mean turning angles measured in one owl remained almost constant when binaural correlation was varied from 1 to 0.3. This finding suggests that the consistency with which the owl can detect phantom sources is unaffected by changes in binaural correlation of this magnitude. When binaural correlation was further reduced to 0.2 and 0.1, the owl could no longer localize phantom sources reliably. Thus, human beings and owls are similar in performing similar perceptual tasks.

Neural correlates for the above perceptual phenomenon can be studied in owls. The nucleus laminaris is the first site where interaural time differences are detected. The response of laminaris neurons to the most favorable interaural time difference changed almost linearly as binaural correlation was varied from -1 (negative correlation) to 1. Similar responses to variation in binaural correlation were observed in neurons of higher-order nuclei that receive direct and indirect input from the nucleus laminaris.

However, the response of neurons in the external nucleus of the inferior colliculus and the optic tectum to their best interaural time differences declined sharply as binaural correlation was reduced from 1 to 0.3 or 0.4. Thus, the behavioral threshold for binaural correlation could be accounted for by the sensitivity of single neurons to binaural correlation.

449. The effect of a monaural plug on sound localization by the barn owl
Roian Egnor

Sound localization in humans is supported by a variety of cues, both binaural and monaural. The study of sound localization in barn owls has focused on two binaural cues: interaural time differences (ITDs) and interaural intensity differences (IID). Monaural sound localization, however, has not been demonstrated in barn owls. The results of some early studies of barn owls with monaural plugs showed localization deficits consistent with a shift in the binaural cues used for sound localization. The observed shift was believed to be a result of the attenuating and delaying effect of the plug on the normal IID and ITD. However, these studies either used plugs which were incomplete or behavioral tests which did not map the deficit adequately. Current work with monaural plugs suggests that in the absence of binaural cues owls orient consistently to the side of the unoccluded ear, regardless of the location of the sound source. In humans, monaural localization is based on the location-specific filtering of sound by the convoluted pinna (outer ear) of the unoccluded ear. The behavior of the monaurally plugged owls is similar to results obtained with monaural plugs in humans after the spectral filtering cues have been removed (by filling in the pinna convolutions). This suggests that owls are not using monaural spectral filtering cues to localize sounds. In addition, these results suggest that sound in only one ear is interpreted by the nervous system as coming from a location ipsilateral to that ear. Preliminary results indicate that this monaural ability may be supported by the circuitry which processes interaural intensity differences.

450. Interhemispheric synchronization of vocal premotor activity in songbirds
Marc F. Schmidt

Songbirds have evolved an elaborate set of song control nuclei to enable them to sing complex learned vocalizations. These nuclei are bilaterally symmetrical and show no obvious anatomical specialization between hemispheres even though song production has been suggested to show hemispheric dominance. While the role of such bilateral symmetry, and possible lateralization, is not known, it is clear that song production requires some degree of bilateral coordination since individual sides of the syrinx (avian vocal organ) can produce distinct but coordinated sounds. To investigate the relationship between vocal pre-motor activity in each hemisphere, I have
simultaneously recorded neural activity in HVc from both hemispheres in awake singing adult male zebra finches. In all cases observed, neural activity in HVc during singing (as well as calls and chirps) showed a strong linear correlation between the left and right HVc implying that pre-motor control of vocalization always involves the simultaneous activation of both hemispheres. A striking feature of these data was the precision of onset and offset of pre-motor activity bursts suggesting a synchronization between both hemispheres. Cross-correlation analyses confirmed this observation. Neural activity between both hemispheres being significantly correlated with a time lag of only 0.2 ± 0.7 msec. Because no known direct interhemispheric connections exist between HVc, or any other forebrain song nucleus, this short time lag suggests that the left and right HVc receive a common synchronizing input. Potential sources for such input include bilateral midbrain vocal areas.

**Publications**


**Associate Professor:** Gilles Laurent

**Research Fellows:** Fabrizio Gabbiani, Leslie Kay, Holger Krapp, Larry Proctor, Mark Stopfer

**Visiting Faculty:** Beulah Leitch

**Graduate Students:** Alex Backer, Ari Hershowitz, Katrina MacLeod, Michael Wehr

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**Summary:** We are interested in information coding in the brain and in the design principles of local circuits involved in processing sensory (olfactory and mechanosensory) and motor information. We are particularly interested in relating the biophysical properties of neurons and synapses to the function of the networks in which they are embedded. We therefore study the cellular, synaptic and network aspects of neural processing, and focused this year on four different model systems: The sensory-motor circuits underlying leg movements in the thorax of insects; the olfactory system of insects and rats; the visual system of insects; and the auditory system of birds. Our work combines experimental (behavioral and electrophysiological) and modeling techniques and aims at understanding functional aspects of brain circuits design and the rules of information coding used by the nervous system.

451. **Biophysical implementation of a multiplication operation in an interneuron of the locust**

Fabrizio Gabbiani, Holger Krapp

The lobula giant motion detector (LGMD) is a wide-field visual neuron responding preferentially to approaching objects on a collision-course with the animal. During approach, the instantaneous firing rate of LGMD is well fitted by a product of two terms proportional to the angular velocity and to an exponential of the size subtended by the object at the retina, respectively (Hatsopoulos et al., 1995; but see Rind and Simmons, 1997). To investigate the biophysical implementation of this multiplication operation, LGMD was stained with cobalt hexamine and Lucifer Yellow (n=14) and the morphology of the cell was reconstructed from stacks of images obtained by 1- and 2-photon confocal microscopy. The membrane time constant and input resistance at various locations in the dendritic tree were determined from current injections in discontinuous current clamp mode.

LGMD shows an anomalous rectification manifest as a sag in the membrane voltage trajectory during hyperpolarizing pulses and subsequent rebound excitation. Depolarizing pulses were used to investigate the dependence of the firing frequency on injected current and time-averaged membrane potential. They revealed a strong firing frequency adaptation which is consistent with the presence of voltage and/or calcium activated K-conductances in the membrane of LGMD.

**References**


452. **Analysis of the variability of temporal response patterns of simultaneously-recorded projection neurons in the olfactory system of the locust**

Alex Bäcker, Michael Wehr

Odors appear to be represented in the antennal lobe of the locust by odor-specific, but overlapping, evolving assemblies of synchronously firing projections neurons. These projection neurons (PNs) fire in temporal patterns which are odor- and cell-specific. We have examined the variability of the responses of these cells across repeated stimulus presentations of the same odor. We found strong correlations (p<0.001) between the variability of spike trains of some, but not all, simultaneously recorded pairs of PNs. In some of these pairs, the firing of the neurons was negatively
correlated: i.e., one neuron fired less than on average on trials when the other neuron fired more than on average. In other pairs, the firing was positively correlated. The correlations in some cell pairs was odor-evoked, and, interestingly, happened only at a particular epoch in a the dynamically evolving response. We also observe significant correlations during periods of no odor stimulation, which disappeared at the onset of the odor responses. In addition, the inter-trial variability of the responses appeared to decrease in successive trials, making later responses more stereotyped. When the variability of the responses was analyzed as a function of time in the trial (assessed as the variance in the firing rate for short time windows) the variability appeared to vary systematically as the response evolved in time, so that a given cell would exhibit periods of high variability and periods of low variability. These results suggest that the effective connectivity of the antennal lobe varies as the response to odors unfolds in time, consistent with previous physiological findings showing the dynamical nature of the assemblies that represent odors.

Reference

453. In vitro investigations of neural coincidence detection
Larry Proctor, Beulah Leitch

Neurons in the cochlear nucleus laminaris (NL) of birds perform a critical function in the neural processing underlying the behavior of sound localization. These neurons are able to detect the coincident arrival of neural impulses with incredible temporal discrimination. Understanding how these neurons are capable of performing this coincidence detection is important to fully elucidate the mechanisms involved in auditory perception, and may possibly help our understanding of the coincidence detections that take place in other parts of the nervous system.

NL receives bilateral excitatory inputs from the cochlear nucleus magnocellularis (NM), which fires action potentials that are phase-locked to a sound stimulus. When the phase-locked action potentials from each NM arrive at a particular NL neuron simultaneously, that neuron is excited maximally. Should the inputs from each NM arrive 180 degrees out-of-phase, then the activity of the NL neuron is less than when it is stimulated from one side alone. The difference between the in-phase and the out-of-phase arrival of an action potential depends on the frequency of the sound stimulus, but can be as little as 200 microseconds in the chick -- roughly one-fifth of the duration of the action potential. We are currently investigating the mechanisms of interaural phase-difference tuning in NL neurons using an in vitro brainstem slice preparation from embryonic chicks.

Excitatory postsynaptic potentials (EPSPs) can be evoked in NL neurons in a brainstem slice by stimulating either the ipsilateral NM or the afferent fiber tract from the contralateral NM with bipolar stainless steel electrodes. The duration of these evoked EPSPs depends on the membrane potential of the NL neuron, varying from 2.5 msec at -80 mV to less than 1 msec at -45 mV. The EPSPs are not affected by the NMDA antagonist APV, but are blocked completely by the AMPA receptor antagonists CNQX and DNQX. Stimulating the ipsilateral and contralateral inputs simultaneously or with variable time delays results in increasing EPSP amplitude with decreasing time delay. When the time delay is less than 100 microseconds the EPSP is maximal and it then calls off towards the EPSP amplitude obtained with unilateral stimulation with larger time delays. This indicated that coincidence detection is a property of individual neurons rather than a network of neurons, and that it does not depend on a continuous barrage of phase-locked synaptic potentials.

NL neurons receive GABAergic input from the superior olive. In the collaborative study with Beulah Leitch from the University of Durham, we are examining the distribution and function of these GABAergic inputs. Combined electron-microscopy and immunocytochemical staining reveal GABAergic synapses onto both the soma and dendrites of NL neurons. These inhibitory synapses occur all around the soma and on most dendrites. In the presence of blockers of excitatory synaptic transmission, inhibitory synaptic potentials (IPSPs) can be evoked by stimulation with bipolar stimulating electrodes placed ventrolaterally to NL, presumably activating afferents from the superior olive. These IPSPs have a much longer duration than the evoked EPSPs, lasting between eight and ten milliseconds. We now intend to characterize the effect of this inhibitory input on coincidence detection.

454. Multiple single unit recording in the olfactory nervous system of awake-behaving rats
Leslie M. Kay

This project was designed to study the mechanisms of sensory perception and memory in the olfactory system of the rat. Previous and ongoing studies in the locust have indicated that oscillating neural cell assemblies are activated upon exposure to odorants and that odors may be coded sparsely by these oscillating assemblies (Laurent, 1996). Other work has indicated a complex cascade of events that can be extracted from field potentials in the olfactory bulb, pyriform cortex, and hippocampal formation during olfactory perception (Kay, 1995, 1996). These sets of findings guide the present experiments in rats, designed to study mechanisms of neural coding during different phases of learning and perception. We examine the firing rate, correlation structure, and presence of oscillatory activity in small groups of spatially separated mitral cells.
Standard movable microelectrode technology has been adapted for use in the olfactory bulb of a freely moving rat. A drive has been developed that removes the weight from the front of the rat's head and also allows the animal normal sniffing and exploration behavior. Tetrodes (a bundle of four 15 μm tungsten wires attached to movable microdrives) provide a good technology for recording in the neocortex and hippocampus, but for the olfactory bulb, due to the high degree of background coherence in awake animals, these wires have too large a diameter for optimal separation of neurons. The microdrive technology has been adapted for use with movable single etched tungsten electrodes (1 μm tip diameter), which prove useful in isolating the activity of single mitral cells.

After recovery, the rats are trained in a simple associative paradigm to identify and discriminate two or more odors. After the animals are familiarized with the task (one day) mitral cell unit activity is recorded before, during, and after training, in a one-day test period. Field potentials from the olfactory bulb and prepyriform cortex are recorded simultaneously with the mitral cell activity.

The questions addressed in our experiments are: Do small neural assemblies form in the olfactory bulb upon presentation of odors? In what way do these assemblies exhibit themselves (correlation, patterned firing, increase in firing rate, oscillation)? Do such putative neural cell assemblies change with training or during the course of a single perceptual act, and does the "coding" strategy change?

References

455. Sensitivity of Kenyon cells to input coincidence in the locust mushroom body

Michael Wehr

Odors evoke synchronized oscillatory firing in ensembles of projection neurons in the locust olfactory system. These ensembles change in an odor-specific manner on a cycle-by-cycle basis during an odor response, forming a reliable and odor-specific sequence of synchronous ensembles (Wehr and Laurent, 1996). We are interested in the possibility that these odor-specific sequences of ensembles form a temporal code. For a temporal code to be useful, it needs to be read out or decoded by post-synaptic neurons. To address this question, we are investigating synaptic integration by Kenyon cells (KCs) and its dependence on input synchrony and temporal structure.

Projection neurons (PNs) synapse onto KCs in the mushroom body (MB), where odor-evoked field potentials oscillations can be recorded. We recorded local field potentials in the MB evoked by electrical stimulation of different (possibly overlapping) groups of PNs with tow bipolar stimulating electrodes positioned at opposite ends of the antennal lobe.

When both electrodes (and therefore both PN ensembles) were stimulated synchronously, the peak magnitude of the evoked potential was 1.8 times greater than the peak magnitude of the mathematical sum of evoked potentials from each electrode stimulated alone. We varied the delay between stimulation of the two electrodes, and observed that evoked potential amplitude decreased in a complex, non-monotonic fashion with increasing inter-stimulus delays. Delays less than 10 ms evoked supralinear summation, and delays between 30-80 ms evoked sublinear summation. These complex effects of inter-stimulus interval on PSPs could be due to synaptic depression of facilitation, active dendritic conductances, polysynaptic effects such as feedforward or feedback inhibition, or combinations of these. The supralinear summation of evoked potentials with short inter-stimulus interval suggests that PSPs arriving at KC dendrites within a short time window may summate more efficiently. This hypothesis is supported by previous intracellular recordings demonstrating active "boosting" of otherwise passive KC PSPs when a stimulus magnitude threshold is exceeded, or when the KC is held depolarized by current injection (Laurent and Naraghi, 1994). We are currently recording intracellularly from KCs to examine whether the non-linear summation of evoked potentials arising from near-synchronous stimulation of different ensembles of PNs is due in part to these active dendritic conductances in KCs.

References

456. Impairment of odor discrimination by desynchronization of odor-encoding oscillating neural assemblies in the honeybee

Mark Stopfer

Stimulus-evoked oscillatory synchronization of neural assemblies has been observed in the sensory systems of many animals ranging from insects to amphibians to mammals. Recent results from our laboratory show that, in locusts, information about odor identity is contained in the precise timing of action potentials relative to an oscillatory population response. This suggests that oscillations may reflect a common timing signal for coding sensory information.

Although this stimulus-evoked oscillatory phenomenon is reliable, its roles in sensation, perception, memory formation, and pattern recognition remain to be examined in a behavioral paradigm. In previous work we demonstrated that the honeybee (Apis mellifera), an animal well suited for behavioral and cellular studies, employs a synchronous, oscillatory olfactory mechanism like that observed in the locust. In recent work, using intra- and extracellular recording techniques in the honeybee, we found that, as in the locust, the oscillatory synchronization of assemblies of projection neurons is selectively abolished by picrotoxin (drug that blocks the coordinating effects
of the inhibitory transmitter GABA). Then, using a behavioral learning paradigm (in collaboration with Brian Smith and Seetha Bhagavan at Ohio State University), we found that picrotoxin-induced desynchronization impairs the discrimination of molecularly similar odorants, but spares discrimination of dissimilar odorants.

Together, these results provide the first indication that oscillatory synchronization is functionally relevant and essential for precise sensory discrimination. Furthermore, the results suggest that oscillatory synchronization, and the kind of temporal coding it affords, provides important additional dimensions by which the brain can segment spatially overlapping stimulus representations.

457. Functional organization of insect olfaction: Optical imaging and genetic analysis

Ari Hershowitz

From insects to humans, the olfactory system exhibits a conserved anatomical organization. This organization gives tantalizing clues about the functional units which come into play when an animal interprets airborne chemicals as behaviorally relevant odors. We would like to “watch” olfactory processing in the brain as it occurs, using optical imaging to examine the spatial and temporal distribution of activity in an insect antennal lobe. In this way, it may be possible to form a functional interpretation for some of the salient anatomical features of olfactory systems.

Work in this laboratory has detailed the interaction of the two major cell types in the locust antennal lobe. Excitatory projection neurons (PNs) respond to odors with consistent patterns of activity, whose timing is controlled by inhibitory local neurons. Nearly all of the connections between these cells, as well as the input connections from the receptor neurons are concentrated in about 1000 compact bundles of neuropil, the glomeruli. Single projection neurons have discrete dendritic processes in many glomeruli. Using an intracellular calcium-sensitive dye, we hope to compare the activity of these processes to determine how the signals from various glomeruli combine to result in the patterns of activity seen in PNs. We have employed both confocal microscopy and the dedicated calcium imaging set-up in the Caltech Imaging Center, in order to test the usefulness of different calcium sensitive dyes in the antennal lobe. We are also working with Steve Potter and Jerry Pine to apply the high speed CCD camera, which they developed, to

In addition to optical imaging experiments, and as an extension of our interest in the functional organization of the olfactory system, we are working with Leslie Vosshall in Richard Axel’s laboratory at Columbia University to find the genes for olfactory receptors in insects. We developed a method to identify and easily remove single olfactory receptor neurons (ORNs) from locust antennae, which Dr. Vosshall will use to genetically screen for DNA coding for olfactory receptors. The identification of these genes will allow comparison with large families of genes thought to be olfactory receptors in vertebrates and nematode worms. It will also be possible to correlated the distribution, in glomeruli, of the ORN axon terminals to the receptor proteins expressed in the cells. This would allow us to describe the organization of the olfactory system in insects at the molecular level.

458. Effects of antennal lobe neuron desynchronization on odor responses of mushroom body extrinsic neurons in the locust

Katrina MacLeod

Odors appear to be represented in the locust olfactory system by specific but overlapping ensembles of synchronously firing antennal lobe (AL) projection neurons causing fast 20-30 Hz) local field potentials (LFP) oscillations in the ipsilateral mushroom body (MB). Oscillatory neural activity is found in systems across several phylla and has been proposed to play a role in sensory perception, learning, and memory. The synchronization of the projection neurons (PN) arises from reciprocal interactions between them and non-spiking, inhibitory, local interneurons (LN) in the AL. PNs send their output to the next olfactory relay of the locust brain, the mushroom body (MB), a site important for learning and memory in insects. These interactions, and therefore the LFP oscillations, can be abolished by local application of the GABA-receptor antagonist picrotoxin (PCT). PCT specifically abolishes synchronization of the PNs, but does not affect the slower temporal patterns evident in intracellular recordings of PNs, or their odor tuning. PCT is thus an effective tool to probe the specific role of synchronization in olfactory information processing. To investigate this role we have recorded intra-cellularly from one downstream, odor-responsive neurons in one of the output regions of the MB, the beta lobe. These neurons are spiking, often have marked responses to specific odors, and can show significant phase-locking to MB calyx LFP. We characterized the odor responses of these beta lobe neurons (bLN) before and after injection of PCT into the AL. PCT injection and thus desynchronization of PNs did not eliminate odor responses in the bLNs. In almost all cases, however, the bLN response-to-background contrast increased, due to a decrease in resting firing rate, and an increase in odor-evoked response firing rate, and an increased tendency to fire phasically at the onset of an odor pulse. In some experiments, bLNs which responded differentially to tow odors in the control trials continued to respond differentially in PCT trials. In other experiments, the bLNs’ responses were so altered as to make their response to the two odors no longer distinguishable. Finally, some bLNs developed a response in PCT trials to odors to which they
previously showed negligible or no response. These data together suggest that desynchronization of antennal lobe PNs can result in a loss of specificity of downstream olfactory neuron responses.

Publications
Svaramakrishnan, S. and Laurent, G. Postsynaptic receptor characteristics determine fidelity of information transfer at a synapse, submitted.

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The Neurosciences Institute, Hospital of the Good Samaritan
Summary: Our experiments this past year continued to reflect our long-term interest in the functions of the dopamine neurons of the ventral midbrain designated as A9 and A10. In the past two years, we have given primary attention to the A9 group of neurons in the context of the motor function expressed in the normal rat and in the rat model for Parkinson's disease.

The A9 neurons have their cell bodies in the substantia nigra, and their axons ascend to the forebrain via the medial forebrain bundle. The primary target for the A9 neurons is the striatum, a structure encompassing the caudate and putamen. The A9 group of neurons plays a role in the motor function of the CNS. Our aim is to understand the behavioral consequences of the activity of the A9 neurons in terms of their electrophysiological and neurochemical actions at synapses in the basal ganglia, the circuitry of the CNS mediating the motor function.

The striatum is the receiving end of the basal ganglia, a set of nuclei lying below the cortex with reciprocal connections to the cortex. These nuclei show also reciprocal regulation of each others' output. The basal ganglia send their output to the thalamus, and from there, the information is sent back to the cortex. The circuit encompassing cortex/basal ganglia/thalamus/cortex constitutes a loop wherein activity determines whether motor activity will be expressed. Current research aims to identify the factors that regulate the activity in this loop and the factors that cause that activity to become abnormal. The A9 dopamine neurons send a massive projection to the striatum to modulate the cortical input. The dopamine neurons exert their effects in striatum via an inhibitory action, while the cortical neurons exert their action via an excitatory action using glutamate as the transmitter. The loss of dopaminergic striatal input leads to Parkinson's disease (PD), while loss of the striatal neurons that are the targets for the cortical input leads to Huntington's disease. Much effort has been spent in recent years to elucidate the effects of dopamine neuron loss, the primary cause of PD, on the flow of information in basal ganglia, the subcortical set of structures where intervention to alleviate the symptoms of PD is an option. One widely accepted view is that the loss of the dopamine neurons of the A9 group leads to the subthalamic nucleus (STN), a basal ganglia component, to become disinhibited. Its normal function is presumed to be to maintain a balance in the basal ganglia output structures between excitation and inhibition for effective regulation of thalamic activity. The loss of the dopamine neurons impairs that function to the extent that the activity in the thalamic/motor cortex portion of the loop is depressed, leading to reduced motor output.

Our experiments this past year dealt with the effects of a loss of the A9 dopamine neuron on the responses in STN to enhanced dopaminergic activity induced by dopamine agents and on the capacity of pallidotomy and/or transplants of fetal dopamine tissue in striatum to restore, at least in part, the ability of the STN to process information originating with the stimulation of the dopamine receptor and the motor response to these dopamine agents seen in normal, intact animals.

459. The effects of lesions in the entopeduncular nucleus of the rat model for Parkinson's disease on the output of the subthalamic nucleus
Marianne Olds, D. Jacques, O. Kopyov
This study represented the second step in the broadly based project comparing the effects of pallidotomy and transplants on the activity of the subthalamic nucleus (STN) in the rat model for Parkinson's disease (PD). The model for PD used in our project is the rat that had received a unilateral injection of the dopamine-specific neurotoxin 6-hydroxydopamine in substantia nigra to effect a loss of the dopamine neurons present in the pars compacta of this structure. This chemical lesion has two effects. One is that it causes the deafferentiation of the striatum on the lesioned side of the CNS, and the second is that it leads to the development in the rat of a motor syndrome characterized by spontaneous and
drug-induced rotational asymmetry. This syndrome is considered a valid model for Parkinson's disease because it is believed to be due to the deafferentiation of striatum, the condition underlying the human disease. At the clinical level, various procedures are available for counteracting the motor syndrome, but each has its drawbacks. Pallidotomy is one of these procedures. It involves the destruction of tissue in the globus pallidus.

The globus pallidus in humans has two segments. One, the internal segment (GPI) is one of two basal ganglia structures relaying signals to the thalamus, the other being the substantia nigra, pars reticulata. The homologue of the GPI in the rat is the entopeduncular nucleus. The second segment of the globus pallidus in primates is the external segment (GPe), in the rat referred to as the globus pallidus (GP). The GP feeds into the STN whereas the STN feeds into the GPI or entopeduncular nucleus, in the rat. But, the GP also receives input from the STN, indicating reciprocal regulation of activity.

The first study in this project showed that the unilateral loss of the dopamine neurons in substantia nigra reduced the increase in the discharge rate of the STN induced by treatment of the animal with amphetamine and potentiated the increase in the discharge rate induced by treatment with apomorphine, thus under conditions of enhanced dopaminergic activity. The unilateral loss of nigra dopamine neurons was thus expressed in the STN response to two drugs that stimulate the dopamine receptor.

In the present study, the aim was to find out whether these effects of dopamine neuron loss could be counteracted by pallidotomy. The lesions were bilateral and were made in the entopeduncular nucleus. We have completed collecting and analyzing the data and are proceeding with the writing up of this material. The principal finding is that these lesions attenuated the activity in STN evoked by the stimulation of the dopamine receptor in the rat model for PD. The attenuation was not restricted to the activity evoked by amphetamine but generalized to the activity evoked by apomorphine, a compound with a capacity to directly stimulate this receptor. The combination of dopamine neuron loss and bilateral electrolytic lesions of the entopeduncular nucleus thus potentiated the deficit in activity induced by dopamine neuron loss alone on the response to amphetamine, and blocked the increase in activity induced by dopamine neuron loss alone on the responses to apomorphine. The beneficial effects of pallidotomy reported, in PD patients derive, therefore, in all likelihood, from the effects of the GPI lesions on other basal ganglia nuclei. The material collected is being readied for publication.

460. Effects of globus pallidus lesions in the rat model for Parkinson's disease on the output of the subthalamic nucleus
Marianne Olds, D. Jacques, O. Kopyov

The aim of this study was to determine whether bilateral lesions of the globus pallidus (GP; GPe in the monkey) alter the effects of dopamine neuron loss on the responses of the subthalamic nucleus (STN) to amphetamine and apomorphine, compounds which enhance dopaminergic activity in the CNS. The GP is a basal ganglia structure which receives information from striatum, relays it to the STN, and in turn, has its output regulated by the STN. Current thinking about the role of the STN, based on the anatomy of the basal ganglia and the transmitters involved, assumes that the loss of the nigra dopamine neurons leads to reduced output from the GP to the STN. Because this transfer of information is based on an inhibitory action mediated by GABA, the loss of the dopamine neurons, it is argued, leads to the STN becoming disinhibited. Our first study in this series showed that under these conditions, responses in STN to enhanced drug-induced dopaminergic activity were attenuated when the enhancement was produced by amphetamine and potentiated when it was produced by apomorphine. The aim in this study was to find out whether lesions of the GP had the capacity to counteract the effects of dopamine neuron loss on the responses in STN to enhanced dopaminergic activity.

We have completed collecting data. This material is now being readied for publication. The results show that bilateral electrolytic lesions GP had only minor effects on the activity in STN evoked by amphetamine but significantly attenuated the activity evoked in STN by apomorphine. The activation of the dopamine receptor in GP by apomorphine may have been responsible for the attenuation of the responses to the drug in STN. However, because of the debilitating effects of dopamine neuron loss coupled with bilateral GP lesions, it seems possible that the attenuation of the effects of apomorphine on STN activity were due to a metabolic deficit rather than specific effects in the STN. Experiments will be carried out in animals with unilateral instead of bilateral GP lesions, unilateral lesions normally not inducing chronic health deficits.

461. Effects of transplants of fetal dopamine tissue in striatum of the rat model for Parkinson's disease on the output of the subthalamic nucleus
Marianne Olds, D. Jacques, O. Kopyov

In this study, the aim is to determine whether countering the loss of the dopamine neurons in substantia nigra with striatal implants of dopamine fetal tissue can restore the responses to amphetamine and apomorphine seen in the subthalamic nucleus (STN) of the normal, intact rat. In these experiments, the basic preparation is the rat in which the dopamine neurons in substantia nigra have been destroyed by focal
injections of the neurotoxin 6-hydroxydopamine. After the animal has recovered from the chemical lesioning in substantia nigra and been tested for asymmetrical rotation, the behavioral marker for the lesion in substantia nigra having been effective, one group of rotators receives transplants of fetal dopamine tissue in striatum on the side of the brain with dopamine neuron loss. A second group of rotators receives transplants of fetal dopamine tissue in striatum, and at the same time a lesion is made, unilaterally, in the entopeduncular nucleus (the globus pallidus, internal segment, GPi). A third group of rotators receives transplants of fetal dopamine tissue in striatum and at the same time a lesion is made in the globus pallidus (the globus pallidus, external segment, GPe). One month after the transplantation of dopamine tissue, the animal is implanted with recording electrodes in the STN. After recovering from the electrode implantation, the animal is tested for asymmetrical rotation for evidence of changes in the rotation response related to the transplant of fetal dopamine tissue. Next activity in STN is recorded following the administration of amphetamine or apomorphine to enhance dopaminergic activity. We are seeking evidence that the combination of transplants with pallidotomy restores normal responsiveness in STN and normal motor response to amphetamine and apomorphine more effectively than either procedure carried out alone.

Publications

Associate Professor: Shinsuke Shimojo
Research Fellows: Romi Nijhawan, Christian Scheier, Carol Yin
Visiting Research Associate: Al Seckel
Graduate Students: Elizabeth Ho Ching, Yukiyasu Kamitani, Katsumi Watanabe
Research and Laboratory Staff: David Flowers, Johanna Weber

Summary: We are investigating intermediate- to higher-level perception, and its relationship to the control of attention, by testing normal, adult human subjects. In particular, we have studied how automatic and controlled attention affect local visual information processing, and how this is reflected in our visual experience and sensory-motor performance. For example, we have employed particular types of visual illusions, which we had demonstrated earlier to be very sensitive psychophysical tools to measure spatial attention or efficiency of visual information processing, to find out how exactly automatic and controlled attention interplay in the space-time domain. We have also employed the reaction time paradigm, where the subject was engaged in a task while reaction time was measured as an indicator of attention or efficiency.

We have found an intriguing distinction between two basic visual functions. One is vision for detection/orienting responses. The other is vision for detailed feature analyses. This was found by analyzing the reaction time data in terms of location/feature repetition of target stimuli, as we will describe below. Both of these lines of research, together with others, indicate the highly-adaptive and dynamic nature of the human visual system.

We are currently preparing to expand our focus of research towards developmental, neurological and cross-modal aspects.

The following are examples of two current lines of research that we plan to explore further:

Dynamic aspects of attention assessed by the line motion effect. The “line motion” effect is a new visual illusion, where we draw the observer’s attention to a particular location of the visual field, either by a visual cue or by a task demand. Then, we present a line segment between the attended and the unattended locations. Even when the line probe is presented physically simultaneously, the line appears to unfold or expand from the attended to the unattended location, thus reflecting an attention-modulated efficiency gradient in visual information processing across the visual field.

Employing this new psychophysical technique, we investigated the relationship between automatic and controlled attention. Our specific question was how the controlled, or voluntary component of attention overcomes the automatic, or stimulus-driven component of attention. By presenting a pop-out display and instructing the subject to attend to the item which was located opposite the pop-out target, we found that there was an initial transient automatic component of attention which was attracted to the area around the pop-out target, which was then overridden by the controlled component.

In an independent set of experiments, we presented a local cue in non-visual modalities, such as an auditory or somatosensory cue, then presented a visual line probe. The line was perceived to expand from the location of the non-visual cue, thus indicating the existence of a cross-modal representation of space.

Finally, we manipulated the locations of the fixation point and/or the object, between the presentation of the cue and that of the line, in order to isolate the retinotopic, the environmental, and the object-bound components of attention. We have found evidence for the environmental and object-bound components, both of which were dominant over the retinotopic.

Dissociation of visual functions by reaction times. Facilitation of visual information processing at the repeated location of stimuli has been reported in the literature (Facilitation Of Return: FOR), whereas some other studies reported a seemingly opposite
result: that is, inhibition at the repeated location (Inhibition Of Return: IOR). To examine this issue more systematically, we randomly varied the target attributes (location, color, and shape) across trials, and asked the subject to perform either one of the four tasks: (1) detection; (2) location discrimination; (3) color discrimination; and (4) shape discrimination. We further found FOR in other types of feature discrimination tasks, such as Vernier, luminance and size. We also found IOR in other orienting tasks such as the saccadic eye-movement task.

We found a very clear-cut task-dependent dissociation in the pattern of results: FOR in the tasks (1) and (2), while IOR was found in the tasks (3) and (4).

Following-up experiments have further revealed: (1) the same pattern of results in the pop-out, as well as the single-target displays, and (2) cumulative, facilitory effects by repetition of task-relevant feature.

Publications
Biopolymer Synthesis Center  
Supervisor: Suzanna J. Horvath  
Staff: Darcy A. Campbell, Linda S. Dunbar-Palm, John Racs, Kelly R. Redeker-DeWulf

The Biopolymer Synthesis Center has continued to provide high quality chemical synthesis, purification and analysis of a wide range of biopolymers at the request of the scientific community at Caltech. We also provide strong technical support to individual research projects through the introduction and the use of the latest technology in both chemistry and biology.

The DNA/RNA Synthesis Laboratory has been updated and it operates again on the state-of-the-art level. Our mode of operation is now synchronized with the highly increased need of synthetic DNA and RNA molecules in biological and chemical research. We chemically synthesize large number of deoxyoligonucleotides within the shortest period of time the most cost-effective manner.

The purchase and installation of ABI Model:3948 Nucleic Acid Synthesis and Purification System in September, 1996 and the reorganization of our mode of operation provided the key to our continued successful existence. The total number of DNA molecules synthesized in FY'97 (October-May), compared to that of FY'96 shows an 88% increase. During the first eight months of FY'97, we synthesized 6293 deoxyoligonucleotides (DNA) for 42 Caltech faculty members, which represents 166126 bases. Caltech community has welcomed the changes of increased quality; each DNA sample is now purified and provided with UV-quantitation. The 24 hour throughput is strictly maintained regardless of the continuously increasing number of requests.

The primary instrument, ABI Model:3948 is able to synthesize 48 purified deoxyoligonucleotides up to 91 bases in length in every 36 hours. This instrument is also used for routine modifications, such as mixed and inosine containing probes. Three 4-column DNA/RNA synthesizers (ABI Model:394-08) are used for RNA-synthesis and “special” DNA and RNA synthesis with chemical modifications. Our repertoire of chemical modifications became very impressive with a number of new additions to our last year's list. All three of 4-column synthesizers are constantly in use, they are also serving as a back-up system of the 3948-instrument. As a gift of Drs. Gary Hathaway and John Abelson, we received a CE-system (ABI Model: 270 AHT). We are in the process to set up this instrument for quality control analysis of regular and chemically modified DNA and RNA molecules. We have invested in a computer interface; application software, as well as in a series of new capillaries, application kits and reagents.

During the first eight months of FY'97 for five faculty members we synthesized 105 oligonucleotides (RNA), 37 of them with special design. For one faculty member we carried out 230 DNA extractions from cell suspensions.

In our Peptide Synthesis Laboratory there are three automated peptide synthesizers. Our most advanced solid phase automated synthesizer is an ABI Model:433A. It is set up for the application of Fmoc chemistry with the optimized Fastmoc chemical cycles. Every synthesis cycle is automatically monitored by a conductivity cell. There are two automated peptide synthesizers, ABI Model:430A set up for application of t-Boc chemistry. We have synthesized large numbers of short peptides, peptide-protein conjugates to generate heterogeneous antisera or monoclonal antibodies with desired specificity for a large variety of applications. Each peptide made in our laboratory includes CE, HPLC, and MS analysis. MS analysis is usually done at the Protein/Peptide Microanalytical Laboratory (PPMAL). Three groups from chemistry division regularly request HF-cleavages of synthesized t-Boc peptides. In addition to routine Fmoc synthesis we participated in the design and synthesized several Fmoc peptides with unique chemical modifications for Professor Jacqueline Barton’s group. In collaboration with Professor David Sigman (UCLA) we have successfully synthesized histone-peptides which involved the combined application of both t-Boc and Fmoc chemistries. As a collaboration with Professors Harry Gray’s and Tom Mead’s research groups we are involved in pharmaceutical development and challenging structure-function studies. During the first eight months of FY'97, we have synthesized 87 peptides for 18 Caltech faculty members and 8 outside collaborators, which represents 1271 amino acid couplings.

Flow Cytometry and Cell Sorting Facility  
Supervisor: Ellen Rothenberg  
Staff: Rochelle A. Diamond, Patrick F. Koen

This multi-user facility provides expert assistance and advanced instrumentation to researchers analyzing and separating various types of cells. The facility is equipped with two research grade flow cytometer/cell sorters capable of sorting >3000 events/second based on multiparameter analysis of the cell’s characteristics (size, density, and fluorescence intensities of multiple colors). A third non-sorting analyzer (FACScan) is available to researchers for self-service analysis provided that they demonstrate knowledge of the analyzer or take a 3-day training course provided by the facility.

Essentially any cell or cell sized particle can be analyzed for its scatter and fluorescent properties and then sorted by those parameters to separate it from a heterogeneous population. These scatter and fluorescence measurements can give quantitative as well as qualitative information on the levels of given molecules on or in individual cells, which are then correlated together to reveal statistical clusters of characteristics among all the cells in a given population.
Although our instrumentation is not new, we continue to make improvements in the operation and ability of our machines to meet the needs of our user base. One of the major achievements this year was to make four and five color multiparameter analysis and sorting operational, and, in the case of four color, routine. This involved upgrades to the entire front end optics of our Coulter Epics Elite which up to this time had been absorbing a high percentage of our red fluorescence intensity due to the optical coatings. Red sensitive photomultiplier detectors were also installed to increase sensitivity in that range. This now enables the machine to analyze cells that are stained with FITC, PE, and Red613 fluorochromes which are excited at 488nm by an Argon laser that is spatially separated from a Helium neon laser which excites the fluorochrome APC at 633nm. Using a gated amplifier circuit which delays the first signal and combines the information with the second signal, four color detection can be correlated with the scatter signals to provide multiparameter analysis and sorting capacity. Five and six color is also possible in the future, provided that one has available fluorochromes that enable their use in the system such as the new Allo-7 derivative tandem dyes that are not yet commercially available. Another improvement was the addition of a 100 micron sort-sense tip for the Elite which allows for better sorting recovery of slightly larger cells. In addition a color printer has been added to enhance the analysis of multiparameter experiments.

This year the facility has serviced, collaborated, and consulted with 33 researchers representing 13 laboratories in the Biology and Chemistry/Chemical Engineering divisions as well as outside universities, institutions, and companies. Highlights from some of these projects are presented here.

Researchers in David Anderson's Lab have been studying the development of cells in the central nervous system and neural crest of the embryonic rat by preparative sorting to isolate rare pluripotent cells. Sean Morrison has been attempting to identify and separate stem cells from the rat olfactory epithelium and telencephalon by FACS utilizing a unique combination of surface markers. Very small numbers of these cells are sorted into cluster dishes containing growth medium and then allowed to express their post-sort clonal proliferation capacity in culture. Purifying the stem cells from the olfactory epithelium will make it possible to study the regulation of olfactory receptor expression as these progenitors differentiate into sensory neurons. Li Ching Lo has been isolating neural crest precursors separate from the sympathadrenal lineage by developing the proto-oncogene, c-ret, as a new marker to sort by FACS for enteric neural precursors. Pat White has been using these sorted cells for in vivo transfer experiments to determine to what extent they maintain their developmental plasticity.

Yinon Zhang from the Zinn group has been studying the olfactory receptor system by analyzing and screening epitope-tagged olfactory receptors by FACS. The C. elegans olfactory system can detect and differentiate a large number of odorants at low threshold. Odorant molecules are thought to interact with receptors which couple G-proteins resulting either an increase in second messengers such as cAMP or release of Ca$^{2+}$ from the intercellular stores. To study the function of these olfactory receptors they transfect HEK293 cells with Myc-tagged receptor genes and look at the expression level of Myc with FACS. They have identified one receptor, ODR-10, which could be expressed on the transfected cells. They are also studying the expression of vertebrate olfactory receptors including the catfish, F47.

Several groups (Deshaies and Campbell) have been routinely analyzing wild-type and temperature-sensitive mutant yeast for cell cycle utilizing the nucleic acid staining dye propidium iodide in combination with RNase on FACS. The Campbell group has been studying mutations that affect DNA replication at both a cellular and enzymological level. The Deshaies group is deciphering how ubiquitin-mediated proteolytic pathways are deployed to control key transitions in the budding yeast cell division cycle.

The Rothenberg group has been using FACS for years to sort and analyze T-lineage cells in various stages of development. This year their needs provided the impetus to provide routine four color multiparameter analysis and sorting. The increased number of parameters has allowed for the analysis and sorting of rare subpopulations in the immature mouse thymus and fetal liver. Utilizing post-sort RT-PCR techniques, the group has been able to stage the onset of expression of different lineage-specific gene in these early precursor cells.

The Simon group has been analyzing the effects of the constitutively active G-proteins Ga13 and GaQ on programmed cell death in COS and CHO cells. They have been trying to confirm their results using the TUNEL assay, which labels the DNA strand breaks present in apoptotic cells with FITC which is then detected by FACS.

Robbert van der Most of the Strauss Lab is analyzing the interaction between the envelop protein from the flavivirus, yellow fever virus, and its cellular receptor. he is measuring the binding of whole virus particles as well as a baculovirus-produced soluble envelope protein to human cells. The results show that both ligands bind to cells, which now allow us to analyze mutated forms of the envelope protein for cell binding.

**Monoclonal Antibody Facility**

**Supervisor:** Paul H. Patterson  
**Member of the Professional Staff:** Susan Ker-hwa Ou  
**Staff:** Shi-Ying Kou

The Monoclonal Antibody Facility provides assistance to researchers wishing to generate monoclonal antibodies (mAbs), polyclonal ascites Abs,
ascites fluid or other tissue culture services. In addition to the service functions, the Facility also conducts research on the development of novel immunological techniques.

In collaboration with Jose Lebron from the Bjorkman laboratory, we are developing a new tolerization method that utilizes high dose injection of uninteresting antigens in adult mice followed by challenge with interesting antigen. Serum screens show that the immune response in tolerized animals is preferentially against the interesting antigen. Several hybridoma lines were subsequently cloned that produce mAbs that recognize the interesting antigen only.

In collaboration with Jr-Gang Cheng from the Harrington laboratory, we succeeded in obtaining mAbs against human leukemia inhibitory factor (LIF) or mouse LIF by immunizing LIF knockout mice.

In collaboration with Tara Chapman from the Bjorkman laboratory, we succeeded in obtaining mAbs against human CMV heavy chain by immunizing β2m transgenic mice.

In its service capacity, the Facility obtained mAbs for the following groups during the past year: Alice Paquette (Anderson laboratory) obtained mAbs against N terminal of NRSF (neuron-restrictive silencer factor) plus mAbs against C-terminal of NRSF. Hoa Nguyen (Chan laboratory) obtained mAbs against 45 Kd, 26 Kd, 23 Kd subunits of the particulate methane monoxygenase from Methylococcus capsulatus. Chung-Ming Choung (U.S.C.) obtained mAbs against E13 chicken feather filament and E8 chicken epithelium protein. Jose Lebron (Bjorkman laboratory) obtained mAbs against the heavy chain of major histocompatibility complex class I molecule HLA. Matt Wahl (U.C.L.A.) obtained mAbs specific for a phosphopeptide from a regulatory tyrosine residue of a signaling protein. Kaushiki Menon (Zinn laboratory) obtained mAbs against Drosophila synaptotagmin. Ed Rubel (U. of Washington) obtained Abs against chicken basal cochlea. Tim Fritz (U.C. Irvine) obtained Abs against WGA soluble fraction, and some of these can block the chicken growth cone collapse activity of this fraction. Jim Ou (U.S.C.) obtained mAbs against Hepatitis C virus core protein. Kwang Choi (Baylor College of Medicine) obtained a polyclonal ascites against WGA soluble fraction, and some of these can be used in large sequencing projects. A collection of Macintosh software is also available.

A number of researchers use the SGI workstations for molecular modeling. The programs available for this purpose include BioSym, O, Tom, MidasPlus, Setor, Rasmol, VMD, Molscript, XtalView, CCP4, Delphi and GRASP. These machines are also used for the Protein Structure course and for the summer Chemistry animation project.

The lecture notes and homework from the introductory course "Fundamentals of Sequence Analysis" are available on the SAF webserver (http://seqaxp.bio.caltech.edu/). In order to make it easier for users to find the topic of interest, the documentation for all programs is available on the SAF webserver. This server is also the central distribution site for nucleic acid sequences prepared by the DNA sequencing facility.

The Protein/Peptide Micro Analytical Laboratory
Director: Gary M. Hathaway

The protein/peptide micro analytical laboratory (PPMAL) was established in the Beckman Institute as a facility of the Division of Biology for the purpose of providing protein and peptide structure analysis to the campus. The laboratory collaborates with the Caltech...
faculty and (for purposes of efficiency) to a limited extent with the general scientific community. Funded in part by the Beckman Institute, charges are set only to assist in cost recovery. The laboratory is located in room 204 of the Beckman Institute with offices in rooms 232 and 215.

Activity Centers
1. Protein & peptide micro chemical sequence analysis at the low to subpicomole level.
2. Protein & peptide identification through sequence analysis and mass spectrometry.
3. Biopolymer mass analysis by matrix assisted, laser desorption, time-of-flight mass spectrometry (MALDI-TOF).
4. C-terminal sequence analysis by exoprotease digestion and mass spectrometry.
6. Microcapillary HPLC for the separation of peptide and proteins.
7. Protein chemical modification, fragmentation, and purification of peptides.
8. Database search by amino acid sequence, fragment masses, and composition.
9. Consultation on sample preparation and error analysis including the timely dissemination of literature materials pertinent to its operations.
10. Maintenance of the laboratory’s homepage on the world wide web.

Protein/Peptide Sequence Analysis  Protein and peptide sequencing remains a major analytical function of the PPMAL. Currently, it is carried out by both chemical degradation and mass spectrometry. All use the latest chemistries and techniques for PTH-amino acid separation by HPLC with UV detection to the femtomole range, and mass spectrometry (including collisional activated dissociation, and exoprotease digestion ladder sequencing). The lab annually sequences more than 220 proteins and peptides for the faculty at Caltech and the scientific community at large.

Mass Spectrometry of Large Biopolymers The laboratory houses a 2.0 meter time-of-flight instrument from Vestec, a division of Perseptive Biosystems', Inc. equipped with matrix assisted, laser desorption ionization, time-lag focusing, single stage reflectron, and collision cell. Analysis of proteins and peptides is carried out routinely with mass accuracy ranging from 0.1% to 0.003% and sample loads down to the attomole scale. Resolution exceeds 5000 FWHM with peptides to mass 3 kDa. The instrument has been used to gain structural data and sequence information on peptides by employing the collision cell or by ladder sequencing following enzymatic exoprotease digestion. In other experiments we have identified unknowns starting with as little as 5 picomoles of material in an SDS polyacrylamide gel slice or polyvinylidene difluoride membrane. This year we began using direct collection of entire HPLC fractions to the mass spectrometer sample stage by means of an X/Y robotic collector. It is estimated that when coupled to capillary HPLC, less than 1 picomole will be required to identify a protein using in situ digestion. In addition to proteins and peptides, hundreds of modified oligodeoxy- and oligoribonucleotides are analyzed. This year over 1500 proteins, peptides, oligonucleotides, and small organic molecules will be analyzed for Caltech.

Amino Acid Composition Analysis  The method requires as little as 0.5-1.0 µg of sample and the analytical data have been used successfully to identify unknowns by searching protein databases (see for example, PropSearch at EMBL at http://www.embl-heidelberg.de/aaa.html).

Sub Millimeter High Performance Liquid Chromatography  Micro bore HPLC is used for the separation of nanogram amounts of peptides.

Preparative, reverse phase chromatography is carried out using 0.3 to 0.5 mm ID columns with flow rates of 4-10 µl/min. Detection is by absorbance in the low UV. Peak fraction collecting is achieved in volumes of one to four microliters.

Chemical and Enzymatic Digestion of N-terminally Blocked Proteins For Internal Sequence Analysis and Identification  The laboratory carries out the fragmentation of protein samples using both chemical and enzymatic methods. Less than 5 picomoles of protein have been digested, the resultant peptides separated by microcapillary HPLC, and peptides analyzed by mass spectrometry. This methodology is designed to allow unambiguous identification of biologically important factors which have been traced to a physiological modification of interest. It is also employed when the protein amino terminus blocked, or when the molecular weight excludes it as a candidate for direct N-terminal chemical sequence analysis.

Database Search and Analysis  The laboratory maintains access to protein databases for searching with sequence information obtained by chemical sequence analysis, or by searching peptide libraries with mass data obtained on peptides and their fragments. These searches often combine sequence and mass information achieved by chemical and collisional degradation and are available on our lab computers as independent software, and our connection with the Internet through the Campus Computing Organization.

Homepage on the web  We have continued to expand the utility of the homepage with more detailed information and links to other sites. Recently we have introduced a bulletin board where we post the latest information and references. A newer link has been created which allows printing forms of high quality. As a result, one can now access information regarding sample preparation for submitral, cost recovery schedules, forms, and other technical information on our operation through our homepage, as well as the latest technical developments:
http://www.caltech.edu/~ppmal

In addition, the homepage contains a section which provides the reader with a means to address comments.
and questions directly by computer. If desired, hard copy may be obtained on the do's and don'ts of analysis by direct down loading to your computer or by requesting a copy by emailing us at: ppmal@caltech.edu, or calling 818-395-6388.

Collaborations and Accomplishments

Interfacing Mass spectrometry and chemical sequencing

Major successes continued utilizing the new instrument's ability to detect peptides in mixtures from tryptic digests at high sensitivity. This "peptide fingerprinting" technique permits the identification of physiologically important unknowns through a database search analysis. One collaboration resulted in the successful identification of a peptide from a digest of a 100 kDa protein. The theoretical number of peptides generated as calculated from the sequence was 88. More than 65 peptides were counted from the trace fingerprinting mass spectrometry and chemical sequencing of the HPLC chromatogram. A single peptide was isolated and identified from the mass spectrum profile containing a phosphorylated residue. Its non-phosphorylated counterpart was also identified based on the predicted molecular weight and the mass spec. data. The PPMAL utilized the Internet to access multiple software programs for this type of analysis, and have used this approach to identify an unknowns starting with less than 5 picomoles in a polyacrylamide gel slice. The PPMAL also participated in a study sponsored by the Association of Biomolecular Resource Facilities’ Internal Sequence Analysis Committee. Thirty eight resource labs like PPMAL participated in a test designed to measure their ability to obtain internal sequence of a protein and use the information to identify the protein. The sample was present in an SDS polyacrylamide gel electrophoresis slice, and also as the sample blotted to a polyvinylidene difluoride (PVDF) membrane. As we did last year, the PPMAL opted to analyze both media. Participants were asked to digest the sample, separate the peptides by HPLC, locate and sequence a target peptide. Mass analysis of the putative HPLC fraction containing the target peptide revealed the presence of two peptides. Since further separation by HPLC was impractical because of the small quantity, the impure peptides could not be sequenced by N-terminal chemical sequence analysis which requires at least 80 mole per cent purity. However, using the collision cell of the mass spectrometer, we were able to obtain both the correct sequence and subsequent identity of the peptide from analysis of the mixture. This technique of timed ion selection allows each peptide in a mixture to be analyzed in turn without having to resort to purifying the individual peptides. The sequence was especially difficult since it contained both Lysl, glutamyl and glutaminyl residues. Nevertheless, the PPMAL was one of four labs who successfully completed the test with 100% accuracy. Also, members of the lab traveled to Palm Springs in June for the 45th ASMS conference on mass spectrometry and allied topics.

As stated previously, PPMAL was established to provide the campus with techniques for protein and peptide analysis. This consisted primarily of chemical sequencing using Edman degradation with automated instrumentation. Development of this analytical capability has proceeded by validation and modification of state of the art techniques and chemistries and by the development and setting in place supporting methods, technologies, and hardware such as amino acid analysis, microcapillary HPLC and time-of-flight mass spectrometry. With the advent of the post genome sequence era, protein identification as opposed to the more traditional uses for the protein sequence information (such as database construction) has begun to emerge as a major activity in the biological sciences. The study and analysis of the entire complement of proteins expressed by an organism's genome at any point in time has been dubbed "Proteome Science". This type of science places stringent demands on issues of analytical sensitivity and throughput such that many facilities are now being asked to identify unknowns starting with less than 5 picomoles in a gel slice or on a membrane with a few specialized labs being able to analyze at the one or two picomole level. In accordance with this, high sensitivity methods coupled with both chemical sequencing and mass spectrometry have become the focus of lab activity. This rising demand for increased sensitivity and throughput has led to the introduction of new and exciting techniques, many of which involve mass spectrometry. More and more peptide mass data and mass fragmentation data obtained through collisional fragmentation within mass spectrometers are being used to search appropriate databases with little or no de novo sequence assignment. This year we put in place an X/Y robotic collector which directs whole HPLC fractions to the stage of the mass spectrometer. This one method which is helping to address the major stumbling block to high sensitivity analysis, i.e. the losses accompanying sample digestion and purification.

Further improvement in MALDI-TOF instrumentation including delayed extraction or time lag focusing has increased our mass accuracy to better than 30 parts per million with future improvements expected to give resolution to 10,000 at mass 5 kDa and accuracy to 5 ppm. This level of accuracy is critical for the sequencing of peptides e.g. distinguishing glutamine from lysyl residues which differ by only 0.036 mass units, and enhanced resolution increases the upper size limit for proteins which can be C-terminally sequenced by mass spectrometry.

The newly acquired triple quadrupole mass spectrometer will couple mass analysis with microcapillary HPLC for direct mass analysis of liquid fractions. This instrumentation will be crucial for the identification of post translational modifications such as myristylation, sulfation, phosphorylation, and glycosylation. Following modification, the mass
spectrometer will operate in the “nanospray” mode for MS/MS analysis of peptide materials.

Transgenic and Knockout Mouse, Core Facility
Director: Shirley Pease, F.I.A.T
Mouse Facility Manager: Bruce Kennedy, Msc, LATG
Staff: Diane D’Agostin, MSc
   Hector Ferero
   Alba Granados
   Prim Kanchanastit, Bsc, ALAT
   Jorge Mata, ALAT
   Jose Mata
   Juan Silva, Bsc, LAT
   Alicia Sosa

Summary: The transgenic technique for gene addition in the mammalian system (Gordon et al. 1980) was established at Caltech in 1984. More recent technological advances in this field have provided for targeted disruption of specific genes. This technique involves the manipulation of pluripotent embryonic stem cells in vivo and their subsequent return to the embryonic environment, for incorporation into the developing embryo (Zijlstra et al. 1989). In some cases the resulting chimeric mouse born is useful for its tissue contribution from two sources: 1) the host embryo and 2) the manipulated embryonic stem cells, but in the majority of cases, the success of the technique rests upon the generation of wholly transgenic descendants from the embryonic stem cell contribution to the germ line of the chimeric mouse. In 1993, we were successful in establishing this technology at Caltech, and the facility in collaboration with Anderson, Simon, Wold and Varshavsky laboratories has generated multiple transgenic, knockout and knockin mouse strains in the intervening years.

As to the management of the mouse colony, we have now established a large pathogen free mouse facility containing 51 Tg and Ko lines. The newly refurbished, barrier maintained Transgenic Mouse Production Facility was opened up and restocked with pathogen free strains in February of 1995.

We have continued to produce new transgenic lines while re-deriving pathogen free stocks of existing strains and in all, since February 1995 have produced 14 new transgenic lines for gene addition and 9 new knockouts with others currently in process. All embryo manipulation involved in the production of these new lines has been executed by core facility staff. Micromanipulation equipment has been set up within the barrier facility, which operates on restricted access as part of the “barrier” itself. The Department has also allocated a room outside both animal facilities that is used primarily to teach grad students, technicians and postdocs the techniques involved in transgenic mouse production. This area has been in use since July, 1996. Investigators have the option to do their own micromanipulation of embryos here, rather than take advantage of the service available from the Transgenic Core.

In addition to producing a total of 23 new animal models since February, 1995, we have evaluated in vivo two E.S. cell lines and re-derived 28 pre-existing Transgenic lines of mice. Also, we are about to embark upon a program to safely store embryos from all our transgenic and knockout lines, by cryopreservation, and have recently succeeded in establishing a protocol that gives us excellent embryo recovery from liquid nitrogen.

The production of pathogen free animals enables Caltech to exchange valuable transgenic mouse models with other academic groups around the world. As more and more mouse models become available and the exchange of animals more frequent, it is essential for Caltech to be in a position to deal effectively with animal lines generated in the facility and in other laboratories. To this end, the Department is this year funding the refurbishment of two quarantine animal holding areas. This will enable us to safely contain and “clean up” other mouse models coming in from other institutions, without putting our own colony at risk. In the coming year we expect to eliminate MHV from residual conventional stocks. The conventional holding area in the Alles Building is due to be refurbished in the fall of this year. We will restock the area with pathogen free animals and will make it our goal to maintain them in such a condition while investigators enjoy full access to the facility. Thus, we expect by early 1998 to have reached one of our long term goals in animal management by having cleaned up all our mouse stocks and effectively separated mouse production from animal use and both from the potential threat presented by the introduction of new stocks from other institutions.

Eight principal investigators and their postdoctoral fellows or graduate students are presently using the transgenic facility. They are: PI-David Anderson (Z.F. Chen, Qui Fu Ma, Amy Greenwood, Hai Wang, Nirao Shah, Jae Kim, Sebastian Gerety); PI-Paul Patterson (Lisa Banner); PI-Melvin Simon (Jeannie Chen, Jason Chen, Stefan Muller, Pam Eversole-Cire); PI-Ellen Rothenberg (Susan Ward); PI-Barbara Wold (Dawn Cornelison, Susan Wang, Xin Yu, Jeong Yoon); PI-Scott Fraser (David Crotty); PI-Alexander Varshavsky (Yung Tae Kwon); PI-Giuseppe Altardi (Catherine Lin). Marianne Bronner-Fraser (Martine Garcia, Sung Kil, Andy Groves); Henry Lester (Purnima Deshpande, Hong Dang, Paolo Kofugi).

References
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Bachelor of Science

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Keely Llewellyn Walker
Jane Chia-Pen Wei*
Barbara Ann Weir
Michelle Sweeney Wiegand

*Graduates whose names are followed by an asterisk graduated with honor in accordance with a vote of the faculty.

Master of Science

Sandra Ann Chin
Katherine Anne Hannon

Joshua Daniel Eisenberg
Hyejin Kang

Doctor of Philosophy

Hyejin Kang (Cellular and Molecular Neurobiology)
Modulation of Synaptic Function by Neurotrophic Factors in the Adult Hippocampus.

Robert P. Lane, Jr. (Molecular Biology and Biochemistry)
Evolution of the Neural Immunoglobulin Supergene Family and Functional Studies of One of its Members.

James William Lewis (Integrative Neurobiology)
The Intraparietal Sulcus of the Macaque and Connected Cortical Regions: Anatomical Parcellation and Connections Throughout the Hemisphere.

Frank Miskevich (Molecular Biology and Biochemistry)
Expression, Characterization, and Ligand Studies Involving Domains of the Chick Cell Surface Protein Neogenin.

Mark Paul Running (Biology)
Molecular Genetics of Floral Patterning in Arabidopsis thaliana.

Nirao Mahesh Shah (Biology)
Mechanisms of Cell Fate Determination and Differentiation in the Mammalian Neural Crest.

Martin Bernard Stemmler (Computation and Neural Systems)
Information Maximization and Stochastic Resonance in Single Neurons.

Giuseppe D. Tocchini-Valentini (Biochemistry)
E. coli tRNA Leucine Identity and Recognition Sets.

Kellie Lynn Whittaker (Developmental Biology and Molecular Biology and Biochemistry)
RNA Localization in Drosophila Oogenesis and Early Embryogenesis.

Katherine Woo (Developmental Biology and Cellular and Molecular Neurobiology)
Pattern Formation in the Central Nervous System of the Zebrafish (Danio rerio).

Kyuson Yun (Biology)
Murine Twist is a bHLH Regulator that Inhibits Myogenesis by Multiple Molecular Mechanisms

(When more than one field of study is listed in the Division of Biology, it indicates a dual major.)
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Zeneca Seeds
Author and Group Index (by page number)

Carlos, Rose, 57
Carpenter, Philip, 27
Celniker, Susan, 67, 68
Chan, Carolyn, 229
Chang, Chieh, 42, 103
Chang, G.C., 222
Chang, Mi, 46
Chapman, Tara, 148, 149
Chaudhry, Prem, 19
Chee-Ruiler, C., 231
Chen, Ching-Kang, 94
Chen, Pei, 87
Chen, Hong-Jung, 184
Chen, Robert, 87
Chen, Wen, 101
Chen, X-N, 182
Chen, Xuemei, 71, 72, 74, 75
Chen, Yu-Juin, 96, 98
Chen, Zhoufeng, 170, 171
Cheng, Alan, 108
Cheng, Jc-Gang, 204
Chiu, Yong, 22
Choe, Wonchae, 153
Chomyn, Anne, 137, 142, 143
Chuang, Chiou-Fen, 75, 76
Clandinin, Tom, 107
Coffman, James A., 40
Colayco, Enrique, 96, 97, 98
Cole, Susannah, 66
Coleman, Thomas, 28
Collazo, Andres, 34, 50
Copeland, Jeffrey, 104
Cornelson, Dawn, 119, 120
Correll, Craig, 22
Crick, Francis, 236
Crotty, David, 49
Crowell, J.A., 224
Csete, Marie, 122
Dahiyat, Bassil, 157, 158
Dang, Hong, 189, 190, 191
Dang, Yan, 194
Davidson (E.) Lab, 37
Davidson, Eric, 37-47
Davidson, Norman, 196, 197, 206
Davydov, Illia, 115, 116
Dawson, Phil, 57
DeModena, John, 104, 108
DeRose, James, 28, 29
Desai, Chand, 211, 212
Deshaies Lab, 20
Deshaies, Raymond, 20
Deshpande, Purnima, 189, 190, 191
Diamond, Rochelle, 84, 85, 92
Dixon, Mary, 53
Ding, Yan, 97, 98
Dougherty, Dennis, 190, 191, 192, 193
Doupnik, C.A., 193, 197
Dreyer Lab, 179
Dreyer, William, 179
Du, Fangyong, 112
Dua, Rajiv, 154
Dunphy Lab, 24
Dunphy, William, 24-28, 116
Dvorak-Carbone, Hannah, 209
Echeverria, Annabel, 97
Eckstein, Dan, 121
Edens, Jean, 95
Egnor, Roian, 243
Ehringeruber, Markus, 197
Elssasser, Suzanne, 155
England, Pamela, 191
Enriquez, Jose, 136, 142
Eversole-Cire, Pamelan, 92, 95

Feldman, Renny, 22
Felix, Marie-Anne, 102
Fernandez-Silva, Patricio, 140, 141, 142
Fidan-Nowak, Melihat, 189
Fisher, William, 67, 68
Fleischer, Kurt, 59
Fletcher, Jennifer, 75, 79, 80
Forss, Jenny, 223
Frazer Lab, 47
Fraser, Scott, 34, 48-62
Friedrich, Markus, 177, 178
Frolich, Michael, 182
Gabbiani, Fabrizio, 236, 237, 244
Gadient, Reto, 202, 203
Galak, Andrew, 129
Gale, Nicholas, 50
Gallivan, Justin, 191, 192
Garcia, L. René, 101, 108
Garcia-Castro, Martín, 36
Garvey, Justine, 197
Gerety, Sebastian, 170
Chatzipia, Ioanna, 108
Ghosh, Pratik, 219
Gilissen, Emmanuel, 219
Gomez, Christie, 97
Gonzalez-Serricchio, Aldy, 105
Gordon, David Ben, 158
Gray, Harry, 57
Greenwood, Amy, 172
Gregorian, Armenia, 42, 43
Grieve, K.L., 222, 225
Groves, Andrew, 35
Grunewald, A., 223
Guan, Min-Xin, 136, 137
Gundappa-Sulur, G., 228
Guo, Zijian, 26
Gupta, Garun, 29
Hadijarygrou, Michael, 204, 205
Hajdu-Cronin, Yvonne, 101
Hamburger, Zsuzsa, 149, 151
Harada, Yoshito, 42, 44
Harrington, Michael, 40, 46
Harrison, Reid, 140
Hartmann, M.J., 229
Hawkins, Christine, 65, 66
Hay Lab, 63
Hay, Bruce, 63-66
Hayran, Archana, 175, 177
Helm, Mark, 138
Hernandez-Hoyos, Gabriela, 87, 88
Hershowitz, Ari, 247
Higgins, Charles, 249
Hofmann, Ulrich, 232
Holt, Gary, 236
Hopper, Neal, 104, 105
Hotz, Ray, 68
Hu, Yi-Hui, 91
Hua, Jian, 81, 82
Huang, Jerry, 156
Huber, Martina, 56
Ito, Toshiro, 70, 77
Itti, Laurent, 239, 240
Jacobs, Russell, 56, 58, 61, 219
Jacobsen, Steven, 73, 78
Jaeger, Dieter, 233
Jagadish, Venkat, 233
Jankowski, Joarna, 203
Jasek, M.C., 196, 197, 198
Jeo, Richard, 217, 220
Jia, Lin, 208
Jiang, Lily, 104, 106, 107
Johnson, Jennifer, 144, 149
Johnson, Marty, 98
This modified figure is taken from René Descartes' *Treatise of Man*, published in 1664. Descartes believed that retinal images led to stimulation of the pineal gland, the teardrop shaped organ depicted in the subject's brain. He believed the pineal gland then activated nerves which resulted in a limb movement. We have shown that the posterior parietal cortex combines retinal image signals with information about the direction of gaze in order to represent visual stimuli in body-centered reference frame, which can then be used to guide limb movements (see Andersen abstracts starting on page 222).