CALTECH Biology Annual Report 1991
FRONT COVER

Photoreceptor axons project into the optic lobe of *Drosophila*, in a precise array. Axons stained with horseradish peroxidase. Preparation by Rosalind Young in Seymour Benzer's laboratory.

BACK COVER

Localized RNA in the *Drosophila* egg (see Abstract Nos. 113 and 114). The figure depicts the distribution of a maternally synthesized transcript that is localized to the posterior pole of the *Drosophila* egg and early embryo. The upper embryo shows the localization of the maternal transcripts shortly after fertilization. The RNA is highly concentrated at the posterior pole and forms a gradient towards anterior. The lower embryo is at cellular blastoderm stage, the posteriorly localized maternal RNA has been incorporated into the pole cells; in addition, zygotic transcription has been activated in the anterior third of the embryo. In both cases, anterior is to the left, dorsal to the top. The RNA distribution was detected by *in situ* hybridization of non-radioactive probe to whole mounts of embryos. The picture was produced by pseudocoloring video images of such embryos. Red indicates the highest level of staining.
A REPORT FOR THE YEAR 1990-91
ON THE RESEARCH AND OTHER ACTIVITIES
OF THE
DIVISION OF BIOLOGY
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CALIFORNIA INSTITUTE OF TECHNOLOGY
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Constance R. Katz - Annual Report Secretary

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RESEARCH REPORTS

Much of the research work summarized here (covering the period 5/90-6/91) has not yet been reported in print, in many instances because it is not yet complete. For that reason this 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 August 1990-August 1991, are listed separately at the end of the research reports of each group.
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INTRODUCTION
THE FACULTY

Professor Scott E. Fraser has joined the faculty of the Division this year as the Anna L. Rosen Professor of Biology. Scott received his B.S. in Physics from Harvey Mudd College in 1976 and his Ph.D. in Biophysics from the Johns Hopkins University in 1979. He came to Caltech from the University of California at Irvine where he was Professor and Acting Chair of the Department of Physiology in Biophysics. In spite of his youth, he has achieved a preeminent position in embryogenesis and developmental neurobiology, with special emphasis on the analysis of cell lineage and cell migration. He will bring a special expertise in two areas of biotechnology: confocal microscopy and very recently NMR microscopy. Fraser will strengthen the whole field of image analysis at Caltech. His laboratories as well as the Biological Imaging Center that he will co-direct are housed in the Beckman Institute building.

HONORS AND AWARDS

Professor John Allman has been recognized by the 1990 Golden Brain Award of the Minerva Foundation in Berkeley, California.

Professor Seymour Benzer has been recognized by an award of the highly prestigious Wolf Foundation Prize for Medicine. He follows in the footsteps of Professor Edward Lewis who received this prize in 1989.

Assistant Professor Pamela Bjorkman has been selected as a co-recipient of the Cancer Research Institute 1991 William B. Coley Award for Distinguished Research in Fundamental Immunology.

John Bowman, who completed his Ph.D. work this year with Professor Elliot Meyerowitz, received the Lawrence L. and Audrey W. Ferguson Prize for the best Ph.D. thesis in Biology this year. He was also recognized by selection as recipient of the Milton and Francis Clauser Doctoral Prize for the most original Ph.D. thesis at Caltech for this academic year.

EVERHART BOWMAN BUSH

Senior Research Associate Andrew Cameron and Associate Professor Mary Kennedy have been elected Fellows of the American Association for the Advancement of Science in recognition of their contributions as members "whose efforts on behalf of the advancement of science or its applications are scientifically or socially distinguished."

Dr. Anna C. Glasgow, Visiting Associate, has been named as a recipient of an NSF Presidential Young Investigator Award.

Research Fellow Dr. Min Han has been selected as a recipient of the prestigious Lucille P. Markey Scholar in Biomedical Science.
HONORS AND AWARDS (continued)

Professor Leroy Hood received the Franz Groedel Medal of the American College of Cardiology. He gave the following honorary lectureships: the Jeanette Piperno Memorial Lectureship at Temple University; the Microbiology Graduate Students Distinguished Lecturer at Iowa State University; the Basil Staples Visiting Professorship at the University of Maine; the Bren Fellows Lectureship at the University of California, Irvine; the Leo S. Weil Memorial Lecturer, Touro Infirmary, at the Tulane Medical Center and Louisiana State University Medical Center in New Orleans; the Jessie & John Danz Lectureship at the University of Washington; the Kosuge Memorial Lectureship at the University of California, Davis; the Franz Groedel Lecture at the Open Plenary Session of the American College of Cardiology; and the Hoffman-LaRoche Lectureship in Microbiology at the Waksman Institute of Rutgers University. He has become a member of the Ediotorial Board of Current Opinion in Biotechnology and a Member of the Science Advisory Board of the Discovery Museum of Orange County, the Scientific Advisory Board for Biological Sciences of Molecular Simulations, Inc., the Advisory Board of the Network of Centres of Excellence, University of British Columbia, and the Advisory Board of the Human Genome Center at the Lawrence Berkeley Laboratory.

Professor Masakazu Konishi has been appointed a Non-Resident Fellow of the Salk Institute. He has received the highly prestigious 1990 International Prize for Biology from the Japan Society for the promotion of science. He has given the following named lectureships: The 1990 Baule Distinguished Lecture in Neuroscience; The 1990 Robert J. Terry Lecture; the Harvey Lecture; The 1991 Anton J. Carlson Memorial Lecture.

Assistant Professor Gilles Laurent was awarded a McKnight Scholars Award and a Sloan Research Fellow Award.

James Mazer and Andrew Huber were chosen for awards for their teaching of Bi110 and Bi11, respectively, by the Biology Undergraduate Student Advisor Committee.

Professor Elliot Meyerowitz was elected a Fellow of the National Academy of Arts and Sciences; he has been appointed Chairman of the Science Steering Committee for the Multinational Coordinated Arabidopsis thaliana Genome Research Project and appointed to the Committee on Plant Sciences of the National Research Council.

Professors Paul Patterson and David Van Essen were chosen by the Biology Undergraduate Student Advisory Committee as recipients of the 1989-90 Excellence in Teaching Awards.

Professor Jean-Paul Revel has been selected as Chairman of Section G of the American Association for the Advancement of Science.

Professors Jean-Paul Revel and Eric Davidson each received a Lawrence L. and Audrey W. Ferguson Teaching Prize. Teaching Assistant Shubha Tole also received a Ferguson Teaching Prize. A modest cash award is attached to this recognition for contributions in teaching.

Senior Research Associate S. Barbara Yancey is serving as a Member of the Program Organizing Committee for the International Bi-Annual Gap Junction Conference.
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Norman Davidson, Executive Officer
Paul H. Patterson, Executive Officer

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Summary: The bending of cilia and flagella is generated by active sliding between the doublet microtubules that are the dominant structural features of these organelles. This active sliding is generated by the axonemal motor molecule, dynein. The active sliding process must be regulated in a variety of ways: to provide for the intermittent activity that is the basis of oscillation and bending wave propagation and to vary the bending pattern to meet the needs of the cell.

Much of our experimental study of flagella exploits the ATP-reactivated movements of demembranated flagella. This in vitro assay system retains all of the motile capabilities and some of the regulatory capabilities of intact flagella. Photographic recording of the movements provides high spatial and temporal resolution, which is exploited by computerized image analysis to extract quantitative data on flagellar behavior. Our recent work has included analysis of regulatory mechanisms involving phosphorylation, calcium, and calmodulin, with the aim of finding out how these regulatory mechanisms interact with critical control points in the basic mechanisms for oscillation and bend propagation.

1. Direct measurements of microtubule sliding in beating flagella

Charles J. Brokaw

The use of computerized image analysis to measure the positions of 40 nm gold bead clusters attached to the exposed outer doublets of demembranated sperm flagella, in order to obtain direct measurements of microtubule sliding associated with flagellar bending, has now been extended to spermatozoa of the tunicate Ciona. Results of measuring 234 pairs of bead clusters distributed over the length of the flagella show that the amplitude of oscillatory microtubule sliding is not uniform along the length. Instead, there is a clear minimum in the sliding amplitude between 20-25 µm from the base of the flagellum, corresponding to the minimum revealed by calculating sliding from measurements of angular orientation of the flagellum, relative to the axis of the sperm head. Therefore, in Ciona spermatozoa, the sperm head is rigidly attached to the basal end of the flagellum, and measurements of flagellar angle relative to the sperm head axis give an accurate indication of sliding between the flagellar microtubules. This contrasts to the result obtained previously with sea urchin spermatozoa, where the head appears to have a flexible attachment to the basal end of the flagellum.

The non-uniform amplitude of sliding verified by these measurements indicates that propagation of constant amplitude bends along the flagellum is not accomplished by propagation of a wave of oscillatory sliding of constant amplitude, and therefore appears to require a mechanism for monitoring and controlling the bend angle as bends propagate.

2. Phosphorylation associated with activation of Ciona sperm motility

Chinmoy S. Dey, Charles J. Brokaw

A high molecular weight dynein ATPase polypeptide and an 18-20 kDa dynein light chain of Ciona sperm flagella are strongly phosphorylated during in vitro activation of motility or in vitro activation of motility by incubation with cAMP. These proteins also become thoroughly phosphorylated during incubation of washed, demembranated spermatozoa with catalytic subunit of cAMP-dependent protein kinase, under conditions where there is no activation of motility until a supernatant component is added. Therefore, phosphorylation of these dynein polypeptides is not sufficient for activation of motility. In vitro activation of motility by incubation with cAMP can be completely inhibited by a random copolymer of glutamate and tyrosine, which inhibits tyrosine kinase activity. Under these conditions, much of the protein phosphorylation associated with activation of motility, including phosphorylation of the dynein polypeptides, is also inhibited. These results suggest that regulation of motility of these spermatozoa may involve a multicomponent kinase cascade rather than a simple phosphorylation of a protein "switch" by the cAMP-dependent kinase. A 53 kDa axonemal phosphoprotein
band shows the strongest correlation with activation of motility in these experiments.

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Summary: The higher eukaryotic cell undergoes a precisely controlled reorganization of many of its most complex structures during mitosis. The replicated genome is packaged into chromosomes for orderly transmission to each daughter cell. The nuclear membrane and its supporting lamina are dismantled. Organelles of the secretory machinery such as the endoplasmic reticulum and the Golgi apparatus are partially or completely fragmented. Finally, the cytoskeleton is refashioned to form the mitotic spindle.

During the cell cycle, proliferating eukaryotic cells employ regulatory enzymes which guarantee that mitosis occurs at the appropriate time. The ultimate target of these regulatory factors is maturation-promoting factor (MPF), a pivotal protein kinase that directs the cell to enter mitosis by phosphorylating numerous structural and regulatory proteins. For example, MPF appears to phosphorylate the nuclear lamins and histone H1 directly, thereby eliciting lamina disassembly and DNA condensation. In addition, MPF can phosphorylate other kinases such as c-src, which in turn bring about ultrastructural changes such as reorganization of the cytoskeleton.

MPF is a dimeric complex consisting of the cdc2 protein kinase and a larger regulatory subunit known as cyclin. As the name implies, the abundance of cyclin varies throughout the cell cycle, with the peak occurring at mitosis. In contrast, the concentration of the cdc2 subunit is roughly constant throughout the cell cycle, although cdc2 is active as a kinase only during mitosis. The periodic accumulation of cyclin, however, only partly explains the cyclical nature of cdc2 kinase activity. In addition, cdc2 undergoes a complex series of post-translational modifications during the cell cycle.

Many details about the regulation of cdc2 remain to be elucidated, but it is well established that one critical type of regulatory mechanism involves the inhibitory phosphorylation of cdc2 during interphase. In particular, the phosphorylation of cdc2 on a specific tyrosine residue leads to suppression of its activity prior to mitosis. Thus, the signals that trigger cdc2-specific tyrosine dephosphorylation control the timing of MPF activation and the ensuing entry into mitosis. For example, several lines of evidence have suggested that the tyrosine phosphorylation/dephosphorylation of cdc2 plays a role in coupling the activation of MPF to the faithful completion of DNA replication. One focus of our lab has been to examine the biochemistry of this tyrosine phosphorylation pathway.

The biochemical mechanisms regulating the activation of the cdc2 protein can be studied conveniently in extracts of *Xenopus* eggs and oocytes. In extracts from activated eggs, the periodic alternation between S phase and mitosis can be recreated *in vitro*. During each cycle, the DNA in newly formed nuclei undergo disassembly following activation of the pre-MPF that had accumulated during interphase. In lysates from *Xenopus* oocytes, a stored supply of pre-MPF can be activated in a cell-free reaction. The activation of pre-MPF entails tyrosine dephosphorylation of cdc2 and activation of its capacity to
phosphorylate mitotic substrates. Several negative regula-
tors of this reaction have been described. One inhibitory
molecule is fission yeast p13 (the suc1 gene product).
p13 binds directly to the cdc2 protein and can effectively
block the process of tyrosine dephosphorylation. Using
extracts from Xenopus eggs and oocytes, we are engaged
in characterizing the various factors that regulate the
activation of cdc2. In the long run, we would like to
understand how the cell can monitor events such as cell
growth, centriole duplication and DNA replication so that
mitosis can occur only after all of the molecular
prerequisites for cell division have been satisfied.

Due to their crucial function, the proteins that control
the dephosphorylation of cdc2 have attracted wide interest.
Most of our efforts over the past year have been centered
in this area. Genetic and biochemical studies have
indicated that the cdc25 protein is a central player in the
process of cdc2-specific tyrosine dephosphorylation. In
yeast and fruit flies, the cdc25 protein determines the
timing of mitosis by serving as a rate-limiting activator of
cdc2. Significantly, yeast mutants that lack active cdc25
protein accumulate the tyrosine-phosphorylated form of
cdc2. Other perturbations of the cdc25 control system
also have deleterious consequences. For example, over-
production of the cdc25 protein in yeast can result in the
inappropriate initiation of mitosis before the completion of
S phase. Below, we describe our progress in unravelling
the molecular regulation of the cdc25 protein, as well as
our beginning efforts at understanding broader aspects of
how the cell cycle is regulated in eukaryotes.

3. Biochemistry of the cdc25 protein
Akiko Kumagai, William G. Dunphy

Genetic studies in fission yeast have indicated that the
cdc25 protein serves as a rate-determining activator of the
cdc2 protein kinase. To examine the biochemistry of the
cdc25 protein and its regulation during the cell cycle, we
have introduced bacterially-produced cdc25 protein into
Xenopus extracts. In particular, we have devised a
procedure to renature the Drosophila cdc25 protein from
the inclusion body fraction of overexpressing bacteria.
We find that the recombinant cdc25 protein efficiently
induces the tyrosine dephosphorylation of the cdc2 protein
and activation of its capacity to phosphorylate mitotic
substrates. Moreover, in extracts that cannot enter mitosis
owing to inhibition of DNA synthesis, the addition of the
active cdc25 protein efficiently elicits the mitotic state by
inducing premature dephosphorylation of the cdc2 protein.
Strikingly, the cdc25 protein can also elicit de-
phosphorylation of cdc2 even after purification by affinity
chromatography on p13-agarose. This reaction occurs in
the absence of ATP and suggests that cdc25 acts directly
upon the cdc2 protein. These experiments offer the pros-
tpect for elucidating the mechanism of cdc25-dependent
dephosphorylation of cdc2 and for identifying additional
factors that might regulate the dephosphorylation process.

4. The cdc25 protein contains an intrinsic
phosphatase activity
William G. Dunphy, Akiko Kumagai

Genetic and biochemical studies have indicated that the
cdc25 protein controls the entry into mitosis by triggering
tyrosine dephosphorylation of the cdc2 protein kinase.
We have shown that the bacterially-expressed cdc25
protein can catalyze the hydrolysis of p-nitro-
phenylphosphate in the absence of any other protein. The
cdc25-dependent cleavage reaction closely
resembles dephosphorylation by known tyrosine phosphatases: the
reaction requires a reducing agent; shows high sensitivity
to sodium vanadate; and proceeds efficiently in the
presence of metal chelators. Moreover, the phosphatase
activity of the cdc25 protein is eliminated by treatment
with N-ethylmaleimide or by alteration of a single
conserved cysteine residue by site-directed mutagenesis.
These observations indicate that the cdc25 protein is a
cysteinyl-requiring tyrosine phosphatase. Consequently,
the cdc25 must act by dephosphorylating cdc2 directly
without the aid of any other protein.

5. Isolation of Xenopus cdc25 homologs
Akiko Kumagai, William G. Dunphy

In order to study the regulation of the cdc25 protein
throughout the cell cycle in Xenopus egg extracts, we
have undertaken a molecular cloning of the cdc25
homolog from Xenopus. Using a polymerase chain
reaction-based strategy, we isolated three closely related
cDNAs that encode proteins with great structural similarity
to cdc25 homologs from other species such as yeast,
Drosophila, and human. Moreover, all three putative Xenopus cdc25 homologs can rescue a yeast mutant containing a temperature-sensitive mutation in the single identified yeast cdc25 protein.

The mRNAs for all three Xenopus cdc25 proteins are present at appreciable levels throughout the meiotic and early embryonic divisions. Likewise, the Xenopus cdc25 protein is present at easily detectable levels during this period. Surprisingly, the cdc25 protein is present at a concentration that is quite similar to that of the cdc2 protein, the physiological target of cdc25. Presently, we are engaged in elucidating the posttranslational modifications that regulate the activity of cdc25 during the cell cycle. Already, we have demonstrated that cdc25 is inactivated by a posttranslational modification when DNA synthesis is blocked. By characterizing the molecular nature of this and other modifications of cdc25, we expect to discern how the activation of cdc25 and the ensuing activation of MPF are coupled to earlier events in the cell cycle such as completion of DNA synthesis and attainment of adequate cell size.

6. Cloning of cell cycle genes by complementation

Paul R. Mueller, William G. Dunphy

We are interested in isolating and characterizing factors that regulate the progression of the cell cycle in Xenopus. The initial approach will be to isolate genes for these factors by interspecies complementation. This will allow production of unlimited quantities of factors to be used with Xenopus extracts in reconstitution assays or in the production of antibodies. The eukaryotic cell cycle is highly conserved from yeast to vertebrates, and many factors are homologous and interchangeable between species. This allows the cloning of cDNAs from higher eukaryotes by complementing temperature-sensitive lethal yeast mutants. Several cell cycle genes have been isolated using this approach, including the human cdc2 and Drosophila cdc25 homologs. 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. Therefore, in addition to isolating the homologous wild-type gene, suppressors or genes of bypass pathways can be cloned.

Presently, we are constructing Xenopus cDNA expression libraries to be used in curing several Schizosaccharomyces pombe mutants that are deficient in their cell cycle regulation. At the restrictive temperature, some mutants enter mitosis before DNA replication is completed, and others cannot enter mitosis. Both of these are lethal phenotypes. Because Xenopus oocytes contain large stores of mRNA that are used throughout maturation and early development, they will used as the source of RNA for the library construction. The library is being constructed so that it can shuttle back and forth between expression in yeast and growth in a bacteria. For expression in yeast, different promoters will be used to modulate the level of expression. 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. Following isolation of the Xenopus homologs, we plan to assess their biochemical function in extracts from Xenopus eggs.

7. Modification of cdc25 protein upon entry into mitosis

Nathan C. Rockwell, William G. Dunphy

Entry into mitosis is dependent upon the activation of MPF, a protein complex consisting of a cyclin and the cdc2 kinase. cdc2 is itself regulated by phosphorylation; in particular, the dephosphorylation of tyrosine 15 is necessary for the transition to M phase. A number of proteins have been shown to act as positive or negative regulators of this dephosphorylation, including the cdc25 protein in yeast and Drosophila.

Our studies have shown that the cdc25 protein is itself modified by entry into mitosis. By adding bacterially-produced cdc25 to Xenopus oocyte extracts, we are able to trigger the onset of M phase. The relative molecular weight of cdc25 subsequently increases, as analyzed by Western blotting, and two modifications of approximately equal size are observed. We have explored these modifications further, and have found that they are slowed by addition of sodium vanadate but not by Zn$^{++}$ ion up to 2 mM concentration. We also plan to examine the modification of cdc25 in Xenopus egg extracts, where even further modification is seen. The modifications are
largely reversed by phosphatases. Currently, we are mapping the sites of the modifications with the eventual aim of altering them by site-directed mutagenesis in order to assess their impact on cdc25 activity.

8. Characterization of cdc2-specific tyrosine kinase activity
Zhaohua Tang, William G. Dunphy

We have previously demonstrated that when DNA synthesis cannot be completed, the activation of cdc2 is prevented by inhibitory phosphorylation on a specific tyrosine residue. Although it appears likely that the cdc25 protein, the phosphatase that acts upon cdc2, is inactive under these circumstances, little is known about the regulation of the cdc2-specific tyrosine kinase during the cell cycle. The genetic studies of Nurse and colleagues have implicated the wee1 protein kinase as a critical negative regulator of cdc2. For example, overexpression of the wee1 protein lengthens the cell cycle, whereas reduced production of wee1 results in an accelerated cell cycle. More recently, Beach and coworkers have identified a closely related kinase known as mik1. Based on work in several laboratories, it now appears that wee1 and mik1 are tyrosine kinases whose most likely target is the cdc2 protein. To make use of the advantages afforded by the Xenopus extract system that we employ, we have undertaken an effort to introduce the wee1 protein into Xenopus extracts. Currently, we are utilizing a bacterial system and also a eukaryotic system (using baculovirus vectors) with the aim of producing unlimited quantities of the wee1 protein for biochemical studies. Our ultimate goal is to elucidate the biochemical mechanisms that repress and stimulate the kinase activity of wee1 so that cdc2 is turned off and on at the appropriate times during the cell cycle.

9. Biochemical characterization of the Xenopus p9suc1 protein
Thomas R. Coleman, William G. Dunphy

In addition to cyclin, p34cdc2 also exists as a complex with a 13 kDa protein identified in fission yeast as the product of the suc1 gene. Previous experiments with suc1 have revealed complex phenotypic manifestations. suc1 is an allele-specific suppressor of certain cdc2 mutations. Overexpression of suc1 delays mitotic onset; deletion of suc1 causes mitotic arrest. Addition of p13suc1 to Xenopus extracts inhibits p34cdc2 tyrosine dephosphorylation and MPF activity. Moreover, p13suc1 agarose affinity columns deplete Xenopus extracts of MPF. Thus, the role of p13suc1 in regulating cdc2 appears to be multifaceted.

We are studying the biochemistry of p13suc1 in frog extracts. In collaboration with S. Reed (Scripps Clinic), the Xenopus suc1 gene has been isolated and sequenced. Xenopus suc1 encodes a 9 kDa protein (p9suc1) with high homology to both the central domain of yeast p13suc1 and the entire human p9suc1 proteins. Like the other characterized suc1 proteins, Xenopus p9suc1 can bind MPF and inhibit tyrosine dephosphorylation of p34cdc2. Furthermore, Xenopus p9suc1 can displace pre-MPF from p13suc1 agarose affinity columns. We are in the process of characterizing polyclonal antibodies made against bacterially-expressed Xenopus p9. Given its complex genetic and biochemical traits, p13suc1 may regulate p34cdc2 at multiple points in cell cycle. We anticipate that antibodies against the Xenopus homolog of this protein will facilitate our understanding of this interesting molecule.

10. Role of multiple cyclins in the regulation of cdc2
Jerry L. Haney, William G. Dunphy

The active form of the cdc2 protein kinase is invariably associated with a larger regulatory subunit known as cyclin. Although cyclin is unquestionably essential for the kinase activity of cdc2, the precise biochemical basis for the requirement of cyclin is still mysterious. Moreover, there are multiple classes of cyclin molecules. In particular, in Xenopus there are at least two families of mitotic cyclins designated A and B. Both types of cyclin can drive the cell into mitosis at a sufficiently high concentration, but cyclin A may in addition play a role in the initiation or the continuation of S-phase. We have produced biologically-active cyclin A and cyclin B in bacteria. After addition to Xenopus extracts, both proteins bind to cdc2 and activate its H1 kinase activity. We expect that this biochemical system will facilitate a molecular understanding of how cyclin A and cyclin B separately activate cdc2. Moreover, we are in a position to understand the molecular differences between each class of cyclin either in the activation process itself or in the biochemical action of their activated complexes with cdc2.
PUBLICATIONS


Albert Billings Ruddock Professor of Biology: Jean-Paul Revel

Senior Research Associate: S. Barbara Yancey

Senior Research Fellow: Scott A. John

Research Fellows: Dale W. Laird, Kasturi L. Puranarn

Graduate Student: Jan H. Hoh

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Summary: The cloning of Cx31 by Jan Hoh and Scott John has given us the opportunity to look at the organization and phylogeny of connexins. Comparison of Cx31 to the other 13 known connexins reveals them to be in two large phylogenetic groups, which we refer to as α and β for convenience. While the criteria for distinguishing the two groups has to do with sequence, it turns out that the α connexins are all of estimated mass 38 kDa or more, and the β connexins are between 26 and 32 kDa. Some regions of the molecule appear to evolve rapidly, while others are highly conserved. Comparisons of sequences show that gap junctions and K channels, but probably not Na channels, have a common ancestry. In addition, there seems to be some immunological (and sequence?) similarities between some plant plasmodesmatal proteins and gap junctions. This would suggest that today's gap junction proteins are descended from rather ancient proteins.

S. Barbara Yancey's continuing work with the distribution of gap junction proteins in the embryo has brought up the interesting, if as yet inexplicable, fact that Cx43 is particularly abundant in tissues in which embryonic inductive interactions are taking place. We hope that further work will shed some light on the role of gap junctions in embryonic development. Dale Laird and Kasturi Puranarn have shown that during translocation and insertion of Cx in the plasma membrane, there are different forms of Cx43 differing from each other by number and, perhaps, location on the molecule of phosphorylation sites. One phosphorylation step takes place after leaving the ER, but before exiting the Golgi compartment; another type is required when leaving the Golgi or right afterwards. Use of inhibitors of translocation stops the progress of Cx through the secretory machinery, and pools of protein are seen to accumulate in different compartments, where they can be visualized in the confocal microscope.

In an unexpected and very promising turn of events, we have been able to visualize the cytoplasmic face and the extracellular face of gap junctions, using the atomic force microscope. To achieve this, Jan Hoh and Scott John, in collaboration with Ratneshwar Lal (formerly in my lab) and Morton F. Arnsdorf at the University of Chicago, managed to dissect the gap junction in the microscope itself to reveal structural detail at the extracellular face of the junctions, which has never been seen before, since it is buried in the narrow gap separating junctional membranes.

From the personal side, this report marks the end of an era in my laboratory—Jean Edens has retired, a second time; Barbara Yancey will be leaving in October; Jan Hoh graduates this June; and Scott John will go to another postdoctoral position, following Kasturi Puranarn. I do
not know how to thank you all for the wonderful times we have had together, but wish you the very very best in your careers.

11. Atomic force microscopy and dissection of gap junctions
Jan H. Hoh, Ratneshwar Lal, Scott A. John, Jean-Paul Revel, Morton F. Arnsdorf

We have used an atomic force microscope to study the structure of isolated hepatic gap junctions in phosphate buffered saline. Imaging with subnanometer height resolution, gap junctions adsorbed to glass appear to be 14.4 nm thick, close to the dimensions determined by electron microscopy and X-ray diffraction, and have an overall morphology similar to that previously described.

In order to investigate the effect of the force applied by the microscope tip on the structure of the membrane, gap junction plaques were imaged using a series of increasing and decreasing forces. Under these conditions, the top membrane of the gap junction can be "dissected" away leaving the extracellular domains of the bottom membrane exposed. This single membrane is 6.4 nm thick, slightly less than half of the full thickness of the gap junction. We call this process "force dissection."

Examination of the cytoplasmic surface of the gap junctions reveals no visible substructure. However, when "force dissection" is carried out on samples that are both trypsinized and glutaraldehyde fixed, the hexagonal array of gap junction hemi-channels as they protrude from the extracellular side of the membrane is revealed, at a lateral resolution of 1-2 nm, with a lattice constant of 9.1 nm. Some of these channels appear to have a depression in the middle which may represent the aqueous pore of the channel. These images represent the first visualization of the extracellular domains of the gap junction as they protrude from the membrane, and are the first images of an ion channel acquired by atomic force microscopy.

Similar images of cardiac gap junctions have also been acquired, though these appear substantially thicker than hepatic gap junctions, consistent with larger cytoplasmic domain of the major cardiac gap junction protein connexin 43. If these gap junctions are trypsinized to remove these cytoplasmic domains, they appear 14-15 nm thick.

12. Phylogenetic analysis of the gap junction gene family
Jan H. Hoh, Scott A. John, Jean-Paul Revel

We have analyzed the phylogenetic relatedness of 14 members of the connexin gap junction gene family. This analysis reveals two major branches corresponding to the α and β type connexins. Connexin45 appears to be an α type but in our analysis does not clearly fall into this group and may form a new branch of γ connexins. Estimates of divergence rates predict that the α and β connexins diverged 1.3-1.9 billion years ago, well before the origin of multicellular organisms. This raises the fundamental question of what function a molecule that we know as a cell-to-cell channel performed in unicellular organisms (and maybe still does!). The presence of two separate genes prior to multicellularity would appear to preclude that these molecules performed some other function and were recruited to form cell-to-cell channels, since the α and β connexins would have to have been recruited independently to this new function.

13. Intracellular maturation of connexin43 in neonatal rat cardiac myocytes
Dale W. Laird, Kasturi L. Puranam, Jean-Paul Revel

In previous studies, we have shown that neonatal rat cardiac myocytes contain a parent 40 kDa form and phosphorylated 42 and 44 kDa forms of the gap junction protein connexin43 (Cx43). We have continued to analyze the posttranslational modifications of Cx43 by perturbing the normal biosynthesis and maturation of the protein with cycloheximide, monensin or Brefeldin A (BFA). We also have followed the turnover of unassembled and assembled pools of Cx43 in the presence of these drugs.

Immunocytochemical labeling of monensin-treated cells shows Cx43 accumulating in the membrane of Golgi cisternae. Our results suggest that the nonphosphorylated 40 kDa form of Cx43 is phosphorylated to a 41 kDa form before or during its passage into the trans cisternae of the Golgi apparatus. Phosphorylation of Cx43 to the 42 and 44 kDa forms occurs later as the protein translocates towards the plasma membrane. Cells treated with BFA and cycloheximide for up to 15 hr maintain prominent, immunocytochemically identifiable gap junction plaques and functional competence. However, these drugs do not act by prolonging the half-lives of temporal late pools of
we are currently monitoring the early steps of Cx43 maturation in freshly dissociated cardiomyocytes that are allowed to recover and begin Cx43 biosynthesis. Prior to the onset of substantial junctional coupling, cardiomyocytes synthesize 40 and 41 kDa forms of Cx43. This 41 kDa form of Cx43 appears to be biochemically distinct from the phosphorylated 41 kDa form of Cx43 identified in monensin-treated cardiomyocytes. To date, we have not identified the origin of this 41 kDa form of Cx43 in reassociating cells.

14. Expression of connexin43 is upregulated at sites of inductive interaction during mouse embryogenesis

S. Barbara Yancey, Jean-Paul Revel

Connexin43 (Cx43) is a member of the family of channel-forming proteins that make up the gap junction. Gap junction channels are believed to form conduits for the direct cell-cell exchange of developmental signals. As background for experiments to test the biological function of gap junctions during embryogenesis, we have recently used indirect immunofluorescence to determine the spatial and temporal patterns of distribution of Cx43 in the post-implantation mouse embryo (Biology 1990, Abstract No. 28). We have now extended these studies using confocal microscopy to map more precisely the loci of Cx43 type junctions. We have identified several areas of high level expression of Cx43 that are particularly intriguing with regard to functional implications because they represent sites of inductive interaction between proximate tissues: the eye lens and retina, the infundibulum which interacts with Rathke's pouch, and the apical ectodermal ridge which interacts with limb bud mesenchyme. These systems will serve as potential candidates for experimental manipulation.

15. Disulfide bonding of RCx43

Scott A. John, Jean-Paul Revel

We have continued to examine the precise topological arrangement of the heart gap junction protein (RCx43) within the membrane as part of our efforts to find out how gap junctions work. RCx43 has two highly conserved extracellular loops. Within each loop there are three cysteine residues. We have now characterized the disulfide bonds present within RCx43. When RCx43 is isolated in the presence of the alkylation reagent iodoacetamide, no intermolecular disulfide bonds are found. However, the alkylated RCx43 does have at least one intramolecular disulfide bond. By using antibodies directed against the N terminus and the second extracellular loop and proteolytic cleavage, at least one intramolecular disulfide bond is shown to be present between the two extracellular loops of RCx43. This arrangement places structural constraints on the RCx43 molecule and the conformation it adopts within the membrane. It also implies that the structural integrity of the functional unit of gap junctions, the "connexon," formed of six protein subunits, is maintained by non-covalent bonds.

PUBLICATIONS


DEVELOPMENTAL BIOLOGY AND GENETICS

Roy J. Britten
Eric H. Davidson
Leroy E. Hood
Edward B. Lewis
Howard D. Lipshitz
Elliot M. Meyerowitz
Ellen Rothenberg
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Summary: The major interests of this laboratory are the mechanisms by which differential genomic expression is first established in the various spatial domains of the early embryo, and the evolution of genomic regulatory circuitry. Sea urchin eggs, embryos, and larvae serve as the main experimental systems. Other biological problems that we work on include molecular aspects of spermiogenesis, the basis of species-specific recognition of sperm and egg at fertilization, and the nature of immune effector molecules expressed in adult sea urchin coelomocytes. In addition, cloned genes of humans and other primates are being utilized in evolutionary studies. Generally, projects concerned with the molecular biology of sea urchin development are carried out in our Pasadena laboratory, while those at the Kerckhoff Marine Lab are centered on genomic evolution.

Recently developed methods for gene transfer in the sea urchin are being exploited in studies of differential gene expression in the early embryo. The major issue in this work is the molecular basis of the spatial and temporal regulation of marker genes in the various lineage elements of the early embryo. Our own studies of the embryonic lineage, in the context of classical observations on this long-studied system, show that the lineage is invariant from embryo to embryo, but that the specification of individual lineage elements depends conditionally on intercellular interactions. We are actively seeking the molecular components that mediate these interactions, i.e., the ligands and their receptors. The cis-regulatory elements that determine expression of marker genes in specific early lineage elements are used to analyze specific interactions with trans-acting protein factors that can be extracted from eggs and embryos. A major effort in our laboratory is now being devoted to the purification, cloning and characterization of these embryonic gene regulatory factors. We regard the developmental origin, activation, and spatial distribution of such regulatory factors in respect to the cell lineage as a route to a causal interpretation of early development.

16. A sea urchin gene shows an increase in transcripts after immune challenge

L. Courtney Smith

We have isolated and partially characterized a single copy gene (Spcoel) that is expressed in coelomocytes of the purple sea urchin (Strongylocentrotus purpuratus). It shows a 4 kb transcript in coelomocytes with very low expression in blastula stage embryos and ovary. The cDNA has a 598 bp 5' untranslated (UT) region, a 702 bp open reading frame (ORF), and a 3 kb 3' UT end, with functional start and stop sequences as revealed by in vitro translation. An internal stop codon appears in the ORF after 142 codons which is followed by 92 more codons before the second stop appears. Before the internal stop, a deduced 15.2 kDa protein shows significant amino acid
sequence similarity to CD45 (15/39 residues in register, or 38.5%, with the second phosphatase domain; as well as to a region of the extracellular domain where 12/50 residues are identical, or 24%). This portion of the ORF also has amino acid sequence similarity to profilin, an actin-binding protein involved in the second signal for cell division (71/142 residues compiled from four species, or 50%). If the stop codon is read through, a 25.9 kDa protein would be produced by the addition of a transmembrane-like region with a short internal domain displaying sequence similarity with the equivalent region of the LDL receptor (17/63 residues identical and in register, or 27%).

Western blots, however, show that coelomocytes do not read through the first stop codon and contain the smaller protein.

In titrating the numbers of Spcoel transcripts per coelomocyte, there is a severalfold increase in transcript number in cells pooled from sea urchins receiving xenogeneic challenge. Smaller responses are observed on wounding and injection of self-coelomic fluid. These increases in absolute Spcoel transcript concentrations begin to occur on a time scale of several hours, with a maximum at 1 day, and returning to the normal range by 6 days. Spcoel transcription is thus a functional response to coelomocyte stimulation. We are currently isolating genomic clones so that the structure of the gene and the promoter can be investigated.

17. Comparison of the bindin proteins of three sea urchin species

Joseph E. Minor, David R. Fromson

Bindin is the sea urchin sperm acrosomal protein that is responsible for the species-specific adhesion of the sperm to the egg. The bindin gene of Stronglylocentrotus purpuratus was previously isolated and analyzed in this laboratory. Two new bindin cDNA sequences that contain the entire open reading frame for the bindin precursor have now been cloned: one from S. franciscanus and one from Lytechinus variegatus. Both contain inverted repetitive sequences in their 3' untranslated regions, and the S. franciscanus cDNA contains an inverted repetitive sequence match between the 5'-untranslated region and the coding region. The middle third of the mature bindin sequence is highly conserved in all three species, and the flanking sequences share short repeated sequences that vary in number between the species. Cross-fertilization experiments were carried out with the species S. purpuratus, S. franciscanus, L. variegatus and L. pictus: a barrier to cross fertilization exists between the sympatric Stronglylocentrotus species but there is no barrier between the allopatric Lytechinus species.

18. Species-specific inhibition of fertilization by bindin peptides

Joseph E. Minor

Bindin is the sperm acrosomal protein that is responsible for the species-specific adhesion of the sperm to the egg for the sea urchin species Stronglylocentrotus purpuratus and S. franciscanus. The regions of the bindin molecule responsible for forming the contact between the sperm and the egg were investigated by assaying the ability of bindin-derived peptides to inhibit fertilization. Twenty-nine peptides were studied: seven based on the S. purpuratus bindin sequence, eleven based on the S. franciscanus bindin sequence, and six control peptides. The concentration of peptide necessary for 50% inhibition of sperm contacts (IC50) averaged 220 µM for the control peptides. Five bindin-derived peptides had IC50 values less than 20 µM; the most potent peptide (Sfo) had an IC50 value of 2.2 µM. One of the peptides (Sfr) inhibited fertilization species-specifically. The peptides inhibit fertilization with a steep dose response, which probably reflects the requirement for multiple bindin monomers for the initiation of the sperm-egg bond. These results demonstrate that specific regions of the bindin molecule are involved in the sperm-egg contact, and that these regions mediate the species-specificity of the interaction.

19. Do ligand-receptor interactions affect development of sea urchin embryos?

Louis J. DeFelice, R. Andrew Cameron, L. Courtney Smith

Classical experiments in which cleavage stage blastomeres were cultured alone or in abnormal rearrangement suggest that the fate of cells in the sea urchin embryo is conditional. The modern interpretation of such experiments is that surface interactions between blastomeres, mediated by ligand-receptor contacts, determine the five distinct tissue territories in the blastula. If correct, this
interpretation implies that membrane-bound receptors in one blastomere are activated by soluble (or membrane-bound) ligands from an adjacent cell. This interaction then initiates a second messenger cascade within the cell that results in the activation of transcription factors. We wish to know what these ligand-receptor systems are and which signal transduction systems they use. Our approach to these questions is to introduce a known ligand-receptor system into the zygote and to challenge the receptor at specific stages of early cleavage. If our experiments show that the surrogate receptor system activates endogenous G proteins, and if that activation modifies early embryo development, they will lend support to the idea that surface membrane complexes play a role in controlling development. For this study, we have selected a well-known ligand-receptor system that has already been shown to insert and couple to endogeneous pathways in the starfish egg. We have produced mRNA from mouse serotonin (5HT) 1c receptor (Ser-R) cDNA obtained from Prof. H. A. Lester's laboratory. This mRNA injected into Xenopus oocytes gives a Ca-induced Cl current in response to 50 nM 5HT. We have injected this mRNA into Strongylocentrotus purpuratus zygotes and exposed zygotes or early embryos to various acute or chronic concentrations of 5HT. Injecting ~10 pl of 0.1 µg/µl mRNA into zygotes, and exposing them constantly to 10 µM 5HT, results in abnormal blastula and pluteus stage embryos. Chronic exposure to 50 µM 5HT (~72 hr) is usually lethal. Neither serotonin-only (up to 10 µM) nor mRNA injected-only zygotes show these responses. To investigate which signal transduction pathways are involved, we will co-inject Fluo-3 or h-aequorin into sea urchin zygotes and look for Ca-induced light flashes in response to 5HT. In separate experiments, we will also co-inject CAT reporter genes with Cy IIIa or SM50 promoters to look for abnormal expression in response to 5HT. By these experiments, we hope to (a) identify the second messenger pathways that couple to the Ser-R, and (b) produce phenotypic responses by application of the ligand to specific cleavage stages. The ultimate goal of this research is to show that ligand-receptor-mediated signal transduction plays a role in development.

20. Characterization of a sea urchin endoderm gene
Andrew Ransick, Pedro Martinez, Ann E. Cutting

An ongoing project in this laboratory is to isolate and characterize genes that are expressed by specific embryonic lineages. Recently, S. Ernst's lab (Tufts University) identified an endoderm-specific gene (ENDO-16) and produced a cDNA clone corresponding with the 3' end of ENDO-16 mRNA. Using this cDNA as a probe, R. Franks (now at NIH) isolated a genomic clone from a cosmid library and we are using it to characterize the coding and regulatory domains of the gene. Restriction mapping, sequencing, Northern and primer extension analyses were used to estimate the 5' end of the coding region. We are currently constructing fusion genes in which various upstream sequences of the ENDO-16 gene are covalently linked to reporter genes (i.e., bacterial CAT or β-galactosidase), and a transgenic-embryo expression assay is being used to identify those sequences that promote correct spatial and temporal expression of the reporter constructs. Ultimately, the cis-trans interactions in the control region of the gene will be mapped and characterized, and the corresponding gene regulatory factors will be isolated, sequenced and cloned.

21. Analysis of endoderm specification
Andrew Ransick

The sea urchin embryo is well known for its regulative abilities in experimental circumstances. These responses require the respecification of embryonic cells to new lineages, which must involve reprogramming the batteries of genes that particular cells express. One good example is using the inducing power of micromeres to reprogram cells that have been specified as ectodermal into endodermal cells. We would like to understand this respecification process (as well as the normal specification mechanisms) at all levels. We hypothesize that a plausible cellular mechanism underlying such a regulative response involves a cascade of events triggered by a cell-cell interaction, amplified via some signal transduction pathway and culminating with the activation of cytoplasmic factors that regulate gene expression. Our approach to analysis of the specification process is guided by where we can get a handle on the system, which is the DNA regulatory domain of the ENDO-16 gene (a gene expressed in
embryos exclusively in endodermal cells). Initially, it must establish what elements of the regulatory domain become occupied with DNA binding factors as a result of endodermal specification. The initial experiments address the questions of whether specific sequence elements are necessary for regulative gene expression, and whether these same elements are used in the normal activation of the END0-16 gene. To address these questions, we are using an experimental bioassay: chimeric embryos will be constructed microsurgically such that some embryonic cells undergo the respecification from ectoderm to endoderm. After being respecified as endodermal cells, those that are carrying a CAT-reporter gene construct driven by the entire END0-16 regulatory domain should express CAT. This experiment will be repeated after eliminating defined regions of the END0-16 regulatory domain from the CAT constructs, and should reveal which sequence elements are required for the expression of END0-16, and which, if any, are specifically needed during the regulative response. The normal activation of END0-16 can be studied in parallel by looking for endodermal-specific expression of constructs in whole injected embryos. The next phase of the project will involve isolation of the DNA binding factors and determination of the modifications that they undergo when activated [i.e., (de-)phosphorylation]. Depending on what these modifications are, we will have clues as to what (signal transduction) pathway might be involved. This knowledge, in turn, should point toward a candidate cell-surface receptor involved in the cell-cell interaction.

22. Interactions of DNA-binding proteins with a sea urchin skeletal matrix gene

Kellie L. Whittaker

The sea urchin embryo relies on both autonomous and conditional specification to set up initial patterns of differential gene expression. At fourth cleavage, a quartet of small cells, known as the micromeres, is formed at the vegetal pole. The progeny of these cells construct the larval skeleton and can be shown to do so autonomously in vitro, i.e., without requiring contact with other cell types. Expression of SM50, a gene coding for a 50 kDa skeletal spicule matrix protein, begins not long after the micromere lineage segregates. The SM50 promoter, a 560 bp domain extending from -440 to +120 relative to the transcription initiation site, contains a total of 16 binding sites for seven different DNA-binding proteins. Three of these factors (P3A, P4, P5) are known to also regulate genes from other expression territories. The functions of the other four factors have yet to be determined. All of these factors are being purified using sequence-specific oligonucleotide affinity chromatography. In addition to protein sequencing and cloning of the genes for these factors, investigation of their function in SM50 regulation will be carried out by in vitro promoter mutagenesis and gene transfer experiments.

23. SM50 expression in haploid, non-cleaving sea urchin embryos

Kellie L. Whittaker, Antonio Garcia-Bellido

We are investigating whether SM50 is expressed in parthenogenetically activated, haploid sea urchin eggs that undergo DNA replication without undergoing cleavage. The eggs are activated by a 7-10-minute treatment with A23187, a calcium ionophore, which causes them to raise fertilization membranes and begin DNA replication. Staining the eggs with DAPI shows that replication continues in about 40% of activated eggs until at least the 48-hour stage. We are quantitating the amount of DNA in these eggs by DAPI fluorometry, in order to determine how many rounds of replication they are actually undergoing, and will assay SM50 transcript levels by single-strand probe excess titration.

24. Fluorescent detection of trans-gene products in sea urchin embryos

Robert W. Zeller, R. Andrew Cameron, Roberta R. Franks

A reporter gene consists of a regulatory region of interest fused to a suitable gene whose product is easily detectable in an in vivo system. One common reporter gene is the bacterial enzyme chloramphenicol acetyltransferase (CAT). We have used CAT reporter genes to examine the regulatory regions of several genes expressed in the sea urchin embryo and have developed a new method for the detection of the protein product from these fusion genes. By using a commercially available anti-CAT antibody and video microscopy or confocal microscopy, we can visualize embryonic CAT reporter
gene expression in whole embryos. Three reporter genes have been examined in pluteus stage embryos: SM50-CAT, CyIIa-CAT and CyIIIA-CAT. Each of the endogenous genes is expressed in specific regions of the embryo. The spicule matrix protein gene SM50 is expressed in skeletogenic mesenchyme cells, the cytoskeletal actin gene CyIIIA is expressed in the aboral ectoderm and CyIIa is expressed in the mesenchyme and gut cells. CAT protein expression patterns for SM50-CAT or CyIIIA-CAT reporter genes are similar to previously described RNA in situ hybridizations with radioisotopes. The interpretation of CAT expression patterns attained by the antibody labeling method is superior, however, and has shown for the first time that the ciliated band of the pluteus also expresses CyIIIA-CAT. We also examined the expression of the new fusion construct CyIIa-CAT and show that CAT protein is expressed in the gut and primary skeletogenic mesenchyme cells as expected from the localization of CyIIa mRNA.

STUDIES RELATED TO THE REGULATORY DOMAIN OF THE SEA URCRIN CyIIIA ACTIN GENE

The 5' promoter region of the CyIIIA cytoplasmic actin gene (Figure 1) regulates the expression of this gene in the sea urchin embryo and larva. Our laboratory has been analyzing the properties and interactions of this domain for several years, using a variety of experimental approaches. In addition, we are investigating in detail a number of protein factors that interact with the specific sites depicted in Figure 1. The following five reports all refer to aspects of these studies.

25. P6, a member of the Kruppel family of zinc finger DNA binding proteins

David Guo-wei Wang

The CyIIIA cytoskeletal actin gene is specifically expressed in the aboral ectoderm in the embryos of the sea urchin Strongylocentrotus purpuratus. A 2.7 kb cDNA clone contains about 2.3 kb open reading frame, which encodes a protein with a predicted molecular weight of 80 kDa. This protein, P6, binds specifically to the corresponding binding site within the CyIIIA 5' regulatory region (Figure 1). It contains a DNA binding domain consisting of 12 zinc fingers. Comparison of the P6 finger sequence with other zinc finger proteins indicates that it has consensus features representative of the Kruppel family of zinc finger proteins. Further investigations into the developmental function of P6 protein are in progress.

26. In vivo tests of individual CyIIIA actin regulatory elements

Barbara R. Hough-Evans, Ann E. Cutting

We are testing the protein-binding DNA sequences of the CyIIIA 5' region (Figure 1) for promoter activity, in order to shed some light on the functioning of individual protein factors that interact with the gene. In the CyIIIA-CAT plasmid, the CyIIIA 5' region regulates the expression of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. Embryos that develop from sea urchin eggs microinjected with CyIIIA-CAT incorporate the injected DNA into their nuclei and express CAT mRNA at the developmental stage and in the embryo tissues in which the CyIIIA actin gene is normally expressed. In our experiments, we substitute particular fragments of the 5' region for the "wild-type" 2.3 kb included in CyIIIA-CAT. The assay consists of injecting the various constructs into sea urchin eggs and analyzing for CAT enzyme activity in the 24-hr embryo. Parallel injections of CyIIIA-CAT provide a positive control.

In tests of the proximal binding sites (i.e., sites 5 through 1), we have discovered some general requirements for transcription. P1 alone will not activate the gene, but a combination of P5, P3B and P1 will. When two copies of the P5 site are included, expression approaches that of the positive control. The P5 transcription factor apparently requires the presence of binding sites P3B and P1 to act as an enhancer. Binding sites P3A, P3B, P2, and P1 also activate the gene, but only when present in the correct order and orientation. Thus, it appears that P2 also interacts with P3B. We have determined in addition that both active constructs express CAT at the correct stage of early development. Our conclusion at this point in the investigation is that interactions of factors in the proximal CyIIIA promoter region play an important role in gene expression.
Figure 1. The sea urchin CyIIIa actin 5' control region, showing sites of regulatory interactions. The solid boxes indicate the location of specific sites in the DNA where DNA-protein interactions occur. P1 to P8 designate the nuclear proteins that bind at each particular site. Open boxes show the locations of putative interactions for which extensive evidence is lacking. The arrow indicates the start and orientation of transcription. The diagram includes about 2.3 kb of upstream DNA sequence. From: Thézé, N., Calzone, F. J., Thiebaud, P., Hill, R. L., Britten, R. J. and Davidson, E. H. (1990) Mol. Reprod. & Dev. 25, 110-122.

27. Localization of two negative gene regulatory factors in sea urchin embryos

Robert W. Zeller

Recent experiments have suggested that two regulatory sites, P3A and P7II, function as negative regulators of the cytoskeletal actin gene CyIIIa (Figure 1). The P3A site is also present in the promoter of the spicule matrix protein gene SM50, which is expressed in the skeletogenic mesenchyme cells, although it is not known if the site also functions negatively. Two proteins, P3A1 and P3A2, are known to bind the DNA sequence of the P3A site. Proteins produced from pET T7 expression vectors have been used as antigens to generate polyclonal antibodies. Each antibody is specific to its antigen and there is no cross reaction with other proteins. Immunofluorescent studies are being used to localize each regulatory protein in whole embryos; their staining patterns appear to be identical. At the pluteus stage, both proteins are localized to the nucleus of every cell in the embryo. These antibodies have also been used to quantitatively measure the amounts of each protein present in isolated nuclei from several stages in development. Both proteins are present in the egg, although they do not appear to be functional at this time and do not show DNA binding activity in vitro in gel mobility assays (Calzone et al., 1988; personal communication). P3A2 is present in nuclei at approximately the same number of molecules per embryo as measured by Calzone et al. (1988). Recent experiments have indicated that P3A2 interacts with several other proteins believed to be transcription factors that also regulate the CyIIIa gene, although the mechanisms of CyIIIa regulation are not understood.

Reference:

28. Sequencing factors that bind to the P3A sites of the CyIIIa and SM50 promoters

Roger F. Anderson

To further our understanding of the regulatory factors involved in early development, we are looking to understand the regulation of two factors that have the same spatial distribution and DNA binding sites. These two factors, P3A1 and P3A2, have been shown to bind the P3A site of the CyIIIa actin gene promoter region (Figure 1), as well as similar sites in the regulatory region of the SM50 spicule matrix protein gene. Genomic clones for these factors have been isolated from a λ-EMBL3 library using probes from the cDNA clones isolated previously in this laboratory. Two different alleles were found for P3A1, based initially on their restriction map differences. After sequencing, they were found to differ in several respects. The size of the first intron is approximately 1 kb larger in the first allele and this allele also has several sites of inserted sequence, some as large as 1.5 kb between regions of >90% homology that extend for approximately 600 bases. Over 14 kb of upstream region has been characterized using the technique of transposon-facilitated DNA sequencing (Strathmann et al., 1991). About 7 kb of each upstream region for the two alleles has been sequenced to make this comparison. The P3A2 clone is now being sequenced. Its sequence will be used to find regions of homology which may indicate shared regulatory factors. We will also use the sequence to generate overlapping fragments for a gel-retardation assay screen to find the binding sites for the factors that regulate P3A1 and P3A2 expression. Using the sequences for the binding sites, we hope to isolate the factors that are responsible for regulating the expression of P3A1 and P3A2.

Reference:
29. Automated sequential affinity chromatography of DNA binding proteins

James A. Coffman, Frank J. Calzone¹, Cydia Keller, James G. Moore

We have developed an automated method of using a tandem sequence of oligonucleotide affinity columns to purify transcriptional regulatory proteins. A robot (the Gilson ASPEC) was adapted to affinity-purify up to 12 different sequence-specific DNA binding proteins in a single run, which may then each be isolated by methods such as preparative gel electrophoresis. We have employed this technique to isolate several factors that bind specific cis-regulatory sites of the CyIIIa cytoskeletal actin gene (see Figure 1) of the sea urchin embryo Strongylocentrotus purpuratus. Amino acid sequences obtained from the purified proteins are being used to clone the corresponding genes.

¹Department of Development and Cell Biology, University of California, Irvine, CA 92717.

30. Analysis of DNA binding proteins by two-dimensional gel electrophoresis

James A. Coffman, Michael G. Harrington

We have found that a number of sea urchin embryonic transcriptional regulatory proteins can be quantitatively enriched by chromatography on the cation-exchange resin Biorex 70. Of eight different factors tested, all were found to bind to the Biorex 70 column. We have employed high-resolution two-dimensional electrophoresis to analyze the population of nuclear proteins that are enriched by such chromatography, which is likely to include most if not all of the transcriptional regulators found in the nuclei of sea urchin embryos. Enumeration of the chromatographically-enriched spots on a two-dimensional gel shows that there are probably only 100-200 such proteins. A number of regulatory factors that were previously identified have been located within the two-dimensional pattern, and we are currently mapping the remaining known regulatory factors onto the pattern. We are using this method to analyze the temporal and spatial variation of individual as well as the global population of such proteins, and to detect protein modifications that may play a role in developmental transcriptional regulation.

31. TGF-β genes possibly involved in intercellular signaling

L. Courtney Smith, J. Richard Whittaker, Jose-Luis Micol

The results of some classical and modern experiments involving surgical excisions and recombinations of cells and tissues support the suggestion that intercellular signaling is involved in the permissive specification of early embryonic pattern formation in sea urchin development. Polypeptides of the transforming growth factor-β family (TGF-β) have been implicated as ligands in cell interactive processes of other organisms (e.g., decapentaplegic in Drosophila, Veg-1 growth factor of Xenopus). Our objective is to isolate and characterize members of this family from sea urchin embryos and to study their possible role in inductive cellular interactions.

PCR primers were designed to correspond to the best conserved sequences of TGF-β family members, and one of these primers was used to produce PCR fragments with sequence similarity to Veg-1. We are currently sequencing several clones from the 12-hour embryonic cDNA library of Strongylocentrotus purpuratus. In addition, this project will continue to screen libraries of different developmental stages for this and other clones similar to gene sequences of TGF-β family members.

32. Sea urchin cell lineage: The ciliated band

R. Andrew Cameron

The embryonic ectoderm of the sea urchin Strongylocentrotus purpuratus is constituted from two tissue territories, the oral and the aboral ectoderm, which are made up of squamous epithelial cells. The squamous epithelia are separated by a region of columnar ciliated cells termed the ciliated band. Whole mount antibody labeling of the protein product of aboral ectoderm-specific reporter genes reveals that the ciliated band is expressing this product. We wanted to know the exact lineage history of all the cells that make up the ciliated band.

In order to ascertain the other contributors to the ciliated band, we have undertaken a series of injections of adjacent cells at the two-cell stage and the 16-cell stage employing lineage tracing dextran with two different fluorochromes. These preparations consistently showed that the central, pre-oral portion of the ciliated band, destined to become the oral hood, derives from progeny of the animal half aboral eight-cell stage blastomere. The
lateral portions of the ciliated band received contributions from the lateral animal half eight-cell stage blastomeres, as well as the animal half oral blastomere. Contributions to the band vary slightly from embryo to embryo and sometimes one of the boundary pair contributes cells across the entire band for a short stretch. The post-oral region of the band between the mouth and anus derives from the vegetal half oral blastomere.

In summary, animal half blastomeres which contribute to the aboral ectoderm also contribute to the ciliated band in the pre-oral and lateral regions. That these ciliated band regions also express aboral ectoderm-specific reporter genes is consistent with their cell lineage origins. The variable source of the cells that make up the lateral ciliated band suggests that the developmental decision to become a ciliated band cell occurs late and as a result of position rather than lineage history.

33. Expression of ribosomal protein genes in early mouse embryos

*Lajos Piko, Kent D. Taylor*¹

Following fertilization, protein synthesis in the mouse zygote depends mostly, if not entirely, on maternally inherited components. A shift from maternal to embryonic control occurs during the two-cell stage (second day of development) when the zygote nuclei become fully active in RNA synthesis and most of the maternal mRNA is degraded. To analyze the pattern of change in the mRNA population during this transition, we previously produced a random cDNA library from late two-cell embryos. This work showed that a significant number of clones were not represented measurably in egg RNA but became increasingly abundant during cleavage. Three clones showing this pattern of expression have now been sequenced and identified as the mouse homologs of ribosomal protein genes S15, L7a and L15a. Northern blot analysis has revealed a single band of the expected size, and transcription termination signals. Immediately upstream of this is a string of eight pyrimidines, a feature shared with certain retrotransposons and plant abundance parallels the rise of ribosomal protein synthesis in the early mouse embryo. The low level of ribosomal protein mRNAs in the egg suggests that, unlike the bulk of the maternal mRNA, these mRNAs are not stored in a stable form in the oocyte and thus become depleted once their synthesis is arrested upon completion of oocyte growth. After the first cell division, the transcription of ribosomal protein genes becomes activated at the same time when ribosomal RNA synthesis also begins. A high rate of ribosome production continues throughout cleavage, resulting in an approximately sixfold increase in the cytoplasmic concentration of ribosomes by the blastocyst stage.

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**Britten Subgroup**

34. The first retrovirus-like element in marine invertebrate DNA

*Mark S. Springer, Roy J. Britten*

As described in last year's Annual Report, a 5 kb long sea urchin repeated sequence was shown to include a reverse transcriptase coding region. The complete sequence has been obtained showing that it is a retrovirus-like element, meaning that it has long terminal repeats (LTRs) and most of the same genes in the typical order for retroviruses. This first abstract deals with the sequence and LTRs and subsequent abstracts describe other aspects. The example that has been fully sequenced from the Hawaiian sea urchin *Tripneustes gratilla* is 5266 bp long, has a 5 bp target site duplication (CCACC) of host DNA and identical 254 bp LTRs. The LTRs contain TG...CA terminal inverted repeats, a putative promoter region, and transcription termination signals. Immediately interior to the 5' LTR is a putative tRNA primer binding site for minus-strand synthesis that begins with the sequence TGG. Sea urchin tRNAs have not been characterized, but starting with the TGG sequence it shares 11 of 12 bases with the methionine tRNA primer binding site of both retroviral-like elements 'TAL-3' of *Arabidopsis* and 'del' of lily. Interior to the 3' LTR, in turn, is a 13 bp poly-purine region (AAAGAAAGGGAGG), a characteristic of plus-strand priming sites in retroviruses. Immediately upstream of this is a string of eight pyrimidines, a feature shared with certain retrotransposons and plant
pararetroviruses. The internal domain contains a single 3927 bp open reading frame (ORF) and the predicted 1309 amino acid sequence includes both the gag and pol regions. A single ORF including both the gag and pol genes is unusual but is known in 'copia' and its close relatives. The pol region encodes protease, reverse transcriptase, RNase H, and integrase functions in the same order as in most retroviruses, based on amino acid sequence similarities.

35. Sea urchin retrovirus-like element had a long, active past

Mark S. Springer, Roy J. Britten

Continuing with the sea urchin retrovirus-like elements (SURL elements), the presence of stop codons and divergence within the subfamily suggest that most SURL1 subfamily members are inactive inserted copies that have been subject to drift. However, the DNA and amino acid sequence comparison of SURL1-3 from Tripneustes gratilla (Tg) and SURL1-21 from Strongylocentrotus purpuratus (Sp) shows a seven to thirteenfold excess of synonymous substitutions versus those causing amino acid replacement (per site) over the entire open reading frame including gag and pol genes. This shows that these coding regions have evolved under sequence-dependent selection. In addition, a 702 bp region of the reverse transcriptase and RNase H genes was sequenced for a member of another SURL subfamily from the very distant sea urchin Eucidaris tribuloides (Et). There is an eighteen-fold excess of synonymous versus nonsynonymous substitutions for comparisons with the same region of the Sp element coding region, providing further support for the concept that the RT protein evolved under strong selection over much of the evolutionary history of echinoids. Estimates for the lower bounds on divergence times between Sp and Tg or Et are 45 or 250 million years. These estimates give rates of synonymous substitution of 0.49, and 0.62% divergence/lineage/million years. These rates are similar to the rate of 0.5% divergence/lineage/million years calculated from single-copy DNA hybridization studies. Thus, it appears that synonymous rates of change among reverse transcriptase genes of sea urchin retrotransposons are about equal to the single-copy drift rate, which is a very slow rate compared to that of the known vertebrate retroviruses. We may conclude from the freely occurring synonymous substitutions and the selection against amino acid replacements that active copies, dependent on these genes for replication, have been the main line of inheritance of SURL elements during echinoid evolution.

36. Occurrence and subfamily structure

Mark S. Springer, Roy J. Britten

Retroviral-like (RL) elements are known in a wide variety of organisms, including yeast, dipterans, lepidopterans, gymnosperms, angiosperms, and vertebrates (where they are recognized as retroviruses or endogenous retroviruses). RL elements are apparently also widely distributed among echinoids. We have observed the reverse transcriptase gene coding region of RL elements in the DNA of all sea urchin species that we have examined, including one cidaroid (Eucidaris tribuloides [ET]) and seven euechinoids (Strongylocentrotus purpuratus [Sp], S. franciscanus [Sf], S. drobachiensis [Sd], Tripneustes gratilla [Tg], Lytechinus pictus [Lp], L. variegatus [Lv], Arbacia punctulata [Ap]). There are approximately 400 copies of the reverse transcriptase coding region interspersed in the genome of Sp, forming a family that consists of subfamilies containing from a few to 50 members. The subfamilies have been distinguished by the DNA sequence divergence of a conserved segment of the reverse transcriptase coding region. Members of each subfamily have closely similar sequences (1-10% divergent), whereas this DNA sequence region of each subfamily is greatly divergent from that of other subfamilies (30-45%). Interestingly, examples of intermediate divergence (10-30%) are rare or nonexistent. The implication is that copies of the RL elements that have been inserted into the genome drifted in sequence and the observation of stop codons in required coding regions suggests that many pseudogenes or "pseudocopies" are present. A similar pattern (termed superfamilies) has been observed for the 'Ta' elements of Arabidopsis that are similar to 'copia' of Drosophila and less similar to retroviruses than are the SURL elements.
37. Is the sea urchin retrovirus-like element a retrotransposon?

Roy J. Britten, Mark S. Springer

To assess this question we start in the gag gene region and successively make amino acid sequence comparisons with other retrovirus-like elements. First, the nucleocapsid gene: in common with most retroviruses, both SURL elements we have sequenced contain two RNA binding sites, each possessing a presumptive zinc-finger pattern of cysteines and histidines. Previously reported retrotransposons either lack RNA binding sites, as is common among Drosophila retrotransposons related to 'gypsy,' or contain a single site. Next, a short region of the presumptive protease gene including the conserved pattern of D(S,T)G, which is part of the active site of the enzyme that cleaves the gag-pol polyprotein, and here the closest is the sequence of murine leukemia virus. Next, a representative 100 amino acid region of the reverse transcriptase gene comparison suggests that the SURL elements are more closely related to a branch of retrotransposons that includes gypsy of Drosophila, Ty3 of yeast and plant pararetroviruses than they are to representative retroviruses. The majority of the sequence comparisons are in agreement with this conclusion. Next, a portion of the integrase gene is more similar in amino acid sequence to certain retroviruses than to retrotransposons, with the exception of the 'Mag' retrotransposon from the silkmoth. Finally, in retroviruses the env gene is in a separate ORF just 3' of the integrase gene, while in SULR1 (Tg), there is no such separate ORF that appears to be a candidate for an env gene. The evidence leans toward classifying the SURL elements with retrotransposons while suggesting a complex history. However, the original question may not be well formed since less conventional infectious processes could occur among retrotransposons; for example, the lepidopteran retrotransposon 'TED' may be capable of expanding its host range via insertion in a vaculovirus genome that shuttles it to a new host. "Helper" interactions may exist with other viruses and a new domain such as sea urchin RL elements may contain surprises.

PUBLICATIONS


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Summary: Our group is divided into three major categories. One group is concerned with the biology of mammalian T-cell immune responses, the structure and function of nonclassical class I proteins encoded by the major histocompatibility complex (MHC), and the biology of myelination. The second group is concerned with developing tools for modern molecular biology, including new ways to isolate and characterize proteins, novel approaches to the analysis of nucleic acids and computational techniques associated with these new chemistries and instrumentation, as well as the analysis of proteins and genes. A third group is concerned with large-scale mapping and sequencing of the human and murine T-cell receptor (TCR) loci. These last two groups represent the NSF Science and Technology Center for Molecular Biotechnology.
Our T cell efforts focus on immunologic recognition and autoimmune disease. We have developed two murine models of immune diseases. Experimental allergic encephalomyelitis (EAE) is induced by injecting certain inbred strains of mice with myelin basic protein (MBP) to create the pathology that resembles human multiple sclerosis. The second model system is induced by injecting collagen II into certain inbred strains of mice and creating the chronic arthritis that resembles in many features rheumatoid arthritis. For both of these systems, we are characterizing TCR repertoire usage, the nature of the antigenic T-cell epitope, and the use of anti-TCR antibodies to block and reverse these model diseases. In addition, we have created a transgenic mouse that expresses predominantly the major TCR specific for MBP. This mouse gives us the opportunity to explore tolerance induction to a self antigen and the induction of autoimmune diseases. From the studies of these model systems in mice, we have developed the hypothesis that certain polymorphisms in MHC molecules and TCR variable (V) regions predispose animals to autoimmune diseases. We have begun to test this hypothesis in human disease through collaborations with the group for large-scale genetic mapping. As a part of this effort, we are creating highly detailed genetic maps of all the human TCR and MHC loci. We plan to use newly developed automated techniques for genetic mapping to analyze populations of humans that have various autoimmune diseases to determine whether particular polymorphic MHC or V genes do predispose to disease. In this regard, we have already initiated studies on multiple sclerosis and have observed that a disposition to this disease maps both to one or more genes in the human TCR β locus and the human class II MHC locus.

Our studies on nonclassical class I molecules and genes have focused on the Qa-2 antigen, which appears to play dual roles in the expansion or deletion of specific subsets of cells in the immune system. We have recently demonstrated that the Qa antigen is attached to the cell surface by a phospholipid anchor and are now in the process of exploring the structural basis for this attachment as well as its regulation. In addition, we are attempting to express this molecule in large quantities so that we can identify the ligands that it might bind and ultimately delineate its three-dimensional structure by x-ray analysis after crystallization.

The molecular neurobiology group focuses on delineating the control elements that govern the expression of the MBP gene. In addition, we are in the process of carrying out "knock-out" experiments for myelin-associated glycoprotein (MAG) genes. We have used a gene encoding the large T antigen driven by an MBP promoter to generate interesting oligodendrocyte and Schwann cell lines with two distinct phenotypes.

The Science and Technology Center for Molecular Biotechnology has as its major objective the development of tools for molecular biology. The protein isolation and analysis group concentrates on the use of two-dimensional gel electrophoresis for the isolation and characterization of complex mixtures of proteins. We are developing techniques that will permit proteins to be sequenced directly from two-dimensional gels. The protein chemical microsequencing group is focused on the exploration of a new chemistry for microsequencing, the thioacetylation chemistry, because it has two striking advantages: the potential for extended sequencing runs, and the possibility of microsequence analysis at the low femtomole levels, some thousand times more sensitive than current technology. In addition, we are in the process of developing a new solid-phase protein sequenator that can be employed in conjunction with conventional Edman-reagent microsequencing as well as the thioacetylation chemistry. The mass spectrometry group has three instruments: a triple quadrupole with electrospray ionization, an ion trap, and a time-of-flight instrument. Each of these instruments offers unique opportunities for the characterization of both proteins and nucleic acids. The nucleic acids chemistry group focuses on efforts to improve both genetic mapping techniques and the large-scale DNA sequencing techniques. The computational group has a variety of interests including the development of a laboratory information management system for large DNA sequencing, better logarithms for calling DNA bases from the automated fluorescent DNA sequencing technology, the development of models for optimizing large-scale DNA sequencing strategies, and the development of systems for networking together all of the genetic and physical mapping and DNA sequence data. We are also
interested in computational approaches to the protein folding problem. The large-scale analysis of the genetic polymorphisms group has focused on developing the oligonucleotide ligase assay which together with polymerase chain reaction permits the automation of the analysis of diallelic genetic polymorphisms. In addition, clustered and adjacent diallelic polymorphisms can often create a multiplicity of distinct haplotypes that provide a high degree of genetic informativeness. In addition, an ambitious attempt to create a 5 centimorgan genetic map of human chromosome 14 by developing new approaches for the rapid identification and analysis of diallelic polymorphisms and use of the PCR/OLA system for genetic analysis is now under way. These techniques have all been applied to creating a multiplicity of genetic markers across the immune receptor gene families (TCR loci, immunoglobulin loci, and MHC loci). The large-scale DNA sequencing group is attempting to establish an organization that will permit megabase sequencing per year within the next three years. This group will concentrate on the complete sequence analysis of the human and murine TCR loci α/δ, β and γ. We have already obtained approximately 250,000 bases of DNA sequence across these TCR loci. Thus, the large-scale genetic mapping and DNA sequencing groups interact affectively with the group interested in immune recognition. Likewise, new techniques developed by the Science and Technology Center are being employed by the mapping, sequencing and biology groups to generate striking interdisciplinary collaborative interaction.

38. A model system for studying tolerance to self antigens and autoimmunity using transgenic mice expressing myelin basic protein-specific T-cell receptor genes

Joan M. Goverman, Dennis M. Zaller

We are employing transgenic mice in which the majority of T cells express a receptor specific for the self antigen, myelin basic protein (MBP), to investigate various aspects of tolerance induction and autoimmunity. We have shown that 5th generation mice (transgenes back-crossed into the appropriate major histocompatibility complex [MHC] H-2b background) fall into three categories with regard to thymus T-cell development: 1) animals that exhibit complete deletion of mature (CD4+) T cells; 2) animals that exhibit a partial deletion of mature T cells; and 3) animals that exhibit no deletion of mature T cells. Peripheral tolerance was present in mice of the first category, but was lacking in animals of the second and third categories. In these, spleen cells from immunized transgenic mice were capable of responding to MBP. The presence of these self-reactive T cells did not result in spontaneous induction of disease.

We are continuing our studies of this system in order to address several questions concerning the establishment of tolerance to self antigens. First, we will attempt to determine whether the three thymic phenotypes arise from genetic or environmental causes. Obviously, the answer to this question will determine how we proceed with further characterization of this system. Second, we will characterize further the development of T cells in transgenic mice that either do or do not express MBP. In addition to the T-cell accessory molecules CD4 and CD8, we will analyze the expression of the α chain of the transgenic T-cell receptor and other developmental markers in T cells that accumulate in the thymuses of mice undergoing negative selection. We will compare these populations to those in the thymuses of mice that are positively selecting the transgenic T cells as well as to T cells of mice that are neither positively nor negatively selecting the transgenic T cells. Third, we will examine the ability of transgenic thymocytes undergoing negative selection in an MBP-expressing animal to differentiate further into mature T cells when introduced into the thymus of a non-MBP-expressing animal. This analysis may help pinpoint the stage of differentiation at which commitment to negative selection occurs. Finally, we will carry out various experiments designed to investigate the nature of the antigen that mediates the negative selection of the transgenic T cells, the source of the antigen and the cell types that might present this antigen in the thymus. Fourth, we will determine whether the MBP-reactive T cells in the periphery are the result of "leakiness" in the thymic negative selection or arise from seeding of T cells in the periphery before the onset of MBP expression and negative selection. We will also investigate why these T cells are not deleted or inactivated in the periphery following MBP expression.
Epitope dominance in mouse experimental allergic encephalomyelitis
William K. Funkhouser, Jr., Dennis M. Zaller, Michael I. Nishimura, Joan M. Goverman

Murine experimental allergic encephalomyelitis (EAE) is a T lymphocyte-mediated autoimmune disease that targets normal neural myelin. It does not occur spontaneously, but rather develops after immunization with syn- or xenogeneic myelin basic protein (MBP). Multiple T-cell epitopes of MBP have been defined for each of a number of individual murine major histocompatibility complex (MHC) haplotypes, and these epitopes vary as a function of MHC haplotype. Some haplotypes do not respond to syngeneic MBP at all. Present models would suggest that response is a function of MHC haplotype, antigen processing, and activatable antigen-specific peripheral T-cell density.

At least three different T-cell epitopes (NAc1-20, 31-50, 121-140) can be estimated by the response of B10.PL (H-2b) mouse lymph node cells to mMBP synthetic peptides in vitro after mMBP immunization. Most of the response is to one of the three peptide epitopes, NAc1-20. If these peptides accurately reflect the response to processed and presented self MBP, then this epitope is dominant with respect to the other epitopes in the in vivo response to mMBP immunization. The basis for this dominance phenomenon is unknown, but will presumably involve differences in peptide-MHC affinities, differences in T-cell receptor-(MHC-peptide) affinities, an artifact due to the use of synthetic peptides to estimate actual (unknown) epitopes, and/or differences in the prevalences of activatable peripheral T cells specific for the different epitopes.

At present, it appears that 121-140 is subdominant because of 1) tolerization to MBP and 2) competitive binding of NAc1-20 and 121-140 for I-A^u. It is not clear why 31-50 is subdominant. It is restricted by the I-E^u rather than the I-A^u molecule, is not displaced by either NAc1-20 or 121-140, and appears to have a similar T-cell responder prevalence to that of NAc1-20. Estimates of binding affinities for these three peptides are under way.

Mechanisms of tolerance induction in transgenic mice expressing T-cell receptors specific for myelin basic protein
William K. Funkhouser, Jr., Dennis M. Zaller, Joan M. Goverman

We have studied the T-cell response to myelin basic protein (MBP) in B10.PL mice, which express major histocompatibility complex (MHC) molecules of the H-2^b haplotype, and in shiverer mice, which also express H-2^b MHC. B10.PL mice express MBP beginning around day five after birth and are tolerant to the extent that these mice do not spontaneously activate MBP-specific T cells. However, T cells specific for MBP can be isolated from B10.PL mice following immunization with the protein. These T cells primarily recognize the first 20 aminoterminal residues of the antigen. Shiverer mice do not synthesize MBP due to a deletion of much of the gene encoding this protein. Thus, the repertoire of peripheral T cells in these mice is presumably not impacted by the expression of MBP. In shiverer mice, the dominant response to MBP is to the epitope comprised of MBP 121-140, although a response to MBP 1-20 comparable to that of the B10.PL mouse is also observed. Thus, the dominant epitope in the antigen-negative mouse, MBP 121-140, appears to be tolerized in the wild-type mouse. However, T cells responsive to the 1-20 epitope do not appear to be well tolerized and they proliferate equally well in the wild type or shiverer animal.

We are interested in investigating why the 121-140 epitope of MBP is largely tolerized in a mouse expressing MBP endogenously, but the response of T cells recognizing 1-20 does not appear to decrease in the same animal relative to the shiverer. Previous work has shown that the T cells recognizing the 1-20 epitope employ a limited set of V genes in their receptors. The V-gene usage in the receptors of T cells recognizing 121-140 is not known. We will prepare a panel of hybridomas specific for this epitope and characterize the T-cell receptor usage. This information is necessary to determine if the ability to tolerize T cells responsive to a particular epitope is related to the heterogeneity of the T cells responding to that epitope or the particular V genes involved in the response. We will also use these hybridomas, as well as T-cell hybridomas specific for 1-20, in an assay system to determine which cells in the thymus may be responsible
for presenting these different epitopes to thymocytes during the process of negative selection. We will examine whether potential differences in the presentation of the epitopes may account for differences in the degree of tolerization to them. Ultimately, we will make transgenic mice expressing a T-cell receptor specific for the 120-140 epitope, analogous to our transgenics specific for the 1-20 epitope, and compare the process of negative selection of transgenic T cells in these two types of mice.

41. F1 T-cell receptor usage in EAE: Dominance of one parental response over another
Gregory J. Reynard, Joan M. Goverman

Experimental allergic encephalomyelitis (EAE) is an autoimmune disease that can be induced in susceptible strains of mice by immunization with myelin basic protein (MBP) in adjuvant. Autoreactive T cells are responsible for demyelination of the central nervous system, which leads to paralysis. The T cells responsible for this response have been characterized by the sequence of the T-cell receptor (TCR) genes used. Two such susceptible strains, B10.PL and PL/J (both H-2b major histocompatibility [MHC] haplotypes), have had their MBP-specific T cells characterized in this way. While these strains share a common MHC, they use different TCRs in their response. B10.PL uses Va2.3 and 4.2 gene segments, while PL/J uses Va4.3 and 4.4 gene segments. Preliminary observations suggest that when these strains are crossed, the response in the F1 generation is highly skewed toward that of the B10.PL parent. If confirmed, this type of simple dominance would be interesting in that immunotherapy by elimination of detrimental T cells might be more feasible than previously imagined. We intend to characterize this phenomenon further and then address the question of what its basis might be. Possible explanations might be the induction of tolerance in the F1 generation resulting in negative selection of the PL/J receptors, or kinetic differences in the formation of antigen/MHC/TCR complexes favoring those complexes found with B10.PL T-cell receptors.

We have begun by addressing the question of what genetic material is available to the mouse before rearrangement of the TCR locus. We have constructed several subgenomic libraries containing DNA from each strain. We have screened for the members of the Va2 subfamily and will sequence these to determine what is available to each parental strain on a genomic level.

Possible future experiments include bone-marrow chimeras to investigate the possibility of tolerance induction, as well as investigating the kinetics of various TCR-MHC-antigen complexes.

42. Dominant inheritance of T-cell receptor V gene in murine autoimmune encephalomyelitis
Kuang-Chuan Cheng, Gregory J. Reynard, Kai Wang, M. K. Lee1, T. J. Yoo

Experimental allergic encephalomyelitis (EAE) is an induced central nervous autoimmune disease in rat and mouse models. This T cell-mediated disease is induced by immunization with myelin basic protein (MBP). Restrictive usage of T-cell receptor (TCR) has been reported in H-2d diseased mice (B10.PL and PL/J). The dominant expression of the Vb8.2 (~80%) chain has been observed in both strains of EAE mice. Of the three Va gene segments used in the autoimmune response in these mice, two are unique to B10.PL (Va2.3 and Va4.2) and one to PL/J (Va4.3). We would like to determine whether the germline deletion of certain Va genes is the cause of the different usage of Va genes in these diseased mice. To establish the presence or absence of these Va gene segments, we have amplified either genomic DNA or cDNA from thymus and lymph nodes with Va-specific primers by the polymerase chain reaction (PCR). The results suggest that both strains of mice have the Va4.3 gene segment in genomic DNA and expressed in both thymus and lymph nodes. However, the Va4.2 and Va2.3 gene segments are not present in PL/J. The deletion of these Va gene segments is also confirmed by genomic blots. These observations suggest that deletion of certain germline V gene segments may be the cause of different V gene usage in these two different mouse strains.

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43. Construction of an antibody-toxin fusion protein directed against the Vβ8.2 T-cell receptor

Lee S. Rowen, Dennis M. Zaller

In vivo administration of large doses of F23.2, a monoclonal antibody directed against mouse Vβ8.2 T-cell receptor, prevents or reverses symptoms of experimental allergic encephalomyelitis in about three-fourths of mice tested. We were thus motivated to examine the therapeutic effectiveness of a single-chain polypeptide containing the Fv portion of F23.2 and the cytotoxic portion of exotoxin A from Pseudomonas aeruginosa. To produce the polypeptide, the DNA construct was assembled from the coding regions for the heavy and light variable regions of the antibody and domains II and III of exotoxin A. The resulting 66 kDa protein was overexpressed in E. coli, renatured, and purified to near homogeneity. Although the toxin activity of the protein could be restored, as judged by ADP-ribosylation assays, the immunologic activity could not be, as judged by binding and killing assays with a cell expressing the Vβ8.2 T-cell receptor. We do not know whether our negative result is due to failure to renature the antibody portion of the polypeptide or to the production of an Fv that has too low an affinity for binding the T-cell receptor. This project is now being pursued in collaboration with Cary Queen at Protein Design Inc. in Palo Alto.

44. The T-cell repertoire in collagen type II-induced arthritis

Gamal E. Osman, Mark Feild, Kuang-Chuan Cheng, Paul Patengale

Collagen type II-induced arthritis (CIA) in animals has been shown to be an experimental animal model system of the human autoimmune disease rheumatoid arthritis. CIA is induced in susceptible animal strains by intradermal injections of collagen type II. In contrast, other joint tissue components such as collagen type I and proteoglycans are nonarthritogenic. Although prior work indicates that CIA is associated with a high level of both cellular and humoral autoimmunity to collagen type II, the role of antibodies and T lymphocytes in the pathogenesis of the disease is ill defined. In addition, high levels of collagen immunity also have been documented in certain strains of mice and rats without the development of arthritis. This finding has led to the suggestion that an immune response to one epitope or perhaps to a limited number of epitopes on the collagen molecule is essential to the development of arthritis. Several other collagen type II epitopes are immunogenic but not arthritogenic in most nonsusceptible animals.

Since helper T lymphocytes play a pivotal role in immune responses, the overall objective of our study is to understand the molecular basis of immune recognition in the collagen arthritis model. We are examining components of the trimolecular complex: T-cell receptor (TCR), arthritogenic peptide fragment(s), and major histocompatibility complex molecules. This study may lead to approaches for the elimination of pathogenic T-cell subsets.

Our attempts to map the arthritogenic epitope(s) have revealed that the major antigenic determinant(s) resides in a 25 kDa cyanogen bromide polypeptide fragment. Further digestion using trypsin indicated that the reactivity is retained in two different polypeptides. We are in the process of confirming this observation and generating synthetic peptides corresponding to the two tryptic polypeptide fragments.

We have generated T-cell hybridomas as well as T-cell lines against bovine collagen type II using DBA/1 LacJ mouse strain (H-2q). DNA sequencing data obtained from 11 independently derived T-cell hybrids indicate that three α chain- and four β chain-subfamilies are utilized by these hybridomas. Thus, the T-cell repertoire in CIA seems to be limited. Prevention and/or treatment of CIA using monoclonal antibodies directed against TCRs are under way.

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45. Immunomodulation of collagen-induced ear diseases by anti-TCR antibodies

T. J. Yoo, Kuang-Chuan Cheng, Kai Wang, M. K. Lee

Collagen autoimmunity has been described in association with several disorders characterized by hearing loss: relapsing polychondritis, Meniere's disease, otosclerosis and autoimmune sensorineural hearing loss. Type II collagen-induced autoimmune ear disease (CIAED) in animals has been shown to have histopathologic and immunological resemblance to human autoimmune ear diseases.
We are proposing to study the roles of the T-cell and CIAED by antibody blocking of appropriate T-cell receptors (TCRs) to ameliorate the CIAED. We have studied H-2q mice and found that TCRs to the CB-11 peptide are restricted. One of the antigen epitopes is a 26 mer peptide extending from residues 122-147 in collagen. We found that H-2r mice employ a different epitope from that of H-2q mice. We will test the following possibilities for our animal model.

1. T cells from CIAED ear lobes might have high expression of gamma and delta of TCRs in restrictive fashion and the pattern of expression of T cells in ear lesion is different from the one in joint tissues from collagen-induced arthritis.

2. CIAED could be blocked by either anti-TCR antibodies or peptides from the second hypervariable regions of the Va and/or Vp chains.

We have generated H-2r T-cell lines and their structures of TCR will be identified to produce anti-TCR antibodies. These antibodies to TCR will be used to test our hypothesis.

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46. T-cell receptor repertoire in Meniere's disease

T. J. Yoo, Kuang-Chuan Cheng, Kai Wang, M. K. Lee

Collagen autoimmunity has been described in Meniere's disease. It is a chronic progressive cochlear and vestibular disorder. Some patients with idiopathic Meniere's disease showed a high level of serum antibody against type II collagen.

To dissect the role of T cell's involvement, we are analyzing the Va, Vb, Vg and Vd gene segment expressions from surgical samples from these patients and the T-cell epitopes of type II collagen possibly involved in the specificity of these T cells.

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47. Joint and ear lesions induced in transgenic mice by ectopic expression of interferon-gamma (IFN-γ)

T. J. Yoo, Marc Krug1, Rosa Ong1, Jim Thompson1, Kuang-Chuan Cheng, Carol Readhead

The ectopic expression of class II major histocompatibility complex (MHC) molecules on non-immune cell types has been documented in a variety of autoimmune states. Although the significance of this is unknown, it has been speculated that the aberrant MHC class II expression could result in tissue destruction by self-antigen presentation. Although the cause of ectopic expression of MHC class II molecules is not known, interferon-γ (IFN-γ) can induce class II expression on a wide variety of cell types in vitro.

An intriguing potential pathway for autoimmune ear disease or arthritis is as follows. Local viral infection or environmental factors can lead to the activation of T cells and subsequent local production of T cells which then induce the expression of class II MHC molecules not only on normal antigen-presenting cells, but on local ear tissues or joint tissue cells. These could initiate autoimmune lesions in the joint and ear tissues.

Experiments using transgenic mice may allow us to test the hypothesis that both collagen-induced autoimmune ear disease (CIAED) and collagen-induced arthritis (CIA) can be induced in transgenic mice when gene constructs consisting of promoters of type II collagen with IFN-γ genes are expressed in a tissue-specific manner. We have made a DNA construct of the type II collagen promoter linked to the IFN-γ gene. We will produce transgenic mice with this gene construct and ear and joint lesions will be examined and will be compared with the lesions produced by antigen immunization.

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48. Molecular analysis of the T-cell repertoire in allorejection

Kuang-Chuan Cheng, Gamal E. Osman, T. J. Yoo

We would like to determine whether T-cell receptor repertoire for allospecific T cells in a mouse undergoing allorejection is restricted. This study would determine whether allo T-cell responses are restricted, and if so, whether antibody therapy could be employed to circumvent allorejection. The difference between the coisogeneic B6 (I-Ab) mouse and the mutant class II MHC (I-Abm12) mouse resides entirely in the second hypervariable region of the β chain of the I-Ab molecule. This I-Abm12 molecule differs from the wild-type I-Ab
molecule by three amino acid replacements: Ile to Phe67, Arg to Gin70 and Thr to Lys71. In spite of these limited substitutions, skin graft rejection and lymphocyte responses in mixed lymphocyte cultures have been reported.

We have generated 30 T-cell hybridomas in bm12 animals that react to B6 spleen cells. The nucleotide sequences from several TCR \( \alpha \) and \( \beta \) genes indicate that \( \nu_\beta \) usage is restricted. This observation is confirmed by FACS analysis of these hybridomas with appropriate \( \nu_\beta \) antibodies. In vitro, a single monoclonal antibody could inhibit the proliferation of bm12 T cells in the presence of B6 antigen presenting cells by 50%. Our study may lead to an antibody strategy for elimination of alloreactive T cell in vivo.

49. The isolation and characterization of cDNAs expressed by CD4-CD8- (double negative) T cells but not by mature lymph node T cells

_Sally L. Orr, Elaine F. Gese_

We have constructed a subtractive cDNA library to identify cDNA clones corresponding to RNAs expressed during early T-cell development. The library contains clones that are differentially expressed by CD4-CD8- (double negative) T cells but are not expressed by mature lymph node T cells. The subtractive library was screened using differential screening techniques to identify relatively abundant cDNA clones. The library was also screened with labeled cDNAs derived from RNA bound to membrane-bound polysomes to identify clones that encode cell surface molecules. The cDNAs were classified into three groups according to the screening results and tested for tissue-specific expression by Northern analysis using poly(A)+ RNA from various cell types. Of the 50 individual clones tested on Northern blots, approximately one-half of the clones could not be detected and may represent rare single copy clones. While 20% of the clones were expressed by a variety of cell types including the double-negative T-cell line used to construct the library and other cell lines including monocytes, T cells and B cells, nine of the cDNA clones appear to be specific for double-negative T cells as determined by Northern analysis. While the Northern analysis was in progress, 72 clones representing each of the three expression groups were subjected to limited DNA sequence determination. Interestingly, none of the cDNA clones were of known sequences in the Genbank or EMBL databases. Two cDNA clones, D51 and M517, which were specifically expressed by double-negative T cells, were observed to contain limited protein homologies to a leukocyte adhesion protein and members of the immunoglobulin gene superfamily, respectively. Therefore, this method of cDNA subtraction can be successful in isolating rare genes that may be involved in early T-cell growth or differentiation.

50. The characterization of a human T-cell receptor \( \nu_\beta 8.1 \) promoter binding protein

_Yu-kang Wang_

A cDNA clone, ht16, encoding a zinc finger protein which binds to the promoter region of human T-cell receptor \( \nu_\beta 8.1 \) gene, was cloned from a human peripheral blood T-cell cDNA library. By subcloning ht16 cDNA into PET vector, we expressed ht16 in _E. coli_. The ht16 binding site in \( \nu_\beta 8.1 \) promoter has been characterized by DNase I footprinting assay to encompass about 21 nucleotides. Besides human \( \nu_\beta 8.1 \) promoter, ht16 protein can bind to other T-cell receptor gene regulatory sequences. The ht16 gene is expressed not only in T-cell lines but also in B cell, macrophage, HeLa cell, and other lines. The regulatory functions of this DNA binding protein are now being studied.

A mouse cDNA that is highly homologous to ht16, especially at the zinc finger region, was isolated with the polymerase chain reaction method. We are interested in determining whether it is involved in mouse T-cell receptor gene expression.

51. T-lymphocyte antigen receptor V gene segment conservation among primates

_William K. Funkhouser, Jr., Ben F. Koop, Patrick R. Charmley_

The degree of evolutionary conservation of the T-lymphocyte antigen receptor (TCR) locus is unknown. It is known that the natural ligands for the TCR proteins, the major histocompatibility complex class I/II proteins, are highly allelic and this suggests that the TCR proteins may also be highly diverse. Since the variable portions of the TCR proteins result from \( V, (D) \) and \( J \) gene segment DNA rearrangements, TCR-protein variability might result from
germline, junctional and/or combinatorial diversity. To examine germline diversity, human Vp8.1 and Vp8.2 gene segments were compared to orthologous sequences in other primates and to homologous sequences in other humans.

Human primers were used to amplify 2.0 kb fragments containing either Vp8.1 or Vp8.2 gene segments from human, chimp, gorilla and rhesus, and internal human primers were used to sequence the cloned PCR fragments. Sequence comparisons of human Vp8.1 and Vp8.2 gene segments showed about 7.5% (126/1675) differences within the noncoding sequences, and 2.0% (6/286) differences within the coding sequences. Comparisons of the chimp and human sequences revealed that chimp lacks the hVp8.2 homolog. Comparisons of gorilla and human Vp8.1sequences revealed both hVp8.1 and hVp8.2 homologs. Comparisons of rhesus and human sequences revealed two gene segments, but it is not possible to tell which is orthologous to the Vp8.1 versus Vp8.2 gene segments. Comparisons of rhesus "AP8" to human Vp8.1 revealed 9.2% (155/1675) differences within the noncoding sequences and 6.0% (18/298) differences within the coding sequences; comparisons to Vp8.2 revealed 8.2% (137/1675) differences within the noncoding sequences and 6.0% (18/298) differences within the coding sequences. Comparisons of rhesus "AP9" to human Vp8.1 revealed 6.9% (116/1675) differences within the noncoding sequences and 5.7% (17/298) differences within the coding sequences; comparisons to Vp8.2 revealed 8.7% (146/1675) differences within the noncoding sequences and 6.4% (19/298) differences within the coding sequences. "Neutral," or freely substituted, rates of change since the divergence of these species are 1.5% for human-chimp, 1.8% for human-gorilla, and 6.8% for human-rhesus. Our non-coding sequence data are consistent with these benchmark "neutral" rates. In each case, orthologous coding sequences are slightly more conserved than neutral.

A similar study of a sample of ten unrelated humans revealed no nonsynonymous substitutions within Vp8.1 or Vp8.2 gene segments, suggesting few polymorphisms within these V gene segments.

These two observations together suggest that the basis for TCR protein variability is due not to germline diversity, but rather to junctional and/or combinatorial diversity.

52. Production of soluble T-cell receptor and MHC in Escherichia coli
Joan A. Kobori, Caryn D. Tong, Elizabeth E. Burnell

Peptide antigen bound to a major histocompatibility complex (MHC) molecule is the antigenic determinant recognized by a T-cell receptor (TCR). We have succeeded in expressing the α and β chain of a lysozyme peptide-specific TCR and the α and β chain of the relevant MHC molecule, I-Ek, using inducible promoter vectors. The transmembrane and cytoplasmic domains were removed from full-length cDNA clones and in some expression constructs, the murine signal sequences were replaced with E. coli signal sequences. We are now attempting to express heterodimeric structures (αβ) by producing a single transcript able to produce both chains in the same cell. We hope that by exporting the proteins, we can improve our chances of forming the appropriate disulfide linkages. In addition, we are attaching "flag" sequences to the amino-terminal ends of our proteins to facilitate detection on Western blots and purification by affinity chromatography. Detection of the flag by a specific monoclonal antibody and cleavage of the sequence by enterokinase permit the production of highly-purified protein. We plan to use the protein for biochemical studies of TCR, MHC, and peptide antigen.

53. Studies on somatic hypermutation in B cells
Joan M. Goverman, Lisa N. Larson, Michelle Arcinue, Helen Lee

Determining how the diversity of B- and T-cell antigen-specific receptors is generated is a fundamental question in immunology and is critical for understanding
many aspects of immune disorders. The process of somatic hypermutation scatters random point mutations throughout rearranged immunoglobulin V genes and has a major impact on the specificity and diversity of antibody molecules. Several features suggest that somatic hypermutation arises from a unique genetic mechanism with two notable features: an extraordinarily high rate of mutation, and tight localization to the surrounding V-gene region. Our objective is to define the sequences associated with variable region genes that are responsible for targeting these genes for somatic hypermutation. This has been difficult to address because B cells undergoing this process have not been isolated in vitro. Our approach requires determining the ability of various target genes to undergo somatic hypermutation in transgenic mice. The target gene design takes advantage of two observations: T- and B-cell receptor V genes are very similar, but the sequences flanking them are quite different, and T-cell receptor (TCR) genes do not exhibit somatic hypermutation. We are generating constructs from portions of T- and B-cell receptor genes that represent the combinatorial possibilities of three regions: the 5' promoter region, the V gene itself, and the intron between the rearranged V and C gene.

We have begun by making transgenic mice using a rearranged TCR coupled to an Ig enhancer sequence, assuring transcription of the TCR gene in B cells. Several transgenic lines have been established with low copy number of the transgene. B-cell hybridomas have been generated from the mice and are being screened for those that activated the process of somatic hypermutation. The transgenes will be sequenced to determine if mutation has occurred. Lack of mutation in the TCR gene would suggest that there are Ig-specific sequences, either within, 5' or 3' to the V gene that define the Ig template for mutation. The presence of somatic mutations in the TCR transgene suggests that V gene-specific sequences alone are sufficient to specify the template for mutation, independent of the flanking sequences. This result allows us to study the nature of mutations that occur in the absence of antigenic selection on a single template in many B-cell clones.

54. Effect on tumor cells of cytotoxic T cells transfected with chimeric T-cell receptors specific for tumor antigens

Wang Rong Qiu, Chia Chen, Joan M. Goverman, Walter E. Lang

T and B cells participate in the immune response through their recognition and elimination of foreign antigen (Ag). B cells bind soluble antigen through immunoglobulin receptors expressed on their cell surface which interact by molecular complementarity with the antigen. T cells recognize only Ag presented on a cell surface in the context of class I or class II molecules encoded by the major histocompatibility complex (MHC) known as MHC restriction. The antigen receptors for both B and T cells are heterodimeric structures in which both chains are composed of a variable (V) and constant (C) region. The V domains of both receptors are involved in antigen recognition, are encoded by genetic elements distinct from those that encode the C domain, and appear to share similar tertiary structures. The C regions link the receptor to the cell surface and, in the case of the T-cell receptor, to other cell-surface accessory molecules, including a complex of polypeptides called CD3.

Tumor cells, although expressing tumor-specific antigens (TSA), escape recognition and elimination by cytotoxic T cells through MHC-dependent Ag presentation. Thus, human neuroblastomas lack MHC expression and small cell lung carcinomas (SCLC) express TSA separate from MHC.

B cells, recognizing Ag independent of MHC, readily produce antibodies binding to TSA. Therefore, chimeric TCR, in which the V gene region is replaced by a V gene coding for monoclonal antibodies specific for TSA, should, after transfection into a cytotoxic T cell, recognize and bind to the corresponding tumor cells, with subsequent cytolysis. This hypothesis is supported by our previous finding that chimeric TCRs for the hapten phosphocholine recognize and bind to immobilized hapten after transfection into EL-4 cells. In addition, binding to phosphocholine resulted in activation of the transfected T cell with interleukin-2 release (Goverman et al., 1990).

We will investigate the possible cytotoxic effect of the cytotoxic T-cell hybridoma (SPH-1) transfected with a chimeric TCR containing the variable region of the neuroblastoma-specific hybridoma CE-7 (H. Amstutz,
Berne, Switzerland) on various human neuroblastoma cell lines. In addition, we are cloning the variable region genes of the SCLC-specific hybridomas SWA20 and SWA11. After construction and transfection of specific chimeric TCR into the cytotoxic T-cell hybridoma, their effect on SCLC cell lines will be evaluated in vitro using the chromium release assay and in vivo in nude mice growing SCLC.

These studies may lead to a novel immunotherapy of certain human cancers.

Reference:

55. Self-nonself recognition in the immune system: Nonclassical transplantation antigens
Iwona Stroynowski

The vertebrate immune system uses a multitude of different mechanisms to identify and eliminate pathogenic organisms and other "nonself" substances. The concerted interactions between self and nonself components are encoded genetically and include humoral, or antibody-mediated, responses as well as cellular responses mediated by specialized receptors on target and effector cells. We study the interplay between cytotoxic T cells expressing T-cell receptors and target cells identified by major histocompatibility complex (H-2) class I, or class I-related, receptors. This year we have concentrated our efforts on the biology of the nonconventional class I antigens in mice, called Qa-2 molecules. These polypeptides, like classical H-2 antigens, consist of heavy chains associated with β2-microglobulin. They are presumed to fold into structures that bind protein fragments derived from nonself polypeptides. Qa-2 antigens can be recognized by T-cell receptors and function as guiding molecules for allogeneic cytotoxic reactions. They can also transduce activation signals leading to the proliferation of target cells. Thus, the Qa-2 antigens appear to perform a dual role as mediators of expansion and/or deletion of specific subsets of cells in the immune system.

We have recently demonstrated that Qa-2 antigens are attached to cell surfaces via phospholipid anchor, the property that is encoded within exon 5 of Qa-2 genes. Upon T-cell activation, Qa-2 mRNAs undergo alternative splicing that deletes exon 5 and leads to the synthesis of soluble, β2-microglobulin-bound Qa-2 molecules. We are currently involved in studying cellular and molecular mechanisms that signal alternative splicing of Qa-2 transcripts and result in "switching" from membrane-bound to soluble Qa-2 isoforms. The physiological functions of these molecules will be analyzed after expressing sufficient quantities of both of these forms in vitro, purification of proteins and injection into live mice. The regulation of expression and the sites of constitutive and inducible production of these molecules are analyzed in various mouse strains. We are also interested in the mechanism of molecular interactions between Qa-2 and T-cell receptors. Studies are under way to analyze peptides extracted from Qa-2 complexes and to identify antigens from which they are derived. We hope that these experiments will contribute to the understanding of the cytotoxic reactions, T-cell activation and alternative splicing as well as to the development of new concepts in immunology. The specific experiments aimed to address these questions are described in the next 10 abstracts and the following references.

References:

56. Detection of soluble forms of Qa-2 antigens in mouse sera
Nusrettin Ulker, Piotr Tabaczewski, Keith D. Lewis, Iwona Stroynowski

Concanavalin A (Con A) stimulation of murine splenocytes induces alternative splicing of Qa-2 gene transcripts (Ulker et al., 1990). In addition to the canonical mRNA encoding phospholipid-anchored Qa-2 antigens, activated T cells and transfected cell lines synthesize a truncated
version of Qa-2 mRNA lacking exon 5. Previous studies demonstrated that the alternatively spliced transcripts encode soluble molecules that are actively secreted into the culture supernatants (Ulker et al., 1990). We have now produced antisera against the predicted C terminus of the soluble molecules that is directed against amino acids spanning the fusion of exon 4 with exon 6. This antiserum was used in ELISA assays to detect soluble Qa-2 products in the culture supernatants and in the sera of Qa-2 positive mice. In addition to the actively secreted Qa-2 form, mouse sera and cell supernatants from activated splenocytes contain Qa-2 proteins lacking C-terminal epitope. We hypothesize that it is derived from PI-anchored Qa-2 precursors. Studies are under way to identify whether the two soluble forms contain the same peptides and if they stimulate/anergize the same subsets of cytotoxic T cells.

Reference:

57. Immunomodulation of soluble Qa-2 levels in mice
Piotr Tabaczewski, Iwona Stroynowski

Qa-2 proteins are members of the murine major histocompatibility complex (H-2) molecules. In contrast to the classical H-2 antigens, which exist only as membrane spanning proteins, the Qa-2 antigens are attached to the cell surfaces by glycosylphosphatidyl inositol (GPI) anchors. They exist also as actively secreted forms due to alternative splicing of their transcripts. Both of these forms are expressed in Con A-stimulated mouse splenocytes in vitro and both give rise to soluble Qa-2 polypeptides found in culture supernatants. To determine the relevance of these findings to the in vivo system, we have screened mouse sera for soluble Qa-2. Using the ELISA method, which discriminates secreted form from GPI-derived form of Qa-2, we have estimated that the total circulating Qa-2 level in adult healthy C57BL/6 mice is of the order of 100 ng/ml. The actively secreted form constitutes less than half of the total soluble Qa-2. It appears that different cell subsets contribute to the Qa-2 pool in serum since all the C57BL/6 mutants that demonstrate severe dysfunctions of immunological cell systems (beige, nude, scid, motheaten) contain Qa-2 in blood, albeit at different levels. The level of the soluble Qa-2 is age dependent: it is lowest at birth, reaches maximum at 3-5 months of age and then declines. The amount of serum Qa-2 is strongly enhanced in polyI:C-injected mice and is decreased in hydrocortisone-treated animals, suggesting dependence on immunomodulation. We are planning to investigate further how the levels of Qa-2 molecules in mouse serum change upon such stimuli as viral and bacterial infections and tumor challenge. We hope that these experiments will help to clarify physiological functions of soluble and membrane forms of Qa-2 antigens in vivo.

58. Purification of class I-like molecules for biological, biochemical and crystallographic studies
Piotr Tabaczewski, Kathleen N. Blackburn, Iwona Stroynowski

We are interested in the function of molecules related to the classical major histocompatibility complex antigens. Murine genome encodes at least 30-50 such molecules having different degrees of homology to the H-2 transplantation antigens. It is possible that these class I-like molecules perform a multitude of unrelated tasks, but on theoretical grounds, it is predicted that they resemble peptide-binding transplantation antigens and play a role during cell-cell interactions. Our goal is to study the structure of some of those molecules and to identify putative peptides bound to them. The purified proteins will be tested in vitro and in vivo to determine their biological activity. We have overexpressed proteins encoded by secreted as well as membrane versions of Qa-2 cDNAs from C57BL/6 mice using different eukaryotic expression systems. At the moment, we are able to purify enough material by FPLC for biological and peptide identifications studies. The efforts to increase expression for crystallographic analyses are under way.

59. Characterization of Qa-2 alternative splicing in lymphoid cell subsets
Laura T. Lebow, Piotr Tabaczewski, Iwona Stroynowski

Qa-2 molecules are closely related to class I H-2 K, D and L antigens and are among the best characterized nonclassical major histocompatibility complex antigens. The membrane-attached Qa-2 antigens can be detected serologically on cells from lymph node, spleen, and bone marrow. They also exist in mouse serum as soluble
forms. Membrane forms are expressed in highest quantities on mature T cells and in lesser amounts on B cells, NK cells and immature T cells, while soluble forms are known to be secreted by Concanavalin A (Con A)-activated splenocytes (Ulker et al., 1990). In contrast to classical H-2 antigens, which are transmembrane proteins, the Qa-2 antigens are attached to cell surfaces through glycosylphosphoinositol (GPI) lipid anchors. And like other GPI-linked proteins, Qa-2 molecules can transduce activation signals. The peptide regions that mark Qa-2 for GPI modification are encoded by exon 5, the transmembrane encoding exon in classical H-2 genes.

Recent studies show that mitogenic stimulation of splenic T cells with Con A induces synthesis of soluble Qa-2 molecules derived from alternatively spliced transcripts lacking exon 5 (Ulker et al., 1990). This truncated mRNA species lacks the signal for GPI attachment and encodes a protein with a highly charged C terminus. In Con A-activated T cells, the level of the truncated transcript constitutes 60% of the canonical full-length transcript level that encodes the membrane-bound isoform. The physiological function(s) of the switching from membrane to soluble form is not known, nor is the phenotype of the cells that secrete Qa-2 molecules constitutively and/or after immunostimulation. An aim of this work is to identify cell subsets (CD4+ versus CD8+ mature and immature T cells, B cells and NK cells) that express truncated Qa-2 mRNA and secrete Qa-2 molecules. To achieve this goal, polymerase chain reaction (PCR) assays, ELISA and flow cytometric techniques will be used. We hope that this information will be useful in delineating in vivo functions of the soluble and membrane isoforms of Qa-2. We have now demonstrated that in addition to activated splenic T cells, the expression of truncated Qa-2 transcripts can be detected in Con A-stimulated thymocytes and lipopolysaccharide-stimulated splenic B cells. Purification of specific cell subsets within thymus and spleen is under way. So far the data show that the expression of soluble Qa-2 antigens in various cell types tested is dependent on cell activation. This suggests that Qa-2 molecules may play a role in modulating immune responses.

Reference:

60. Identification of cis-acting elements involved in the regulation of alternative splicing of Qa-2 mRNA

Laura T. Lebow, Iwona Stroynowski

Successful presentation of antigen to T cells activates a cascade of signaling processes which ultimately leads to modification of immune responsiveness and induces changes in T-cell gene expression. Some of these changes result from new alternative splicing decisions. To date, three different genes have been shown to undergo alternative splicing of their transcripts after T-cell activation. These include the nonclassical major histocompatibility complex Qa-2 genes (Ulker et al., 1990) and cell surface receptor genes CD45 (Birkland et al., 1989; Saga et al., 1990) and CD8 (Norment et al., 1989). In all three cases, alternative splicing results in a deletion of specific exons in mature messages. In the case of Qa-2 genes, the activation-specific truncated form of mRNA encodes soluble Qa-2 molecules derived from alternatively spliced transcripts lacking exon 5. In the canonical message, this exon encodes signals required for attachment of Qa-2 to the plasma membrane through glycosyl phosphoinositide linkage.

The nucleotide sequences that interact with factors involved in activation-induced alternative splicing of any of these genes have not been defined. Analysis of Qa-2 genes offers a unique system to study regulated alternative splicing and the signals leading to the change in Qa-2 expression. By taking advantage of the high homology between Qa-2 genes and the classical H-2 genes, which do not undergo alternative splicing, the region(s) controlling exon 5 expression can be analyzed. We are constructing a series of hybrid Qa-2/H-2 genes and Qa-2 mutant genes using site-directed mutagenesis that will be used to generate stable transfected cell lines for the analysis of Qa-2 alternative splicing patterns. These experiments may permit the determination of nucleotide sequences that function in splicing decisions within cells of various lineages or states of differentiation in response to different activation signals. In addition, comparison of sequences important for controlling Qa-2 exon 5 expression to sequences of other genes regulated by alternative splicing may reveal general cis-acting elements required for exclusion of specific exons during T-cell activation.
I examine the expression of the Q6d gene product, Hepa-
Alternatively, Q6 protein may exist as a membrane-bound
possibility that secreted Q6d molecules can induce CTL.

(hepatoma) cells were transfected with the
antigen capable of
hybrid antigen
terminal regions of H-2Ld (Mann and Forman, 1988). We
(CTL) raised against Qa region molecules recognize a
Previous studies have shown that cytotoxic T lymphocytes
function of the Qa-Tla products is not known,
believed that they may
express on virtually all adult tissues. More recently, a
second group of class I-like molecules, encoded by the
Qa and Tla regions of the MHC, was identified. These
molecules share considerable structural homology with the
H-2 classical antigens but are nonpolymorphic and have a
restricted tissue distribution. Although the physiological
function of the Qa-Tla products is not known, it is
believed that they may play a role in antigen presentation
during self-nonself recognition by cytotoxic T cells. We
have undertaken a study of one of the nonclassical
molecules encoded by the Qa region gene, Q6d. DNA
sequence analysis indicates that the three external domains
of the predicted Q6d molecule, α1 α2, and α3, encode a
polypeptide similar to typical H-2 N-termini. The putative
transmembrane region of Q6d contains hydrophilic
residues, suggesting that Q6d molecules may be secreted.
Previous studies have shown that cytotoxic T lymphocytes
(CTL) raised against Qa region molecules recognize a
hybrid antigen (Q6d/H-2Ld) expressing the amino-terminal
α1 and α2 domains from Q6d and the α3 and carboxy-
terminal regions of H-2Ld (Mann and Forman, 1988). We
have demonstrated that Q7- and Q9-specific CTL do not
cross-react with Q6d/H-2Ld-bearing targets. This raises a
possibility that secreted Q6d molecules can induce CTL.
Alternatively, Q6 protein may exist as a membrane-bound
antigen capable of eliciting CTL responses. In order
to examine the expression of the Q6d gene product, Hepa-1
(hepatoma) cells were transfected with the Q6d gene. The
resulting transfectants express Q6d-specific PCR products
and ~40 kDa membrane-bound molecules recognized by a
monoclonal antibody that reacts with hybrid Q6d/H-2Ld
antigens. These transfectants are susceptible to Q6-
specific CTL but not Q7-specific CTL. Unlike the Qa-2
antigen encoded by the Q7 or Q9 genes, the Q6 gene
product is not attached to the membrane by a phosphatidylinositol anchor. Therefore, the Q6d gene appears to
code a novel, membrane-bound antigen. Experiments
are in progress to examine the nature of membrane attachment.

Reference:
1813.

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62. The Q7 α3 domain alters T-cell
recognition of class I antigens
Carla J. Aldrich1, L. C. Lowen1, Donald Mann2,
Michael I. Nishimura, Iwona Stroynowski,
James Forman4

We have analyzed the role of the α3 domain of class I
molecules in T-cell recognition. Using the laboratory
engineered molecules LLQQ (α1/α2 from Ld, α3 and
phosphatidylinositol [PI]-linked C terminus from Q7) and
LLQL (α1/α2 from Ld, α3 from Q7, transmembrane [TM]
and cytoplasmic domains from Ld), we show that these
molecules are not recognized by primary Ld-specific
cytotoxic T lymphocytes (CTL). The cell membrane
expression of both Ld and LLQL are upregulated by co-
culture with an exogenously supplied murine cytomegalovirus-derived peptide indicating that the Q7 α3
domain does not interfere with binding of antigen (Ag) to
α1/α2. However, only peptide pulsed Ld but not LLQL
target cells are recognized by Ld-restricted peptide-specific
CTL. In contrast to the above results, LLQL and LLQQ
molecules can be recognized by bulk alloreactive anti-Ld
CTL and 2/3 of CTL clones derived from in vivo primed
mice. The fact that these secondary CTL recognize LLQQ
indicates that a PI linkage is permissive for presentation of
class I epitopes to alloreactive CTL. These secondary
CTL are resistant to blocking at the effector stage by mAb
against CD8 and express relatively low levels of
membrane CD8 molecules compared to CTL from

References:
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Norment, A. M., Lonberg, N., Lacy, E. and Littman, D.
Ulker, N., Lewis, K. D., Hood, L. E. and Stroynowski,

61. Structure and function of the class I-like
molecule Q6d
Michael I. Nishimura, Kathleen N. Blackburn,
Keith D. Lewis, James Forman1, Iwona Stroynowski

Genes located within the major histocompatibility
complex (MHC, designated H-2 in mouse) encode
proteins required for immune recognition and response to
foreign antigens. Classical H-2 class I antigens are
highly polymorphic cell surface glycoproteins that are
expressed on virtually all adult tissues. More recently, a
second group of class I-like molecules, encoded by the
Qa and Tla regions of the MHC, was identified. These
molecules share considerable structural homology with the
H-2 classical antigens but are nonpolymorphic and have a
restricted tissue distribution. Although the physiological
function of the Qa-Tla products is not known, it is
believed that they may play a role in antigen presentation
during self-nonself recognition by cytotoxic T cells. We
have undertaken a study of one of the nonclassical
molecules encoded by the Qa region gene, Q6d. DNA
sequence analysis indicates that the three external domains
of the predicted Q6d molecule, α1 α2, and α3, encode a
polypeptide similar to typical H-2 N-termini. The putative
transmembrane region of Q6d contains hydrophilic
residues, suggesting that Q6d molecules may be secreted.
Previous studies have shown that cytotoxic T lymphocytes
(CTL) raised against Qa region molecules recognize a
hybrid antigen (Q6d/H-2Ld) expressing the amino-terminal
α1 and α2 domains from Q6d and the α3 and carboxy-
terminal regions of H-2Ld (Mann and Forman, 1988). We
have demonstrated that Q7- and Q9-specific CTL do not
cross-react with Q6d/H-2Ld-bearing targets. This raises a
possibility that secreted Q6d molecules can induce CTL.
Alternatively, Q6 protein may exist as a membrane-bound
antigen capable of eliciting CTL responses. In order
to examine the expression of the Q6d gene product, Hepa-1
(hepatoma) cells were transfected with the Q6d gene. The
resulting transfectants express Q6d-specific PCR products
and ~40 kDa membrane-bound molecules recognized by a
monoclonal antibody that reacts with hybrid Q6d/H-2Ld
antigens. These transfectants are susceptible to Q6-
specific CTL but not Q7-specific CTL. Unlike the Qa-2
antigen encoded by the Q7 or Q9 genes, the Q6 gene
product is not attached to the membrane by a phosphatidylinositol anchor. Therefore, the Q6d gene appears to
code a novel, membrane-bound antigen. Experiments
are in progress to examine the nature of membrane attachment.

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unprimed mice. Further, culture of unprimed CTL precursors in the presence of CD8 mAb also allows for the generation of CD8-independent CTL that recognize LLQL. Taken together, these data indicate that the a3 domain of Q7 (Qa-2) prevents CD8-dependent CTL from recognizing Ld4, regardless of whether the class I molecule is attached to the cell surface by a PI moiety or as a membrane-spanning protein domain. We hypothesize that this defect in recognition is most likely due to an inability of CD8 to interact efficiently with the Q7 a3 domain and could account for why Q7 molecules do not serve as restricting elements for virus and minor H-Ag-specific CTL.

63. Recognition of phospholipid-anchored class I antigens by cytotoxic T cells

Michael I. Nishimura, Kathleen N. Blackburn, Iwona Stroynowski

Major histocompatibility complex (MHC) class I molecules are cell surface glycoproteins involved in the rejection of virus-infected cells, tissue allografts, and tumor cells by cytotoxic T lymphocytes (CTL). Classical MHC class I molecules are attached to the cell surface by a hydrophobic transmembrane polypeptide while non-classical Qa-2 molecules are attached by a phosphatidyl-inositol (PI) anchor. Previous studies have shown that H-2Ld-specific CTL do not efficiently recognize hybrid MHC class I molecules (LLQQ) consisting of the a1 and a2 domains from H-2Ld and the a3 and the carboxy terminus from Q7d (Mann et al., 1989). Failure to recognize these hybrid LLQQ molecules may be in part due to the inability of the Q7d a3 domain to interact with CD8 molecules on the CTL (Aldrich et al., 1991). The present studies examine the effect of PI or transmembrane attachment of MHC class I molecules on recognition by H-2Ld or Qa-2-specific CTL. Hybrid H-2Ld (LLQQ) and Qa-2 (QQQL) genes were transfected into Hepa-1 and BRL3a hepatoma tumor cells, and high expressing lines were selected. Susceptibility to CTL lysis of LLQQ and QQQL transfected cells was compared to wild type LLLL and QQQL transfected cells. Bulk anti-H-2Ld CTL efficiently lyse both QQQL and QQQL bearing targets. However, preliminary results indicate that QQQL bearing target cells are more susceptible to lysis by Qa-2 reactive T-cell lines maintained on QQQL expressing stimulator cells than are QQQL bearing cells. These results suggest that at least two classes of Qa-2 reactive T cells exist and these T cells can distinguish PI-linked and transmembrane-linked Qa-2 molecules. Both classes appear to be present in bulk CTL populations since bulk effectors efficiently lyse both QQQL and QQQL bearing target cells. Only after long-term culture can one class of T cells be expanded preferentially over the other. Differential recognition of PI-linked molecules may be due to: 1) intracellular signals via the PI anchor, 2) different mobility of PI-linked molecules in the membrane, or 3) the presence of different antigenic peptides in the Qa molecule since PI-attached molecules may traffic differently through the cell. Experiments in progress designed to test these hypotheses include establishment of long-term anti-Ld reactive T-cell lines to independently confirm the anti-Qa-2 data and isolation of anti-Qa-2 T-cell clones capable of distinguishing between PI- and transmembrane-linked Qa-2.

References:

64. The a3 domain of class I molecules affects both negative and positive selection of antigen-specific cytotoxic T lymphocytes

Carla J. Aldrich1, Robert E. Hammer2, Sharon Jones-Youngblood2, Ulrich Koszinowski3, Iwona Stroynowski, James Forman1

The a1 and a2 domains of major histocompatibility complex class I molecules function in the binding and presentation of foreign peptides to the T-cell receptor and control both negative and positive selection of the T-cell repertoire. While the a3 domain of class I is not involved in peptide binding, it does interact with the T-cell accessory molecule, CD8. CD8 is important in selection of T cells since anti-CD8 antibody injected into perinatal mice interferes with this process. We previously used a hybrid class I molecule with the a1/a2 domains from Ld and the a3 domain from Q7b and showed that this molecule binds an Ld-restricted peptide but does not interact with CD8-dependent cytotoxic T lymphocytes...
Expression of this molecule in transgenic mice fails to negatively select a subpopulation of anti-Ld CTL. In addition, positive selection of virus-specific Ld-restricted CTL does not occur. We conclude that besides the α1/α2 domains of class I, the α3 domain plays an important role in both positive and negative selection of antigen-specific cells.

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65. Differences between the lateral organization of conventional and inositol phospholipid-anchored membrane proteins. A further definition of micrometer scale membrane domains

Michael Edidin, Iwona Stroyowski

Plasma membranes of many cells appear to be divided into domains, areas whose composition and function differ from the average for an entire membrane. Fluorescence photo-bleaching and recovery were previously used to demonstrate one type of membrane domain, with dimensions of micrometers (Yechiel and Edidin, 1987). The presence of membrane domains is inferred from the dependence of the apparent mobile fraction of labeled molecules on the size of the membrane area probed. We now find that by this definition classical class I major histocompatibility complex molecules, H-2Db, are concentrated in domains in the membranes of K78-2 hepatoma cells, while the nonclassical class I-related molecules, Qa-2, are free to pass the boundaries of these domains. The two proteins are highly homologous but differ in their mode of anchorage to the membrane lipid bilayer. H-2Db is anchored by a glycosyl-phosphatidyl inositol (GPI) anchor. A mutant class I protein with its external portion derived from Qa-2 but with transmembrane and cytoplasmic sequences from a classical class I molecule shows a dependence of its mobile fraction on the area of membrane probed, while a mutant whose external portions are a mixture of classical and nonclassical class I sequences, GPI-linked to the bilayer, does not show this dependence and hence by our definition is not restricted to membrane domains.

Reference:

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66. Dysmyelination in SV40 large T transgenic mice: Strategies employed in the regulation of the major myelin genes in oligodendrocytes and Schwann cells

Niels A. Jensen, George Smith, H. David Shine, Justine Garvey

Oligodendrocytes and Schwann cells are myelin-forming cells of the central (CNS) and the peripheral nervous systems (PNS), respectively. The two cell types are morphologically distinct, derive from different regions of the embryonic neuroectoderm and seem to use very different strategies in the myelination of axons. Oligodendrocytes and Schwann cells are thought to rely on cell-cell interactions as well as a variety of growth factors as they proceed to terminal differentiation. In order to develop a system for the study of cellular regulatory events that occur in oligodendrocytes and Schwann cells at terminal stages of their differentiation, we have blocked the differentiation of these cells in transgenic mice by introducing the gene encoding the large T antigen coupled to the myelin basic protein (MBP) promoter. We next derived immortalized cell lines from the CNS and the PNS of these transgenic animals and are presently using these lines in detailed regulatory studies.

The expression of the large T (LT) antigen from SV40 virus was targeted to myelinating cells in the CNS and PNS in transgenic mice using a 3 kb MBP promoter. Transgenic founders harboring the T antigen developed one of two distinct neuropathies. One group of founders developed a general shivering that progressed in 1-2 weeks into severe convulsions and early death. No walking abnormalities could be observed in these mice. A second group of transgenic founders developed a severe hindleg ataxia that progressed in weeks into complete paralysis of the hindleg region. No generalized shivering was observed. This second group of mice also lived significantly longer. Neuropathological examinations of the mice showed that different regions of the nervous system were affected in the two groups of mice. Mice with the generalized shivering phenotype suffered from a
severe dysmyelination of the CNS while the PNS appeared normally myelinated. In contrast, the ataxic mice suffered mainly from PNS dysmyelination, but myelin pathologies in the CNS could also be observed in some of the mice. This suggests that the ataxic phenotype may suppress the shivering phenotype in mice with a dual pathology.

Large T immortalized Schwann cell and oligodendrocyte lines have been established from transgenic mice with the ataxic phenotype and from mice with the shivering phenotype, respectively. Presently, most studies have been done on the Schwann cell lines. Individual clonal lines of the Schwann cells differ with respect to morphology and growth rate and all lines express high levels of the MBP promoter-driven expression of the LT gene shown by Northern blotting and immunofluorescence. Neither of the lines tested expressed either the endogenous MBP gene or the Po gene (Po is the major myelin protein in Schwann cells). The observation that the exogenous MPB promoter is active in Schwann cells that do not permit expression from the endogenous MBP promoter, suggests that transcriptional activation of the MBP locus is at least a two-step mechanism with the appearance of positive factors prior to modulation of a negative-acting factor(s). We are presently pursuing this possibility by transfecting different CAT constructs into the cell lines. Similar studies are also in progress using our immortalized oligodendrocyte cell lines.

67. Regulation of myelin basic protein gene expression during mouse development

Peter M. Mathisen, Carol Readhead

During the development of the central nervous system (CNS), myelin basic protein (MBP) is synthesized by the oligodendrocyte and is concomitant to the myelination of the CNS. MBP mRNA is first detected 7 days postnatally, peaks at 18 days and is activated in a caudal to rostral manner. Although MBP is expressed in the peripheral nervous system (PNS) by the Schwann cells, it is only 5-15% total myelin protein, whereas in the CNS it is 30-40% of the myelin protein. In the mouse mutation shiverer, there is a deletion of 60% of the MBP gene, which results in mice with a shivering gait and reduced life span. Thus, MBP is a gene product that is tightly regulated in its expression and results in a differentiated cell-type that provides a physiologically essential function in the development of the nervous system.

In order to study that mechanism controlling the temporal and spatial regulation of MBP gene expression, we have constructed a series of transcriptional fusions containing sequential deletions of the MBP 5' flanking sequences. Beginning with the 3.5 kb MBP 5' flanking sequences, a series of 5' deletions have been fused to the bacterial reporter gene lacZ and used to produce a series of transgenic mice. Such promoter deletion analysis with extended amounts of 5' flanking sequences in transgenic mice offers the opportunity to delineate control elements that would go undiscovered in other types of transfection assays. In addition, as a means of a positive control for X-gal staining of lacZ activity, we have also produced transgenic mice with the SV40 enhancer and promoter fused to lacZ, which we expected will be expressed in many organs of the mouse. We are in the process of examining the expression pattern of lacZ expression in each of these transgenic lines by sectioning the brain of 18-day-old mice and staining with X-gal. In this way, the cis-acting elements that are involved in the spatial and temporal regulation of MBP gene expression will be examined.

68. Gene disruption of the myelin-associated glycoprotein by homologous recombination in embryonic stem cells

Peter M. Mathisen, Carol Readhead

Myelination results in the formation of the insulating sheath that surrounds the axons of the nervous system and facilitates the conduction of electrochemical stimulation along the axon. One of the components of the myelin sheath implicated in the first interactions between the glial cells and the neuronal axon is myelin-associated glycoprotein (MAG). MAG is an integral membrane protein that is a member of the immunoglobulin gene superfamily, which most closely resembles cell-adhesion molecules. We are currently investigating the role of MAG in the initiation and perpetuation of the myelin sheath by using gene-targeting technology to produce a mouse line mutated in the MAG gene (MAG-). This technology relies on the use of permanent cultures of
Glutamate receptors termed kainate receptors. The methyl-isoxasole propionic acid (AMPA) define a KN
subunits and there appears to be five or more genes classified based on their activation by different agonists. The agonists kainate (KA) and α-amino 3-hydroxy-5-
neurotransmitter receptors in the mammalian brain and are

Molecular cloning and chromosomal information concerning the molecular mechanisms responsible
for the initialization of myelination in the nervous system. As it is hypothesized that MAG is essential in the first interactions between the glial cells and axons, the oligodendrocytes and Schwann cells may not be able to make initial contact with the axons and produce no myelin sheath in MAG− mice. However, if there is a myelin sheath on the axons, we will examine it carefully to determine if it is structurally abnormal. Besides studying the disruption of the myelin sheath, one can address MAG’s role in development. Since it is known that oligodendrocyte differentiation proceeds through a discrete set of precursors cells, if these cells are stalled at any particular stage in MAG− mice, this would indicate that MAG is required at some point during development. Thus, this project employs gene-replacement technology to produce a useful model system to study the role of MAG during myelination and will yield important information concerning the molecular mechanisms responsible for the initialization of myelination in the nervous system.

Molecular cloning and chromosomal localization of one of the human glutamate receptor subunits

Carmie Puckett, Christopher M. Gomez, Julie Korenberg

Glutamate receptors are the predominant excitatory neurotransmitter receptors in the mammalian brain and are classified based on their activation by different agonists. The agonists kainate (KA) and α-amino 3-hydroxy-5-
methyl-isoxasole propionic acid (AMPA) define a class of glutamate receptors termed kainate receptors. The KA/AMPA receptors in rats are composed of heteromeric subunits and there appears to be five or more genes encoding these subunits. We have isolated and sequenced the first human glutamate receptor cDNA (GluHI) and determined the chromosomal localization of its gene. The predicted amino acid sequence of GluHI would encode a 907 amino acid protein which has a 97% identity to one of the rodent kainate receptor subunits. The extreme conservation between the human and rat kainate receptor subunits suggests that a similar gene family will encode human kainate receptors. The GluHI mRNA is widely expressed in human brain, but most abundantly expressed in the cerebellum. The human gene encoding the GluHI glutamate receptor subunit is located at 5q33. While the GluHI gene is not located near the map locations of any human neurogenetic disorders, the homologous region on mouse chromosome 11 contains five neurologic mutants. Two of these mutants, vibrator (vb) and spasmodic (spd), demonstrate cerebellar pathology, a region where this receptor subunit is abundantly expressed. Using the GluHI cDNA, we have now isolated mouse cosmids for the mouse kainate receptor subunit. We will use these cosmids to determine if the mouse gene maps near either vibrator or spasmodic.

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The identification of genes involved in growth cone formation and function

Carmie Puckett

During neuronal differentiation, the neuron extends long processes termed neurites with a specialized elaboration called the growth cone. The goal of this research is to identify genes expressed by the neuron during neurite outgrowth that encode proteins involved in the formation and function of these structures. This approach utilizes the rat adrenal tumor cell line PC12. Following exposure to NGF, PC12 cells display a short latency response associated with NGF activation. PC12 cells also display a long latency response to NGF which occurs over several days in which they stop dividing, sprout neurites, and develop a phenotype similar to sympathetic neurons. Utilizing the SWAJ vector system (Palazzolo and Meyerowitz, 1987), a subtractive cDNA library was made from PC12 cells induced for six days with NGF (a long induction was chosen to select against messages associated with the short latency response). A
subset of these clones was screened with subtractive cDNA probe made from the microsomal fraction of NGF-induced PC12 cells. Current efforts are directed at the analyses of several clones that demonstrate induction by NGF and are represented in the microsomal fraction of cytoplasmic mRNAs. These clones will be used to isolate full-length cDNAs and to make antibodies to study the cellular localization of the corresponding proteins.

Reference:

71. Genomic structure and polymorphisms of the human prion protein gene
Carrie Puckett, Patrick J. Concannon1, Christine G. Casey

Scrapie and Creutzfeld-Jakob disease are transmissible degenerative disorders that occur, respectively, in sheep and man. A closely related disorder in man, Gerstmann-Straussler syndrome (GSS), is characterized by familial occurrence, progressive ataxia, dementia and death. The pattern of transmission within families suggests an autosomal dominant pattern of inheritance, yet this disorder can be transmitted to non-human primates and rodents by intracerebral inoculation of brain homogenates from affected individuals. The brains of affected individuals accumulate a protein termed prion protein (PrP) that can be shown to be closely associated with transmissibility in animal models.

The gene encoding this protein in man has been shown to be on the short arm of chromosome 20 (20pter-p12), an area with few known genes or markers. Several groups of investigators have demonstrated that non-synonymous substitutions in the PrP gene are associated with GSS. We have determined the genomic structure of the human PrP gene. We have also identified a frequently heterogenous polymorphic marker which should be a useful marker for this region of chromosome 20. The genomic clone that we have isolated contains an interesting 24 bp deletion of an 8 amino acid repeat in the coding region of this allele. This deletion is very likely to represent an uncommon polymorphic variant of this gene, since a deletion in this same region has been described in a North African pedigree. We have distributed these clones to investigators studying families with Gerstmann-

 Straussler syndrome.

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72. Characterizing protein changes in complex biological systems
Michael G. Harrington, David M. Gunderman, Thomas E. Zewert, Jerry E. Solomon, Susan Eberlein, Wei Wu, Miki Yun, Jina Yun

The emphasis of our work is on the development of technologies that are required for ongoing studies of sea urchin nuclear proteins, T-cell activation, PC12 differentiation and human cerebrospinal fluid from patients with dementia. The methods are focused on making optimal use of the high resolving power of two-dimensional electrophoresis (2DE) of proteins. The problems that we are addressing are the reproducibility between gel runs, the sensitivity of protein detection, the analysis of the complex 2DE patterns, and sample transfer from 2DE gel for amino acid sequence analysis.

We have built and tested a prototype device that performs the 2DE separation in a mechanically controlled manner. The isoelectric focusing dimension is precisely connected to the SDS-PAGE dimension, and the spatial reproducibility of proteins between two gels is improved two to threefold compared to the standard methods. A second version of this device is now being designed that will enable multiple 2DE gels to be run simultaneously.

The limit of sensitivity of protein detection after 2DE has been improved to 1,000,000 molecules by sensitive silver staining, digitization on a laser densitometer followed by image processing. This has enabled 200 proteins to be visualized from a single mouse oocyte, and comprehensive 2DE protein analysis of over 3,000 proteins from 10,000 lymphocytes (previously 1,000,000 cells were required). In order to improve the sensitivity to enable 2DE analysis of a single cell, we are investigating methods to attach signal molecules (both fluorescent and radioactive) to proteins after 2DE separation.

We have designed and implemented a complete basic 2DE image analysis system, GALtool, that performs the functions necessary for analyzing and displaying gel data. These functions include the fundamentals of image acquisition, calibration (optical density and radioactivity), finding all protein spots automatically, and matching any two gels to identify the differences and similarities. We
have used GALtool to perform subtractive analysis of two complex systems. The sea urchin study carried out in collaboration with Prof. Eric Davidson has enabled us to estimate the total nuclear DNA-binding proteins from the relative enrichment of these proteins on a cation exchange column: a total of approximately 100 binding proteins were identified in the 24-hour stage of the embryo. In a study of human cerebrospinal fluid (CSF), 20 abnormal proteins were identified in the CSF of patients with dementia compared with appropriate controls. Furthermore, the brain origin of 10 of these abnormal proteins was determined by subtractive comparison with a normal brain 2DE gel pattern.

Sample preparation related to 2DE separations has been improved for obtaining chemical sequence analysis at low picomole quantities of starting material. A cyanogen bromide cleavage method for obtaining multiple protein fragments of reasonable size for sequence determination has been refined.

73. Cataloguing the integral membrane proteins of synaptic vesicles

David M. Gudeman, Michael G. Harrington, Thomas E. Zewert, Reinhardt Jahnl

Synaptic vesicles are membrane enclosed concentrations of neurotransmitter found in the synaptic terminal. The vesicles undergo exocytosis during neurotransmitter release. Three synaptic vesicle integral membrane proteins have been characterized at the molecular level, but analysis of this cohort of proteins is hindered by the extreme difficulty in separating these hydrophobic molecules. Since our objective is to comprehensively separate and identify the major integral membrane proteins of synaptic vesicles, developing a method for the separation of hydrophobic integral membrane proteins must first be addressed. We have chosen two-dimensional polyacrylamide electrophoresis for our separation technique because cataloguing and protein sequencing techniques are well worked out for this method. Indeed, conventional two-dimensional electrophoresis of a synaptic vesicle preparation yields a relatively well resolved silver-stained protein pattern. However, when the same two-dimensional array of proteins is stained with an antibody specific for a known membrane protein, an extremely poorly resolved pattern is obtained. Although it has been estimated that small synaptic vesicles contain 258 proteins, from our data we have concluded that this number includes predominantly hydrophilic, nonmembrane proteins. We have developed a system based on isoelectric focusing in organic solvents that resolves a hydrophobic protein, p29, and thus appears to have overcome the resolution problem. Preliminary results suggest that this system will allow the cataloguing of integral membrane proteins from a complex-protein mixture. Whether sequence data can be obtained from proteins separated by this method has yet to be determined.

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74. Polyethylene glycol methacrylate polymers as matrices for electrophoresis in organic solvents

Thomas E. Zewert, Michael G. Harrington, David M. Gudeman

The properties of gels composed of polyethylene glycol methacrylate and copolymers of polyethylene glycol methacrylate and acrylamide have been studied. These novel electrophoresis matrices are amphigels (swellable in water and organic solvents) which have unprecedented organic solvent compatibility. Hydrophobic proteins which are poorly solubilized in aqueous detergent systems (e.g., zein) are well resolved in these gels with hydroorganic solvents. Variations of crosslinker, buffer, and solvent systems in these gels have been explored. Also, microscopic and macroscopic structural properties of these amphigels have been investigated.

75. Thioacetylation method of protein microsequencing

Mark. L. Stolowitz, Chin Sook Kim

A reinvestigation of the thioacetylation method of protein sequencing has revealed that 2-methyl-5(4H)-thiazolones, prepared by trifluoroacetic acid catalyzed cleavage of an N-terminal amino acid from an N-thioacetylated polypeptide, were found to react instantaneously with an equivalent of an aromatic sulfonic acid chloride to yield a stable derivative suitable for analysis by high performance liquid chromatography (HPLC). NMR studies revealed the product of the derivatization to be the
corresponding 5-O-arylsulfonyl-2-methylthiazoles, which result from reaction with the tautomeric 5-hydroxy-2-methylthiazoles. A preliminary HPLC separation of 2-methyl-5-O-(4'-nitrobenzenesulfonyl)thiazole derivatives was developed and the feasibility of this approach to protein sequencing demonstrated by solid-phase degradation of the oxidized insulin B chain.

Our attention has now turned to the development of suitable instrumentation for the automation of this process, as well as the optimization of a detection strategy for subpicomole sequencing. Components of a commercial protein microsequenator were integrated into a new instrument designed to support the chemical requirements of the thioacetylation method.

A detection scheme in which 2-methyl-5(4H)-thiazolones are derivatized by reaction with pentafluorobenzene sulfonyl chloride to afford 2-methyl-5-O-(pentafluorobenzenesulfonyl)thiazoles has been adopted. These derivatives are suitable for analysis by ultratrace capillary gas chromatography with electron capture detection (GC/ECD). Pentafluorobenzenesulfonyl derivatives have been detected by GC/ECD in quantities of <0.5 femtogram. An interface between the protein sequenator and gas chromatograph is undergoing optimization. The interface exploits normal-phase microbore HPLC removal of excess derivatizing reagent followed by direct injection of the HPLC eluent (100-150 µL) into the GC through a cold on-column injector connected in series with a 20 meter, 530 µM deactivated retention gap and a 12 meter, 320 µM HP-5 capillary column vented to a single electron capture detector. The potential of this approach to afford routine subpicomole sequencing will soon be evaluated for the analysis of protein electrophoretically resolved from two-dimensional electrophoretic gels.

76. Covalent immobilization for solid-phase protein microsequencing

Mark L. Stolowitz, Chin Sook Kim

In our continuing effort to improve the efficiency and ease of covalent immobilization for solid-phase microsequencing, we have undertaken the optimization of an approach in which proteins electroblotted from two-dimensional electrophoretic gels onto polyvinylidene difluoride (PVDF) membranes are covalently entrapped in a macromolecular polymeric network. The network results from copolymerization of a polyamine with a sequencing compatible crosslinking reagent. Components evaluated thus far have included polyallylamine (6,000-10,000 molecular weight range) in conjunction with phenylisothiocyanate and either para-phenylenediisothiocyanate or [bis-(S-carboxymethyl)dithio]dihexanoate. Optimum ratios of the aforementioned reagents have been determined to obtain high recovery of the N-terminal PTH amino acid. Amino acid analyses of entrapped proteins has been routinely obtained despite the published report that such analyses were problematic. Our attention has now turned to the development of a cleavable crosslinker for use in the covalent entrapment approach. Such a crosslinker would prove invaluable in the recovery of blocked proteins and peptides generated by chemical cleavage of entrapped proteins. To this end, we are preparing the cleavable crosslinker [bis-(S-carboxymethyl)dithioethyl]succinate. This crosslinker is cleaved by hydroxylamine hydrochloride under mild conditions. A model system involving the recovery of peptides derived from cyanogen bromide cleavage of entrapped cytochrome c will be employed for optimization of the cleavable crosslinker system.

77. Structural characterization of proteins by mass spectrometry

Patrick R. Griffin, John R. Yates, III

New methodology for the rapid and sensitive analysis of proteins by tandem mass spectrometry has been developed. Presently, in our laboratory we have three mass spectrometers: a triple quadrupole instrument, a quadrupole ion trap, and a laser time-of-flight system. Utilizing the three instruments, a general sequencing strategy was developed that has enabled us to obtain partial amino acid sequence on proteins starting with 100 pmol or less sample. The basic component of the strategy is the coupling of capillary high performance liquid chromatography (HPLC) with electrospray ionization on the triple quadrupole mass spectrometer and on the quadrupole ion trap.

The combination of packed capillary HPLC with tandem mass spectrometry has the potential to redefine approaches for molecular analysis of proteins. As much as 50% of a protein's amino acid sequence can be characterized in the time required to perform three cycles
of automated Edman degradation at sample levels below those currently required by commercial sequencers. The configuration used in our laboratory for coupling capillary HPLC consists of an ABI 140 syringe pump, preinjection split, and on-line UV detection. Eluent from the HPLC is fed directly into the electrospray tandem mass spectrometer (triple quadrupole or ion trap).

Analysis of a tryptic digest of a DNA polymerase (90,000 daltons [Da]) isolated from thermophilic bacteria afforded over 60 peaks in the reconstructed ion chromatogram. The early eluting peaks are from peptides in the mass range 500-5000 Da and the later eluting peaks are from large partially digested fragments (7000-40,000 Da). The large fragments are detected and the spectra are easily deconvoluted. Thus, the mass range for this technique is large, from 400 to 40,000 Da. In addition, using a capillary C4 HPLC column coupled with electrospray, molecular weights of native proteins were recorded at the low pmol level.

Protein tryptic digests were analyzed at sample levels as low as 800 fmol and collision-activated dissociation (CAD) was used to obtain amino acid sequence information on peptides at the 5 pmol level. Preliminary results using computer-aided interpretation of CAD mass spectra show that it is possible to have near real-time interpretation of data obtained on this system. Finally, this technique is compatible with in situ digestion of proteins bound to membranes or in SDS-PAGE gel slices.

78. Mass spectrometry studies of peptides using electrospray ionization and a novel quadrupole ion trap
Karen Furer-Jonscher, Patrick R. Griffin, Jae Schwartz1, Ian Jardine1, John R. Yates, III

The development of high sensitivity approaches for biopolymer sequencing has important implications for the study of complex biological systems. For example, biological events are often dependent on transitory fluxes of specific proteins to initiate regulatory processes. Powerful separation methods such as two-dimensional gel electrophoresis in conjunction with computer imaging and subtractive analysis can help to identify proteins involved in regulatory processes. These proteins are often present at the 100-1000 molecule/cell level and are difficult to characterize by conventional protein sequencing techniques without tedious isolation procedures. Separated proteins can be isolated from electrophoretic gels using electroblotting techniques, but the small quantities of protein recovered discourage further characterization. New approaches for the structural characterization of small quantities of proteins in conjunction with powerful separation techniques will allow the systematic study of complex biological systems.

The application of the quadrupole ion trap to the analysis of biomolecules has yielded extraordinary results (Cooks and Kaiser, 1990; Hail et al., 1990; Vanberkel et al., 1990). In addition to the impressive sensitivities of analysis observed, the ability to perform MSn experiments and ion-molecule reactions strongly suggests that the ion trap will become an important tool for the determination of amino acid sequences of proteins. We are investigating the use of the ion trap in our protein sequencing methodology.

A unique quadrupole ion trap utilizing components of an ITMS and TSQ 700 has been constructed in the Research and Engineering department at Finnigan MAT. In order to accommodate an electrospray ionization source, the ion trap has been placed in the TSQ 700's vacuum chamber to allow for differential pumping. A 20 keV conversion dynode electron multiplier is used to detect ions ejected from the trap. The ion source lens potentials can be optimized for maximum ion transmission from the DecStation 2100 using the ICIS operating system and instrument control language. A tube lens has been added to the end of the capillary to focus ions into the skimmer. Data is acquired on a PC and transferred over a network to the workstation for storage and manipulation with the ICIS software.

To investigate the performance of the ion trap on peptides, pyridylethylated tryptic fragments derived from a newly-characterized class of neurotoxin, r-bungarotoxin, were analyzed. Mass assignments for molecular weights were accurate to .01%. High resolution separation of the isotopes of the quadruply-charged ion allowed determination of the charge state. The resolution obtained was ~5650. The daughter ion mass spectrum exhibited several peaks providing structural information.

Open tubular liquid chromatography experiments were performed on Angiotensin I by concentrating 1 pmol of the peptide in .5% acetic acid onto a C18 derivatized 50 µm
i.d. capillary column. The peptide was eluted isocratically with a 1:1 mixture of 0.5% acetic acid/methanol flowing at 0.5 µL/min. The reconstructed ion chromatogram showed that the peptide was eluted in one peak with a "peak width" of 30 scans. When optimized using lower elution flow rates, this technique will be useful for MS/MS experiments on sample-limited peptides.

The combination of HPLC and ESI provides a powerful technique for the direct analysis of complex mixtures of peptides. Packed capillary HPLC analysis of a tryptic digest from the partially characterized toxin Dendropis Angusticeps was performed on the ion trap. Approximately 20 pmol of the digest was loaded onto a 0.5 µL sample loop and injected onto a 320 µm i.d. 15 cm packed capillary. Conventional HPLC solvents were used with on-line UV detection. The selected ion chromatogram revealed a number of peaks matching known tryptic fragments. The mass accuracy for the fragments of known sequence was ~0.03%.

Results of these and other experiments indicate that the ion trap has promise for the structural analysis of unknown proteins and peptides.

References:

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79. Computer-aided interpretation of low energy MS/MS mass spectra of peptides
John R. Yates, III, Youbin Mao1, Joe X. Zhou2, Patrick R. Griffin

The development of tandem mass spectrometry for the high sensitivity determination of internal amino acid sequences in proteins will make a significant impact on the field of structural biology. The ability to directly analyze complex mixtures of peptides derived from proteolytic digests of proteins will increase the rate at which amino acid sequences can be determined. However, real-time or near real-time interpretation of the data would make such an approach a powerful tool for the molecular dissection of key biological processes.

We have developed two approaches for the interpretation of low energy MS/MS spectra of peptides. The first approach was designed for the interpretation of the singly-charged mass spectra produced by MS/MS of peptides ionized by LSIMS. Peptides analyzed by this method are rich in fragment ions of type b and y". The second computational approach has been optimized for the interpretation of mass spectra produced by MS/MS analysis of doubly-charged peptide ions from electrospray ionization. In this type of mass spectrum, y"-type ions are frequently the most abundant. Peptides derived from tryptic digests of proteins will consist predominantly of doubly-charged peptide ions. The fragmentation patterns of doubly-charged peptides are more consistent and easier to interpret than those of higher charge states.

The strategy used in the interpretation of LSIMS MS/MS is to sequentially subtract the weights of each of the amino acids from the (M+H)+ ion and compare the predicted fragment ions with the mass spectrum. A final score is calculated from three factors: 1) the number of peaks in the mass spectrum (free acid and methyl ester, if used) accounted for in the mass spectrum, 2) the average intensities of predicted fragment ions, and 3) the continuity of sequence ion occurrences within a given fragmentation scheme. Any known sequence information can be provided to the program such as the presence or absence of amino acids, the extension directions (N to C, or C to N) and any known residues. The program also allows graphical analysis of the results obtained by comparison of the predicted fragments for a sequence with the mass spectrum.

A different computational approach was used for the interpretation of ESI MS/MS spectra. The mass spectral data are smoothed and ions below a set threshold are eliminated from the spectrum. The spectrum is then divided into sections of approximately 50 daltons each. The fragment ion of the greatest intensity in each section is identified and the boundaries are shifted to those ions classified as y"-type ions. Amino acid sequences are then determined for the molecular weight differences between boundaries. Where continuous y"-type ions have been found, the differences between the ions will correspond to the weight of a single amino acid. In cases where the differences between boundaries correspond to two of three
Given amino acids, all combinations corresponding to the weight difference are determined and their predicted fragment ions calculated. An average score based on predicted fragment ion intensity is calculated and the sequences sorted. Computer-aided interpretation of MS/MS spectra represents a promising approach for increasing the throughput of protein sequencing by tandem mass spectrometry.

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80. High sensitivity detection of DNA using europium chelates and time-resolved fluorescence detection

Robert J. Kaiser, Angelo J. Alfano

We are continuing to explore the use of organic chelates of rare earth metals, primarily europium, as labels for the high sensitivity nonisotopic detection of DNA. Such chelates typically have large Stokes' shifts, narrow emission lines, and long fluorescence lifetimes, thus affording very large signal-to-noise ratios.

Our initial work centered around the investigation of 4,7-bischloro-sulfophenyl-1,10-phenanthroline-2,9-dicarboxylic acid as the chelate. This molecule forms a strong complex with europium, and can be used to covalently label biopolymers (proteins, nucleic acids) containing available primary amino groups. We have developed procedures for the labeling of synthetic oligonucleotides with this chelate and are beginning to test the use of such labeled probes in the oligonucleotide ligation assay. In this regard, we have designed and assembled a prototype instrument for measuring fluorescence using time-resolution. The device consists of a nitrogen laser as the excitation source, a fiberoptic to deliver the laser light to the sample chamber, some excitation and detection optics, a gated photomultiplier tube as the emitted radiation detector, and gating electronics to sample the fluorescence data and relay it to a PC. Gating delay, sampling time, and number of repeated samplings can be set by the user. The sampling chamber is suitable for a diversity of assay formats, including 96-well microtiter plates and solid surfaces such as membranes. We are currently optimizing this hardware using standard chelates for maximum detection sensitivity. We are also synthesizing and characterizing new, more highly fluorescent chelates based on the same phenanthroline core structure. Our goal is to be able to perform the ligation assay directly on small samples of genomic DNA, bypassing the time-consuming and expensive polymerase chain reaction amplification step currently required when employing nonisotopic detection. This will require low attomole sensitivity, which we feel we can obtain using optimal chelate chemistry, multiple labels per probe, and optimized detection hardware.

81. An automated strategy for genetic mapping using polymorphic sequenced-tagged sites

Deborah A. Nickerson, A. Cecilie Boysen, Stephen D. Lappin, Pui Kwock, Judy Buckingham, Robert Moyzis, Maynard Olson, Helen Donis-Keller

The development of methods to rapidly and automatically type DNA sequence polymorphisms could greatly increase the speed and efficiency of genetic linkage analysis. We are exploring the feasibility of a genetic mapping system using polymorphic sequence-tagged sites (pSTSs) containing single nucleotide substitutions. We have selected a poorly mapped human chromosome, chromosome 14, as a model to test this approach. Using an M13 library of flow-sorted chromosome 14 and automated fluorescence-based sequencing, we have rapidly obtained random DNA sequences from chromosome 14 and have developed a large number of chromosome-specific STSs. Direct sequence analysis of amplified DNA samples from six different individuals has revealed the presence of common biallelic DNA polymorphisms in many (60%) of these STSs. Once identified, pSTSs can be rapidly typed using a semi-automated system combining DNA amplification by the polymerase chain reaction (PCR) with the analysis of sequence polymorphisms using a colorometric oligonucleotide ligation assay (OLA). Our analysis of DNA polymorphisms in STSs has shown that multiple biallelic polymorphisms (3 to 4 polymorphisms) in partial linkage disequilibrium can be located within short distances of one another, 1000 to 2000 bp. These physically-linked biallelic polymorphisms can be used to generate highly informative haplotypes with heterozygosities of 70 to 90%. A genetic map of chromosome 14 using pSTSs and PCR/OLA typing is currently under development. Once a genetic map of the pSTSs markers is obtained, their PCR...
primers and OLA probes can be used to rapidly identify the corresponding YAC clones which can serve as a framework for a physical map of human chromosome 14.

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82. Solid-phase oligonucleotide ligation assay
Leigh Clawson, Deborah A. Nickerson, Robert J. Kaiser

Solid phase oligonucleotide ligation assay (OLA) is another variation of the assay developed in this laboratory used to detect DNA variants arising from a single nucleotide substitution. In every OLA reaction, there are three oligonucleotide probes used. Two are complementary to the two possible allelic variants, and the third is complementary to the invariant sequence immediately 3’ to the other two. In solid phase OLA, this third oligonucleotide, common to both alleles, is covalently attached to a chemically modified polystyrene microtiter plate. This allows for the entire assay to be performed in the microtiter plate and eliminates the transfer of the OLA reaction mixture to the microtiter plate for biotin-avidin capture of the ligation product. Elimination of this step further permits the biotin-avidin system to be exploited for detection purposes. This allows for the use of a wide number of well-studied detection assays. In this system, the two reporter probes are labeled at the 5’ end with biotin, and the results are visualized using streptavidin-linked alkaline phosphatase. Furthermore, elimination of the transfers step allows the process to be fully automatable. The functionalized microtiter plates may be prepared and stored for long periods of time with no perceived decrease in activity.

83. Multiplex oligonucleotide ligation assay
Leigh Clawson, Robert J. Kaiser

We have adapted technology developed for fluorescent DNA sequencing for extending oligonucleotide ligation assay (OLA) to the simultaneous analysis of single nucleotide sequence variants at multiple genetic loci with polymerase chain reaction (PCR)-amplified DNA targets. The amplification of the target sites can be done singly or multiply for some combination of sites. For each genetic locus, the assay uses a set of three oligonucleotides. Two of the oligonucleotides are complementary to the two possible allelic variants of interest and are each color-coded with a different fluorophore. Alleles are therefore distinguished by the color of the product of the ligation reaction. The third oligonucleotide is complementary to the invariant sequence immediately 3’ to the other two. The length of this oligonucleotide determines the length of the ligation product of the assay and is varied for each genetic locus. All of the OLA probes and amplified DNA for the desired loci are mixed in a one-pot reaction, an aliquot of which is loaded directly onto a sequencing gel. Ligation product length and color determination is carried out by electrophoresis of the assay mixture using the Applied Biosystems 370A automated DNA sequencer. In addition to being able to analyze several sites at once, one OLA identifies the sample as being homozygous for one or the other allele, or heterozygous. We have recently worked out conditions for the simultaneous analysis of seven loci using known genetic mutation sites and polymorphisms in the various T-cell receptor loci.

84. An automated PCR-based sequencing system to detect single-nucleotide polymorphisms in genomic DNA samples
Thomas D. Yager, Robert J. Kaiser, Deborah A. Nickerson

We are developing an automated sequencing method that employs polymerase chain reaction (PCR)-generated DNA templates. We require the DNA sequence determination to be accurate enough that one single-nucleotide polymorphism could be detected in 500 nucleotides of sequence data. There are three aspects to this project. 1) Development of a statistical model to predict the success of detecting single-nucleotide polymorphisms by DNA sequencing. We have developed a model that allows the rational design of a strategy for sampling DNA from individuals in a population. We have found that the "reading length" of a DNA-sequencing run, the way in which "sequence defects" are distributed over this reading length, and the minimum heterozygosity required of a polymorphic marker are key variables in the model. 2) Testing of current fluorescence-based methods for sequencing PCR products that are generated from genomic DNA. Thus far, we have evaluated the biotin/streptavidin solid-phase capture protocol for the
preparation of single-stranded sequencing templates, and the protocol for cycle-sequencing with thermostable Taq polymerase. By combining these two protocols, we currently can obtain approximately 250 nucleotides of high-quality sequence data from each end of the amplified DNA target (or 500 nucleotides per pair of genome-specific primers). 3) Development of an algorithm for detecting single-nucleotide polymorphisms in multiple aligned DNA sequences. The algorithm must address the problem of discriminating true single-nucleotide polymorphisms from "sequence defects." The confidence with which one claims to have detected a single-nucleotide polymorphism is directly related to the quality of the raw sequencing data. We therefore are examining the factors that limit the resolution, signal-to-noise ratio, and multicomponent fluorescent properties of raw sequencing data generated with the Applied Biosystems 373A DNA sequencer. Various experimental parameters (different gel matrices, different sequencing enzymes) are also being tested to optimize the quality of the raw sequencing data.

85. Forensic analysis by the oligonucleotide ligation assay
Claire Delahunty, Michael Waterman\textsuperscript{1}, Simon Tavare\textsuperscript{1}, Stephen D. Lappin, Deborah A. Nickerson

The use of DNA sequence polymorphisms to create DNA "fingerprints" has recently gained acceptance for forensic analysis. DNA sequence polymorphisms are generally of two types: 1) single-nucleotide point mutations which occur approximately 1 in 300 bp and are usually biallelic in the population, and 2) variations in the number of repeated sequence fragments such as variable number tandem repeats (VNTRs) or simple dinucleotide repeats. Repeat sequences are somewhat less frequent in the genome and often display many alleles.

To date, most DNA fingerprinting is performed using VNTRs detected as restriction fragment length polymorphisms (RFLPs) using the Southern blot technique. More recently, polymerase chain reaction (PCR) amplification has allowed non-radioactive detection of VNTRs as length polymorphisms on polyacrylamide gels. These methods require an electrophoretic step which is difficult to interpret because of gel artifacts such as band shifting and possible DNA artifacts. Furthermore, electrophoresis is not automatable and the results are not easily interpreted by computer.

Our goal is to assemble a collection of biallelic polymorphisms which will be analyzed by the oligonucleotide ligation assay (OLA). Biallelic markers are intrinsically less informative than polymorphisms with multiple alleles but a modest number of markers may be combined to achieve a highly informative set. In fact, only 15 unlinked markers with a 50:50 distribution in the population will give identity for 1 in 1,000,000. We are developing small PCR-amplified regions containing single-nucleotide polymorphisms (pSTSs) on each chromosome. These polymorphisms are then analyzed by PCR/OLA, a semi-automated system for examining pSTSs. The entire assay is performed in microtiter wells and results in a simple plus or minus readout which is easily interpreted by a computer. Currently, 1200 ligation assays per day can be performed using a robotic workstation. Increased throughput will be possible with advances in multiplex formats.

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86. Identification of DNA sequence polymorphisms in the human \(\alpha\) and \(\beta\) T-cell receptor gene complexes
Patrick R. Charmley, A. Cecilie Boysen, Deborah A. Nickerson

T cells play a pivotal role in the regulatory and effector pathways of the immune response against either foreign or self antigens. Thus, it has been suggested that certain inherited polymorphisms in the T-cell receptor (TCR) genes themselves could contribute to differential immune responsiveness, e.g., autoimmunity. We are developing tools necessary to test the hypothesis that germline DNA polymorphism is associated with certain human autoimmune diseases. To detect DNA polymorphisms in the TCR \(\alpha\) and \(\beta\) gene complexes, we have compared the DNA sequences from several individuals using both direct DNA sequencing of these regions, and also the technique of denaturing gradient gel electrophoresis. In this way, we have examined noncoding regions for polymorphisms using random sequences derived from cosmids clones, and more than 60 different TCR gene segments. At present, we have detected at least 12 polymorphisms that are commonly found in the general population. Work is
under way to determine the specific DNA base changes involved in all of these polymorphisms. Our goal is to develop a panel of DNA polymorphisms located across the α/β and β loci. These polymorphisms will be useful for studying the TCR genes that may contribute to genetic susceptibility towards certain autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease. These polymorphisms will also be useful as genetic markers to trace the inheritance of the TCR gene complexes for the purpose of genetic mapping these regions (see Abstract No. 87).

87. Analysis of recombination in the human T-cell receptor gene complexes

Patrick R. Charmley, A. Cecilie Boysen, Deborah A. Nickerson

The human T-cell receptor (TCR) α- and β-chain gene complexes each contain 50-100 TCR gene segments distributed across up to 1000 kb of DNA. Significant progress has been made in constructing physical maps of these loci by analysis of large cloned pieces of DNA in cosmid and yeast vectors, and via pulse field gel electrophoresis. By using DNA polymorphisms in these loci (see Abstract No. 86), we hope to compare the physical map with a genetic recombination map. Because the frequency of meiotic recombination in these loci may be very infrequent (<1%), a large number of meioses must be analyzed. We intend to use the automated oligonucleotide ligase assay (OLA) which will allow us to study a large number of human meioses in the form of families, or alternatively the nonlimiting supply of single sperm.

As an indirect measure of recombination frequencies, we have examined the degree of allelic association between a limited set of polymorphisms in both the α and β complexes. For example, we have already defined two adjacent cosmids from the TCR β complex in which one cosmid appears to include an area of increased recombination, and the other does not. In the near future, we plan to directly study TCR meiotic recombination across such possible hot and cold spots by analyzing recombination in single sperm. The automated analysis of several thousand meiotic products will determine whether the distribution of recombination in these regions is indeed nonrandom. This information will have application in understanding more specifically which TCR genes are involved in human autoimmune diseases that have been associated with the TCR gene complexes.

88. Analysis of DNA sequence polymorphisms in mouse T-cell receptor loci

Masa-aki Toda, Deborah A. Nickerson

The physical organization of T-cell receptor (TCR) genes in the mouse has been extensively analyzed. However, efforts to obtain genetic maps of these loci have been hampered by the restricted gene pool found in inbred mouse strains. Highly polymorphic (CA)n repeats are found in many mammalian genomes and serve as useful markers for genetic studies. We have analyzed (CA)n repeats in TCR genes of several inbred mouse strains and wild mice, Mus spretus. Analysis of three repeats in the α/β locus suggests that (CA)n repeats may be highly polymorphic even in inbred strains and could provide effective markers for genetic mapping studies. DNA sequence polymorphisms, predominantly single nucleotide substitutions, have also been detected by single-stranded conformational analysis, or by denaturing gradient gel electrophoresis. Based on the distribution of these DNA polymorphisms in the TCR Vα/β and Vβ, most inbred strains of mice can be placed into two groups. To date, we have sequenced polymorphisms in five Vα, 11 Vβ, one Vγ and one Vδ gene. The levels of polymorphism between inbred M. musculus and M. spretus mice suggest that genetic mapping may be possible using F1 hybrids of M. musculus and M. spretus mice. We will analyze meiotic products from F1 hybrids to determine the recombination frequencies between polymorphisms using single-sperm typing by polymerase chain reaction. Comparison of genetic and physical distances between these polymorphisms will provide a method to locate and eventually sequence hot spots of recombination in mouse α/β, β and γ TCR loci.

89. Application of the oligonucleotide ligation assay for class II HLA typing and characterization of other informative polymorphisms within this region

Louis N. Martin, Deborah A. Nickerson, Robert J. Kaiser, Stephen D. Lappin

The components of the human HLA complex, encoding genes for the major histocompatibility antigens, are spread across approximately 3500 kilobases (kb) of
DNA within the short arm of chromosome 6. The HLA region encoding the class I antigens spans about 1500 kb, the class II region spans about 1000 kb, and the class III region spans another 1000 kb. Most of the class I and class II genes are highly polymorphic. These extensive polymorphisms have been useful for identifying genetic linkages of numerous diseases. About 70 diseases have demonstrated genetic linkages to one or another HLA gene. In particular, susceptibilities to a number of autoimmune diseases are associated with certain class II alleles or linked haplotypes (e.g., type I diabetes, rheumatoid arthritis, celiac disease, pemphigus vulgaris, multiple sclerosis, lupus erythematosus and Graves disease). In most instances, where genetic linkages of disease susceptibilities with HLA alleles have been demonstrated, the particular genes directly responsible for disease have not been identified. In fact, many other genes are interspersed among HLA genes. For example, at least 19 genes, most of whose functions are unknown, have recently been identified in the 600 kb region lying between the class I and class III HLA regions. Since the HLA region is so broad and contains many unidentified interspersed genes, evaluation of other polymorphisms within the region could assist in studies of the genetic linkages of diseases as well as be useful for forensic applications.

We have begun to use the previously described oligonucleotide ligation assay (OLA) to characterize the class II HLA genes. We have synthesized PCR primers for use in amplifying HLA-DQA, HLA-DQB, and HLA-DRB genes to be used as DNA targets for OLA diagnostic reactions. For typing HLA-DQA, we have designed and synthesized 11 probe pairs, each consisting of a biotin-conjugated probe and a digoxygenin conjugated probe which abut on polymorphic nucleotides that characterize the different alleles. The 11 OLA reactions are capable of identifying all eight known DQA alleles and of characterizing genotypes in all allelic combinations. Typing cell lines characterized in HLA workshops are being used to validate the typing results we obtain with the OLA procedure. We are also designing and synthesizing OLA probe pairs for 20 reactions which should identify and characterize 15 HLA-DQB alleles and all allelic combinations.

We have begun seeking other biallelic polymorphisms within introns of the HLA region by designing and synthesizing PCR primers for amplification of three intron regions within the TNF α gene. Other such polymorphisms will be identified in each major HLA region using a similar strategy. By amplifying and sequencing these regions from different individuals, we should be able to identify a series of biallelic polymorphisms, joined in linkage disequilibrium, that can be used for more detailed studies of disease associations.

90. Construction of a fine structure genetic map in the human immunoglobulin heavy chain region

Honghua Li

A fine structure genetic map can be defined as a map within which all the genetic markers are spaced within 1 cM (a genetic distance corresponding to about 1% genetic recombination fraction) of one another. Such a map is useful in the following aspects: 1) facilitating localization of disease-causing gene(s) in the corresponding region; 2) discovering and localizing genetic recombination hot spot(s); 3) providing useful information for physical mapping; and 4) comparing genetic and physical distances.

The human immunoglobulin heavy chain (IGH) cluster has been estimated to span 2-3 megabase (Mb) pairs in the subtelomeric region of chromosome 14 (band 14q32.3). Because of its subtelomeric location and the existence of various repetitive DNA sequences, the IGH region has been proposed to contain genetic recombination hot spots. Because of this possible existence of genetic recombination hot spots, the genetic distance across this region could be several times greater than that for an average chromosomal region (2-3 cM, based upon the equation 1 cM = 1 Mb) of the same physical distance.

Three major steps are involved in constructing a genetic map: 1) defining genetic markers; 2) determining the genetic distances between the markers; and 3) placing the markers on the map. Genetic markers are currently being defined. This study involves amplification of DNA segments by polymerase chain reaction (PCR) from human genomic DNA samples collected from different individuals. Detecting DNA sequence variation is carried out by directly sequencing the PCR products of the same DNA segment from different DNA samples and comparing the DNA sequences obtained. The variation
91. Determination of the genetic elements involved in the susceptibility to multiple sclerosis: Family studies

Steven S. Beall, Dale E. McFarlin1, Henry F. McFarland1, William E. Biddison1

Fifteen multiplex multiple sclerosis (MS) families comprising first degree, second degree, and third degree affected pair relationships have been studied to determine if there is linkage to the T-cell receptor (TCR) α and β chain complexes and/or major histocompatibility complex (MHC) and susceptibility to MS. We determined genotypes at seven different positions throughout the variable and constant regions of the TCR β-chain locus and at four different positions throughout the variable, joining, and constant regions of the TCR α-chain locus using Southern-based and polymerase chain reaction (PCR) based methods. In addition, molecular methods and classical serotypic methods were used to determine genotypes within the MHC. Segregation of the molecular genotypes (n = 1056) along with classical serotyping for MHC allowed determination of haplotypes at the three loci in the affected and unaffected members of 12 families. Seven affected sib pair (ASP) and two affected cousin pair families were informative for haplotypes which could be unequivocally assigned as being identical by descent (IBD). The results of the ASP families (n = 7) are not statistically significant for linkage to TCR-β (p = 0.22), HLA (p = 0.17) and TCR-α (p = 0.25). The results of the second degree relationship families approach significance (assuming haplotypes are IBD) for linkage to TCR-α (p = 0.15), and TCR-β (p = 0.08). Presently, we cannot make firm conclusions from these data and studies are in progress to establish the haplotypes as IBD in the second degree relationship families using additional molecular and statistical methods. We are also collecting additional unaffected and affected members of families to increase the power of this study.

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92. Susceptibility to multiple sclerosis is determined by inheritance of a 175 kb region of the TCR-β chain locus in conjunction with HLA class II genes

Steven S. Beall, Dale E. McFarlin1, Henry F. McFarland1, William E. Biddison1

We have attempted to find and map a susceptibility gene(s) in the germline T-cell receptor (TCR) Vβ region of multiple sclerosis (MS) patients. Six restriction fragment length polymorphisms (RFLPs) spanning approximately 600 kb of the TCR Vβ region were used to define TCR haplotypes in 197 Caucasian normals and 83 Caucasian MS patients in the chronic progressive stage of the disease. Comparisons of the genotype frequencies between the MS and normal populations were significantly different only at the Vβ11 RFLP (corrected p = 0.004). The distribution of TCR subhaplotype frequencies was significantly different (p = 0.0005) only in the ~175 kb region between RFLPs defined by Vβ8.1 and Vβ11. Stratification of the MS patients into HLA-DR2+ (n = 51) and HLA-DR2- (n = 32) populations demonstrated that the subhaplotype frequencies differed from the normal population only in the HLA-DR2+ (corrected p = 0.00007) and not in the HLA-DR2- (corrected p = 0.46) population. Within this region, subhaplotypes which are rare in the normal population are over-represented in the MS patient population and confer a relative risk of 4.06 (X2 = 17.72; p << 0.00025). These results confirm and extend our previous study that had indicated the existence of an MS susceptibility gene within the TCR Vβ region, and provide new evidence for gene complementation between an HLA class II gene and TCR Vβ gene(s) in conferring susceptibility to MS.

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93. Vβ and Vα gene usage in DPw2-specific T cells

Steven S. Beall, Bernard Zaller, William E. Biddison1

We previously demonstrated that the response to a single allogeneic class II HLA gene product is characterized by predominant usage of a Vβ14 gene segment (Beall et al., 1987). A bulk culture from a DPw2-specific MLR showed a predominant rearrangement involving the Vβ14 gene segment. Nine cytotoxic T-cell clones were
derived from the MLR. Eight of these clones (8.2 and 8.4-8.10) express the CD4+CD8+ phenotype, whereas one clone (8.3) is CD4+CD8-. Analysis of the specificity of these clones demonstrated that eight of them are strictly DPw2-specific: they all kill nine out of nine DPw2+ targets but do not kill 16 out of 16 DPw2- targets. Clone 8.4 was distinct from the others in that it lysed all DPw2+ targets but also lysed four DPw2- targets. The dominant clonotype was that of clones 8.5, 8.7 and 8.9, all of which used the Vβ14 gene segment. The other clones use unknown Vβ gene segments.

The present study is designed to answer the following questions. 1) Is there a dominant usage of a particular Vα gene segment in this panel of DPw2-specific T-cell clones? 2) Do clones 8.5, 8.7 and 8.9 possess β chains that are derived from independent rearrangements or are they descendants from the same precursor cell? and 3) Does clone 8.3 (CD8+ phenotype) utilize a different Vα and/or Vβ gene segment than the clones with the CD4+ phenotype? In order to answer some of these questions, cDNA was prepared from 2 µg of RNA from clone 8.9. We determined the α chain variable gene of clone 8.9 (Vα22.2) using the single-sided polymerase chain reaction (Zaller et al., in preparation). Clones 8.5, 8.7 and 8.9 rearrange this variable gene as determined by Southern analysis. A Vβ14-specific oligonucleotide has been prepared that successfully amplifies cDNA from clone 8.9 and will be used to determine the joining and diversity region segments as well as the sequence of N-region diversity in clones 8.5 and 8.7. An analogous oligonucleotide will be prepared from the cloned Vα gene from 8.9 in order to determine the Vα genes in these T-cell clones. After these experiments, the TcR α and β chain variable genes will be determined in the remaining T-cell clones.

Reference:

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94. Construction of a physical map ordering the human TCR β genes; detailed mapping of cosmids for high-throughput DNA sequencing of the TCR β locus

Donald Seto, Chia-lam Kuo, Lee S. Rowen, Ben F. Koop, Kai Wang

One major step in understanding multigene families is to define the number of and relationship among members belonging to these families, as well as to determine their physical location relative to one another at a defined chromosomal locus. The four families of the T-cell antigen receptor (TCR) genes have been localized to three distinct genetic loci. This project specifically targets the locus containing the entire repertoire of the human TCR β genes located on approximately one megabase of nucleotides. The eventual goal is to generate the ultimate fine map of the entire one megabase locus, i.e., nucleotide sequence.

We have identified and isolated cosmids (in pWE15A) containing 35-45 kb of TCR β-containing genomic inserts. These were generated from a series of overlapping yeast artificial chromosome (YAC) clones, containing a major part (>500 kb) of the human TCR β locus on chromosome 7, as well as from total genomic (fibroblast) DNA. Currently, we are using existing human cDNA TCR β probes to identify which cosmids contain these TCR β genes. We are mapping, i.e., aligning and overlapping, the entire TCR β locus using these two cosmid libraries. The existing probes will allow the identification and recovery of previously uncharacterized and uncloned TCR β genes along with their associated flanking regions. This in turn will lead to a continuous, physically-linked, cosmid-based library containing the entire human TCR β locus in preparation for high throughput DNA sequencing.

95. The mouse T-cell receptor gamma locus

Bernard Vernooij, Johannes A. Lenstra, Kai Wang

The mouse T-cell receptor (TCR) γ locus codes for the γ chain of the γ/δ T-cell receptor. The germline organization of the γ locus is like that of the mouse immunoglobulin (Ig) γ light chain, and unlike that of the other TCR and Ig loci, including the human TCR γ locus. There are four J-C pairs, each with its own V gene(s) in the genome of BALB/c mice.

We set out to study the organization of this locus in detail. Field inversion gel electrophoresis shows that the
entire locus is 360 kb or smaller. Cosmids covering 220 kb of the locus were isolated from a BALB/c cosmid library, using C and V probes. These cosmids fall in four contigs, which contain all the known gene segments. The distances between these contigs, as determined by field inversion electrophoresis, are 10 kb, 50-100 kb and 20-30 kb. Our current effort is directed at closing the smaller two gaps by cosmid walking. Cosmid end probes are being generated for this purpose. A yeast artificial chromosome (YAC) library will be screened as well, in an attempt to isolate the entire locus on a YAC clone(s). In addition, human V gene probes will be hybridized to the isolated cosmids, in an attempt to isolate new mouse Vγ gene segments.

96. Large-scale cloning and sequencing summary
Ben F. Koop, Kai Wang, Lee S. Rowen, Donald Seto, Chia-lam Kuo

A mouse cosmid library consisting of approximately 30 genome equivalents has been constructed, and clones have already been obtained using probes derived from known mouse T-cell receptor (TCR) α chain V genes. A human cosmid library consisting of 25 genome equivalents has been constructed and five cosmids spanning 100 kb of the TCR Cβ-Cα region have been isolated and mapped. Approximately 70% of the TCR β locus (1-1.5 Mb) has been obtained from a total human cosmid library and five yeast artificial chromosome (YAC) clones obtained from Maynard Olsen's YAC library. The 3' YAC contig consists of four YACs spanning 800 kb. Ninety-one cosmids have been isolated from a limited cosmid library made from these YACs. Efforts are currently directed towards ordering all of the present cosmids and identifying additional clones that will link together existing contigs.

From the mouse cosmid library, over 250 positive clones have been obtained using probes derived from known mouse TCR α chain V genes. Overlap analysis of these clones in conjunction with pulse-field gel mapping indicates that well over 70% of the approximately 1 Mb mouse Vα chain locus has been cloned and placed in overlapping contigs.

In our efforts to sequence the region spanning the constant δ and constant α gene segments in mouse and the corresponding region in human, we have 1) completed 94 kb of mouse sequence, and 2) compared 53 kb of human sequence with another 35 kb nearing completion. The J region in mouse contains 50 joining gene segments of which 42 have been shown to be transcribed. Consensus recombination signals 5' of the joining gene segment are found to correspond closely to the signals for immunoglobulins, although some differences are apparent. Comparisons of human and mouse sequences have revealed: a) approximately 34% sequence divergence in noncoding regions, b) no duplication of Js in the last 85 million years (since the divergence of mouse and man), c) corresponding human and mouse J gene segments are between 65-80% similar while mouse J gene segments are 30-60% similar, and d) one block of 120 bp located about 5 kb 5' of Cα is 96% conserved (this single copy sequence contains stop codons in all three frames on both strands, hybridizes to fish, Xenopus, tunicate and Drosophila DNA at low stringencies, binds T-cell nuclear proteins, and can be shown to alter RNA transcription levels in vitro).

An outline of the strategy we have adopted is as follows: 1) fragment whole-cosmid clones, 2) repair fragments, 3) size-select DNAs, 4) clone blunt-ended fragments in M13 vector, and 5) sequence 600-700 fragments (average 450 bp per fragment). Using this strategy, we have already generated more than 1 million raw bases over the course of a little more than a year. However, this represents only about 15% of the ABI DNA sequencer's capability. The largest problem is that of consistency over long periods of time. We have succeeded in establishing a strategy that 1) will produce large amounts of sequence data quickly, and 2) identify a number of steps that could dramatically increase output.

97. Sequence analysis of single- and double-stranded DNA clones using four-color fluorescence detection and cycle sequencing
Robert J. Kaiser, Sara MacKellar, Barbara J. Otto-Perry

We have continued our development and testing of protocols for use with the Applied Biosystems 373A fluorescence-based DNA sequencer. In collaboration with scientists at ABI, we have investigated a new sequencing reaction protocol that takes advantage of the thermal
stability of Taq DNA polymerase. This protocol, termed cycle sequencing, utilizes repeated thermal cycling of the dideoxy sequencing reactions using Taq polymerase to produce a near-linear increase in the quantities of terminated fragments produced in a manner analogous to amplification by the polymerase chain reaction. This linear amplification allows one to start with much smaller quantities of single-stranded template DNA, on the order of 100-200 ng of an M13 clone, for example. Additionally, repeated denaturation of the reaction mixture allows one to efficiently and simply sequence double-stranded templates, such as plasmid and cosmid clones. We have spent considerable time and effort optimizing the protocol for use with cosmid clones, and can routinely obtain greater than 300 bp of sequence from the ends of inserts cloned in cosmid vectors using specific dye-labeled primer sets synthesized in our laboratory. We are currently utilizing this technique to obtain end sequences from a library of cosmid clones derived from a 250 kb yeast artificial chromosome (YAC) clone, with the intent of using these sequences to align the cosmids with respect to the original YAC using a sequence-tagged site (STS) mapping strategy.

98. Genomic nucleotide sequence determination of the murine TCR Va and Vδ junction, and adjacent Va and Vδ genes; identification and analysis of sequences relevant to cross-expression of Va and Vδ gene segments

Donald Seto

The major form of the T-cell antigen receptor (TCR) consists of α and β polypeptide chains, and appears in thymocytes and mature T lymphocytes. A second class of TCRs is also produced. This class is found in thymocytes and peripheral blood T lymphocytes and consists of δ and γ chains. The expression of these two heterodimers is mutually exclusive: a cell has either one or the other (e.g., αβ or δγ) expressed on its surface, but not both. It is interesting to note that these two forms appear to be differentially expressed both spatially and temporally.

The assemblage of these receptors results from similar gene rearrangements of specific gene segments. In general, the segments of genes comprising each α, β, δ, or γ gene are exclusive, e.g., a Va gene segment is not rearranged with a Cδ gene, etc. The α and δ genes are similar in that both are assembled from gene pools of Va (or Vδ for δ genes) and joining (J)α (δ) gene segments, and Ca (δ) genes to form a rearranged gene that encodes a unique polypeptide chain that will associate with a unique β polypeptide chain (or γ in the case of δ). As families, these four sets of gene segments (α, β, δ, and γ) are organized into three genetic loci on different chromosomes. The β and γ each form one locus; α and δ appear to form the third locus with the δ gene segments appearing as an island in the midst of α gene segments in the mouse. This locus is organized with the Vα gene segments most distal to the Ca gene and the Vδ gene segments located between the Vα gene segments and Ca genes. In apparent contrast to the generally observed mutual exclusion of non-homologous gene segments, certain rearranged and expressed mouse TCR genes appear to incorporate Vδ gene segments with Ca genes.

To begin to understand how this type of rearrangement occurs, we have determined the genomic nucleotide sequence of this putative junction of the Vα and Vδ gene segments by using a random M13 shotgun cloning strategy complemented by a semi-automated fluorescent sequencing mode. This 45 kb cosmid (2-47) containing the junction, along with several Vα and Vδ gene segments and their the flanking sequences, is being analyzed. Comparison of the orthologous region from human, as well as comparisons with other murine Vα and Vδ gene segments, will provide insight as to whether this unique cross-expression of Vα and Vδ gene segments is determined by certain unknown flanking sequences. The comparison with human sequences will elucidate any conserved sequences which are presumably important in other aspects as well. Finally, the nucleotide sequences of the Vα and Vδ gene segments and of this junction from both mouse and human genomes will be integrated into the eventual sequencing of the entire one megabase TCR α and δ locus.

99. Sequence determination of human Cδ and Jα T-cell receptor genes

Lee S. Rowen, Stephen L. Howard, Erik J. Wilk, Purnima G. Deshpande, Kai Wang, Ben F. Koop

Three restriction fragments containing a total of 30 kb were subcloned from a cosmid selected by hybridization of human Cδ DNA. The fragments were sequenced with Sequenase and fluorescent primers, using the ABI 373
machine for automated sequencing. The completed contig contains the exon for C5 and several Jδ coding regions. Based on a dot matrix comparison, it shows a strong homology to the analogous region of mouse genomic DNA. We anticipate that a full alignment of the mouse and human sequences for this region will reveal interesting similarities in regulatory elements. Over the next year, we hope to extend the total length of our mouse and human C5 sequence stretches by sequencing cosmids containing Jδ gene segments. Work to this effect is already under way for mouse DNA.

100. Functional studies of conserved-sequence block in the mouse and human T-cell receptor a/δ chain gene
Chia-lam Kuo, Ben F. Koop, Donald Seto, Bernard Vernooij, Kai Wang

In the sequencing studies of the human a/δ-chain gene constant region, we identified a noncoding 125 bp sequence highly conserved between human and mouse. The conserved region may reflect regulatory or chromosome structural functions (e.g., translocation, folding, replication, etc.). To begin to address this biologically interesting question, a mobility shift assay was performed to first determine if there is a protein-binding site(s) in the conserved-sequence region. The preliminary results show that a certain protein(s) in crude nuclear extracts binds to the conserved-sequence block and the pattern of DNA-protein mobility shift is different between B and T cells. DNase I footprinting assay and transfection experiments with conserved-sequence block-linked CAT genes are under way to define the protein-binding site(s) and possible promoter/enhancer function of this region.

101. Strategies of information management in large-scale sequencing
Josie Zhang, Alan Blanchard, Stephen Speicher, Tim Hunkapiller

Large-scale science of any type, including large-scale sequencing, places particular demands on computational resources for data management and analysis. It is seldom just an issue of scaling up methods adequate for much smaller projects, but rather requires redesign of the basic tools. This process is itself iterative with developing the strategies for the processes of data generation as well. Thus, there will need to be considerable effort aimed at the problems of sample management, data acquisition and maintenance. Most available methods of fragment data management and assembly are file-based systems capable of handling projects of no more than cosmid size, on the order of 50 kb or less. Still, these methods require considerable user intervention and time and fail to maintain complete project records. The rate at which data must be generated in order for very large projects to be practical preclude significant day-to-day intervention in these processes. Also, for sequencers to be able to take advantage of the data they are generating in real time relative to the sequencing process, the throughput rate of the assembly and analysis procedures must be greatly increased. These throughput needs require that the decision processes of these procedures (the base calling and assembly previously discussed, process quality control, etc.) become much more automated. Thus, megabase sequencing projects will place untenable stress on the current computational tools. Therefore, a major concern of ours is the development of practical, affordable computational tools to facilitate the management of this as well as similar projects.

In order to maintain high data generation rates, the researchers must be able to track the processes involved. In order to accomplish this, we will take advantage of laboratory information management systems (LIMS) technology to more nearly automate the enormous record-keeping needs of the project. This will allow greater control of costs, the development of more efficient sequencing strategies and more robust abilities for diagnosing problems with reagents and instrumentation as they arise. Also, these abilities will provide the first thorough bookkeeping of the actual costs in projects of this nature. Perhaps most important, however, a properly designed information management system imposes a strict adherence to the established project organization and process protocols. Because of the distributed nature of our operation (multiple individuals and instruments), we have chosen to design a client/server-based tool using the Sybase relational database system. It will reside on a high-speed Hewlett Packard workstation/server. All instruments will be directly connected via serial links. Each researcher will have a data input terminal of some
kind directly at their work areas. Work on developing the needed schema is presently under way. We are employing schema design tools developed at the Lawrence Laboratory at Berkeley that allow for a more object-oriented view of the data structure. We are also investigating the use of experimental object-oriented database systems from Hewlett Packard Laboratories.

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102. Consensus sequence determination in large-scale sequencing efforts

Wang Qin Chen, Alan Blanchard1, Meichun Shen, Tim Hunkapiller

In order to evaluate the best sequencing strategy, it is first critical to define criteria for the determination of the acceptable level of confidence in the final results. It is interesting that essentially no sequence has been published along with the rules with which the raw or final sequences were defined (other than stating the number of times each clone or region was sequenced). These final sequences have been the result of the personal confidence the researcher had in his or her raw data along with generally ill-defined (at least not stated) rules for consensus. Very large projects can no longer rely on these subjective, hands-on approaches. Even this task (or perhaps, particularly this task) must be automated. The task is too large for individual researchers to check all raw data. We must rely on high quality, redundant data to provide confidence and the methods by which we can automate the base-calling process. The strategies we employ must provide both.

In order to automate the consensus base-calling process, we must define objective rules that quantitatively described the likelihood of each base call. These rules must be predefined such that the final answer is not a consensus at all, but rather a probabilistic definition for each base position determined, e.g., at position 1031: A-97%, G-2%, C-.5%, T-.5%. These rules must also establish quantitative estimates of error likelihood. In general, the error rate should be some order less than the empirically-seen level of variation in the population (or loci) being studied. As experience increases, the consensus rules can be further refined, the data resubmitted to analysis and an improved consensus view generated.

An extensive effort is under way to identify and quantify sequence- and protocol-specific errors that are not randomly distributed or a factor of where they occur along the raw fragment data. Variation in base-to-base signal strength indicates significant local effects of the template sequence on the ability of polymerases to discriminate analog from normal nucleotides. By doing rigorous correlative studies (using neural network and numerical methods) between known sequence and associated raw data, we are defining objectively the effect of local sequence on this signal variation. A better understanding of these relationships will allow us to make more confident decisions when the data are less than robust. Also, each enzyme and protocol is susceptible to unique specific errors related to local template sequence. For example, we have discovered a relationship between dyad symmetry and "dropped" base calls (relative deletions) in cycle-sequencing protocols. The analysis of more data will allow us to define a statistical probability of correctness for any base called within any possible dyad structure we can define.

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103. BISP-VLSI solutions to sequence comparison problems

Glenn Herrmannsfeldt, Edward T. Chou1, Michael Waterman2, Tim Hunkapiller

Our research has been focused on redefining the dynamic programming paradigm such that not only could these methods be implemented effectively in silicon, but also provide, in that context, the flexibility required of a robust biological tool. Our biological information signal processor (BISP) represents a systolic implementation of a dynamic programming algorithm based on that of Smith and Waterman, optimizing its ability to define local similarities at extremely high speeds. There are three fundamental functional differences between the BISP and previous efforts: 1) BISP is optimized for the determination of any number of local similarities between pairs of sequences (although the algorithm is also capable of returning global comparison values); 2) BISP returns values that will allow for the reconstruction of the alignment; and 3) BISP is specifically designed to employ complex, user-definable similarity rules. Most
significantly, BISP supports user-selectable alphabets of up to 128 characters, complete indel penalty definition and completely individually-defined similarity values between characters in the chosen character set, thus allowing the direct definition and search for profiles. The system is implemented in <1 micron CMOS technology and operates at a maximum speed of 12.5 MHz. A board-level system operating in a standard UNIX workstation can provide rigorous comparison to a database at rates exceeding that of any current supercomputer. The system is being incorporated specifically into very large-scale sequencing efforts going on in this laboratory. We are currently beginning the process of designing the next generation of the BISP technology. This will require extensive simulation and involve the use of concurrent supercomputers operating on campus.

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104. Hierarchical approaches to protein tertiary structure prediction
Jerry E. Solomon, Wei Wu, Steven D. Bishop, Alan M. Mathiowitz, William A. Goddard, III

The de novo protein structure prediction problem is one of the current computationally challenging problems in molecular biology and biochemistry. Although the primary sequence of amino acids is known to determine the final folded state of a given protein, current methods are still incapable of computing the protein's three-dimensional structure given only the sequence information. The major problem encountered in such computations is the enormous number of configurational degrees of freedom (corresponding to local minima in the free energy surface) that must be explored in order to find the native structure. It is generally recognized that successful approaches will be those that can efficiently restrict conformational space to a relatively small region which includes the native conformation. Thus, a popular method has been the so-called "homologous protein" approach which attempts to use a protein of known structure, whose primary sequence possesses a 50% or greater similarity with respect to the protein of unknown structure, as the starting point for traditional molecular dynamics or energy minimization algorithms for structure determination.

We have been developing a hierarchical approach to the structure prediction problem which uses a coarse-grained lattice model to provide the necessary constraints on the regions of conformational space which must be explored in later stages of the calculation. In this approach, candidate Cα backbone conformations for a given input sequence are generated on a face-centered cubic (fcc) lattice where the number of lattice points is chosen so as to confine these conformations to being close to maximally compact. Of the large number of candidates generated in this fashion, several tens or a few hundred are selected for relaxation off-lattice to a free-space backbone structure, and then further refined with the addition of side-chain atoms to find the final structure of lowest free energy. The lattice model, although a relatively crude representation of the free-space structure, provides a very efficient computational device for exploring a constrained region of conformational space and allows us to introduce a number of additional general constraints such as hydrophobic-hydrophobic interactions, interior-exterior distribution of non-polar/polar residues, etc., into the chain generation and candidate selection process.

At present, we have implemented two basic methods for candidate chain generation on the lattice: 1) exhaustive enumeration with incorporation branch-and-bound heuristics as well as geometric constraints such as disulfide bridges; and 2) a modified Monte Carlo chain generation method known as "guided replication," which allows efficient incorporation of constraints that are required to produce realistic folded conformations. The latter method is currently being validated using comparisons to exhaustive enumeration of compact chains on the two-dimensional square lattice before running on full three-dimensional cases where we expect to be able to treat cases up to about 100 residues. Both of our basic methods are easily parallelized to run on large-scale concurrent computers such as those available in the Caltech Concurrent Supercomputing Facility, and we are currently developing a collaboration with Dr. Carl Kesselman of the Caltech Computer Science Department, to explore efficient concurrent implementations of these approaches.

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PUBLICATIONS


The fifth abdominal and more posterior segments of the animal. We are testing hypotheses that might explain why clusters of homeobox genes homologous to the bithorax cluster (and the Antennapedia complex as well) have remained intact and retained colinearity of map location and body expression in mice and human beings as well as in flies. One possibility that we are investigating is that there is sharing of cis-regulatory regions (Celniker et al., 1990).

Not only do the cis-regulatory regions require proximity within the chromosome for their normal functioning, but when the chromosomes are paired as in the somatic cells of Drosophila, we find that they function more nearly normally than they do when the chromosomes are unpaired. We report here on some new studies of this phenomenon of transvection. We report on further progress in analyzing cis-regulatory regions 5′ and also 3′ to the transcription units of the Abdominal-B (Abd-B) domain of the BX-C. This domain specifies the fate of the fifth abdominal and more posterior segments of the animal.

Reference:

105. The effects of a suppressor gene on transvection
Maria-Paz Capdevila, Edward B. Lewis

Ectopic expression of the Ultrabithorax (Ubx) gene in the wing imaginal discs elicited by the Contrabithorax (Cbx) mutation is subject to transvection (Lewis, 1954). In Cbx1 Ubx1 / + + flies, Cbx1 is able to induce ectopic expression of the wild-type Ubx allele when there is pairing between the homologs, visualized in the discs by the antibody against the Ubx protein products (Castelli-Gair et al., 1990). The resultant phenotype is a weak Cbx effect consisting of partial conversion of posterior wing to haltere. Babu et al. (1987) reported that when the wild-type chromosome in this case is replaced by a variety of different bithorax mutant combinations, certain of the resultant genotypes show a disappearance of the Cbx effect, as if transvection is suppressed in such cases. In order to retain full viability, all genotypes in our studies as well as those of Babu and colleagues, carried a duplication for the wild-type bithorax complex (namely, Dp-68 in the X chromosome); such duplications essentially lack an effect on the Cbx phenotype since the latter is a gain-of-function phenotype. The only double mutants that show loss of the Cbx effect are those involving two gypsy transposons in cis. A possible explanation is that the two gypsies pair with one another through their association with the protein produced by the suppressor of hairy-wing, su(Hw)+, gene. [The su(Hw)+2 mutant partially suppresses gypsy-induced mutations (Lewis, 1981; Modolell et al., 1983) and is known to cause a loss or a reduction in the amount of a protein that binds to gypsy DNA (Spana et al., 1988).] The resultant configuration would leave the Ubx+ gene in a loop where it would be unable to pair with its homolog and no longer subject to ectopic expression by Cbx in the homolog. To test this hypothesis, we have constructed a number of such double mutant combinations that are at the same time homozygous for the su(Hw)+2 mutant. Our results indicate that these combinations have the Cbx effect restored, suggesting that the su(Hw)+2 protein is the "glue" that binds the gypsies to one another; without this glue, the postulated loop would fail to form and transvection would be restored.

References:
106. Regulation of ABD-B protein expression

Seema K. Sharma, Susan E. Celniker

The Abdominal-B (Abd-B) domain of the bithorax complex specifies the identity of the fifth through the ninth abdominal segments in the adult and the corresponding neuromeres (10-14) in the developing embryo. The domain codes for two proteins, ABD-BI 54 kDa and ABD-BII 36 kDa. Using a monoclonal antibody specific to an epitope shared by the two proteins, we have shown that ABD-B is expressed in a gradient in neuromeres 10 through 14 in the central nervous system (CNS).

We have analyzed ABD-B expression in two dominant gain-of-function mutants, Mcp (Lewis, 1978), and Sab (Sakonju et al., 1984) and the double mutant combination. In homozygous and heterozygous Mcp embryos, ABD-B expression expands into neuromere 9. This phenomenon is interpreted to result from the deletion of repressor-binding sites in the iab-5 region. Homozygotes for Sab show very weak ABD-B staining in neuromere 8 but no detectable staining in neuromere 9. This result is consistent with the interpretation that Sab deletes repressor sites in iab-5 that are distinct from and distal to those deleted by Mcp (Sab has been mapped genetically to lie just distal to Mcp). In double mutants Mcp Sab, ABD-B expression is extended into neuromeres 8 and 9.

Pc is a negative trans-regulator of ABD-B. Embryos that are mutant for Pc express ABD-B in the ectoderm and CNS of all the body segments. To dissect this expression, we have examined embryos homozygous for the double mutant combination Pc3{iab-8D14}. In this genotype, ABD-B is ectopically expressed in all of the neuromeres of the CNS. Since iab-8D14 disrupts the transcription unit for ABD-BI but leaves the transcription units for ABD-BII intact, we infer that it is ABD-BII that is misregulated in that genotype (Biology 1990, Abstract No. 133).

References:

107. Molecular analysis of the Abdominal-B domain

Peter Chang, Daniel J. Keelan, Susan E. Celniker

The Abdominal-B (Abd-B) domain spans 100 kb (from +100 to +200 kb) at the distal end of the bithorax complex (BX-C). In addition to protein coding regions giving rise to two proteins, ABD-BI and ABD-BII, and spanning some 50 kb, the domain contains cis-regulatory regions mapping both 5' and 3' to the transcription units (Celniker et al., 1989, 1990).

We have continued our molecular analysis of the Abd-B domain by sequencing genomic DNA directly upstream of the previously reported (Biology 1990, Abstract No. 132) P3 and P4 promoters corresponding to the infra-abdominal-8 (iab-8) and iab-9 regions. Our sequence data extends 10 kb from +160 to +170 kb and 10 kb from +190 to +200 kb. We are now performing DNA sequence analysis.

References:

108. Mapping of infra-abdominal (iab) mutants

iab-386, iab-3793, iab-7770 and iab-74098

Michael A. Charles, Susan E. Celniker, Edward B. Lewis

We have continued the transvection suppression screen previously described (Biology 1990, Abstract No. 134) to obtain rearrangement breakpoints within the bithorax complex. All transvection suppression mutations are analyzed for a bithorax phenotype over a deficiency (Df P9) for the entire bithorax complex.

During the year, in addition to finding new mutants within the bithorax complex, we have undertaken a cytological and molecular analysis of four infra-abdominal (iab) mutants: two iab-3's and two iab-7's. Cytological analysis shows that three of the iab mutants contain inversions with one breakpoint in the BX-C complex at 89E and another as follows: at position 62C in iab-386; at position 96A in iab-3793; and at position 68BC in iab-7770. The iab-74098 mutant is associated with a translocation with breakpoints in 50AB and 89E. Using Southern analysis, we have molecularly mapped the breakpoint position of all four mutants within the BX-C: iab-386 was mapped to a 8 kb HindIII restriction fragment.
from +52.4 to +60.6; iab-3793 was mapped to a 5 kb EcoRI restriction fragment from +65 to +69.6; iab-7770 mapped to a restriction fragment from +129.2 to +130; and iab-74098 mapped to a restriction fragment from +124.6 to +126.2.

PUBLICATIONS

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Summary: In multicellular organisms, the determination of anterior-posterior (A-P) embryonic polarity is fundamental to subsequent development, since the entire body plan is constructed relative to this axis. The major focus of research in our laboratory is on the molecular genetic analysis of A-P axis formation in Drosophila and the transduction of this axial information into pattern and form. There appear to be three major classes of genes that are active in the maternal germline and are involved in establishing the A-P axis of the embryo: the "anterior" genes which specify anterior segmented regions, the "posterior" genes which specify the abdominal region (as well as the germline) and the "terminal" genes which specify the largely asegmental terminal regions of the embryo.

We are conducting a genetic and molecular analysis of certain of these maternal effect pattern loci and their downstream zygotic genes. Previously, a major focus of our genetic analysis was on the terminal gene hierarchy. We assigned 14 zygotically expressed genes to this hierarchy on the basis of a genetic interaction test with gain-of-function alleles in a key maternal terminal gene, torso, which encodes a transmembrane receptor tyrosine kinase. These genes could be subdivided into three phenotypic classes, each of which represents a distinct aspect of terminal development and evolution. We have focused our molecular analysis on one of these classes, which controls the morphogenetic process of germband movement. One of the genes, hindsight, has been cloned and the molecular cloning of a second germband movement locus, serpent, has been initiated. These loci appear to specifically control morphogenesis and have no obvious role in pattern specification; consequently, we hope to gain general molecular insights into the mechanisms by which cell shape changes and movements are controlled during animal development.

In addition to these studies, we have made progress in the phenotypic analysis and molecular cloning of the maternal effect locus, valois, that is required for cellularization of the embryo at the blastoderm stage as well as for specification of the germline. We suspect that this gene product plays a key role in establishing or maintaining the appropriate topographic relations between the cytoskeleton, cell membrane and associated organelles/molecular complexes.

Apart from these studies, which move from the genetic to the molecular level, we have chosen to proceed from an interesting molecular phenotype to genetic analysis. Consequently, we have chosen to search for cDNAs representing transcripts localized to either the anterior or posterior pole of the unfertilized egg and early embryo. To date we have identified cDNAs representing five distinct polar-localized RNAs and expect to identify
additional cDNAs as we proceed with higher-sensitivity molecular screens. Our analyses are focused on mutational dissection of the genes encoding these RNAs, the molecular mechanisms of RNA localization per se and the possible role of the "posterior" RNAs in germline determination.

After the A-P axis is established, various genes act to subdivide the developing embryo into periodic repeats and, ultimately, into segments. The homeotic genes combine axial and periodic information in order to specify the identity of certain structures specific to particular segments. A secondary interest of ours is analyzing the genetic and molecular basis for cis- and transvection in the Ultrabithorax domain of the bithorax homeotic gene complex as well as the functions of the transcripts encoded by the bithoraxoid regulatory region of the complex.

Finally, in addition to these studies on Drosophila development, Senior Research Fellow Susanna Lewis is independently studying the mechanisms of antigen receptor V-(D)-J joining.

109. Genetic dissection of the Drosophila terminal developmental pathway

Teresa R. Strecker, Man Lun R. Yip

The formation of the anterior and posterior ends of the Drosophila embryo requires the activity of the maternal terminal genes. Loss-of-function mutations in the receptor tyrosine kinase-encoding torso terminal gene result in the absence of embryonic structures derived from both the anterior and posterior terminal regions of the embryonic fate map. In addition, such embryos exhibit abnormalities in germband movement as well as dorso-ventral pattern defects (Strecker et al., 1991). In contrast, females homozygous for gain-of-function alleles of torso produce embryos that lack central structures, while the anterior and posterior ends are present (Strecker et al., 1989; Strecker and Lipshitz, 1990). We previously demonstrated that the gain-of-function torso phenotype could be suppressed by mutations in the zygotic terminal gene tailless (Strecker et al., 1989). This suggested that other zygotic terminal pathway genes could be identified based on suppression or enhancement of gain-of-function torso alleles. Twenty-six zygotic pattern/morphogenetic mutants with developmental defects in the termini were tested in this way and 14 were found to reside in the terminal genetic hierarchy (Strecker et al., 1991). These results suggest that multiple zygotic genes mediate the complex functions associated with terminal development in the Drosophila embryo. The phenotypes associated with these genes fall into three classes, each of which represents a distinct aspect of terminal development and evolution: (i) the specification of terminal versus central cell fates, (ii) pattern specification along the antero-posterior and dorso-ventral axes within the termini, and (iii) control of morphogenetic events that occur in the termini. We are pursuing the molecular analysis of representatives of the last class of genes (Abstract Nos. 110 and 111). We hope in this way to gain insights into the molecular coordination of the cell shape changes that underly morphogenesis.

References:

110. Molecular genetic analysis of morphogenesis: The hindsight locus

Man Lun R. Yip, William W. Fisher

Morphogenesis in Drosophila involves a series of coordinated cell shape changes and movements. The hindsight mutation is one of four zygotic loci that specifically affect the morphogenetic process of germband retraction. Hemizygous hindsight embryos fail to retract their germbands and so have a characteristic "tail-up" phenotype. There are no obvious pattern defects in these embryos. Therefore, hindsight appears to be involved in programming a specific morphogenetic process.

We have shown that hindsight mutations suppress the torso gain-of-function phenotype (Strecker et al., 1991). Suppression occurs by the blastoderm stage, suggesting that the hindsight gene product must be active by 2-3 hours after fertilization although germband retraction does not commence for another 4-5 hours. Since torso encodes a receptor tyrosine kinase, these results also raise the possibility that hindsight is regulated in part by an intracellular phosphorylation cascade initiated by the torso kinase.
We hope that genetic and molecular analysis of hindsight will yield insights into the control of morphogenesis in Drosophila. The hindsight locus maps to 4C5.6 on the X chromosome, proximal to optomotor-blind and distal to shaven baby. We have identified a 25 kb region that is transcribed in 0-3-hour embryos and exhibits spatial and temporal expression patterns consistent with that expected for hindsight. Isolation and sequencing of cDNAs, transformational rescue of the hindsight mutant phenotype and further molecular analyses are in progress.

Reference:

111. Molecular genetic analysis of morphogenesis: The serpent locus
Joanne Topol

The serpent (srp) gene is one of a group of zygotic genes involved in controlling and mediating morphogenetic movements during early Drosophila development. Embryos homozygous for a loss-of-function srp allele fail to retract their germ band; as a consequence, the posterior end of the mutant embryo remains on the dorsal side, and the embryo dies before hatching with a "tail-up" phenotype. The phenotype of srp mutant embryos is very similar to that of embryos lacking a functional rail-up (tup), u-shaped (ush) or hindsight (hnt) gene. All four zygotic loci appear to have little or no impact on the process of pattern specification during embryogenesis (cuticular preparations of mature mutant embryos reveal the characteristic structures found along both the anterior-posterior and dorsal-ventral axes of the wild-type organism). Thus, rather than altering morphogenetic movement indirectly through changes in pattern or tissue specification, it is likely that this class of genes plays a direct role in the control and/or mechanics of cell movement and cell shape change. Three of these genes (tup, ush and hnt) have also been shown to mediate the functions of the maternally expressed terminal gene, torso, by their genetic interaction with torso gain-of-function alleles (Strecker et al., 1991).

We have initiated the molecular cloning of the srp gene in order to determine the nature of its product, its pattern of expression and its role in the process of germ band movement. In addition, we are interested in determining at the molecular level whether srp acts downstream of the torso receptor tyrosine kinase gene in the terminal regulatory hierarchy.

Reference:

112. Molecular genetic analysis of early embryonic cytoarchitecture: The valois locus
Susan R. Halsell, Cheryl Napier, William W. Fisher, Andreja Volenec

The maternal effect gene, valois (vls), results in two classes of defects in embryos derived from mutant mothers: (i) abnormal abdominal segmentation and no pole cells in 10-20% of the embryos (the "posterior" defect); and (ii) failure of somatic cellularization in 80-90% of the embryos (the "cellularization" defect) (Schüpbach and Wieschaus, 1989). Gene dosage experiments indicate that all known vls alleles behave as amorphs; therefore, it is likely that the variability in the vls mutant phenotype results from the vls gene product functioning in a redundant pathway. We suspect that the vls gene product plays a key role in establishing and/or maintaining the integrity of the embryonic cytoarchitecture. We are conducting a combined phenotypic and molecular analysis of the vls locus.

The vls mutant phenotype is being analyzed using time-lapse videotapes of live embryos as well as by examining fixed material. The latter includes visualization of cytoskeletal components using anti-cytoskeletal antibodies. Videotape analyses show that aberrations occur early; examination of fixed embryos shows that some form relatively normal syncytial blastoderms, but that a range of phenotypes is detected at cellularization. As expected, some embryos complete cellularization normally and go on to gastrulate. In many cases, however, cellularization occurs only in patches; no particular region of the embryo appears to be highly sensitive to defects.

Finer analysis of the cellularization defect is being made using antibodies directed against α-tubulin. In wild-type embryos, tubulin is initially distributed evenly throughout the cortex of the embryo. At the onset of cellularization, the pattern resolves into a regular hexagonal array surrounding each blastoderm nucleus.
The nuclei are evenly spaced, equal in size, and tightly packed. It is at this stage that defects in vlIs mutant embryos are detected. As expected, there is a lot of variability in the staining of embryos produced by mutant females, with some embryos having apparently wild-type patterns of staining. In extreme cases, highly disorganized patterns are seen; these include gaps, irregular connections and regions completely devoid of tubulin staining. Irregular rings of tubulin may also include several nuclei. In the last case, the nuclei are irregularly shaped; some appear to be squeezed out of the cortex. Our analysis is continuing using antibodies directed against actin, α-spectrin, and non-muscle myosin.

The molecular nature of the vlIs gene product is of great interest, and we are in the process of cloning the vlIs gene. A number of cloning strategies are being employed, including chromosome jumping and walking in wild-type cosmid and lambda phage libraries and in libraries subcloned from a 260 kb yeast artificial chromosome derived from the region. Thus far, our walk spans 140 kb. We are completing the walk, mapping rearrangement breakpoints, conducting P element-mediated transformational rescue experiments and determining which regions of the walk detect transcripts present in 0-1-hr embryos.

Reference:

113. Isolation of cDNAs representing polar-localized RNAs in the Drosophila egg
Dali Ding

We are interested in the possibility that certain of the RNAs that encode proteins involved in specifying the A-P embryonic pattern might be non-uniformly distributed in the egg. In addition, we wish to understand the mechanisms by which RNAs become asymmetrically localized in cells. Using a miniature guillotine, we cut the anterior poles off 7,500 frozen early embryos (0-30 minutes old) and the posterior poles off another 7,500 frozen early embryos. We purified 100-200 µg of total RNA from each of these collections of A- and P-poles. In collaboration with the Meyerowitz lab, we constructed a new set of directional cDNA cloning vectors that allow automatic phage-to-plasmid conversion mediated by the Cre-loxP recombinase and were designed to facilitate differential and subtractive screens with limited amounts of source RNA (Palazzolo et al., 1990). The vectors were used to construct A- and P-specific cDNA libraries from 20 µg each of total A- and P-RNA.

Using A- and P-specific probes generated from plasmid A- and P-cDNA libraries automatically converted from the phage libraries, a whole early embryo library constructed in Agt10 was differentially screened. Of approximately 100,000 plaques screened, 700 putative A- or P-differential plaques were isolated and rescreened using Southern blots of insert DNA obtained from these plaques by polymerase chain reaction. Over 300 clones rescreened positively; these were sorted into 50 cross-hybridizing classes. Whole mount RNA tissue in situ hybridization on embryos, using as probes representative members of each cross-hybridizing class, revealed at least four classes of cDNAs that detect maternally localized RNAs. Further studies are focusing on defining the developmental functions of these RNAs and their products, as well as on the mechanisms of RNA localization per se (see Abstract No. 114).

Reference:

114. Mechanisms of RNA localization in the Drosophila egg
Dali Ding, Mark P. Running

A number of maternal mutations that disrupt polar granule integrity in the Drosophila egg are also known to delocalize posteriorly-localized RNAs such as cyclin B and oskar. Embryos produced by female flies homozygous for mutations in seven such polar granule integrity genes (capuccino, spire, tudor, valois, staufen and oskar) were used for whole mount RNA tissue in situ studies using as probes three posteriorly-localized RNAs (P-RNAs) identified in our differential cDNA screen (see Abstract No. 113). The posterior localization of all three P-RNAs is completely abolished in all such mutant embryos. This indicates that the same machinery implicated in localizing previously identified posteriorly-localized RNAs is also involved in localization of the these three P-RNAs. Since polar granules are not formed in all of the above mutants, it is likely that these three P-RNAs
are components of, or closely associated with, the polar granules (the germline determinants). Further analysis of the distribution of these RNAs in wild-type and mutant ovaries will provide us with details of the RNA localization process as well as help us define the detailed role of each of the polar granule mutations in this process.

Interestingly, the gene encoding one of the three maternally localized P-RNAs is also zygotically transcribed in the anterior region of the embryo at the cellular blastoderm stage. We have shown that zygotic expression of this gene is absent in embryos derived from bicoid mutant mothers. Current efforts are directed towards determining whether the bicoid gene product, a homeodomain-containing transcription factor, is directly involved in activating the zygotic expression of this gene.

115. Developmental functions of the hairy class of helix-loop-helix transcriptional regulators

Susan M. Parkhurst

The Drosophila pair-rule segmentation gene, hairy (h), is required during two distinct developmental steps: (i) embryonic segmentation, and (ii) the establishment of adult bristle pattern during larval/pupal stages. h encodes a nuclear protein with a helix-loop-helix (bHLH) motif. This protein motif, found in a family of proteins, consists of two amphipathic helices (separated by a short loop) that have been implicated in protein dimerization, and an adjacent region of basic amino acids necessary for DNA binding. In vitro experiments have suggested that HLH transcription factors may act primarily as protein heterodimers. Although h protein normally plays no role in sex-determination, we have shown that ectopic h expression causes female lethality by inhibiting initiation of the master sex-determination control gene Sex-lethal (Parkhurst et al., 1990). This lethality is suppressed by altering the dosage of other HLH transcription factors known to act in chromosome counting, suggesting that X chromosomes are counted through heterodimers of HLH transcription factors that act synergistically to initiate Sxl expression. During embryonic segmentation, h acts as a negative transcriptional regulator, presumably because it forms transcriptionally inactive heterodimers. Their lack of activity could be due to an inability to bind DNA or to their failure to activate transcription. DNA-binding activity in HLH proteins and other heterodimeric transcriptional activators (e.g., jun/fos) appears to depend on conserved basic domains that lie adjacent to potential heterodimerization helices. This basic domain is lacking in two other HLH repressors, emc and Id, leading to suggestions that they may repress by forming heterodimers that do not bind DNA (Benezra et al., 1990). h may represent a different class of HLH repressor as it retains a basic region such that h heterodimers retain DNA-binding but lack transcriptional activation.

We have modified the h gene using site-directed mutagenesis and "domain-swapping" with other HLH proteins. By ectopically expressing these modified h genes in transgenic flies, we are analyzing the role of the h class of HLH regulators and, in particular, the function of h's basic domain in vivo.

References:


116. Developmental functions of the Ultrabithorax and Abdimal-A domain of the bithorax complex

David R. Mathog

Drosophila mutants for bithoraxoid (bxd) often have two thoracic-like legs in the first abdominal segment of the adult (giving 8 pairs of legs) (Lewis, 1955). The only known function of the bithoraxoid region is as a cis-regulatory element of Ultrabithorax (Ubx). I have recently completed a genetic study that showed that the dosage of abdominal-A is the primary determinant of ectopic leg development in bxd backgrounds (Mathog, 1991). The same studies indicated that the bxd region produces no trans-acting product, and that even though bxd is adjacent to abd-A, bxd does not cis-regulate abd-A. These results suggest that the gene product most directly responsible for the suppression of abdominal legs may be the abd-A homeodomain protein rather than the Ubx one. Such an interpretation is consistent with the observation that homozygous Ubx mutant ovaries do not present ectopic legs (Lewis, 1963). I am testing this theory by producing gynanders deleted for Ubx and/or abd-A.

An interesting spontaneous mutation has recently been recovered from a Cbx stock. Provisionally named
segmental pigmentation (spg), this mutation, which is not completely penetrant, results in the appearance of a pattern of intensely black patches in the adult cuticle. Except for the patterning, spg resembles the melanotic tumor mutations. In each of the second abdominal and more posterior tergites, dark patches are organized into a stripe that covers what would normally be the junction of the pigmented and unpigmented cuticle. The first abdominal segment has a pattern resembling back-to-back parentheses [ ) ( ]. Ventrally, a small dark patch is centered among the sternites. Dark patches also occur at a much reduced frequency in the legs, thorax, and head. The mutation appears to be on the third chromosome, but due to the variable penetrance, it has not yet been accurately mapped. Cbx is not near spg, since there is frequent recombination between them. The spg pattern does not require Cbx.

References:

117. Molecular genetics of the late bithoraxoid gene in the bithorax complex

J. Coire Angus

The 40 kb bithoraxoid (bxd) region of the bithorax complex encodes a set of differentially spliced poly(A)+ RNAs that are expressed transiently at the time of transcriptional activation of the Ultrabithorax domain of the bithorax complex. In addition to these "early bxd" RNAs, the bxd region encodes a single 0.8 kb mRNA that is transcribed late, from the mid-third larval instar through the adult stages of development. Analysis of the sequence of the "late bxd" RNA suggests that the late bxd gene may code for a 101 amino acid protein (Lipshitz et al., 1987).

In an attempt to localize spatial and temporal expression of the late bxd gene, we have utilized whole-mount RNA tissue in situ hybridization. Previous work has shown that the late bxd RNA is transcribed at successively higher levels in third instar larvae, pupae and adults. Dissected third instar larvae and adult flies were probed with Abd-B cDNA and late bxd cDNA probes. The Abd-B probe, a positive control, hybridized to the genital disk, as well as the hindgut and portions of the ventral nervous system. At present, results using the late bxd probe are inconclusive, although we have some indications that the gene may be expressed in abdominal histoblasts in late larvae.

A second approach is to determine whether the location and sequence of the late bxd gene has been evolutionarily conserved. Mouse HOX-C genomic clones, genomic libraries from various Drosophila spp., and genomic DNAs from various Drosophila spp. are being analyzed to test for possible conservation of late bxd-homologous sequences.

Reference:

118. Mechanism of antigen receptor gene assembly

Joseph T. Meier, Susanna M. Lewis

In order to manufacture antigen receptors, T and B cells must assemble the corresponding genes through a series of site-directed DNA rearrangement events. One feature of this recombination process is distinctive: unlike other site-directed rearrangements of nucleic acid (such as, for example, RNA splicing or phage λ integration), V(D)J joining is fundamentally imprecise. The fine structure of the junctions vary from one recombinant to the next; typically a small number of bases have been subtracted from the precursor gene segments, and often a few residues of unknown origin have been inserted. On the basis of this property, one could guess that V(D)J joining is a different type of transaction than those in which strand breakage and joining are energetically coupled. It is even conceivable that cooperation between more than one enzymatic apparatus (not all of which are specialized, in terms of antigen receptor gene assembly, and none of which may qualify as a "recombinase per se") may be involved. Because the sequences of junctions vary from one to the next, it is reasonable to ask whether any patterns can be deciphered, and to use these as clues in modeling the recombination mechanism.

Relatively recently, it was reported by two different laboratories that the inserts found in a subset of V(DJ) junctions were not altogether random (McCormack et al., 1989, Lafaille et al., 1990). Specifically, in junctions where there has been no base loss from one or both
component gene segments, inserted residues found just adjacent to the "non-nibbled" sequence appear to be the inverse complement of that sequence. The total number of bases that adhere to this pattern is small (one or two) and may represent only a portion of the entire inserted sequence. These apparently patterned residues have been termed "P (for palindromic) nucleotides" (Lafaille et al., 1990).

The data used to support the existence of P nucleotides is a compilation of the sequences of rearrangements of endogenous gene segments, as well as of recombinants derived from introduced rearrangement substrates. While provocative, this evidence is basically anecdotal in nature. In murine junctions, one or two presumed P nucleotides occur against a backdrop of essentially random base insertion: both random and non-random residues are found within the same junction.

A more critical test of the existence of P nucleotides would be to make small, defined changes at the ends of one or both coding segments, and then to determine whether the junctional inserts found upon recombination adhere, in each case, to the pattern predicted for P nucleotides. To this end we are attempting to determine whether the identity of the inserted residues found adjacent to non-nibbled gene segments varies as predicted for P nucleotides when the sequence of the gene segment is altered. We have made use of an extrachromosomally replicating plasmid substrate designed by Hesse et al. (1987), in which recombination of V(D)J joining signals activates a selectable marker (recombinants are scored in E. coli). A feature of this substrate, of particular use in the present study, is that transiently-introduced plasmids allow one to examine the recombination process apart from its physiological context, thus excluding the possibility of selection for certain products within the immune system. We have constructed two nearly-identical variants in which only the sequence just adjacent to one or both joining signals has been altered.

Data from the first substrate provide some evidence for P nucleotides, but the pattern of their occurrence is not fully consistent with previous reports. Studies with the second two substrates are under way to confirm and clarify the relationships between inserts and adjacent sequences. Preliminary evidence points to a significantly different type of rearrangement mechanism than those that have been proposed to date.

References:

PUBLICATIONS
Summary: We continue to seek molecular and genetic explanations for pattern formation, and answers to the question of how the two-dimensional information stored in DNA is converted to a developmental pattern with three spatial and one temporal dimension. Most of our work is an attempt to find the molecular steps in flower development in the flowering plant Arabidopsis thaliana, which we have chosen because of its small size and rapid life cycle.

We have identified many mutations whose phenotype is disruption of flower development. Three classes of mutation seem to have particularly profound effects on flowers. The first are organ identity genes, whose mutations cause homeotic conversions of sets of floral organs. We have identified three classes of these genes and have developed a model in which the overlapping expression of these three gene classes dictates the position of the four types of organs found in Arabidopsis flowers. The model makes a series of genetic and molecular predictions, many of which have been tested and shown to be correct. Genes from two of the three classes have been cloned and sequenced, and work is in progress on cloning of a gene from the last remaining class. Remarkably, both cloned homeotic genes share homology, being members of the same family of DNA-binding proteins. The homeotic mutations so far identified are apetala2, pistillata, apetala3 and agamous, with the last two being the cloned members.

The second class of mutations under study are those that change the initial pattern of expression of the homeotic organ identity genes. An example is superman, a mutation that causes misregulation of the homeotic apetala3 gene, and as a consequence causes the ovary of each flower to be reduced in size, and the floral region occupied by stamens to be enlarged. The last class of genes under intensive study are meristem identity genes. Mutations in these cause meristem conversions. For example, in leafy and apetalal mutants, floral meristems are partially converted to inflorescence meristems. So far, we have cloned and sequenced one gene in this class (the LEAFY gene).

To some degree, the answers to the questions posed by developmental pattern formation must come from an understanding of the basic processes of cell biology. This is true because many developmental processes, such as developmental induction and patterned cell division, rely largely on machinery that exists in most cell types and that is activated differentially. One process critical to communication between adjacent cells is endocytosis. We are studying this in Drosophila, by analyzing a gene (shibire) whose product is essential for one step in endocytosis. The cloning and sequencing of this gene led to the surprise result that it codes for Drosophila dynamin, a microtubule-associated GTPase. An additional series of experiments is directed to understanding cellular communication in plants, by cloning a gene whose product is involved in signaling by the plant hormone ethylene, studying a new class of transmembrane protein kinases in plants, and analyzing the regulation of gene expression in plant embryos.

119. Genetic analysis of the flower control gene LEAFY
Detlef Weigel, John Alvarez1, David R. Smyth1, Lindee Goh

Mutations in the Arabidopsis gene LEAFY alter the...
inflorescence as well as individual flowers.

Eleven mutant LEAFY alleles have been isolated and studied on the macroscopic level, and by scanning electron microscopy. All mutant alleles produce more cauline leaves than wild-type plants. As in wild-type, flowers are ultimately formed, although they are abnormal. The number of cauline leaves produced and the effects on flower development allow the ordering of the alleles in a phenotypic series. In weak alleles, the first and fourth whorl of organs (sepal and carpels) are largely normal, but the number of second and third whorl organs (petals and stamens) is reduced. In addition, these organs are often mosaic, e.g., sepal/petals, petal/stamens, and stamen/carpels. In strong alleles, multiple whorls of sepals, which have some leaf-like characteristics, arise and an abnormal gynoecium forms in the middle. The pattern in which these organs arise is intermediate between the whorled mode of wild-type flowers and the spiral mode of inflorescences, indicating that the floral meristem is partially transformed into an inflorescence meristem. The flowers in strong alleles are subtended by bracts, a structure not normally found in Arabidopsis plants. The phenotype indicates that LEAFY has at least three different functions: 1) it contributes to the switch from vegetative to floral development; 2) it suppresses bract formation; and 3) it controls the pattern and identity of organs within the flower.

One striking result is that even in amorphic alleles (see Abstract No. 120), the switch from vegetative to floral development is only delayed, but not completely eliminated. In order to identify factors that interact with LEAFY in the vegetative to floral switch, we are currently screening for mutations that enhance the leafy mutant phenotype.

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120. Molecular analysis of the flower control gene LEAFY
John L. Bowman

In order to clone the LEAFY gene, we have placed the gene on the available restriction fragment length polymorphism (RFLP) maps. Starting with an RFLP marker located about 2 centiMorgans distal of LEAFY, we initiated a chromosomal walk using cosmid and yeast artificial chromosome libraries. After the isolation of over 500 kb of contiguous genomic DNA, we obtained a probe for the snapdragon gene FLORICAULA, mutations in which have a phenotype similar to leafy mutations. We cloned the Arabidopsis homolog of FLORICAULA by low stringency hybridization, and found that it mapped within our chromosomal walk to a position where no recombination with LEAFY among 374 chromosomes was found.

The Arabidopsis gene has a coding potential for a 420 amino acid protein and is very similar to the snapdragon gene, with both introns in identical positions and 69% of the amino acids of the deduced protein conserved. We compared the DNA sequence of wild-type and five mutant alleles and found that all mutant alleles are associated with single base pair changes, indicating that the Arabidopsis homolog of FLORICAULA is identical to the LEAFY gene. The sequence alterations correspond well to the phenotypic classification of the alleles: three of the five sequenced alleles are strong alleles, and all have a stop codon which prematurely terminates the open reading frame. Two intermediate alleles have missense mutations in positions that are conserved between Arabidopsis and snapdragon. This analysis also shows that at least two of the alleles are putative null alleles since the protein is terminated after only 30 amino acids. Interestingly, the leafy mutant phenotype appears to be weaker than the floricaula mutant phenotype, suggesting that these homologous genes play similar but not identical roles in two different species of flowering plants.

To characterize the expression of LEAFY, which is expressed in developing flowers as determined by Northern blot analysis, in more detail, we are currently performing in situ hybridization to tissue sections.

121. Making cauliflower out of Arabidopsis
John L. Bowman

Studies on the development of apl-1 flowers in both the Landsberg erecta and Wassilewskija genetic backgrounds were performed. The apl-1 mutation was originally induced in the Landsberg erecta background. In the Landsberg erecta background, apl-1 flowers have axillary buds arising from the axils of the first whorl
organs, which are quite leaf-like. These axillary buds also have axillary buds, and so on. The buds are not developing in place of any floral organ primordia since all floral organs can be present and the axillary buds still form. Interestingly, the development of axillary buds is independent of formation of first whorl organs, which are often missing in ap1-1 flowers. In this case the axillary buds seem to develop right out of the pedicel of the primary flower. In addition to the first whorl organs being leaf-like, the second whorl organs of ap1-1 flowers are usually absent.

When this strain (ap1-1 in the Landsberg erecta background) was crossed to ag-2, which was isolated in the Wassilewskija (Ws) ecotype, plants with an enhanced ap1 phenotype were found. The enhancement of the ap1 phenotype is independent of the ag-2 mutation (the enhancing locus is also independent of apetala2). The enhanced phenotype has been termed 'cauliflower'. In the 'cauliflower' flowers, each floral primordium produced by the inflorescence meristem behaves as if it constituted an inflorescence meristem, producing primordia in a phyllo-tactic spiral on its flanks. These primordia also behave like inflorescence meristems. After two to three rounds of this, the primordia produced do develop into flowers, with an ap1 phenotype but with little production of axillary buds as observed in the Landsberg erecta background. Perhaps there is a second locus that is mutant or missing in Ws with which AP1 interacts (or conversely, a suppressor of ap1 in the Landsberg erecta background). Crosses with the 'cauliflower' phenotype into another ecotype, as an initial step in RFLP mapping the enhancing locus, have been performed. Additionally, genetic experiments to determine whether the enhancer is a single locus and its dominance/recessive character are in progress. A final additional point of interest is that the 'cauliflower' phenotype of ap1 is morphologically similar to a variant of Brassica oleracea (var. botrytis), the common cauliflower of our supermarkets.

122. Superman, a candidate for a gene controlling the spatial expression patterns of Pistillata and Apetala3
Detlef Weigel, Thomas P. Jack, Hajime Sakai, John L. Bowman

A simple model has been proposed to explain how four homeotic genes, acting alone and in combination, could specify the identity of each of the four whorls of floral organs (see Abstract No. 123). That AGAMOUS and APETALA3 are initially expressed in complex spatial and temporal patterns suggests that these genes are responding to a prepattern. Genetic and molecular experiments have demonstrated that at least AGAMOUS and APETALA2 interact antagonistically to help define each others' spatial domain of activity. However, the mechanisms by which PISTILLATA and APETALA3 are confined to the second and third whorls have not been addressed. One candidate for a gene controlling APETALA3 and PISTILLATA is SUPERMAN. superman-1 flowers have a normal outer three whorls, four sepals, four petals, and six stamens, but in the fourth whorl, which is normally occupied by the gynoecium in wild-type flowers, up to 20 additional stamens are present. Thus, SUPERMAN may be repressing or preventing APETALA3 and/or PISTILLATA activity in the fourth whorl. In superman-1 flowers, APETALA3 and PISTILLATA activity would expand to include the fourth whorl, thus resulting in the specification of stamens in the fourth whorl. Double and triple mutant strains with superman-1 and the floral homeotic mutants support this hypothesis. pistillata-1 and apetala3-1 are epistatic to superman-1 in terms of specification of organ identity. The first whorl carpels that develop in apetala2-2 superman-1 flowers suggest that superman-1 is not merely causing a homeotic transformation of carpels to stamens.

The SUPERMAN gene maps to the third chromosome approximately 20 centiMorgans distal to glabra1. The closest restriction fragment length polymorphism marker analyzed so far lies within a 2 cM region of the SUPERMAN locus. This genetic distance corresponds to about 0.5 Mb in Arabidopsis thaliana. Now, to clone the gene SUPERMAN, chromosome walking from this marker is in progress.

123. Genetic interactions among floral homeotic genes of Arabidopsis
John L. Bowman, David R. Smyth

Allelic series for four loci, mutations in which result homeotic conversions in two adjacent whorls in the Arabidopsis thaliana flower, were analyzed. Both the
structure of the mature flower and its development from the initial primordium were analyzed by scanning electron microscopy. Mutations at the APETALA2 locus, ap2-1, ap2-2, ap2-8 and ap2-9, cause homeotic conversions in the outer two whorls: sepals to carpels (or leaves in the case of ap2-1) and petals to stamens. Mutations of PISTILLATA, pi-1, pi-2, pi-3, and APETALA3, ap3-1, cause second and third whorl organs to differentiate incorrectly. Homeotic conversions are petals to sepals and stamens to carpels. AGAMOUS mutations ag-1, ag-2 and ag-3 affect the third and fourth whorls and cause petals to develop instead of stamens and another flower to arise in place of the gynoecium. In addition to homeotic changes, mutations at the APETALA2, APETALA3 and PISTILLATA loci may lead to reduced numbers of organs, or even their absence, in specific whorls. The bud and flower phenotypes of doubly and triply mutant strains, constructed with these alleles, were also analyzed. Based on these results, a model is proposed that suggests that the products of these homeotic genes are each active in fields occupying two adjacent whorls, AP2 in the two outer whorls, PI and AP3 in whorls two and three, and AG in the two inner whorls. In combination, therefore, the gene products in these three concentric, overlapping fields specify the four types of organs in the wild-type flower. Further, the phenotypes of multiple mutant lines indicate that the wild-type products of the AGAMOUS and APETALA2 genes interact antagonistically. AP2 seems to keep the AG gene inactive in the two outer whorls while the converse is likely in the two inner whorls. This field model successfully predicts the phenotypes of all the singly, doubly and triply mutant flowers analyzed.

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124. Towards the cloning of APETALA2, a homeotic gene in Arabidopsis flower development
Zhongchi Liu, John L. Bowman, Elliot M. Meyerowitz

Mutations in the APETALA2 (AP2) gene of Arabidopsis transform sepal into carpels and petals into stamens. Genetic analysis of ap2 mutants (Bowman et al., 1989, 1991) suggests that AP2 has two functions in regulating flower organ differentiation: 1) specifying sepal and petal development in the first and second whorls of a wild-type flower; and 2) negatively regulating AGAMOUS (AG) (Yanofsky et al., 1990) in the same whorls of a wild-type flower. Among all ap2 mutations isolated thus far (10 alleles), some appear to lose only one of the two functions (such as ap2-1); others appear to lose both functions (for example, ap2-2).

Understanding the molecular basis of ap2 function inevitably requires the molecular cloning of AP2. A new ap2 allele (ap2-10) was generated in Dupont by Ti insertion (Feldmann et al., 1989). Southern analysis of the ap2-10 mutant revealed that the insertion is a rather complex one consisting of at least seven partial copies of the T-DNA, yet with all copies tightly linked to each other. Using Ti plasmid border fragments as probes, we have isolated a flanking plant DNA by screening a lambda library made with the ap2-10 genomic DNA. We are in the process of determining the genetic position of this DNA by restriction fragment length polymorphism mapping, to verify whether this DNA is linked to AP2. In addition, we have searched for flower-specific transcripts in this piece of flanking DNA and detected only one transcript present in flowers and vegetative tissues, but absent in siliques. Once ap2 is cloned, we plan to detect molecular lesions in different ap2 alleles to understand how the two ap2 functions are differentially affected by different ap2 mutations. We also plan to analyze ap2 expression in wild-type and other homeotic mutants to test how ap2 and other homeotic flower genes interact to specify flower patterning in Arabidopsis.

References:

125. Molecular and genetic analysis of the APETALA3 gene of Arabidopsis thaliana
Thomas P. Jack, Laura L. Brockman, Elliot M. Meyerowitz

Mutations in the APETALA3 (AP3) gene of Arabidopsis thaliana result in homeotic transformations of stamens into carpels and petals into sepals. We have cloned the AP3 gene from Arabidopsis by using the cDNA of the
DEFICIENS (def) gene of Antirrhinum majus as a molecular probe. The sequence of two ap3 mutant alleles and restriction fragment length polymorphism mapping data show that this gene is AP3. The AP3 gene product contains a MADS box and likely acts as a transcription factor. AP3 also shares extensive similarity with DEF in protein coding regions outside the MADS box. In situ hybridization to wild-type flowers shows that AP3 transcripts are expressed only in stamens and petals, the organ types that fail to form correctly in mutants; in other tissues of the plant, AP3 is not expressed. In the homeotic flower mutants agamous, apetala2 and pistillata, the AP3 expression pattern remains confined to second and third whorl primordia. In flowers mutant for the SUPERMAN gene, however, the domain of AP3 expression expands into the fourth whorl.

To identify genes that interact with AP3, we are undertaking a temperature-sensitive mutagenesis of the ap3-I allele. At low temperatures (15°C), ap3-1 homozygotes form morphologically normal and fertile flowers, while at high temperatures (29°C), all four petals are transformed into sepals and the six stamens are partially transformed into carpels. We plan to mutagenize ap3-1 seeds with EMS, grow them at 29°C, and look for dominant suppressors of AP3 in the M1. After screening through the M1 plants at 29°C, we plan to shift the plants to 16°C and harvest the M1 seeds. To screen for recessive suppressors and enhancers or AP3, we will grow the M2 plants at both high and low temperature and screen for wild-type plants at 29°C and mutant plants at 16°C.

126. A molecular and genetic analysis of PISTILLATA, a homeotic flower development gene in Arabidopsis thaliana

Koji Goto

One of the homeotic flower genes, PISTILLATA (PI), affects second and third whorl developmental specification in Arabidopsis. In pi mutant flowers, like ap3, the second whorl organs develop as sepals rather than petals, and the third whorl organs develop as carpels rather than stamens. From genetic analysis, PI and AP3 gene products seem to have similar functions. To determine the molecular basis of PI gene product action, and of the interactions between PI and other homeotic flower genes and their products, especially AP3, we are undertaking the molecular cloning of the PI gene.

We are using two approaches, as follows. The first one is chromosome walking to the PI locus from the nearest markers that have mapped restriction fragment length polymorphisms (RFLPs). Since Arabidopsis has small genome size and few repetitive sequences, chromosome walking using a yeast artificial chromosome (YAC) library is a useful tool to clone mapped genes. PI maps between two RFLP markers whose genetic distance is about 10 centiMorgans (approximately 1500 kb). The direction of the walk is determined by using RFLP of plants with meiotic recombinations between pi and other linked genetic markers. Since each YAC has about 150 kb inserts on average, this distance might be covered by approximately 10 YACs.

The second approach is to use a region homologous with other cloned homeotic flower genes as a probe. The cloned genes AG and AP3 in Arabidopsis, and DEF in Antirrhinum share an homologous region called MADS box. We have screened an Arabidopsis genomic library with the AP3 MADS box region, and got dozens of independent clones. We mapped those clones that have RFLPs in the strains used for the crosses, and found that none was PI. We have also made degenerate oligonucleotides corresponding to the MADS box amino acid sequence. Using these oligos and universal primers that have vector sequence, Arabidopsis flower cDNAs were amplified by polymerase chain reactions (PCRs). We have obtained several PCR products that have the expected length, as measured by agarose gel analysis. We are testing these products to see if any are part of the PI gene.

127. Expression of the Arabidopsis floral homeotic gene AGAMOUS is restricted to specific cell types late in flower development

John L. Bowman, Gary N. Drews

Mutations in the AGAMOUS gene cause transformations in two adjacent whorls of the Arabidopsis thaliana flower. Petals develop in the third floral whorl rather than the normal stamens, and the cells that would normally develop into the fourth whorl gynoecium behave as if they constituted an agamous flower primordium. Early in flower development, AGAMOUS RNA is evenly distributed throughout third and fourth whorl organ...
primordia but is not present in the organ primordia of whorls one and two. In contrast to the early expression pattern, later in flower development, AGAMOUS RNA is restricted to specific cell types within the stamens and carpels as cellular differentiation occurs in those organs. Specifically, AGAMOUS RNA is concentrated in the developing ovules and stigmatic tissue of the carpels, the connective and endothecium of the stamens and the nectaries. AGAMOUS RNA does not appear to be present in either the megagametophyte or microgametophyte lineages. Ectopic AGAMOUS expression patterns in other floral homeotic mutants, such as apetala2-2, suggest that the late expression patterns are established independently of the early expression and that APETALA2, a known regulator of early AGAMOUS expression patterns, does not define late expression patterns. The late expression patterns of AGAMOUS suggest that AGAMOUS may be involved in the specification of cell fate within stamens and carpels in addition to having an earlier role in the specification of identity of floral organ primordia.

128. Analysis of AGAMOUS gene product function

Leslie E. Sieburth

The AGAMOUS gene of Arabidopsis plays a critical role in normal flower development. Mutations in this gene, when homozygous, produce both homeotic and heterochronic defects: the third whorl organs are transformed to petals and the cells that would give rise to the gynoecium instead produce another floral primordium. This gene has been cloned, and sequence analysis reveals homology with the MCM1 and SRF transcription factors (Yanofsky et al., 1990). Polyclonal antibodies recognizing the AG gene product have been produced in rabbits, and are currently being used to identify the tissues that express AG during flower development in wild-type and other floral mutants. The intracellular localization of the AGAMOUS polypeptide in the third and fourth whorls may provide insight into the different functions of AG in these tissues. Another approach toward understanding AG function is the analysis of misexpression of the gene product. Constructs have been generated that place the AGAMOUS gene (cDNA) under the control of a constitutive high level promoter (the Cauliflower Mosaic Virus 3S promoter) and an inducible promoter (the HSP18.2 promoter of Arabidopsis). These constructs are currently being used to transform wild-type plants. Analysis of the developmental repercussions of ectopic expression of AG in floral meristems will increase our understanding of the interactions of this gene product with other regulators of flower development, and effects of expression in vegetative and inflorescence meristems may also provide insight into organization and developmental competence of these meristems. The functions of the AG gene product are also being studied by mutagenesis. The K-box is the first target for site-directed mutagenesis, and transformation constructs bearing mutations in this region are being generated. Future goals also include cloning and characterizing genes downstream of AGAMOUS.

Reference:

129. Analysis of the AG4 allele of Arabidopsis

Mark P. Running, Leslie E. Sieburth

Mutations in the AGAMOUS gene of Arabidopsis affect development of the flower. The gene has previously been cloned and sequence analysis revealed homology with MCM1, SRF, and related transcription factors (Yanofsky et al., 1990). Previously characterized alleles ag-1 - ag-3 cause homeotic transformation of the third whorl stamens into petals and transformation of the fourth whorl gynoecium into a new flower primordia. A new allele, ag-4, has been provided by Dr. D. Smyth (Monash University, Melbourne). The phenotype of plants homozygous for this allele differ from those previously described in that the third whorl is wild type (six stamens) but the fourth whorl still produces a new flower primordium. This allele has been cloned and will be sequenced. Experiments are also in progress to determine the localization of the polypeptide using immunolocalization techniques and to determine the distribution of transcripts in situ. Results from these analyses may provide the first clues to different functional domains of this polypeptide or promoter elements that give rise to expression patterns of this gene in the third and fourth whorls.

Reference:
130. Genetic mosaic analysis of AGAMOUS gene function

Gary N. Drews, Leslie E. Sieburth

The Arabidopsis AGAMOUS (AG) gene is a floral homeotic gene that is necessary for the specification of stamens and carpels in the third and fourth whorls of the developing flower. To determine which cell layers of the floral meristem that AG activity is required in, we plan to carry out a genetic mosaic analysis of AG gene function.

To create mosaic plants, we are using the loxP-Cre site-specific recombination system of phage P1. We are now in the process of creating an Arabidopsis plant that has a Cre recombinase gene under the control of heat shock regulatory sequences, and a second plant that has an AG gene and marker gene flanked by the target sequences of the Cre recombinase (loxP sites). These plants will be crossed to produce a plant containing both constructs. Plants carrying these two constructs will be subjected to heat treatment at various times of development. The heat treatment will induce the expression of the Cre recombinase, which will cause deletion of all sequences between the loxP sites (namely, the AG and marker genes) in some cells. The result will be plants with sectors of cells lacking the AG and marker genes. From an analysis of such plants, we hope to determine whether AG action is cell autonomous, and in which cells the AG gene must be active for normal differentiation to occur. An approach similar to this has been successfully used to create mosaics in Drosophila (Golic and Lindquist, 1989).

Reference:

131. Tagging genes controlling flower development

Hajime Sakai

T-DNA integration into the genome of Arabidopsis thaliana enables us to clone affected genes in a faster and more convenient way than conventional chromosome walking. The junction region of the T-DNA, which can contain pBR322 portions, can be rescued in the form of E. coli plasmids along with the bordering plant DNA. This DNA can then be used as a probe to isolate genomic cosmid DNA and cDNA from the respective libraries, and finally to identify the mutagenized genes.

In collaboration with Dr. Ken Feldmann of Dupont and the University of Arizona, we have obtained a number of T-DNA-induced mutations of Arabidopsis thaliana that show defects in flower development. Two strains, #1086 and #1937, are particularly of interest because of their phenotypes which differ from the homeotic transformations seen in many previously characterized mutants such as agamous, pistillata, apetala2 and apetala3. The strain #1086 has extra organs in a fourth whorl, usually containing a gynoecium of three fused carpels instead of the wild-type two carpels. A similar phenotype (increased numbers of organs in the inner most whorl) is also observed in clavata mutant strains, which have been isolated by EMS mutagenesis. Genetic crosses between these strains will soon answer the question of whether the mutation of #1086 is allelic to those in one of the three known clavata loci.

On the other hand, mutant strain #1937 shows an opposite floral phenotype. It has a decreased number of organs. The mutation specifically affects the second and third whorls, usually resulting in no petal and stamen formation between normally developed sepals and carpels. Occasional appearance of one petal or one stamen indicates that homeotic information of these whorls is not influenced by the mutation. The action of the mutation is rather to restrict the spatial pattern of organ primordia in these whorls.

Considering the phenotypes described above, both mutant strains resemble the mutants of the segmentation genes in Drosophila to some extent. In addition to the gene tagging from both strains that is in progress, the construction of double mutant strains with the homeotic mutants is being done, to uncover possible functional interactions of these classes of genes.

132. Similarity between the Drosophila shibire product, associated with endocytosis, and the mammalian motor-protein dynamin

Alexander M. van der Bliek, Joo Hyun Chang

Temperature-sensitive paralysis of adult flies is the most striking defect of shibire mutants. A reversible block of endocytosis prevents membrane cycling, which is detected as reduced numbers of vesicles at synapses, as well as in the many other tissues affected outside the nervous system. We mapped the genomic environment of shibire by converting a 275-kb yeast artificial chromosome
to a series of overlapping cosmids, and then located the gene within the collection of cosmids using restriction fragment length polymorphism analysis of recombinant chromosomes. A 15-kb fragment of wild-type DNA rescues the mutant phenotype, demonstrating that we have isolated the shibire gene. This gene encodes a protein that is highly similar to rat dynamin; 68% of the amino acid sequence is identical. Dynamin is a GTP-driven mechanochemical enzyme related to mammalian mx-proteins and to yeast vps1. Because shibire and dynamin have extensive similarity, we propose that they are cognate homologs. In vitro, dynamin causes microtubules to slide along each other and in extracts it is associated with a distinct, but not yet characterized, membrane fraction. Drawing from the shibire phenotype, we suggest that, in vivo, dynamin motorizes the vesicular transport that occurs during endocytosis.

133. Progress toward cloning ETR, a putative ethylene receptor gene in Arabidopsis
Caren Chang, Shing F. Kwok, Anthony B. Bleecker

The plant hormone ethylene regulates a wide range of physiological processes during growth and development in higher plants. In order to begin to understand how ethylene is perceived and how the signal is transduced into the observed responses, we are focusing on cloning the ETR gene in Arabidopsis. The ETR locus is defined by a dominant mutation that causes insensitivity to ethylene, as evidenced by the absence of a variety of wild-type ethylene responses. Ethylene binding studies on the mutant and wild-type suggest that the ETR gene may encode a receptor for ethylene. Our strategy for cloning the ETR gene has been to isolate genomic DNA clones from the dominant mutant by chromosome walking, and then to test the clones' ability to confer the mutant phenotype to wild-type Arabidopsis when introduced by transformation. We have cloned over 200 kb of contiguous DNA that is genetically linked to the ETR locus. Wild-type plants transformed with either of two overlapping cosmids from the walk display partial insensitivity to ethylene. From additional transformations, we have delimited the source of this phenotype to a 10 kb DNA fragment contained on both cosmids. Molecular analysis of this DNA fragment has revealed a gene whose DNA sequence strongly suggests that it encodes a zinc finger of the type found in a number of transcription factors. Further analyses are in progress to determine whether this gene is indeed the ETR gene.

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134. A putative transmembrane protein-serine/threonine kinase gene in Arabidopsis
Caren Chang, Shing F. Kwok

In the process of chromosome walking to the Arabidopsis ETR gene, we have stumbled upon a gene that appears to encode a transmembrane protein kinase based on its DNA sequence. Two aspects of this gene are noteworthy. First, although the protein's potential transmembrane structure implies homology to the receptor tyrosine kinase family, the kinase domain sequence is more similar to that of the serine/threonine-specific kinase family (which is rarely known to possess transmembrane domains). Second, the putative extracellular domain consists of a repeated leucine-rich motif which is highly similar to that found in transmembrane and membrane-associated proteins in yeast, flies and humans. These other proteins, none of which are kinases, include yeast adenylate cyclase, Drosophila toll and chaoptin, and human glycoprotein Ib and leucine-rich α2-glycoprotein. A function for the leucine-rich repeat is unknown, but some evidence indicates a role in protein-protein interaction.

To our knowledge, protein-tyrosine kinase genes have not been isolated from plants, although a number of putative protein-serine/threonine kinase genes have been identified. Of the serine/threonine group, only one gene (other than the one described here) contains a potential transmembrane domain. The kinase domain of this maize gene shows a high degree of sequence homology to the gene we have isolated. However, rather than the repeated leucine motif, the extracellular domain has sequence homology to the Brassica self-incompatibility locus (believed to be involved in protein interaction). It may be that transmembrane serine/threonine-specific kinases are widely utilized by plants, serving as receptors for a variety of plant signals. Using PCR, we have cloned a number of other sequences from Arabidopsis that may represent additional members of this newly described kinase family.
Through studying several of these genes, we hope to obtain a clearer picture of the transmembrane protein-serine/threonine kinases, as well as some idea of their role in signal transduction in plants.

135. Selection of mutations in genes necessary for ADH gene expression

Gary N. Drews, Rachel Holmes

The goal of this project is to identify mutations in genes encoding regulatory products necessary for the expression of the *Arabidopsis* alcohol dehydrogenase (ADH) gene. Such mutants can be selected for by exposing plants to allyl alcohol, which is converted into a toxic compound by ADH enzyme activity. To favor mutations in ADH regulatory genes over mutations in the *ADH* structural gene, we have created a strain of *Arabidopsis* containing multiple copies of the *ADH* structural gene. Thus far, we have mutagenized 40,000 M1 seeds in 26 batches, grown up the M1 plants, and harvested the resultant M2 seed. We are now applying the allyl alcohol selection to the M2 seed. So far, we have subjected the M2 seed from 5,000 M1 plants to the allyl alcohol selection, and have recovered several survivors. These survivors can be divided into three classes based on their phenotypes. The first class of survivors displays dramatically abnormal development: the stem is green and has an epidermal morphology that is similar to that of a cotedon, the leaves are twisted, and the root appears to be absent. The second class of survivors is otherwise normal but they do not have roots. The development of the third class of survivors appears to be normal. Over the next year, we plan to continue the mutagenesis and allyl alcohol selections, to verify that the survivors are mutants defective in ADH gene expression, and to further characterize the mutants that we identify.

**PUBLICATIONS**


Summary: Our laboratory is studying how developing T lymphocytes acquire functional responsiveness. There are two aspects of this problem: one is how T cells come to be programmed for inducible gene expression in a way that other hematopoietic cells are not, i.e., how they mature; the other is how different subsets of T cells come to be specialized for carrying out different transcriptional responses to stimulation, i.e., how they diverge. For the past several years, we focused on accumulating data on the detailed molecular properties of these cells at different stages of their development. We found that T-cell "response" genes become accessible to induction at a stage long before the cells possess the recognition molecules that normally trigger "response" gene expression. We found that dramatic shifts in signaling requirements for "response" gene expression mark the major transitions in T-cell development. We also found that different functional subsets of T cells can in fact express each other's characteristic "response" genes, but normally express only their own because of signaling bias. These observations were summarized in last year's Annual Report, and the inferences we drew from them on the plasticity of T-cell developmental choices have been published in a series of review articles (see 1990 Annual Report). We have finally acquired enough data on the properties of T-cell developmental intermediates to be able to confront the molecular and cellular mechanisms that underlie their transformations.

This past year, therefore, has been marked by an intensified focus on two key initiatives. One has been to translate the developmental changes in T-cell behavior into terms of the differential expression of specific transcription factors. The second has been to develop approaches through which we can attempt to perturb the developmental pathway of pre-T cells. Both initiatives are at an early stage, but we have already reaped a harvest from the first.

The T-cell response that we have decided to focus on is the Ca\textsuperscript{2+} and protein kinase C-dependent induction of interleukin-2 (IL-2) expression. The gene encoding the powerful growth hormone IL-2 can be activated transcriptionally for a period of several hours in mature T cells, either in response to antigen recognition or in response to pharmacological signals like the calcium ionophore A23187 + the phorbol ester TPA. IL-2 inducibility provides a valuable monitor of developmental change, because pre-T cells in the thymus progress from 1) IL-2 noninducibility to 2) IL-1 dependent IL-2 inducibility, then back to 3) noninducibility, before emerging as 4) IL-1 independent, IL-2 inducible mature cells. The third of these states is associated with a unique phase in which thymocytes undergo crucial fate-determining interactions with the microenvironment (see Abstract No. 136). We were therefore pleased to find not only that we could distinguish among the patterns of inducible transcription factors in states 2, 3, and 4, but also that we could find clear evidence of unique kinds of responses to stimulation in the cells of the third state, even though they do not result in expression of IL-2. This work, as well as our initial attempts to dissect the interactions and subunits of the transcription factors themselves, is described in Abstract Nos. 142 and 143.

The other major initiative is still just beginning. We have successfully learned to apply the technique of direct intrathymic injection to trace the fate of early T-cell precursors, but we are only now discovering how to transfect the pre-T cells, before injection, with genes that may ultimately be used to direct their development. Initially, as described in Abstract No. 138, we are using this approach to question the commitment of hematopoietic cells to T-lineage vs. non-T-lineage fates. In the coming year, with new postdoctoral fellows in the laboratory, we will also renew our attempts to generate transgenic mice in which the IL-2-producing lineage can be distinguished and
preparatively isolated, and mice in which signals that may control key developmental transitions (e.g., cAMP-dependent kinase activity) are blocked. As background to this intensified effort, we have begun comprehensive analyses of in vivo IL-2 expression patterns (Abstract No. 140) and differences in cAMP metabolism in different T-cell subsets (L. J. Scherer, new work in progress). These studies will provide the controls against which the phenotypes of the transgenics can be compared in detail. Whether by marking cells of one lineage or by altering signaling pathways directly, the transgenic approach will greatly improve our access to the process of T-cell differentiation. Our goal in the next few years is for these present initiatives to converge, ultimately to give us a clean view of the way development and inducible gene expression control each other.

136. Uncoupling functional maturation from T-cell receptor-dependent selection in the thymus

Ellen Rothenberg

Several years ago, experiments in Harald von Boehmer's and Dennis Loh's laboratories revealed that the fate of a developing thymocyte is dictated by the recognition specificity of the receptor for antigen (TcR, for T-cell receptor) that it happens to express. If its receptor reacts too well with self antigens in the thymus, the cell is rapidly killed (negative selection). If it has no receptor at all, or one that cannot react at all, it also dies, more slowly, by default. Only if its receptor interacts just right with environmental structures is the cell allowed to enter the final phase of maturation (positive selection). Even at this point, the type of cell it differentiates into depends on fine aspects of its receptor specificity, controlling whether it will become a CD4+ growth factor-secreting "helper" or a CD8+, growth factor-dependent "killer." Thus, it was a surprise when we found, in recent years, that developing thymocytes could display an ability to make the "helper" product IL-2 even before they had any TcR at all, and thus before their fate could be established. As we described in last year's Annual Report, the immature IL-2 producers are highly active upon stimulation but differ from mature IL-2 producers in that the immature cells require another hormone, IL-1, to trigger them in addition to A23187 + TPA. Once they reach the "cortical stage" where they first express TcR, however, thymocytes enter a phase of deep nonresponsiveness which is only relieved if they are positively selected.

To test whether the changes in IL-2 inducibility are causally linked with TcR-dependent selection events, or merely coincide with them, we have examined IL-2 induction in the thymocytes of scid mice. These animals are congenitally deficient in certain kinds of DNA repair, and once the rearrangement of their TcR genes begins, most scid thymocytes die because they cannot repair the DNA breaks. Thus, scid thymocytes are mostly accumulated in developmental stages that precede TcR expression in normal mice, and they lack TcR expression altogether. As expected, scid thymocytes in vitro include a large proportion of excellent IL-2 producers of the immature, IL-1-dependent type. However, it has been intriguing to discover that they also consistently include cells that make IL-2 in response to A23187 + TPA without IL-1, unlike the purified immature population of normal thymocytes. Thus, it appears that scid thymocytes can undergo a functional conversion to the "mature" IL-2 producer type in the absence of any TcR-ligand interaction. If true, this would suggest that the functional maturation can occur in parallel to, but not dependent on, TcR-mediated selection events. The independence of these two kinds of maturation is in agreement with reciprocal experiments, conducted by Rochelle Diamond last year, on radiation chimeras. We are now extending the test for links between these two processes by studying the responses of thymocytes of transgenic mice that are condemned to a known fate because they are forced to express a TcR of known specificity.

137. Comparative studies of frog and mouse

Patricia M. White

One of the most striking features of the mouse thymus is the massive overproduction of immature thymocytes,
the vast majority of which are going to die. It was this observation that forced immunologists initially to formulate the theory of positive and negative selection. It would be ironic, however, if the mouse were an anomaly in the animal kingdom in this regard. Most classes of vertebrates down to teleost fish have a recognizable thymus, but only rodents have been studied for intrathymic population dynamics so far. It is not clear whether the selection processes, as opposed to simple differentiation events, play nearly so important a role in thymuses of other animals. Therefore, we have done some direct comparisons of the mouse with another common laboratory animal, *Xenopus laevis*, the South African clawed frog. *Xenopus* is an aquatic animal with a similar lifespan to the mouse, but phylogenetically quite distant. Any similarities between the animals point to important considerations in immune development; differences highlight adaptivity and specialization.

We are analyzing the results of a systemic labeling of dividing cells by injecting frogs or mice with 1 µCi per gram of body weight tritiated thymidine three or more times. We measured the rate of loss of radioactivity from the entire thymus, distribution of label in the cells themselves by in situ analysis of cell suspensions, and spatial location of dividing cells by in situ analysis of thymic sections.

The mouse has a much larger cortex than the frog, which is the putative site of positive selection. However, the same fraction of cells are labeled 24 hr after injection in both frog and mouse. Label is very stable in the frog, in that the percentage of highly labeled cells declines slowly, but is not stable in the mouse, where highly labeled cells are chased into moderately labeled forms almost immediately. This may indicate that murine thymocytes divide more rapidly and often. In addition, labeled cells appear in the frog medulla within 24 hr of injection. These cells are most likely either immediate products of cortex division, or recent bone marrow immigrants, proceeding through the medulla before joining the proliferative zone in the outer cortex. The latter explanation is puzzling, but the former would indicate that positive selection in the frog is less time consuming than the mouse. Finally, preliminary results suggest that overall levels of labeling are maintained for days longer in the frog thymus than in the mouse thymus. If this result is confirmed after correction for different body temperatures, it may suggest that frogs produce cortical thymocytes in less excess than mice.

138. Testing the limits of hematopoietic plasticity

*Patricia M. White*

Every modern immunology text contains a lineage diagram for the hematopoietic system. It shows a multipotent, self-renewing stem cell differentiating through orderly steps to produce all mature lymphatic and myeloid cell types. This differentiation is a simple branching tree, where not only each mature cell but also each differentiating precursor falls into a distinct class with rigidly defined phenotype, capabilities and function. We believe this model is simplistic. The molecular view of response holds that physiological stimuli bind receptors, many of which activate second messenger systems, activating DNA binding proteins and eliciting specific genetic responses. While such responses are normally cell type-specific, we have shown that stimulating primary cells *in vitro* with drugs that bypass the surface receptors can generate responses "abnormal" for their cell type. Others have demonstrated that under some conditions, stimulating distinct cell types can produce similar responses (e.g., lymphokine production that can be elicited both from T cells and from mast cells). *In vivo*, the differentiating factors and stimuli that cells encounter are determined by their local environment; if moved to a novel site, it may not be unreasonable to expect a cell to *trans*-differentiate along a wholly novel pathway.

32D is a cloned, non-malignant, bone marrow-derived pre-mast cell line. We have characterized it extensively. It does not produce IL-2 or utilize the IL-2 promoter under normal stimulation conditions. It does not express any mature T-cell surface antigens, although it does express two markers characteristic of immature thymocytes. It contains alcian blue staining granules similar to mucosal mast cells. Under current culture conditions, it is dependent on IL-3 for growth and survival.

We are exploring 32D's *trans*-differentiation potential. We have made stable transformants with β-galactosidase as a reporter gene, driven by the RSV promoter. We inject these cells directly into the thymuses of irradiated congenic
mice and assay for their survival and differentiative response. 32D does not repopulate like the positive control, fresh bone marrow, which can constitute 20% of the thymus at the height of its expansion wave. We are experimenting with weaning 32D from its IL-3 dependence and will assay early time points to catch any response it may make. Furthermore, we are also testing recently derived bone marrow mast-cell cultures for their ability to generate T cells in the thymus. Finally, due to the ease of transfection of the 32D cells, if they prove to survive at all in the thymus we may add in constitutive T-cell genes to promote trans-differentiation.

139. Optimization of β-galactosidase activity in transiently and stably transfected tissue culture cells carrying lacZ constructs

Julia A. Yang-Snyder, Patricia M. White, Rochelle A. Diamond

Constructs containing "recorder" genes encoding proteins possessing readily assayable activities controlled by defined transcriptional regulators are frequently used to study gene expression in vitro and in vivo. One major disadvantage in examining recorder gene expression such as chloramphenicol acetyltransferase, is that protein extracts are used to determine protein activity, and therefore, analysis of activity requires cellular disruption. Additionally, cellular localization of activity involves fixation of cells or tissues and transfection frequencies cannot be easily determined.

One of the benefits using lacZ as a recorder gene is that β-galactosidase activity can be detected without cell lysis and activity can be determined on a per cell basis. We have generated an improved lacZ reporter cassette and optimized conditions of β-galactosidase detection in individual EL4.E1 cells transiently transfected with our RSV/lacZ construct and in stable 32D transfectants generated by electroporation of the RSV/lacZ construct along with a selectable drug-resistance gene. Likewise, these constructs allow us to use fluorescein-di-galactoside (FDG) to characterize viable cells expressing lacZ constructs by fluorescence-activated cell sorting.

Along with our modified RSV/lacZ construct, we have compared the activities of IL-2/lacZ constructs with and without the T cell-specific CD2 locus-activating region. Initial studies indicate that the presence of the CD2 "enhancer" does not substantially increase the amount of β-galactosidase activity in EL4 cells transiently transfected with either an IL-2/lacZ construct or a similar construct carrying the CD2 enhancer. We are currently testing IL-2/lacZ constructs with human growth hormone exons instead of SV40 polyadenylation and splicing signals, to see if enhanced expression will result from more efficient RNA processing. We hope to use the new constructs to generate IL-2/lacZ transgenic mice, thereby using the lacZ gene as a screenable lineage marker to isolate and study the developmental fate of cells that produce IL-2 in vivo.

140. Identification of IL-2 expressing cells in vivo by in situ hybridization

Julia A. Yang-Snyder

Activation of interleukin-2 (IL-2) gene expression is most often studied in vitro using primary cells or established cell lines. Unfortunately, this approach reveals little about the location of IL-2 expressing cells within a given animal or at a particular time during development, and leaves open the possibility that unsuspected cell types may express the IL-2 gene in vivo. These questions can be readily addressed by employing the technique of in situ hybridization to tissue sections using a labeled probe specific for the detection of IL-2 mRNA. I have cryo-sectioned freshly-isolated adult, neonatal and fetal mouse tissues and hybridized the resulting 6 µm sections with a 35S-3' IL-2 probe. This approach has revealed that cells expressing the IL-2 gene in vivo can be identified. IL-2 transcripts are undetectable in most nonlymphoid/nonhematopoietic tissues, including the brain and spinal neurons. In contrast to an isolated report from another laboratory, no sign of IL-2 RNA was found in midgestation placent. For the most part, cells expressing IL-2 RNA have been isolated, round cells, consistent with their being T or pre-T cells. High expressing, IL-2 mRNA positive cells are localized in the cortex of the thymus of adult animals, although they are rare (frequency <1 x 10^-6). However, these cells are absent from the thymus of scid mice, suggesting that these cells are not immature (see Abstract No. 136). IL-2 expressing cells are also found in the gut and dermis of neonatal mice. Likewise, IL-2 expressing cells have been found in day 14 fetal liver (day 1 = day of plug), which is rich in lymphoid
precursors. A small subpopulation of fetal liver cells can also express IL-2 message following 18 hours of culture, but it is yet to be determined whether these IL-2 expressing cells in vitro are identical to those expressing in vivo. I am in the process of characterizing the cell-surface phenotype of these IL-2 expressing cells by combined immunofluorescence and in situ hybridization. Finally, I intend to continue to study the pattern of IL-2 gene expression in vivo during development and during the onset of infection and disease.

141. Physiology of inducible IL-2 expression in immature thymocytes

Ellen Rothenberg

To define the immature thymocyte response phenotype, we first wished to establish whether it was cell-autonomous. Cells of the major immature population (IL2Ra+, HSA++, CD5low, TcR-, CD4·cos·) could be fractionated away from the CD4·CD8· thymocytes and adherent nonlymphoid cells, and still retain unimpaired IL-2 inducibility. The IL-1 effect on these cells appears to be a direct one, not mediated through accessory cells, because the main products that accessory cells make in response to IL-1 (IL-6 and TNFα) have proved to be unable to replace or synergize with IL-1 in repeated experiments. As in our EL4.E1 cell model system (see last year's Annual Report), IL-1 and cAMP antagonize each other in the immature cells. Thus, while the positive response to IL-1 (IL-6 and TNFα) have proved to be unable to replace or synergize with IL-1 in repeated experiments. As in our EL4.E1 cell model system (see last year's Annual Report), IL-1 and cAMP antagonize each other in the immature cells. Thus, while the positive response to IL-1 distinguishes immature from mature thymocytes, the inhibition by cAMP is shared by both. We hope to be able to account for these aspects of pharmacology through analysis of the IL-2 DNA-binding proteins induced under different stimulation conditions.

An intriguing aspect of IL-2 regulation has been its relationship to the cell cycle. T cells newly activated from G0 generally score as much better IL-2 producers than T cells progressing through a full proliferative cycle. We considered the possibility that the high percentage of cycling cells in the immature population might either restrict the percentage of cells able to respond or abort the IL-2 expression once it was induced. The latter might explain the extremely transient kinetics of IL-2 RNA expression in these cells relative to mature T cells. Some support for the possibility of cell-cycle control came from experiments with enoxacin, a DNA gyrase inhibitor. When immature thymocytes were stimulated in the presence of this drug, they exhibited a marked enhancement of IL-2 RNA expression at later times. We hope to pursue this observation further by preparatively fractionating immature cells according to their cell-cycle stage and then assaying for the percentage that can respond to stimulation by expressing IL-2 RNA.

142. Differential regulation of the IL-2 gene transcriptional factors in mouse thymocytes

Dan Chen

The expression of the IL-2 gene is cell type-specific and stimulation-dependent. In a mouse thymus, the IL-2 gene can be induced in immature CD4·CD8·TcR· (double negative) cells and mature CD4+CD8· and CD4·CD8+ cells, but not in cortical CD4+CD8+ (double positive) cells. The fact that the 320 bp sequence 5' to the transcriptional start site is sufficient for transcription-activation and its high degree of sequence homology between the human and mouse imply that IL-2 gene expression relies on the combinatorial interaction of the trans-acting factors whose binding sites are packed in this region. Differential expression of these factors may explain the cell type-specific expression of the IL-2 gene. We used synthetic oligonucleotides from the mouse IL-2 gene 5' regulatory region to detect the existence of individual DNA-binding proteins in the nuclear extracts of the different subsets of mouse thymocytes, with or without in vitro stimulation, by means of gel mobility shift assays. To our surprise, similar to double-negative and single-positive thymocytes, the double-positive cortical thymocytes (see Abstract No. 136) have inducible NF-xB and CD28RC binding proteins at a level even higher than the double-negative (immature) cells, although lower than the single-positive (mature) cells. However, in contrast to the other two types, the double-positive cells express very low or undetectable levels of NFAT protein (a nuclear factor of activated T cells) after stimulation (see Abstract No. 143). Although the quantitative difference of the NF-xB and CD28RC-binding proteins may be part of the reason, it is more likely that the low inducibility of NFAT protein, which is a positive regulator, prevents the double-positive cells from expressing the IL-2 gene. Besides these enhancer elements
in the IL-2 5' flanking region which form the complexes with above-mentioned factors, there are also two putative AP-1 binding sequences in this region; they seem to form DNA-protein complexes with different factors, judged by the different complex mobilities and the results of competition experiments. The factors that bind to the upstream AP-1 site are constitutively expressed in all subsets, while the factors bound to the downstream AP-1 site are undetectable in freshly isolated thymocytes, but are easily seen after in vitro culture for two hours without stimulation. This is more prominent in the double-positive cells, and unlike cells of the EL4 cell lines, the level is not significantly increased after stimulation. Such subtle variation in the downstream AP-1 binding could play a role in turning on or off transcription, depending on when the binding occurs and the precise nature of the components of the "AP-1" complexes in different conditions. Further experiments are under way to resolve this.

143. The NFAT-binding site interacts with multiple factors

Dan Chen

The 30 bp sequence (-290/-260) about 270 bp upstream of both human and mouse IL-2 gene transcriptional start sites binds to an inducible, T cell-specific transcriptional activator called NFAT. While studying the cell-type specificity of the NFAT expression, we discovered that this element can also form a complex with a constitutively expressed, non-T cell-specific factor. With even higher binding affinity, this constitutive factor also binds to a distinct sequence (-320/-300), 10 bp upstream of the NFAT binding element. A very simple-minded assumption would be that under unstimulated conditions, these sites are occupied either cooperatively or independently by the constitutive factor which may act as a repressor; once activated, the NFAT will compete out the constitutive-binding factor or will be superimposed on it or on the same sequence, and activate the transcription. To understand this interaction, we planned to correlate the transcriptional activity with the factor-binding affinity by transfecting mutated sequences with variable binding affinity to either of these factors. While testing the binding affinities of the mutants, we were surprised by the discovery that the constitutive binding factor is a single-strand DNA binding factor, which can bind to both strands from the two sequences with the affinities ten to twentyfold higher than the double-stranded sequences. Two mutations in the upstream sequence decreased the binding affinity to the constitutive-binding factor. Mutations that affect either the NFAT binding or the NFAT and the constitutive factor bindings were also found. Unfortunately, we cannot isolate the mutant with solely reduced affinity for the constitutive-factor binding in the NFAT binding site. This fact might very well indicate that the constitutive-factor binding site is buried inside the NFAT binding site. Therefore, mutations affecting the complex formation with the constitutive factor will also affect the NFAT binding. Functional analyses by testing mutant transcriptional enhancing activity and by in vivo competition for the NFAT binding with the phosphorothioate NFAT-binding oligonucleotide are currently under way to dissect the relationship between the interactions of the cis-elements and the trans-acting factors and the IL-2 gene activation.

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behaviors of the bacterial flagellar motor as well as its interaction with the protein "phosphatase" that removes the phosphoryl group and deactivates the CheY protein.

While the general features of signal transduction in eukaryotic cells are probably similar to those in the bacterium, they are much more diverse. Mammalian organisms and their differentiated cell types can respond to hundreds of different ligands. A family of receptors characterized by their general structure, i.e., their ability to span the membrane of the cell seven times, mediates large numbers of different responses including the response to light, odorants, a large group of neuromodulatory substances, hormones, and a variety of regulatory peptides. We are continuing to characterize the diversity of the proteins that transduce signals from these "serpentine" transmembrane receptors. They generally involve a group of guanine nucleotide binding proteins (GTP binding proteins) that are made up of three polypeptides, an α subunit that binds the guanine nucleotide and a β and γ subunit. We have been able to show that there are 15 different α subunits belonging to at least four different classes. We believe that the heterotrimeric proteins formed from combinatorial associations amongst α, β, and γ subunits generate the specificity for receptors and effectors that results in signal transducing circuits responsible for differentiated cell function. We have been characterizing the Gq class of α subunits. When activated, these proteins appear to modulate the activity of a phospholipase that generates second messengers, e.g., polyphosphoinositides that control intracellular protein phosphorylation and cytoskeletal structure. We have been using transgenic animals that are generated to include mutations in specific G proteins in order to study the effects of changes in the G protein on the physiology of the cell. Specifically, we have been looking at modifications of the G protein involved in the visual system in transgenic mice. Eventually, we hope to understand the specific roles that these signaling systems play in cellular function.

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Summary: We are interested in how cells respond to changes in their external chemical and physical environments. Bacterial chemotaxis continues to provide us with insights into the mechanisms that a simple single cell uses to modify its behavior. These processes are analogous to those that are found in eukaryotic cells. They involve specific cell surface receptors, the activation of protein kinases, and protein phosphatases. In the first step in the process, ligands, e.g., amino acids or sugars, bind to cell surface receptors. Signal processing begins with the receptor itself. The receptor can initiate three kinds of events: (a) it acts to facilitate formation inside the cell of an activated CheA-CheW complex which leads to rapid protein phosphorylation of the CheY protein; (b) it can also act to sequester or inhibit protein phosphorylation; and (c) it can regulate its own activity through receptor modification. The interaction between the control of protein phosphorylation (the excitation process) and the regulation of receptor responsiveness (desensitization) accounts for many of the behavioral features of the organism. We are trying to understand the relationship of changes in ligand concentration to these receptor-mediated processes. We are also looking at the effector end of the signal transduction process, i.e., the nature of the phosphorylation of the CheY protein, the function of the phosphorylated protein and its ability to interact with the bacterial flagellar motor as well as its interaction with the protein "phosphatase" that removes the phosphoryl group and deactivates the CheY protein.
We have been collaborating with Prof. Paul Sternberg and Dr. Jane Mendel in the study of the diversity and function of G proteins in Caenorhabditis elegans. Thus far, we have found and characterized five genes that encode Ga subunits. Jane Mendel has been able to demonstrate the cell-specific expression of some of these genes. We hope to correlate specific G proteins with sensory pathways in C. elegans and to study the role that they play in regulating behavior.

The tremendous diversity of the signaling systems has made it further clear to us that our eventual understanding of these systems is going to depend on our ability to manipulate the genetics of complex organisms. To this end, we have continued our work on rapid gene mapping. We have successfully developed a vector system based on the single copy Escherichia coli F factor. This allows us to clone large fragments (100-250 kb pairs) of human DNA in E. coli. We have shown clearly that these clones are maintained in a stable fashion and that they represent contiguous pieces of a human DNA. We have also been able to prepare chromosome 22-specific libraries of cosmid size DNA (40 kb pairs). We are currently in the process of developing a library of large fragments of the total human genome and a library of clones that are specific to chromosome 22 for pulse field gel electrophoresis and in situ hybridization. We plan to assemble a complete map of human chromosome 22.

Eventually, our understanding of signal transduction will lead to the elucidation of the mechanisms by which these signals regulate gene expression. This will, of course, involve the function of DNA binding proteins. These proteins use DNA as a scaffolding on which to assemble functional complexes. For many years we have been studying the function of the Hin protein, a gene in E. coli that regulates DNA rearrangement. It forms a tetramer that brings together two specific DNA sequences and by interaction with another DNA binding protein, the Fis enhancer, inverts a specific DNA sequence. Recent work in our laboratory has shown that the DNA not only provides a specific sequence upon which the DNA-invertase can be assembled, it also provides physical cues in terms of the superhelical density of the DNA and the structure of the DNA that markedly affect DNA inversion. In other complex systems, collateral sequences and DNA structure may play a critical role in the function of proteins that bind DNA and control its activity. Thus, our continuing studies of the details of Hin recombinase function provide a model that will help clarify the function of DNA binding proteins.

144. Transmembrane signal transduction in bacterial chemotaxis
Andrew A. Pakula, Melvin I. Simon

Chemotactic responses in Escherichia coli begin with the detection of specific chemical substances by one of four transmembrane receptor proteins that span the cytoplasmic membrane. Each of these proteins, known collectively as the methyl-accepting chemotaxis proteins (MCPs), recognizes a distinct set of chemical effector molecules. We have concentrated on one, the Tar protein, which mediates attractant responses to maltose and aspartate and repellent responses to nickel and cobalt ions. Each Tar monomer has a periplasmic ligand-binding domain and a similarly-sized cytoplasmic domain. The latter region communicates directly with soluble intracellular components of the chemotaxis system. Structurally, the periplasmic and cytoplasmic domains are linked by two short membrane-spanning domains. We are interested in understanding their functional linkage. Binding of ligand to the periplasmic domain results in a change in the signaling state of the cytoplasmic domain, although the mechanism by which information is transmitted across the membrane is not yet understood.

The Tar protein is thought to exist as a dimer, and there appears to be no change in its oligomeric state upon binding of ligand. Thus, it is most likely that the mechanism by which the extracellular and intracellular domains of Tar communicate involves changes that occur within this dimer. Furthermore, any such mechanism would necessarily involve motions of one or both transmembrane domains. We are investigating the nature of these predicted conformational changes using two approaches: 1) analysis of missense mutations to identify functionally important residues, and 2) the use of comprehensive disulfide cross-linking to investigate the structural arrangement of the transmembrane regions.

We have isolated two classes of missense mutations in one of the transmembrane regions. Mutations in the first
class appear to mimic attractant binding, while the mutations in the second class cause the receptor to behave as though repellent were bound. The two classes of mutations fall into distinct structural regions—these may represent interaction surfaces important for the conformational change involved in transmembrane signal transmission. On the basis of these results, we have constructed a preliminary model for the nature of this local change. We are currently testing predictions of this model using site-directed mutagenesis.

Structural information for membrane proteins has been notoriously difficult to obtain. We have developed a strategy, involving disulfide cross-linking, for the examination of protein structure. Cysteine substitutions are randomly introduced throughout a protein region or regions and those pairs of cysteine residues that form disulfide cross-links upon oxidation are identified. We have been using this approach to examine the transmembrane domains of Tar. It has been possible to determine which helical faces interact and thus to propose a model for the general arrangement of the four transmembrane helices in the Tar dimer. Furthermore, the most stable interhelical interaction appears to be mediated, at least in part, by side chain hydrogen bonding between helices. We are proceeding to use this method to gain more detailed structural information and to characterize the changes that occur during signal transduction.

145. The role of CheW in bacterial chemotaxis

Lisa A. Alex, Katherine A. Borkovich, Melvin I. Simon

Swimming behavior of bacteria such as *Escherichia coli* and *Salmonella typhimurium* is controlled by the direction of flagella rotation. In a constant environment, these organisms swim smoothly for approximately 0.8 second and then change direction by tumbling for approximately 0.2 second (Berg and Brown, 1972). Smooth swimming is a result of counterclockwise (CCW) rotation and tumbling results from a change to clockwise (CW) rotation. Thus, there is no net migration in any direction over time. As the bacterium senses a change to a more favorable condition (the presence of an attractant), it extends its smooth swimming phase, resulting in a net migration over time.

Chemical stimuli are sensed by one of four transmembrane chemoreceptors. These proteins transmit a signal across the cell membrane which controls the activity of a histidine kinase, CheA. CheA in turn phosphorylates CheY. Phospho-CheY is believed to be the signal to cause CW rotation of the flagellum, generating a tumble. In the presence of a repellent or a decrease in an attractant, the kinase is activated, resulting in high levels of CheY-P. Activation of the kinase by the receptor is dependent on the CheW protein, which appears to act as a molecular switch.

The actual molecular mechanism by which CheW works is unknown. Several avenues are being explored to further our knowledge about CheW function. First, we have generated some random mutants of CheW that result in modified swimming behavior of the organism. We are currently trying to evaluate how this happens by analyzing the properties of the mutant proteins *in vitro*. In addition, as CheW can be purified in large quantities, we have tried to produce crystals to ultimately determine its three-dimensional structure. So far, only microcrystals unsuitable for X-ray diffraction have been obtained.

As the activation state of the kinase is dependent on the receptor and CheW, this suggests that there may be physical interactions of these three components. Indeed, workers have shown evidence of CheA-CheW complexes (Gegner and Dahlquist, 1990). To determine if complex formation can occur between the receptor and CheW and to facilitate analysis of the kinetics of the chemotaxis system, we have tried to reconstitute the purified Tar receptor from *E. coli* into phospholipid vesicles. This has proven to be extremely difficult; therefore, the gene is being engineered to produce only the cytoplasmic C-terminal domain of the protein. This should provide us with a completely soluble system to study.

References:


146. The role of protein phosphorylation in bacterial chemotaxis

Katherine A. Borkovich, Melvin I. Simon

Our work has focused on the signal transduction pathway by which chemical stimuli (ligands) modulate the
swimming behavior of *Escherichia coli*. The pathway is initiated by four transmembrane receptors which bind subclasses of ligands. Binding events at the receptors are converted by the CheA, CheW, CheY and CheZ proteins into chemical signals that change the direction of rotation of the flagellar motor. Counterclockwise rotation of the motor causes smooth swimming, while clockwise rotation results in tumbles.

*In vitro* assays have shown that CheA is an autophosphorylating kinase which transfers its phosphate to CheY, while CheZ is a CheY-phosphate phosphatase. Previously, we demonstrated that addition of a wild-type or tumble mutant, Tar, and the CheW protein to the phosphorylation reactions stimulates phosphorylation of CheY approximately 300-fold. Titration of reactions with ligand inhibits activation of phosphorylation in the concentration range that causes smooth swimming in vivo. Thus, increased production of CheY-phosphate correlates with a receptor capable of generating tumbles in vivo.

We have recently shown that the activation of CheY phosphorylation occurs at the level of CheA autophosphorylation. CheA-phosphate levels are increased in the presence of a wild-type or a tumble-mutant receptor and the CheW protein. This activated CheA-phosphate is very labile, with an *in vitro* half-life of ~10 seconds. We previously described a mutant protein, CheY13DK, that apparently mimics the structure of CheY-phosphate and causes tumbly swimming behavior in the absence of phosphorylation. Much of our effort over the past year has been directed at studying the physical structure of CheY13DK and generating additional mutants with similar phenotypes.

An X-ray crystal structure of CheY13DK has been obtained by Karl Volz (University of Illinois, Chicago) using material supplied by us. The wild-type and mutant structures are very similar. This unexpected result may be due to the fact that both structures were obtained in the absence of bound Mg$^{2+}$, which is required for activity of wild-type CheY. In contrast, Mg$^{2+}$ binding is greatly reduced in the mutant protein and may not be necessary for function. The appropriate comparison to suggest the nature of the phosphorylation-induced conformational change would thus be between the structures of wild-type CheY with bound Mg$^{2+}$ (unfortunately not currently available) and the mutant. A complementary $^{19}$F-NMR analysis of wild-type and mutant CheY structures in solution, carried out in collaboration with Dr. Joseph Falke (University of Colorado, Boulder), is nearing completion.

Additional mutations in cheY that cause tumbly behavior would be useful to our understanding of CheY function. Three approaches have been attempted to generate such mutants. (1) Random mutagenesis followed
by visual screening on swarm plates generated a number of cheY mutants with altered swimming behavior. However, none of the mutants isolated had as strong a phenotype as the original cheY13DK mutation. (2) CheY is a member of a large family of proteins that regulate bacterial response to environmental conditions. A number of mutations that result in constitutive activity of other members of the CheY-family were imported into CheY. Several mutations altering amino acid residues conserved among family members were tested, as were some altering nonconserved residues. No mutations were discovered that activate CheY. (3) The original cheY13DK mutation arose from systematic alteration of amino acid residues believed to be important for CheY function. This strategy is currently being pursued.

148. Structure-function studies of rod and cone transducin α subunits in transgenic mice

Janis Lem, Carol Raport1, James Hurley1, Clint Makino2, Denis Baylor2, Melvin I. Simon

The rod and cone transducins are GTP-binding proteins essential for visual signal transduction. The proteins show 80% amino acid identity, responding to photoactivation of rod or cone opsins and transducing signals via cGMP-dependent phosphodiesterase (PDE) to modulate ion channel closure in the photoreceptor cell membrane.

We have initiated studies to molecularly dissect the transducin genes to study their structure and function. Defined point mutations in the GTPase activity region of human rod (Q200L) and cone (Q204L) transducin cDNAs have been made and expressed in transgenic mice using the previously characterized mouse rod opsin promoter to drive their expression (Lem et al., 1991). In addition, the wild-type transducin genes have similarly been introduced into transgenic mice. Questions we hope to answer are: 1) What are the physiological effects of a mutation in the GTPase activity site of transducin, and 2) can cone transducin function in the rod visual transduction cascade? Using the same strategy with other specific mutations in the transducin genes, we may also ask what sequence features define them as transducins as compared to other G proteins? What sequence features distinguish rod from cone transducin? Answers to these questions will be useful in elucidating the function of G proteins and understanding the role of transducin mutations in human visual dysfunctions.

Transgenic mouse lines containing mutant rod and cone transducin cDNAs or the wild-type cone transducin cDNA driven from the mouse opsin promoter have been generated. We are in the process of generating lines containing the wild-type rod transducin gene. Transgenic animal lines are being examined for changes in PDE activity and levels of GTP binding to assess the activity of the transgene product. In addition, the physiological effects of the transgene products are being assessed by single cell recordings and electroretinograms for changes in photoreceptor sensitivity to flashes of light, integration time of flash response, and kinetics of response. Preliminary studies using mice heterozygous for the normal and mutant cone transducin transgenes show little or no change in GTP binding or PDE activity. Transgenic mice carrying the mutant rod transducin transgene show some increase in GTP binding and PDE activity. Single cell recordings on four transgenic mice have shown no differences as of yet in flash sensitivity, integration time or kinetics. Animals are currently being bred to homozygosity and expanded to allow quantitative biochemistry and a larger sampling size.

Reference:


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149. The functional coupling of G proteins (Gq class) to phospholipase C isozymes in transmembrane signaling mechanism

Chang Ho Lee, Dianqing Wu, Soo Goo Rhee1, Melvin I. Simon

The heterotrimeric G proteins, named Gp or Np, have been implicated as transducing components in the agonist-receptor mediated hydrolysis of phosphoinositides. Studies using permeabilized cells and isolated membranes provided evidence supporting the involvement of GTP-binding proteins in receptor coupling to phospholipase C.

It was reported that functionally distinct G proteins are selectively coupled to different receptor and effector types which activate PI hydrolysis. However, their identity
remains to be defined.

There are many examples of GTP-dependent signaling pathways, particularly those resulting in phospholipase C activation, that are resistant to disruption by pertussis toxin. Recent cloning experiments have provided a variety of new G proteins that may function in those pathways. Eight Ga cDNAs clones belonging to three different classes of Ga subunits were characterized. They include two novel classes, G12 and Gq; all of these lack the cysteine residue four amino acids from the C-terminal end that is the target for PTX-mediated ADP ribosylation.

For the purpose of identifying the functional coupling of these newly cloned G proteins (Gq class) with phospholipase C isozymes in transmembrane signaling mechanism, we have been developing a reconstitution system that consists of various Ga subunit-transfected COS-7 cell membranes, purified phospholipase C isozymes, and phospholipid vesicles. In this system, the level of water-soluble inositol phosphates released from the vesicles was measured as an indicator of functional linkage between phospholipase C isozymes and GTPyS-stimulated Ga subunits. Affinity-purified Ga-specific antibodies were used to block the GTPyS-stimulated PIP2 hydrolysis, which provides another piece of evidence describing these functional relationships. Through these studies, we observed that the Gq class of Ga subunits is coupled to both phospholipase β1 and β2. We will further investigate functional domains of the Gq class which are involved in regulating phospholipase C isozymes in transmembrane signal transduction mechanism.

Gα cDNAs of the Gq class were transiently transfected into COS-7 monkey kidney cells and the level of PLC products (i.e., inositol phosphates) in these transfectants was assayed. We found that all the members of the Gq class of Ga subunits could stimulate the activity of the endogenous PLC in COS-7 cells. Cotransfection of COS-7 cells with cDNAs of the Gq class and different PLC isozymes demonstrated that the Gq class of Gα proteins could stimulate the activity of the β subclass PLC isozymes and not of the PLC δ isoforms.

We also made several mutants predicted to result in constitutive Gq α function based on activation mutations described in the related proteins, Goα and ras. It was found that all of these mutations fully activated the G proteins and remarkably elevated the steady state levels of inositol phosphates in their respective COS-7 transfectants. Experiments to test whether activated mutants will induce abnormal growth or tumorigenesis in NIH 3T3 cells are currently under way.

Finally, we also constructed a series of mutants of PLC-β in an attempt to identify its G protein regulatory domain. Preliminary results suggest that the C-terminal portion of PLC-β is responsible for membrane attachment of this protein and may also be the part of PLC-β1 that the G protein acts on.

Reference:

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150. Activation of phospholipase activity by Gq class GTP binding-protein α subunits

Dianqing Wu, Chang Ho Lee, Soo Goo Rheel1, Melvin I. Simon

The activation of phospholipase C (PLC) by many seven-pass membrane receptors is mediated by trimeric GTP-binding proteins. A new class of G protein α subunits, including the Gq, G11, G14, G15, and G16 α cDNAs, was recently characterized in this lab. These clones are designated as members of the Gq class, based on their deduced amino acid sequence similarity (Simon et al., 1991).

151. G-protein genes in human myeloid cells

Thomas T. Amatruda, David A. Steele, Melvin I. Simon

We are interested in the role of G-protein isotypes in the physiology of hematopoietic cells, focusing on human cells of the myeloid lineage. Myeloid cells are a family of blood leukocytes, derived from pluripotent bone marrow stem cells, which manifest a variety of G protein-mediated responses.

We recently cloned a G protein α subunit, Ga16, which is specifically expressed in cells of hematopoietic lineage. Ga16 is predicted to be resistant to ADP-ribosylation by pertussis toxin. This gene is expressed at highest levels in undifferentiated myeloid cell lines and in T-cell leukemia-derived lines and is not expressed in B
lymphocyte lines with lymphoblast or plasmacytoma phenotypes. Expression of Ga16 RNA and protein varies as a function of myeloid terminal differentiation. In HL-60 cells induced to differentiate to neutrophils, expression of Ga16 decreases by 90% while expression of another α subunit, Gi2, increases. In contrast, Ga16 expression is maintained or increased in human monocytes, macrophages, and monocyte cell lines THP-1 and U-937.

We are determining the receptors and effectors that couple to to Ga16 in myeloid cells, using reagents specific for the Ga16 gene. Studies by our group indicate that Ga16 stimulates phospholipase C when transfected into COS cells. A number of ligands that activate G proteins are known to activate phospholipase C in myeloid cells, including inflammatory peptides, neurokinins, and purinergic agonists. These ligands also cause an increase in intracellular calcium. We are attempting to block the phenotypes induced by these ligands using affinity-purified antisera to Ga16. We have also used site-directed mutagenesis to generate Ga16 mutants predicted to be constitutively activated. We are now developing stable and transient transfection systems to analyze the actions of these mutant G proteins in hematopoietic cell lines.

152. Mutagenesis of G protein α subunits of GTP binding proteins

Vladlen Z. Slepak, Melvin I. Simon

Investigation of structure/function relationships in the G proteins has thus far concentrated on the α subunit and on the use of site-specific mutagenesis designed to modify a few amino acids in the regions that are involved in Gα interaction with guanine nucleotides. Much of the work has been based upon analogies with the extensive mutagenesis of Ras or has focused on the behavior of chimeras of Gs and Gi α subunits.

We are interested in the development of a high resolution missense mutagenesis map of the entire α subunit by means of a series of in vitro assays to screen the behavior of specific clones of an Escherichia coli mutant library. We have chosen G0α as a model target protein for the mutagenesis for two reasons. First, G0α can exchange nucleotides in the absence of the activated receptor which makes it much easier to study the biochemical properties of the protein. Second, in contrast to several other G proteins, G0α can be highly expressed in E. coli in an active form. Many of the characteristics of recombinant G0α are very similar to those of the authentic G0α protein isolated from brain.

The major advantage of working with E. coli rather than, for example, overproducing the protein in the insect SF9 cells where large amounts can be made, is that E. coli can be grown and assayed as individual clones. Therefore, we can prepare and screen hundreds of different mutants and we can use sophisticated techniques of mutagenesis to generate a large number of changes in localized portions of the protein. We have developed a system that allows us to screen colony lifts by incubating them with radioactive GTP or its analogs. Thus, we can characterize in situ the enzymatic activities of rG0α in single colonies. We also can quickly test with the antibody to the C-terminal region to see if the intact protein has been produced. On the basis of this initial screen, we can pick interesting mutants and prepare small volume cultures from each clone. We have shown that 10-20 ml of the soluble cell extract is sufficient to assay by binding and the interactions with receptor analogs. Individual interesting mutants will be sequenced and overproduced in vitro to study their characteristics more carefully. They can be readily subcloned into a variety of vectors for transfection or to prepare RNA for injection into Xenopus oocytes.

These data should allow us to localize specific regions of the protein that may be involved in G0α function. The point mutants generated in our work may be useful in predicting functional mutants in other G proteins and can be helpful in understanding the receptor and effector specificity of G proteins.

153. Characterization of G protein α subunits in the Gq class: Genomic organization and expression in murine tissues, stromal and hematopoietic cell lines

Thomas M. Wilkie, Melvin I. Simon

To explore the diversity of G protein-mediated signal transduction pathways, we developed an approach utilizing the polymerase chain reaction to identify novel cDNA clones that share certain highly conserved amino acid sequences common to all G protein α subunits. We
identified cDNA clones corresponding to eight new α subunits among a total of 16 distinct proteins expressed in mammals. Sequence comparison of all known G proteins indicated that at least four classes of related α subunits were expressed in mammals, termed Gs, Gi, G12 and Gq. These classifications appear to have functional relevance with respect to effector specificity. There are four murine alpha subunits in the Gq class, Gaq, Gα11, Gα14 and Gα15; each of these α subunits activates phospholipase B and is refractory to ADP-ribosylation by pertussis toxin. These characteristics suggest that the Gq class is an important group of G proteins that mediate cellular responses to a wide variety of stimuli. We assayed the distribution of the Gq class in murine tissues and cell lines by RT/PCR and Northern analysis; Gα11 and Gaq are ubiquitously expressed among murine tissues but Gα14 is predominately expressed in spleen, lung, kidney and testis while Gα15 is primarily restricted to hematopoietic lineages. Among hematopoietic cell lines, Gα11 mRNA is found in all cell lines tested, Gaq is expressed widely but is not found in most T cell lines, Gα15 is predominantly expressed in myeloid and B cell lineages and Gα14 is expressed in bone marrow adherent (stromal) cells, certain early myeloid cells and progenitor B cells. The expression patterns that were observed in mouse tissues and cell lines indicate that each of the α subunits in the Gq class may be involved in signal transduction pathways that are fundamental to hematopoietic cell differentiation and function. The genomic organization of the Gq class has been determined in mouse and humans. The genes are encoded in two groups of tightly linked loci in both organism, one containing Gaq and Gα14, the other Gα11 and Gα15. Based on amino acid sequence comparisons of these G proteins in various mammals, it appears that an ancestral duplication of the Gaq gene gave rise to Gα14 and that a subsequent duplication of these genes generated the Gα11/Gα15 locus.

154. The role of negative supercoiling and the effect of a perfectly inverted repeat recombination site, hixC, in Hin-mediated site-specific recombination

Heon M. Lim, Melvin I. Simon

The Hin recombinase is involved in a gene rearrangement that leads to the alternative expression of the H1 and H2 flagellin genes in Salmonella typhimurium known as phase variation. Hin inverts a 993 bp chromosomal segment (H region) which is flanked by two 26 bp recombination sites, hixL and hixR, both of which consist of two imperfect 13 bp inverted repeats.

Biochemical assays were developed and performed to monitor the molecular events that occur during the Hin-mediated DNA inversion reaction. These events can be divided into five different stages: 1) binding of proteins (Hin, Fis, and HU) to DNA, 2) tetramerization of the bound Hin dimers, 3) invertasome formation, 4) DNA strand cleavage, and 5) strand rotation and religation. Topoisomers of the wild-type DNA substrate plasmid (ranging from fully relaxed molecules to those with more than the physiological level of negative supercoiling) were generated and the role of negative supercoiling in each step of the inversion reaction was investigated.

We found differences in the dependence of tetramerization and invertasome formation on superhelical density of the substrate plasmid. Tetramerization of Hin occurs independently from the invertasome formation and a relatively low degree of negative supercoiling is enough to promote maximal tetramerization. However, efficient invertasome formation requires higher levels of negative supercoiling. Therefore, we suggested that negative supercoiling promotes the Hin-mediated inversion reaction at two different levels. First, it increases the local concentration of Hin dimers bound to DNA by shortening the distance between them, therefore promoting the Hin-Hin interaction by increasing the frequency of random collision. Second, it may aid formation of the productive synapsis by promoting physical contact between tetramerized Hin and Fis dimers, which would not be possible by protein-protein interactions. The contact between Hin and Fis at the enhancer would enable Fis to lock up the Hin tetramer in a conformation where concerted DNA strand cleavage could occur.

A hix sequence with two perfect 13 bp inverted repeat (hixC) was constructed using a half site sequence that is identical in both hixL and hixR. This half site has the highest binding affinity to Hin of any half sites. By the challenge phage assay, it has been demonstrated that Hin binds to hixC site slightly better than to hixL. This result is consistent with the notion that Hin dimer, which
possesses twofold symmetry, would bind best to the perfectly symmetric DNA binding site assembled from the two highest affinity half sites. However, when a substrate plasmid with two hixC sites was subjected to the standard Hin-mediated inversion reaction in vitro, the rate of inversion reaction was so slow that almost no inversion was detected. Biochemical assays were performed to investigate which stage of the inversion reaction is blocked on the substrate plasmid. The difference in secondary structure between hixL and hixC sites was probed by KMnO4 and S1 nuclease. We found that the central AT base pairs can form an anomalous DNA structure in hixC. Furthermore, under certain conditions Hin bound to hixC cannot be tetramerized because of the structure in the center of the DNA binding site.

155. Mapping genes on human chromosome 22
Yoshiaki Tachi-iri, Julie Korenberg1, Melvin I. Simon

We are interested in developing systems and procedures for using fluorescent in situ hybridization in mapping genes and chromosomes. We are using video-based imaging technology as well as multicolor in situ hybridization to localize and order probes along human chromosome 22.

We have applied a high sensitive SIT camera and image processing to the detection of fluorescent-labeled probes. Exposure times of less than 2 seconds are sufficient to detect the hybridization of the single copy sequence derived from cosmid DNA. Image processing techniques, such as contrast enhancement, have proven useful in determining precise locations of probes on the relatively small chromosome 22.

Multicolor fluorescent hybridization permits the simultaneous localization of a number of probes on the chromosome. Thus, we can determine the position of a new probe relative to known markers by using different labeling and detection systems. For example, an unmapped cosmid can be labeled using a biotin-avidin system with FITC, while two known probes, serving as standards, can be labeled with digoxigenin-Texas Red. In addition, fluorescent banding techniques permit localization of in situ hybridization signals within chromosome bands.

We are applying these techniques to mapping chromosome 22-specific cosmids and single copy bacterial artificial chromosome clones. We are exploring the use of confocal microscopy and CCD cameras to increase sensitivity and resolution of these techniques.

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156. Physical mapping of human chromosome 22
Hiroaki Shizuya, Bruce W. Birren, Ung-Jin Kim, Valeria Mancino, Tatyana Slepak, Yoshiaki Tachi-iri, Melvin I. Simon

We are interested in new methods for mapping and cloning large fragments of mammalian DNA. As a test of the efficiency and reliability of our methods, we are developing a high resolution map of human chromosome 22 which is represented by an ordered array of overlapping cloned DNA fragments.

We have developed an F factor-based system for cloning large fragments of human DNA in Escherichia coli. In addition to its large size capacity, we have shown that it is capable of maintaining human DNA with a high degree of stability, far greater than that associated with previous bacterial cloning vectors.

The vector consists of a 5.5 kb fragment of the E. coli F factor which contains genes essential for replication and copy control of the plasmid, and provides incompatibility preventing different F molecules from being present in the cell. The vector contains cloning sites, "rare" restriction sites for excising the insert, and the cosN and loxP sites which are used to create fixed points in the circular molecule for mapping by cleavage with lambda terminase and PI Cre protein, respectively. Because it is essential to eliminate as much recombination function as possible from the E. coli host, we have constructed a set of new strains carrying deficiencies in 1) host controlled restriction, 2) methylation-sensitive restriction, and 3) various recombination systems. We have so far been able to generate BAC (bacterial artificial chromosome) clones carrying human DNA with average insert sizes over 100 kb, with clones over 200 kb being isolated.

We have also used the BAC vector to generate a new single copy vector for efficient cloning and stable maintenance of 40 kb average insert sized DNA. The pFOS1 vector contains two cosN sites and can be digested
to generate right and left arms. These are ligated to partially digested, dephosphorylated human DNA and the mixture packaged for transfection. To date, we have prepared two chromosome 22-specific Fosmid libraries. The first used DNA from a hybrid cell line in which chromosome 22 is the only human material. Human clones (about 0.5% of the total) were identified by their hybridization to labeled human DNA. In situ hybridization has shown that all clones tested so far hybridize to chromosome 22. The second library was recently generated in collaboration with Pieter deJong from DNA prepared from chromosomes sorted at Lawrence Livermore National Laboratories. This library represents a 7X coverage of chromosome 22. In spite of existing at single copy levels, Fosmid DNA can be readily prepared and analyzed using the same routine procedures as are used for cosmids.

We feel that the stability of cloned human DNA during propagation in bacteria is so important to the successful use of libraries for mapping that we have begun a rigorous test of the stability of human DNA cloned in our BAC and Fosmid vectors. For comparative purposes, we have performed similar tests for cosmid vectors currently in use.

Twenty randomly picked chromosome 22 Fosmid clones were grown in liquid and diluted daily for about 160 generations of growth. DNA from the initial and final cultures was compared by digestion with three different enzymes. In all cases, the digestion products of the day 8 cultures were indistinguishable from the original cultures, indicating that no detectable rearrangements or deletions had occurred during 160 generations of serial growth. Similar experiments with BACs carrying human inserts as long as 175 kb also showed no rearrangements. In sharp contrast, when this procedure was carried out using the same human DNA cloned in Lawrist 16 or Supercos cosmid vectors, 40% of the clones demonstrated massive rearrangements of the inserted human DNA. In fact, in many cases after just 20 to 40 generations of growth, the original clone had been completely overgrown by deleted products. Generating and picking cosmid libraries and growth for DNA preparation involves at least this number of generations.

We have also established the use of multi-color fluorescent in situ hybridization with high sensitivity image acquisition and processing for chromosome mapping using our clones. We have now begun to utilize all our resources, including our new libraries, pulsed field gel electrophoresis and in situ hybridization to generate a physical map of chromosome 22.

PUBLICATIONS


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Summary: Our laboratory uses a molecular genetic approach to study basic questions in developmental biology and neurobiology: What are the molecular mechanisms by which cells interact with each other to establish a spatial pattern of cell types? How can a cell divide and produce two sister cells that differ in their developmental fates? How are the instructions for innate behavior encoded in the genome? Our major strategy is to identify mutations that make cells or animals misbehave.

We then use classical genetics to study the function of the genes defined by those mutations, and molecular biology to study the structure, expression and function of the genes and their products. Our studies focus on several aspects of the development and behavior of nematodes: induction of the hermaphrodite vulva, maturation of the male tail, and male mating behavior. Another strategy is to clone the nematode homologs of genes encoding evolutionarily conserved cell regulatory molecules, and then use the Caenorhabditis elegans genomic map and transgenic nematode technology to find functions for those molecules in nematodes.

Our major effort this year has been on understanding the development of the C. elegans vulva, which allows hermaphrodites to release eggs and to mate with males. Vulval development in C. elegans is a simple example of inductive pattern formation. A single cell in one tissue, the "anchor cell" of the gonad, induces three of six cells in another tissue, the vulval precursor cells (VPCs) of the epidermis, to generate the vulva. Each VPC has the potential to become any of three cell types (1°, 2° or 3°), defined by the set of progeny cells generated by each precursor cell. Even though the VPCs are multipotent, an invariant pattern of cell types is established: 3°-3°-2°-1°. The pattern of VPC fate is established by three intercellular signals: a graded inductive signal from the anchor cell, a short-range lateral signal from neighboring VPCs, and an inhibitory signal from the HY7 hypodermis. To understand how the fate of each VPC is specified by these two signals, we have defined the following goals: 1) to elucidate the nature of the inductive signal; 2) to understand how the inductive and lateral signals are integrated to specify cell type; and 3) to identify cell type-specific genes regulated by the intercellular signals. Toward these goals, we are studying genes that
control key steps in vulval induction. Three genes necessary for induction, lin-3, let-23, and let-60, are defined by mutations that cause the VPCs to all be 3° and hence prevent formation of a vulva, the "Vulvaless" or "Vul" phenotype (Abstract Nos. 157-161). We have found that let-60 encodes a ras protein whose activity controls VPC fates. Activity of let-60 is controlled by let-23 and lin-3. let-23 encodes a candidate receptor for inductive signal, and lin-3 encodes a candidate ligand for let-23. Mutations disrupting activity of a gene that promotes the 3° fate, lin-15, cause VPCs to become 1° or 2° in the absence of inductive signal, resulting in the formation of extra vulval tissue, the "Multivulva" or "Muv" phenotype (Abstract Nos. 164 and 165). By screening for new mutations that suppress the phenotypes of existing mutations, we have identified two new genes that appear to be negative regulators of let-23 (Abstract Nos. 162 and 163). To understand the cell biology underlying vulval induction, we have begun to characterize the ultrastructure of the anchor cell and the VPCs (Abstract No. 167). To identify new genes involved in vulval development, we are using a subtractive cDNA approach (Abstract No. 166).

Our second major focus has been on the development of the C. elegans male and its mating behavior (Abstract No. 171). The male tail is a specialized structure morphologically elaborated for mating. Development of the male tail involves a series of male-specific cell divisions that produce neurons, muscle and supporting cells. Since C. elegans hermaphrodites are internally self-fertilizing, fertile males are not essential for strain propagation, and thus male mating is amenable to genetic analysis. Our approaches are to study mutants defective in male development or behavior, to develop assays for various steps in male mating behavior, and to assign behavioral functions to each cell in the male tail (Abstract No. 170). The genes involved in signaling during vulval induction have defects in new examples of induction during male tail development (Abstract No. 169).

To understand the establishment of cell polarity, we are studying the 2° VPC cells (Abstract No. 168). We have continued our comparative developmental studies to investigate evolutionary changes in the plasticity of cell fate specification (Abstract No. 175). Our "reverse genetic" approaches have focused on G proteins (Abstract No. 172), the raf protein kinase (Abstract No. 173) and cell cycle control genes (Abstract No. 174).

157. Structure/function studies of let-23, a C. elegans receptor tyrosine kinase
Raffi V. Aroian, Paul W. Sternberg

The let-23 gene encodes a member of the epidermal growth factor (EGF) receptor tyrosine kinase family and is required for five aspects of development, including the hermaphrodite vulva. During vulval induction, let-23, which may be the receptor for the anchor cell inductive signal, is required for two opposing pathways: 1) stimulation of precursor cells to execute vulval rather than hypodermal fates, and 2) inhibition of the inductive process to prevent excessive induction. Also, the let-23 gene may function differently in different tissues since it is possible to obtain let-23 mutations that only disrupt its function in subsets of tissues. Consistent with these genetic results, members of the EGF receptor family have been shown to behave differently in different tissues and to be subject to negative regulation. Mammalian EGF receptor comprises several structural domains. Extra-cellularly, there is a ligand-binding domain surrounded by two cysteine-rich regions thought to form a "pocket." The cytoplasmic portion of the protein contains the tyrosine kinase domain, autophosphorylation sites, and a domain required for receptor internalization. To relate our genetic characterization of let-23 to what is known of EGF receptor structure and to broaden our knowledge of both let-23 and EGF receptor function, we are using hydroxylamine mismatch detection to localize and sequence the mutations associated with 22 let-23 alleles. Fourteen alleles behave like nulls, two behave like hypomorphs, four exhibit tissue-specific defects, and two are defective in the inhibitory vulval pathway. The sequence of these alleles might suggest: 1) residues critical for receptor function, 2) residues responsible for tissue specificity, and 3) residues involved in negative regulation.

We have localized 12 mutations and sequenced six of them. For example, two null mutations alter conserved residues in the tyrosine kinase domain, suggesting that these residues are functionally important. Another
mutation (n1045), which displays a temperature-sensitive defect in negative regulation during vulval induction, is located in an exon/intron boundary and results in both wild-type and C-terminally truncated receptors due to sloppy splicing. We hypothesize that the truncated receptors are either defective in negative regulation (i.e., a dominant positive receptor) or acts in a dominant negative fashion to titrate out wild-type receptor. We plan to use germline transformation of truncated receptor to distinguish between these two models. We have also sequenced the tissue-specific mutation sy97. This mutation also probably results in a truncated receptor. Comparison between the sy97 and n1045 truncations suggests that the C terminus includes regions important for receptor function and negative regulation.

158. The let-60 gene encodes a ras protein whose activity level specifies cell fates during C. elegans vulval induction

Min Han

Genetic analysis suggested that the let-60 gene controls the switch between vulval and hypodermal cell fates during Caenorhabditis elegans vulval induction (Han et al., 1990). We have cloned the let-60 gene and shown that it encodes a gene product identical in 84% of its first 164 amino acids to ras gene products from other vertebrate and invertebrate species. This conservation suggests that the let-60 product contains all the biochemical functions of ras proteins. We have used transgenic nematode technology to examine the effects of increased gene dosage of let-60. Injected DNA is catenated into extrachromosomal arrays that are stably maintained. Extrachromosomal arrays of let-60 ras DNA cause cell-type misspecification (extra vulval fates) phenotypically opposite to that caused by let-60 ras loss-of-function mutations (no vulval fates). High copy numbers of extrachromosomal let-60 ras gene also suppress the vulvalless phenotype of mutations in let-23 (receptor tyrosine kinase) and lin-3 (putative growth factor), both of which are necessary for vulval induction and act upstream of let-60 ras in the pathway (see Abstract Nos. 157 and 160). Thus, the level and pattern of let-60 ras expression may be under strict regulation; increase in let-60 ras activity bypasses or reduces the need for upstream genes in the vulval induction pathway.

Reference:

159. Analysis of dominant negative mutations of the C. elegans let-60 ras gene

Min Han

The let-60 ras gene of Caenorhabditis elegans controls vulval differentiation in response to an inductive signal. Dominant negative mutations of let-60 (let-60(dn)) cause a dominant vulvalless phenotype, and all but one allele also causes a recessive lethal phenotype. Determination of the molecular lesions of these let-60(dn) mutations suggests that all the let-60(dn) mutations likely disrupt the ability of the ras protein to bind guanine nucleotides. Our further study shows: 1) vulval induction and larval growth are very sensitive to changes of dosage of let-60 wild-type or mutant genes; 2) let-60-dependent pathways during early larval growth are less sensitive to decrease in let-60 dosage than is vulval differentiation pathway; 3) the dominant negative effect of let-60(dn) mutations can be suppressed by a gain-of-function mutant let-60 ras protein (Glu-13); 4) although all let-60(dn) gene products interfere with let-60(+) function, some retain their own activity. let-60(Asn-119 dn) product, on one hand, exerts the strongest dominant toxic effect on let-60(+) product; but on the other hand, it retains some constitutive activity. The dominant negative effect of these let-60(dn) mutations might be explained by a competition between the mutant and wild-type let-60 ras protein for another positive factor such as a guanine nucleotide exchange factor (GNEF). The ras protein has been proposed to be activated by GNEF from GDP-bound to GTP-bound state, and be down-regulated by a negative regulator such as the GTPase activating protein (GAP). In the absence of inductive signal, the GNEF catalyzed reaction is limiting, resulting in low ras-GTP concentration. In the presence of the signal (which likely down-regulates the GAP activity through let-23 receptor tyrosine kinase), GAP catalyzed reaction is limiting and resulting in an increase in ras-GTP amount. Therefore, let-60(dn) may titrate out GNEF (limiting) by forming a dead-end complex and a decreased ras(+)GTP amount even in the presence of the signal. On the other hand, high dosage of let-60(+) gene may overwhelm the GAP activity, resulting in a high ras-
GTP amount even in the absence of the signal and causing the gain-of-function mutant phenotype (multivulva).

160. lin-3 is a candidate for a vulval inducing signal
Russell J. Hill, Paul W. Sternberg

Genetic evidence suggests that lin-3 is a candidate for the inductive signal used in vulval development. The extent of vulval induction appears to be sensitive to the level of lin-3 activity. Lowered lin-3 activity results in a vulvaless (Vul) phenotype while multicopy arrays of lin-3 genes can cause a multivulva (Muv) phenotype in transgenic animals. Epistasis experiments suggest that lin-3 acts before let-23, which encodes a protein homologous to the epidermal growth factor (EGF) receptor tyrosine kinase and which is a candidate receptor for the inductive signal. First, the Vul phenotype of let-23 is epistatic to the Muv phenotype of lin-3 multicopy transgenes. Thus, lin-3 needs functional let-23 to stimulate vulval development. Second, let-23 mutations are epistatic to Muv mutations in the gene lin-15, while lin-15 mutations are epistatic to lin-3 mutations. This observation suggests that lin-3 acts before let-23. Moreover, the Muv phenotype of a lin-3 transgene requires the presence of the gonad, which is the source of the inductive signal. Thus, transgenic lin-3 is either made in the gonad or is dependent upon the gonad to be activated.

Our molecular cloning of lin-3 shows that it encodes a putative growth factor belonging to the family that includes TGFr. These growth factors are made as membrane-bound precursors, have a single EGF repeat, and include known ligands for the mammalian EGF receptor. Because the genetic properties of lin-3 indicate that it could act as a signal and because of the molecular structure of lin-3, we hypothesize that lin-3 acts as an inductive signal during vulval development.

161. The evil twins of lin-3 and let-23
Phoebe Tsou

Caenorhabditis briggsae and C. elegans are closely related species. The divergence of these two species has been estimated to have occurred 30 million years ago. Both species consist of males and hermaphrodites and are very similar in their morphology and vulval development. A comparative study of the two species at the molecular and cellular levels will yield insights into the functional domains of the lin-3 and let-23 gene products. lin-3 is a candidate for the inductive signal used in vulval development (see Abstract No. 160). let-23 encodes a protein homologous to the epidermal growth factor receptor tyrosine kinase and is a candidate receptor for the inductive signal (see Abstract No. 157). Comparison of the C. elegans lin-3 and let-23 gene products to their C. briggsae homologs should allow us to define regions of these proteins involved in ligand/receptor interactions. We have used low stringency hybridization to detect the C. briggsae homologs by probing C. briggsae genomic DNA with lin-3 and let-23 cDNAs. We have screened a C. briggsae genomic library and have purified two partially overlapping putative lin-3 lambda DNA clones. In addition, we are in the process of analyzing candidates for the C. briggsae let-23 homolog cloned by polymerase chain reaction.

162. The genetics of sli-1, a candidate negative regulator of vulval induction
Gregg D. Jongeward, Junho Lee

We have isolated more than 40 alleles of sli-1 (suppressor of lineage defect) as silent suppressors of the Vulvaless (Vul) phenotype of weak alleles of let-23. sli-1 mutations suppress the Vul, Male Abnormal and Lethal phenotypes of non-null alleles of let-23, but do not suppress the lethality of a null allele of let-23. Since these mutations are silent and do not suppress nulls, we believe that sli-1 is a negative regulator of let-23, rather than a target of let-23 activity. Certain sli-1 and let-23 alleles show allele-specific interactions, suggesting that these two gene products directly interact. let-23, sli-1 double mutants are generally Hyperinduced ("Hin"; more than three of the vulval precursor cells respond to the inductive signal in a gonad-dependent manner). Hin is believed to be a partial reduction-of-function phenotype of some of the Vul genes. sli-1 suppresses both hypomorphic and null alleles of lin-2 and lin-7 to Hin. Since the sli-1 allele used suppresses a strong hypomorphic let-23 allele to wild-type, sli-1 is not simply increasing the dosage of let-23 or another component of the vulval induction
pathway. sli-1 suppresses weak alleles of lin-3 but does not cause these animals to become Hin. sli-1 does not suppress let-60 or lin-45, two genes acting after let-23 in the vulval induction pathway. Therefore, sli-1 acts near let-23 in the signaling pathway. sli-1 and unc-101, another suppressor of the vulval defect of weak alleles of let-23, show a synthetic vulval phenotype, such that unc-101; sli-1 double mutants can display as much as 130% vulval differentiation, where 100% is wild-type. We believe that sli-1 is a partially redundant negative regulator of let-23 or some other early component of the vulval induction pathway.

163. Role of unc-101 in C. elegans vulval development
Junho Lee, Gregg D. Jongeward

We are interested in the genetics and molecular biology of unc-101 because of its broad range of phenotypes and its role in vulval development. unc-101 mutations confer an uncoordinated phenotype as well as subviability, defecation, male spicule and FITC staining defects. Two unc-101 alleles (sy108 and sy161) were previously isolated in a screen for suppressors of vulvaless phenotype of let-23. unc-101 mutations also can suppress lin-2, lin-7 and lin-10 vulvaless phenotype. lin-3 vulvaless phenotype is not suppressed by unc-101 mutations. A double mutant defective in both unc-101 and sli-1, which is another suppressor of let-23 vulvaless phenotype (Abstract No. 162), displays greater than wild-type induction of vulval precursor cells, while single mutants display wild-type induction. Thus, unc-101 is considered to be active redundantly with sli-1 as a negative regulator of let-23.

We isolated a putative null allele of unc-101. We used trimethyl-psoralen as a mutagen in a screen for mutations that fail to complement unc-101(rh6). One new allele, sy216, was recovered in a screen of 11,000 F1 cross-progeny hermaphrodites. This allele is a homozygous L1 larval lethal. Unlike the visible alleles, the lethality of this allele cannot be maternally rescued. Animals of the genotype sy216/sy108 are less viable than animals bearing sy108 in trans to any visible allele. The sy216/sy108 heterozygote is also a strong suppressor of the let-23(sy1) vulval defect. Therefore, we consider sy216 to be a putative null allele of unc-101. We believe that the null phenotype of unc-101 is lethality and suppression of let-23. sy216 does not delete unc-75, ced-1 and unc-59, nor does it overlap eDF3, consistent with it being a deletion only of unc-101.

We are currently trying to clone unc-101 using parallel restriction fragment length polymorphism (RFLP) mapping with a congenic strain. We obtained 37 Unc-59 nonUnc-75 recombinants from unc-75(e950) ced-1(n1506) + + unc-59(e290)/ + unc-101(sy108) + heterozygotes. By Southern hybridization analysis with Tcl as a probe, we found two RFLPs between ced-1 and unc-101. 1/5 of + + unc-59 recombinants had one of the RFLPs and 2/5 of + + unc-59 had both the polymorphisms. None of 12 + unc-101 unc-59 recombinants had any of the polymorphisms. In addition, we cloned two other RFLPs by inverse polymerase chain reaction (PCR) of the recombinant strain DNAs. By Southern hybridization, we mapped one of the inverse PCR RFLPs (RFLP E) to within ±0.6 m.u. of unc-101. This RFLP hybridized to three yeast artificial chromosome (YAC) clones in the standard ordered set of YAC grids. We will use the RFLPs and the YACs as a starting point for a genomic walk to clone this gene.

164. Molecular genetics of the lin-15 locus
Linda S. Huang, Phoebe Tzou

During vulval induction, lin-15 acts as a negative regulator of vulval cell fate specification. lin-15 hermaphrodites are Multivulva (Muv), such that cells that normally have a hypodermal fate instead have vulva cell fates. This phenotype is inductive-signal-independent and has been shown to be cell nonautonomous (Herman and Hedgecock, 1990).

lin-15 has three different classes of mutations: the mutations that cause a Muv phenotype mentioned above, as well as two silent classes of mutations, A and B (Ferguson and Horvitz, 1989; also see Abstract No. 165). Although there are other synthetic (syn) Muv genes elsewhere in the genome, lin-15 is the only genetic locus exhibiting both a class A and a class B phenotype. We have been unable to obtain null alleles of lin-15 with EMS at a typical frequency.

The molecular analysis of lin-15 suggests that lin-15
encodes two functions. We have rescued both class A and class B lin-15 mutations with a 15 kb plasmid. Deletions (>3 kb) of the lin-15 region are associated with all four N2-derived EMS alleles. Furthermore, eight of the EMS-derived lin-15 syn Muv alleles do not show any polymorphisms detectable on a Southern and only one of the lin-15 syn Muv alleles (n767 class A) shows a small 100 bp deletion. Also, when mutageneses are done for new syn Muv alleles of lin-15 starting with a syn Muv of the opposite class, our laboratory has observed that new lin-15 class A and B alleles are obtained at about 1/3500, a frequency that suggests we are getting knockout alleles. The surprising correlation of the visible Muv phenotype with the large deletions, along with the frequencies of obtaining lin-15 syn Muv alleles suggests that the lin-15 A and B alleles are defective in independently mutable functional regions. To get a visible Muv phenotype, an animal must either have two mutations (one A and one B) or a deletion that removes both the A and the B region. We have identified two noncrosshybridizing classes of cDNAs corresponding to the lin-15 rescuing region. One of the cDNAs resides in a region defined by microinjection as necessary for lin-15B function while the other cDNA is in a region that contains the small 100 bp deletion in the class A syn Muv allele. These two cDNAs pick up transcripts of about 7 kb and 3 kb, respectively, on an RNA blot. We are currently working to further define the molecular genetics of lin-15 and how it negatively regulates cell fate determination in the vulval induction pathway.

References:

165. The search for new class A synthetic Multivulva genes
Mimi Sengupta, Linda S. Huang

There are various genes throughout the Caenorhabditis elegans genome that confer a synthetic (syn) Multivulva (Muv) phenotype. Alleles of these genes have been classified as either class A or class B syn Muvs. This is a functional classification: class A and class B syn Muvs show no phenotype on their own or in combination with a syn Muv of the same class. It is only when a class A allele and a class B allele are combined that the Multivulva phenotype is seen. It is thought that these class A and class B mutations define genes in two functionally redundant pathways needed for negative regulation of vulval induction. To recover more genes in the class A pathway, we undertook an F2 screen for more class A alleles by mutagenizing lin-15 (n744), a class B allele. From about 7,000 mutagenized gametes, we isolated eight new mutations. Two of these mutations were new class A lin-15 alleles (see Abstract No. 164), three were new lin-8 alleles, and the other three are neither lin-15 nor lin-8 alleles and await further mapping and complementation experiments.

166. Molecular cloning and characterization of genes involved in C. elegans vulval development
Paul S. Kayne

Vulval development in Caenorhabditis elegans is initially confined to seven cells. A single gonadal cell (the anchor cell, AC) induces three of six hyperderrnal cells (vulval precursor cells, VPCs) to become vulval tissue. The cell closest to the anchor cell adopts the 1° fate. This cell will divide three times, producing eight descendants, and eventually attaches to the anchor cell. The two cells flanking the 1° cell adopt the 2° fate. They too will divide three times, but produce only seven cells each, which together form the remainder of the vulval structure. The three remaining cells are in the 3° or uninduced state. Induction by the anchor cell begins late in the second larval stage, and all vulval cell divisions are completed by the end of the third larval stage. Many of the genes involved in this pathway have been identified genetically. Several of these genes have now been cloned, including the anchor cell signal and its receptor. However, an unknown number of genes downstream from this receptor-ligand complex remain to be identified. It is possible that we can more readily identify these genes using a molecular screen.

We have employed a subtractive cDNA approach to identify new genes involved in vulval development. We have constructed two libraries from each of the following strains:

Strain 1 (plus strain): AC+; 1°+++; 2°+++  
Strain 2 (minus strain): AC−; 1°; 2°
To ensure that genes involved in vulval induction are active, we made the libraries from strains that have been synchronized in the L3 larval stage, at which time vulval development is occurring. The libraries made from strain 1, the plus strain, should contain all of the gene products involved in vulval development. These same gene products should be absent from strain 2, the minus strain. We then subtracted the minus libraries from the plus libraries. This procedure should enrich for genes involved in the vulval pathway, as the strains are virtually identical except for the vulval structure. We then screened the resulting subtracted library with radiolabeled probe made from the two minus libraries. Any colony from the subtracted library that hybridized with this probe must be present in the minus library and we therefore discarded it. We rescreened the remaining clones using Southern analysis, using probes made from both the plus and the minus libraries. To date, we have identified approximately 60 candidates that hybridize to the plus library probe, but hybridize more weakly, or fail to hybridize to the minus library probe. We are currently checking these candidates using Northern analysis with RNA isolated from both the plus and minus strains, to circumvent any problems with representation in the libraries.

167. Position and fine structure of cells involved in vulval induction and morphogenesis

Lynn K. Carta

We have examined the fine structure of the cells involved in vulval induction. Because cell membranes are not resolved with differential interference optics, observation of the proximity of gonadal and hypodermal cells to one another is needed to support models of vulval induction based on genetic and molecular biological evidence. The nature of the gonadal-hypodermal membrane attachment is also of interest in morphogenesis. The consistent space seen in electron micrographs between the gonadal anchor cell and the P6.p vulval precursor cell (VPC) during induction is consistent with the possibility of a diffusible signal from the anchor cell, although it must pass extracellular matrix. The proposed lateral inhibitory signal from VPCs could work through cell contact, as VPCs can be seen to touch during the early L3 when induction takes place.

168. Polarity in the vulval lineages of C. elegans

Wendy S. Katz, Paul W. Sternberg

The 2° vulval lineages produced by P5.p and P7.p normally produce an array of cell fates with a fixed polarity. This polarity is defined by the pattern of nuclear divisions and migrations. In a normal 2° lineage, the Pn.p granddaughters closest to P6.p fail to divide (denoted N in the figure) or divide transversely (T). The other 2° granddaughters normally divide longitudinally (L). The 2° nuclei migrate toward the primary cells before and after these divisions.

\[
\begin{array}{c|ccc}
\text{P5.p} & \text{P6.p} & \text{P7.p} \\
\hline
\text{LL NN TT TT NN NN LL} \\
\end{array}
\]

The 1° and 2° vulval lineages.

N: cell does not divide.
T: cell divides transversely.
L: cell divides longitudinally and adheres to the ventral cuticle.

Mutations in two genes, lin-17 and lin-18, abolish or reverse the apparent polarity of these lineages. The phenotypic consequence is the presence of two ventral protrusions (bivulva), or a single large protrusion at the vulva. We are investigating the role of lin-18 in establishing and orienting the polarity of the 2° lineages.

We have isolated a deletion of the lin-18 locus and characterized existing lin-18 alleles, to help determine the null phenotype. The two existing lin-18 alleles are incompletely penetrant. When placed in trans to a deletion of the lin-18 locus, the phenotype is more severe than in animals homozygous for either allele. Therefore, the existing alleles are probably not complete loss-of-function alleles, and they would be viable in trans to a loss-of-function allele. We are now using these alleles to screen for null alleles.

To clone lin-18, we have genetically constructed strains marked with restriction fragment length polymorphisms in the lin-18 region. We are now mapping the polymorphisms and will use those most closely linked to lin-18 to help clone the gene.

We are also performing cell ablation experiments to investigate the roles of communication between the gonad and the vulval precursor cells (VPCs), and between the
VPCs themselves, in orienting the polarity of the 2° lineages. \textit{lin-12(d)} mutants form six 2° lineages, even in the absence of inducing signal from the gonad. If the gonad is left intact, the polarities of these lineages tend to be oriented toward the center of the gonad. If the gonad is destroyed by laser ablation, all six lineages are posteriorly-oriented. We are now ablating specific cells in the gonad to determine which are necessary for anteriorly-oriented 2° lineages.

169. Induction during male spicule development
Helen M. Chamberlin, Paul W. Sternberg

We have begun to characterize cell interactions that specify cell fates in the male-specific blast cell B, which generates the spicules in the male tail. The fates of two pairs of bipotent cells, α/β \([B.a(l/r)aa]\) and γδ \([B.a(l/r)pp]\), depend on their relative positions. Thus, they must respond to spatial cues. Our cell ablation experiments indicate that other male-specific blast cells, F and U, provide at least part of such spatial cues. In a wild-type male, F daughters are positioned immediately anterior to the γδ pair (next to γ) and U daughters are anterior to α/β (next to α). Ablation of both F and U results in three B lineage defects: 1) transformation of α to β, 2) abnormal truncated γ lineages, and 3) abnormalities in B.a(l/r)ap. In single ablations of either F or U, the B lineage is either wild-type or occasionally abnormal, with truncated γ lineages (for F·), or α to β transformation (for U·). Variation in the lineages of F·U· ablated animals suggests that we have not completely eliminated the inductive signal(s). However, we have clearly identified candidates for inducing cells in the α/β and γδ equivalence groups.

Genes identified for their role in vulval induction are also involved in induction of the α and γ fates. The lineage defects in mutant males suggest that, as in the vulva, there are two classes of mutations that give two opposite phenotypes. Vulvaless class mutations \textit{lin-3}, \textit{let-23}, \textit{lin-45} and \textit{let-60(dn)} result in transformation of α to β, truncated γ lineages, and abnormalities in B.a(l/r)ap. Thus, the Vulvaless mutations mimic F·U· ablutions in the male B lineage, in the same way that they mimic anchor cell ablation in the hermaphrodite vulva. In contrast, Multivulva class mutations \textit{lin-1} and \textit{let-60(gf)} result in rare β to α transformations and extra divisions in δ.

170. Some male-specific neurons and their role in male mating behavior
Katharine Liu

In addition to the 294 neurons that it shares in common with the hermaphrodite, the \textit{Caenorhabditis elegans} male possesses an additional 87 sex-specific neurons. These additional neurons likely mediate mating behavior, which only the males exhibit. Male mating is a complex behavior that can be broken down into a number of steps. Upon encountering the hermaphrodite: 1) the male responds by backing along the length of her body with his tail; 2) avoids her head and tail by turning over or under her near each end of the body; 3) locates the vulva, at which point he stops; 4) slides back and forth until he inserts his spicules into the vulva; and 4) ejects sperm.

To understand the molecular and neuronal basis of these steps, we have undertaken two approaches: genetic analysis (see Abstract No. 171) and cell ablation. By ablating the male-specific cells, we hope to elucidate their role in male mating behavior via the resulting behavioral defect, if any. Ideally, one would want to ablate cells suspected of participating in the same circuit. However, since reconstruction data identifying possible neuronal connections are not available for the male, we have chosen to follow a lineal approach. That is, male-specific post embryonic blast cells that give rise to male copulatory structures and/or neurons are ablated and the resulting phenotype (mating defect), if any, is determined by observation. Given a defect, the progeny of that blast cell are then systematically ablated until the neuron(s) responsible for the phenotype is identified. This approach has been begun for several structures/lineages with the results shown in Table 1 below.

The fact that we have been able to identify structures/neurons involved in each step outlined above and mutants that affect each step (see Abstract No. 171) strongly indicates that they are valid classifications, delineating subsets of neuronal circuitry. In addition, both ablation results and mutants have indicated that additional substeps may be involved. For instance, ablation of SPVR/L results in some males that while failing to insert their
spicules, eject their sperm anyway outside the vulva. One mutation, *sy192*, has the opposite phenotype. Males do not eject sperm even after successful spicule insertion. Thus, completing the step of spicule insertion and moving on to the next requires not only the physical act but also some feedback to inform the male of his success or lack thereof. SPCR/L, spicule motor neurons, likely mediate the former while SPVR/L, spicule sensory neurons, might at least partly mediate the latter. In the future, we plan to complete this type of analysis for all the male-specific neurons to determine their role in mating behavior.

<table>
<thead>
<tr>
<th>Cell ablated</th>
<th>Structure generated</th>
<th>Behavior affected</th>
<th>Neurons responsible</th>
</tr>
</thead>
<tbody>
<tr>
<td>V5.p and V6.p</td>
<td>V rays</td>
<td>Response to herm.</td>
<td>Not known</td>
</tr>
<tr>
<td>T.ap</td>
<td>T rays</td>
<td>Turning around herm.</td>
<td>Not known</td>
</tr>
<tr>
<td>P10.p</td>
<td>Hook</td>
<td>Vulval location</td>
<td>HOA and HOB</td>
</tr>
<tr>
<td>B.a</td>
<td>Spicules</td>
<td>Spicule insertion</td>
<td>SPC, SPD, SPV</td>
</tr>
</tbody>
</table>

114. Isolation and characterization of copulation-defective mutations

*Yvonne Hajdu, Katharine Liu, Helen M. Chamberlin, William Boorstein*

In *Caenorhabditis elegans*, males but not hermaphrodites exhibit a complex mating behavior, which may be mediated in part by 79 male-specific neurons. This behavior is dispensible due to the hermaphrodite's ability to self-fertilize. In a background of *him-5* (which causes a 33% segregation of males from self-fertilizing hermaphrodites), we are able to isolate and maintain mutations that interfere with male mating ability, both morphological mutants (such as abnormal or missing copulatory structures) and neuronal or behavioral mutants.

Wild-type male mating consists of at least five steps described in Abstract No. 170. To study the genetic basis for male mating behavior, we are isolating and characterizing copulation defective (Cod) mutations. To date, we have screened 4517 F2 clones using a procedure first described by Hodgkin (1983). In this screen, *him-5* animals are mutagenized with EMS, and 20 parents are placed on individual plates and allowed to self. Ten F1 hermaphrodites are pooled from each parent, and 10 F2 progeny are cloned from each F1 pool. The F3 progeny of each F2 line were assayed for mating efficiency (ME) with a qualitative test: 6 males x 6 *unc-52* hermaphrodites (which are paralyzed at adulthood). Those lines that "failed" the test twice with a ME of ≤1% of wild-type were saved and examined at 1250X under Nomarski optics for morphological defects in their copulatory structures. About 25% of all morphologically wild-type lines backcrossed successfully, yielding 28 Cod strains, 12 of which have been assigned to linkage groups.

We assayed the mating defect of each Cod mutant by observing which behavioral step the males failed to perform. Every step in the wild-type mating pathway has at least one corresponding Cod mutation blocking the behavior. *syl66, syl72, syl78, and syl195* fail to respond to the hermaphrodite. *syl95* is especially defective; the males will often crawl away from hermaphrodites and food to die on the side of the plate, a phenotype known as "suicidal males." *sy35* males cannot turn around the head and tail of the hermaphrodite. *sy181* males cannot crawl backwards well, either alone or on the hermaphrodite; instead their tails curl up like a fish hook. (*sy181* hermaphrodites, however, move normally.) *syl57* and *syl174* cannot locate the vulva. *sy38, sy43, syl53, syl56, syl158, syl165, syl176, syl177, syl190, syl191, syl193, and syl194* cannot insert their spicules. *sy36* and *sy192* males cannot transfer sperm. Several mutants, *syl79, syl80, syl84, and sy186*, seem to mate normally, but sire very few progeny. Either they also cannot transfer sperm successfully or their sperm is defective. Finally, one mutant, *sy201*, cannot always retract his spicules, which are left dangling. The fact that we could isolate mutants defective in single steps argues...
that the assignment of male mating behavior into these steps is not arbitrary, but may reflect separate neuronal components underlying each step.

The Cod mutants were examined by polarized light microscopy for abnormalities in the 41 male-specific muscles. sy35, a turning mutant, had aberrant migration of muscle precursor cells, resulting in sex muscle structures located anterior of the male tail (near where the vulva would be if the animal were a hermaphrodite). sy35 hermaphrodites are egg-laying defective, suggesting abnormalities in the uterine or vulval muscles as well. A second mutant, sy43, which fails to insert the spicules, lacks one set of male-specific muscles. syl78, a response mutant, exhibits disorganized myofilament structure in the body wall muscles, though the sex muscles appear normal. Most of the Cod mutants appear to have wild-type muscles and thus are strong candidates for neuronal mutants.

The Cod screen also yielded 15 morphological mutants, whose phenotypes include crumpled spicules, abnormal rays, and a gonad-migration defect. Some of these mutations define new genes, and others are in known genes, including mab-5, vab-3, and lin-25. Four mutants displayed a novel spicule phenotype: the spicules do not autofluoresce, are extremely flexible and, when protracted, flatten out and dangle limply.

Reference:

172. G protein \( \alpha \) genes in \textit{C. elegans}

\textit{Jane E. Mendel, Michael A. Lochrie, Melvin I. Simon, Paul W. Sternberg}

Guanine nucleotide binding proteins, or G proteins, are signal transducing molecules that couple activated cell-surface receptors to intracellular effectors. These G proteins are heterotrimeric made up of \( \alpha, \beta \) and \( \gamma \) subunits. Each subunit exists in multiple forms, with \( \alpha \) showing the greatest diversity. The \textit{in vivo} function of this multiplicity of G proteins is not understood. We are using reverse genetic techniques to analyze the function of several G protein \( \alpha \)-subunit (G\( \alpha \)) genes in \textit{Caenorhabditis elegans}.

Using polymerase chain reaction-based technology as well as standard cDNA and genomic library screens, we have isolated four nematode G\( \alpha \) genes. One of them, \textit{goa-1}, is a homolog of mammalian and \textit{Drosophila} G\( \alpha \), the other three, \( \text{gpa-1}, \text{gpa-2} \) and \( \text{gpa-3} \), appear to be unique to \textit{C. elegans}. We have generated a complete sequence for the cDNA corresponding to \textit{goa-1}, and complete genomic sequences for \textit{gpa-1}, 2, and 3.

We have fused the 5' flanking regions of \textit{goa-1}, \textit{gpa-1}, \textit{gpa-2}, and \textit{gpa-3} to the \textit{E. coli} lac\( Z \) gene using modular vectors (Fire \textit{et al.}, 1990). The staining pattern for the \textit{gpa-1-lacZ} construct indicates that \textit{gpa-1} is expressed in a large number of neurons, both in the pharyngeal nerve ring (the main region of neuropil in \textit{C. elegans}) and the ventral cord. This apparent expression pattern is consistent with the observation that G\( \alpha \) is abundant in neural tissue in other organisms. The staining pattern for the \textit{gpa-1-lacZ} construct indicates that \textit{gpa-1} is expressed in a small number of cells in the pharynx, four cells in the pharyngeal nerve ring, and what appears to be one of the plasmid neurons. In males, staining is also seen in at least one of the spicule neurons. The \textit{gpa-2-lacZ} fusion is expressed in several cells in the pharyngeal nerve ring and in a single cell in the pre-anal ganglion. The \textit{gpa-3-lacZ} fusion is also expressed in several cells in the pharyngeal nerve ring, as well as two pairs of neurons in the tail, posterior to the anus.

These experiments indicate that expression of these G\( \alpha \) subunit genes is confined predominantly to neural tissue. These genes show a great deal of specificity in expression patterns: although all four genes are expressed in the pharyngeal nerve ring, they do not always appear to be expressed in the same neurons. The specificity of expression in the tail is more striking: for example, \textit{gpa-1} but not \textit{gpa-2} or \textit{gpa-3} is expressed in the male spicules. There may also be some overlap in expression, particularly between \textit{goa-1} and the other three genes.

Our efforts in the future will include confirming the lac\( Z \) expression studies with antibody staining, using mutant and ablated animals to precisely identify the staining cells, expressing bacterial toxins such as pertussis and diphtheria with the G\( \alpha \) promoters to determine phenotypes, and attempting to rescue known mutant phenotypes by microinjection of the native G\( \alpha \) genes.

Reference:
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173. A raf homolog in C. elegans
Andy Golden, Phoebe Tzou

Molecular genetic studies have recently demonstrated that three genes essential for vulval development encode products that share remarkable sequence similarity with mammalian signal transduction proteins, including the epidermal growth factor (EGF) (lin-3), EGF receptor (let-23), and the ras gene products (let-60). Studies from other systems have revealed that the EGF receptor functions as a tyrosine-specific protein kinase and, upon ligand binding, transduces these extracellular signals by associating with and/or phosphorylating a number of intracellular proteins. For example, the EGF receptor (as well as other growth factor receptor tyrosine kinases) physically associates with a raf serine/threonine-specific protein kinase. The raf gene product is a cytoplasmic kinase that acts downstream of the receptor tyrosine kinases and the ras gene products, and may be the link between signals at the membrane and at the nucleus.

Using polymerase chain reaction-based technology and degenerate primers designed from conserved regions in the raf kinase domain, we have amplified a cDNA fragment. Using this fragment as probe, cDNAs were obtained from a nematode cDNA library. The longest of these cDNAs appears to code for the entire raf kinase. This Caenorhabditis elegans raf homolog (Ce-raf-1) shares 60% identity in its kinase domain with the human raf family (raf-1, A-raf, and B-raf) and Drosophila raf-1 and is distinct from the nematode daf-1 receptor kinase. Ce-raf-1 also contains the two conserved regions (CR1 and CR2) in its amino-terminal regulatory domain. The CR1 domain contains a "cysteine-box" that has been proposed to form a zinc finger important for binding to regulatory molecules. The CR2 domain is a short serine/threonine-rich region which has been highly conserved among all members of the raf family. By Southern analysis, this nematode gene is single copy.

We have determined the physical map location of the C. elegans raf homolog by hybridization to "ordered" yeast artificial chromosome grids. This gene maps on chromosome IV, near and to the right of unc-44. We are currently attempting to identify a genomic clone of the raf gene so that we can construct variants of the Ce-raf-1 gene. We will test these variant transgenes in wild-type animals and in animals defective in vulval development. These experiments will be performed to determine whether the Ce-raf-1 gene acts in this developmental pathway. In addition, we will attempt to rescue, by complementation, animals defective in known genes that map in this chromosomal region.

174. Molecular cloning of C. elegans cell cycle genes
Hiro Mori

We plan to use the nematode to study how signal transduction events (e.g., the pathway controlling vulval induction) regulate cell division. As the first step of this project, genes that are known to be important for cell cycle control, cdc2 and string/cdc25 homologs, were cloned by polymerase chain reaction. The serine/threonine kinase encoded by cdc2 plays a central role in both G1/S and G2/M transitions. Using a set of degenerate oligos, a nematode homolog of cdc2 was identified and subsequently used to isolate a full length cDNA. Preliminary sequence data have revealed a high degree of overall identity between this gene and other known cdc2 genes, the only surprise being the Ile to Val substitution in the otherwise absolutely conserved "PSTAIR" motif. This gene, tentatively named ncc-1 (for nematode cell cycle), has been physically mapped to the third chromosome.

A similar approach has been taken to clone the nematode homolog of string/cdc25, which has recently been shown to encode a putative tyrosine (and serine/threonine?) phosphatase that activates the p34cdc2. Preliminary data suggest that the cDNA isolated thus far encodes a gene with significant sequence similarity to known string/cdc25 homologs. Quite by coincidence, one of the four genes netted so far by the pilot program for the nematode genome project turned out to be another homolog of string/cdc25 (personal communications, M. Craxton, MRC, UK; and P. Green, Washington University, St. Louis). The limited similarity between the two homologs (~50% in the commonly sequenced region) may be indicative of the functional and/or expression pattern differences.
A collection of the Rhabditida: Candidates for developmental genetics

Lynn K. Carta, Paul S. Kayne, Paul W. Sternberg

To examine the evolutionary transition between conditional and nonconditional specification of cell fate, and between symmetric and asymmetric structures, we have initiated comparative developmental genetic studies of nematode development. We will also be evaluating the relative importance of the different superficial morphological characters with variations in vulval cell lineages.

We have started to collect free-living soil nematodes that have the potential for genetic analysis by virtue of being hermaphroditic with functional males, and that have interesting differences in their anatomy or development. These have been tentatively identified based on stomata, esophageal and male tail characteristics to include Rhabditis sp., Pellioditis sp., Dolichorhabditis sp., a Saproloba sp., and at least two other Rhabditids with leptogrion tails where the spike extends beyond the fan. We also have a Panagrolaimus sp. and at least three isolates of Cephalobus and Zeldia, all of which could not be heat-shocked for males.

For example, Rhabditis sp. (PS70) has 3° vulval cells generating four rather than two progeny as in Caenorhabditis elegans, and 2° lineages generating only four nuclei rather than seven. Either Z1 or Z4 can produce an anchor cell like C. elegans but unlike Panagrellus sp., Pellioditis sp. has an invariant P3.p fate, in contrast to most other species examined. The vulval cell lineages of these nematodes will be used to test the reliability of the hierarchy of anatomical characters used in current classification schemes.

PUBLICATIONS


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Summary: Eukaryotic genes contain introns that interrupt the continuity of the gene. For these genes to be expressed, the intron sequences must be removed. This is done in an RNA processing step called splicing in which the intron is precisely clipped from an RNA precursor and the exons rejoined to give the functional RNA. We have been studying two kinds of splicing: tRNA splicing and mRNA splicing. Our studies are done with the yeast *Saccharomyces cerevisiae* for reasons of the easy genetic and biochemical studies that are possible with this simple eukaryote.

In tRNA splicing, the small introns located in the anticodon loop of about 20% of yeast tRNA genes are removed in a splicing process that requires three enzymes: an endonuclease that clips out the intron, a ligase which joins the resulting two half molecules together and a transferase which transfers a 2' phosphate left at the splice junction to the cofactor nicotinamide adenine dinucleotide (NAD). During the past year, we have made two important discoveries through our studies of the first two of these enzymes. The endonuclease, Feng Miao has found, can cleave the precursor by either of two routes in which the 5' splice site is cleaved first and then the 3' splice site or vice versa. Incorporation of phosphorothioate nucleotides (in which a non-bridging oxygen is replaced by sulfur) into the tRNA precursor can in some cases lead to a selective block in cleavage at one of the splice sites. This technique offers an approach to the understanding of the mechanistic chemistry of the cleavage reaction. Shawn Westaway, in collaboration with Chris Greer's laboratory at UC Irvine, has discovered that ligase unexpectedly has two nucleotide binding sites, one for ATP and one for GTP. Since ligation requires that two high energy bonds be cleaved, this result raises the possibility that both ATP + GTP hydrolysis function in the joining reaction.

In pre-mRNA splicing, we have continued with our effort to understand the assembly of the spliceosome, the components required for splicing and the role of the small nuclear RNAs (snRNAs) in the reaction.

The genetic approach to the understanding of pre-mRNA splicing has been particularly fruitful. In all, more than 30 genes have now been identified but this is certainly not a complete list. We are continuing to search for more splicing mutants by screening a bank of 4000 additional temperature-sensitive mutants (originally we had screened a bank of 1000 ts mutants). This screen should be completed this fall. In the meantime, we continue to study the properties of several of the mutants found earlier. One of the most interesting generalizations to emerge has been the involvement in splicing of a set of proteins called "RNA helicases." These proteins are related in sequence to a translation initiation factor EIF4-A which has been shown to unwind double-stranded RNA in an ATP-dependent reaction. These RNA helicases are found from *E. coli* to man. In studying the yeast splicing "helicases," we have discovered two groups. The first, the DEAD (for a conserved amino acid sequence in the ATP binding pocket), is characterized by a central region of homology of ~400 amino acids. The proteins can be much larger with extension at both the N and C termini. PRP5 and PRP28 are members of this group of proteins and there are at least 13 more DEAD genes in yeast with
different or unknown functions. The other class of "helicase" is the DEAH group which is weakly homologous to the 400 amino acid core of the DEAD proteins and share another 300 amino acids of homology downstream from this core. PRP2, PRP16 and PRP22 are members of this group, and there are at least two more of these genes in yeast with unknown function. We have currently cloned and sequenced the genes for PRP5 (Gloria McFarland), PRP28 (Tien-Hsien Chang), PRP22 (Mahshid Company and Jaime Arenas), CA3 (Jaime Arenas) and DBP3, 4, 5 and 6 (Christine O'Day).

In splicing, these proteins presumably act to alter the secondary structure of RNA in the spliceosome, either the pre-mRNA, the snRNAs or the interactions between these, but in no case has the true substrate of any of these proteins been identified. We assume that each protein has a very specific substrate and that the action of the "helicase" on that substrate mediates a transition step in the assembly of the spliceosome. Figure 1 shows the steps believed to be mediated by each of the five "helicases" involved in splicing. Interestingly, the DEAH proteins PRP2, PRP16 and PRP22 mediate sequential steps at the end of the pathway.

We have currently expressed the PRP5, PRP28 and DPB5 genes in E. coli and have purified the proteins to homogeneity. Each of them is a very weak RNA-dependent ATPase with turnover numbers of one per minute or less. DPB5 is a small protein constituting only the 400 amino acid conserved motif. We hope to determine its three-dimensional structure in collaboration with Doug Rees's group in the Division of Chemistry and Chemical Engineering. This should lead to a better understanding of how this class of proteins functions.

We continue to study the function of the snRNAs in splicing. Complementation assays have been worked out for U2, U5 and U6. These assays allow the in vitro study of mutant variants of each of the RNAs. Previously, this assay was used to study simple nucleotide variants of the RNAs or of deletion or insertion mutations. In the future we hope to make more sophisticated variants of the RNAs. A preliminary approach of this kind was done by Patrizia Fabrizio who substituted phosphorothioate for normal nucleotides in U6 RNA and tested the function of these substituted RNAs in the complementation assay.

Figure 1. ATP and mRNA splicing. The spliceosome assembly pathway is outlined to indicate steps in which ATP is required. ATPase (PRP16) and putative ATPases are members of the "DEAD-box" (PRP5, PRP28) and "DEAH-box" (PRP2, PRP16, PRP22) protein families. The precise step at which PRP28 acts is not yet known. Two additional DEAD-box proteins have been implicated in splicing (DED1/SPP81 and DBP1). The U1 and U4 snRNP's are shaded to indicate their possible loose association with the spliceosome.
Interestingly, the incorporation of (α-35S)CTP into U6 RNA in place of CTP has no effect, (α-35S)GTP and (α-35S)UTP block the first step of splicing and (α-35S)ATP substitution blocks the second step of splicing. The latter result suggests that a phosphate group in U6 has a crucial role in the second step of splicing perhaps through its interaction with a protein or a metal ion. We are currently working out techniques to place single nucleotide analogs into transcripts that can also be used for crosslinking experiments. This will enable us to do a wide variety of experiments directed at understanding the mechanism of the reaction and the location of crucial residues in snRNAs or in the intron relative to other spliceosome components.

We have continued our project to understand the specific recognition of tRNAs by their cognate aminoacyl-tRNA synthetases (AAS). Jeffrey Sampson and Margaret Saks have done a comprehensive study of the recognition of tRNA Ser by Ser AAS. This study builds on and greatly extends the in vivo approach started by Jennifer Normanly who successfully switched the identity of a tRNA Leu amber suppressor to serine identity. A part of their study has focused on the recognition of the acceptor stem. Their results emphasize the importance of G73 and the two base pairs 2-71 and 4-69. The latter base pairs had not been implicated as an identity element in Normanly's work. We now realize that even weak tRNA substrates can be efficient and faithful amber suppressors provided that they are not recognized by other competing AAS. Eventually, we hope to understand in detail the chemical requirements for Ser AAS to recognize its substrate. George Komatsoulis is taking a combined in vitro/in vivo approach to study tRNA Cys identity and Giuseppe Tocchini-Valentini is doing the same for tRNA Leu.

176. Phosphorothioates incorporated into yeast pre-tRNAs specifically inhibit 5' or 3' site cleavage

Reinhard Rauhut

Phosphorothioates used in in vitro transcriptions of RNA are known to generate RNA transcripts that closely resemble the natural nonsubstituted RNA molecule under structural aspects. At the same time, the replacement of an oxygen with a sulfur atom provides a tool to investigate mechanistic aspects of the chemistry of an internucleotide bond involved in recognition or catalysis. We recently purified the nuclear membrane protein tRNA-splicing-endonuclease which catalyzes the endonucleolytic removal of introns from nuclear precursor tRNAs. To analyze the biochemistry of this unusual three-subunit enzyme, addressing questions like order of cleavage events, number of catalytic centers involved, and differences in the chemistry of the two cleavage sites, we use pre-tRNAs transcribed in the presence of α-S triphosphonucleotides in tRNA-splicing reactions with homogeneous tRNA splicing endonuclease. We observe that these transcripts in general are fully functional substrates in the splicing reaction. In several cases, we observe a specific total block of either the 5' or the 3' cleavage, depending on the nucleotide-analog incorporated and the type of tRNA precursor used. Using new sets of pre-tRNA mutants specifically designed to alter the chemistry of the splice sites, we are trying to put together a more complete picture of this particular RNA-protein interaction.

177. Yeast tRNA endonuclease cleaves precursor tRNA in a random pathway

Feng Miao

In the yeast Saccharomyces cerevisiae, about one-tenth of tRNA genes coding for nine different tRNAs have introns. The removal of these introns from tRNA precursors is catalyzed by a tRNA splicing endonuclease that cleaves the precursor tRNA at the two splice sites, generating 5' exon and 3' exon that are subsequently joined by a tRNA ligase. The question I addressed is whether the endonuclease cleaves pre-tRNA in an obligatory or random order.

Truncated pre-tRNA^{Phc}, exons (3' exon, 5' exon) and "2/3" molecules (intron-3' exon and 5' exon-intron) were prepared for the purpose of performing two kinds of experiments. One is ligation of "2/3" with exons to produce the 3' and 5' splice site-blocked pre-tRNA separately by yeast tRNA ligase which provides a 2' phosphate at the ligated junction site. The other is annealing of "2/3" with exon to produce two presumable intermediates: 3' and 5' splice site-cleaved pre-tRNA. Using tRNA endonuclease assay, we found that 1) whichever splice site is blocked, the endonuclease can cleave the other unblocked site of pre-tRNA, indicating two splice sites are cleaved independently, and 2) the
endonuclease can further cleave both the 3' and 5' cleaved pre-tRNA. At the same time, two intermediates observed in kinetics studies provided the additional evidence. These results support that the endonuclease cleaves pre-tRNA in a random order; i.e., there are two routes for removal of intron from pre-tRNA.

The cleavage rates of the 3' and 5' splice sites are compared in several ways. The results showed that the endonuclease cleaves the 3' and 5' sites at almost the same rate in the first cleavage, whereas in the second cleavage the 3' site is cleaved faster than the 5' site, indicating that the rate of the second cleavage is inhibited or enhanced, depending on which splice site is cleaved in the first cleavage. Therefore, the rates of the two routes for cleavage are unequal.

178. Purification of E. coli 2'-5' RNA ligase
Eric A. Arn

All eubacterial transfer RNA (tRNA) genes characterized to date have been found to be free of intervening sequences of the nuclear tRNA type. There appear to be no obvious requirements in these organisms for the specific endoribonuclease and RNA ligase activities necessary for the splicing of introns from eukaryotic nuclear and archaeabacterial tRNA transcripts. Previous research in this laboratory, however, has identified an activity in extracts of a wide range of eubacteria that is capable of ligating the tRNA 1/2 molecules produced by cleavage of yeast tRNA precursors with yeast tRNA endonuclease (Greer et al., 1983). In this reaction, *Escherichia coli* extracts produce a novel 2'-5' phosphodiester bond at the ligation junction. The ligating activity has been shown to be protease-sensitive and ATP- or NAD-independent; it appears to require the 2'-3' cyclic phosphate and 5' hydroxyl end groups which are the products of tRNA endonuclease. It is capable of ligating the 1/2 molecules of only a subset of yeast tRNA precursors produced *in vivo*. The enzyme is able to ligate pre-tRNA-derived substrates transcribed in yeast nuclear extract, but shows only negligible activity towards *in vitro* transcripts produced with viral RNA polymerase unless they are subsequently incubated in yeast or *E. coli* extracts competent for nucleoside modification. The enzyme can also relegate *E. coli* tRNA<sup>Lys</sup> transcripts that have been modified and nicked in the anticodon loop by activities found in *E. coli* S-30 extracts. The ligase has been purified several hundredfold from an S-100 supernatant of *E. coli* extract through ion-exchange chromatography on DEAE-Sepharose and cellulose phosphate, affinity chromatography on Cibacron blue dye and tRNA-Sepharose resins, and hydroxylapatite chromatography. The resulting highly purified fraction contains two detectable polypeptides of molecular weight 13 and 18 kDa which copurify through all chromatography steps but display slightly different sedimentation characteristics on glycerol gradients at moderate salt concentrations. This fraction also contains a nucleolytic activity capable of digesting mRNA transcripts produced *in vitro*. It has not yet been possible to unambiguously assign the two activities to specific polypeptides. The amino termini of both proteins present in the purified fraction have proven unsuitable for direct sequencing. A large-scale preparation of ligase from 4 kg of *E. coli* cell paste is currently under way to produce enough purified material for digestion and isolation of internal peptides for sequence determination. The peptide sequence will be used to design oligonucleotides for cloning of the gene by reverse genetics, providing the starting point for mechanistic and structural studies as well as elucidation of the *in vivo* substrate and function.

Reference:

179. Characterization of two new members of the DEAH family of putative RNA helicases in *Saccharomyces cerevisiae*
Jaime Arenas

A large number of genes have been identified from *Escherichia coli* to man that belong to a highly conserved family of putative RNA helicases. The proteins encoded by these genes share extensive homology over a 300 amino acid domain containing several highly conserved motifs including the invariable sequence D-E-A-D. Two members of this group (the "DEAD family") have been shown to possess an RNA-dependent ATPase and RNA helicase activities *in vitro*, the mouse EIF4-A and the human p68 nuclear protein. In addition to the putative RNA helicase domain, the members of the DEAD family have variable amino- and C-terminal domains that are
unique to each protein. Members of this group play roles in several RNA metabolism pathways including translation, ribosomal RNA metabolism and nuclear mRNA splicing. Two genes from the DEAD family cloned in our laboratory are required for yeast nuclear pre-mRNA splicing, PRP5 and PRP28. A related group of genes has been uncovered in yeast. These encode the PRP2, PRP16 and PRP22 proteins, all three of them required for nuclear pre-mRNA splicing. In these proteins, the conserved boxes in the putative RNA helicase domain are a variation of those found in the DEAD family. Thus, this group has been named the "DEAH family." Interestingly, contrary to the DEAD family, the homology of the DEAH proteins is extended in an additional 300 amino acid C-terminal domain.

The role of ATP in pre-mRNA splicing is unknown. However, the requirement for at least five putative RNA helicases in yeast may explain in part the ATP requirement. Although the specific role(s) of RNA helicases is also unknown, it is likely that they regulate several RNA-RNA transactions that occur during the splicing process. We are interested in the possibility that additional putative RNA helicase genes might be required for splicing in yeast.

In order to identify new members of the DEAH family in yeast, I have performed polymerase chain reaction (PCR) experiments using degenerated oligonucleotides corresponding to the invariable boxes of the putative RNA helicase domain. Sequence screening of the cloned PCR products revealed two new members of the DEAH family, named JA1 and JA2. I have obtained both genomic clones from a YCP50 yeast genomic library and completed full sequence of JA1. Experiments are currently being done to test the essentiality of these genes. In order to find out in which process these genes are involved, I will attempt to make temperature-sensitive mutations by site-directed mutagenesis targeted to several sites in the putative RNA helicase domain. Overexpression of these genes in E. coli will provide a source of protein for in vitro studies with wild-type and mutant gene products.

180. Studies of the role of "Dead Box" proteins in *Saccharomyces cerevisiae*

*Christine L. O'Day*

Recently, a family of proteins has been identified by their conserved amino acid motifs. One conserved amino acid motif, VLDEAD, has coined them the name "Dead Box" proteins. Several members of this family (EIF4-A, SmB and P68) have been demonstrated to possess ATP-dependent helicase activity and/or RNA-dependent ATPase activity, thus giving this large family their putative identity.

Three new Dead Box genes have been identified by polymerase chain reaction techniques, expanding the number of putative helicases found in *Saccharomyces cerevisiae* to 15. We have termed these genes CA9/DBP4, CA10/DBP5 and CA11/DBP6 (Dead Box protein). A previously identified gene, CA4/DBP3, has also been examined. These four genes have been cloned and sequenced.

DBP6 is essential for growth on a non-fermentable carbon source, suggesting that it affects mitochondrial function. It is currently being tested against 210 complementation groups to determine if it is an already established mitochondrial mutant. DBP6 also shares extensive homology to RM31B, a protein expressed during oogenesis in *Drosophila*.

DBP3, DBP4 and DBP5 are essential for cell survival. To investigate the physiological function of these putative RNA helicases, the respective genes were put under galactose-inducible promoter control. By switching growth from a galactose to a glucose medium, the diluted effects of the protein can be monitored. Several processes are being examined to determine the function of these genes. Sequence comparison analysis indicates that DBP5 may be involved in translation initiation, thus making translation a key function to test for this gene. We have also successfully overproduced several of these genes in *Escherichia coli*, and DBP5 has been purified to homogeneity. Its biochemistry is currently being investigated.

181. Purification and biochemical characterization of the *S. cerevisiae* PRP5 protein

*Gloria Dalbadie-McFarland, John N. Abelson*

The *Saccharomyces cerevisiae* PRP5 protein contains a central domain that is closely related in sequence to RNA helicases (Dalbadie-McFarland and Abelson, 1990), proteins that catalyze ATP-dependent RNA unwinding.
PRP5 is required early in the spliceosome assembly pathway and may interact directly with the pre-mRNA substrate as suggested by immunoprecipitation experiments. The absence of functional PRP5 prevents U2 binding to the substrate, but has no effect on U1 binding (S. Ruby, personal communication). Taken together, these observations suggest that the putative PRP5 helicase activity may alter the secondary structure of one or more of these RNA species to allow complex formation.

In order to study the nature of the PRP5 protein's biochemical activity, we have expressed the PRP5 gene in an Escherichia coli system. The protein expressed either by itself or fused at the amino terminus to an eight-residue, unrelated epitope recognized by a monoclonal antibody, is soluble and functional. The functional assay consists of in vitro complementation of heat-inactivated splicing extracts made from a prp5 temperature-sensitive yeast strain. We have purified the protein produced in E. coli and are investigating its properties and biochemical activity. In vitro, RNA helicases hydrolyze ATP in the presence of RNA. An RNA-dependent ATPase activity copurifies with the PRP5 protein, thus setting the stage for understanding its role, substrate specificity and activity requirements.

References:

182. A U5-snRNP protein required only for the second step of splicing

David S. Horowitz, John N. Abelson

The PRP18 gene was discovered by screening a bank of temperature-sensitive mutants of Saccharomyces cerevisiae (Vijayraghavan et al., 1989). In temperature-sensitive prp18 yeast, pre-mRNA splicing intermediates (exon 1 and 2/lariat) accumulate at the non-permissive temperature (Vijayraghavan and Abelson, 1990). Analogous results are obtained in vitro. We have cloned and sequenced the PRP18 gene and have expressed the PRP18 protein in Escherichia coli. PRP18 protein has been purified to homogeneity from E. coli. The purified protein is active; it restores splicing activity to heat-inactivated extracts from temperature-sensitive prp18 yeast. Antibodies to PRP18 have been raised. The antibodies inhibit only the second step of splicing, leading to accumulation of intermediates. The antibodies precipitate the U4/U5/U6 snRNP complex from yeast extracts. Immunoprecipitation of the U4/U6 snRNP requires the U5 snRNP, but precipitation of U5 snRNP is independent of the U6 snRNP. We conclude that PRP18 is part of the U5 snRNP. These observations strongly suggest that the U5 snRNP is involved in the second step of splicing.

References:

183. Recognition of tRNAs by aminoacyl-tRNA synthetases

Jeffrey R. Sampson, Margaret E. Saks

All transfer RNAs (tRNAs) have similar tertiary structures; yet within this structure are elements which ensure that each tRNA will be recognized and aminoacylated by only its cognate aminoacyl-tRNA synthetase (RS). X-ray crystallographic, biochemical and genetic analyses indicate that elements that distinguish one group of isoaccepting tRNAs from another reside in one or more of the following locations: a) the acceptor stem (including position 73), b) the anticodon stem/loop, c) the variable pocket, or d) the extra (variable) stem/loop. In some cases, specificity results from contacts between the functional groups of bases within a particular region and the amino acid side chains of RSs. In other instances, specificity depends on structural elements in the RNA which facilitate contact between its phosphate backbone and the protein.

Our studies focus on determining the regions of Escherichia coli serine tRNAs that are recognized by seryl-tRNA synthetase (SRS). T7 transcripts that model various regions of tRNA\textsuperscript{Ser} are tested in vitro for aminoacylation by purified SRS. One set of studies focuses on the contribution of acceptor stem nucleotides to aminoacylation. The model substrates (minihielices) are composed of the acceptor stem and T\textsuperscript{VC} stem nucleotides of serine tRNAs. These substrates are specifically aminoacylated by SRS, even though their \(k_{\text{cat}}/K_m\) is approximately \(1 \times 10^{-5}\) that of a full-length tRNA\textsuperscript{Ser} transcript. Model RNA transcripts in which various structural features have been eliminated from a full-length
tRNAs are used to examine the contribution of other regions to aminoacylation. One such substrate, devoid of an anticodon stem and loop, is aminoacylated with kinetics similar to that of wild-type tRNA\textsubscript{Ser}. It was expected that the elimination of the anticodon loop would have little or no effect on aminoacylation since there are no nucleotides absolutely conserved among the four anticodons for serine. In a second substrate, the long extra stem/loop that is characteristic of class II tRNAs (serine, leucine and tyrosine in \textit{E. coli}) was altered from 18 to 5 nucleotides to conform to that of class I tRNAs. This substrate is aminoacylated with a \( k_{cat}/K_m \) approximately 3 x 10\(^{-3} \) that of a wild-type tRNA\textsubscript{Ser} and is within a factor of 30 of the wild-type minihelix. A role of the long extra stem/loop in tRNA\textsubscript{Ser} recognition was anticipated since previous results in the Abelson laboratory showed that \textit{in vivo} a tRNA\textsubscript{Ser} with a truncated extra stem/loop is aminoacylated with approximately 95% glutamine and only 5% serine.

More than 60 acceptor stem mutants in the minihelix background have been assayed to date. Each of the 7 base pairs of the acceptor stem was systematically changed to each of the alternative Watson-Crick base pairs, and the wild-type nucleotide G73 was changed to each of the other standard nucleotides. A selected set of double and triple base-pair mutants has also been assayed. Three general conclusions can be drawn from the results of these experiments. First, all minihelices that maintain the consensus acceptor stem sequence of the 5 serine isoacceptors in \textit{E. coli} are the best substrates for aminoacylation by SRS. Second, the effect on aminoacylation of mutations away from the consensus is location-specific. Substitutions at position 73 and at base pairs at positions 2-71 and 4-69 have the greatest effect, whereas substitutions at other positions have small but measurable effects. Third, the absolute effect on aminoacylation at these positions depends on the substitution made. This clearly shows the importance of testing more than one base substitution at a given position when determining sites of recognition. By correlating the chemistry of base functional groups with their effects on aminoacylation, we can infer potential chemical requirements for maintaining specificity between serine tRNAs and SRS. Since we know that recognition elements reside in both the acceptor stem and extra stem/loop of tRNA\textsubscript{Ser}, it is important to determine whether or not these elements contribute independently to aminoacylation. Thus, a subset of acceptor stem mutations is being placed in the full-length tRNA background.

Because of our general interest in the relative importance of acceptor stem and anticodon nucleotides to aminoacylation, we are also continuing studies using model substrates that are composed of a wild-type anticodon, a standard body (that of tRNA\textsubscript{Ala}) and interchangeable acceptor stems. This system allows us to screen efficiently each of the 20 isoaccepting groups to determine whether elements recognized by their cognate RS reside in the acceptor stem, anticodon or some other region of the tRNA. Results to date confirm earlier reports from other laboratories that major recognition elements of tRNAs Met, Val, Lys and Phe reside in the anticodon. The use of this system has also revealed additional recognition elements with small but measurable effects in the acceptor stems of all of the above tRNAs except Phe. Lysine tRNAs may also contain recognition elements outside of the anticodon and acceptor stem. Karin Johnson, a Caltech SURF student, is investigating this possibility. In tRNA\textsubscript{Thr}, nucleotides with large effects on aminoacylation are found in both its anticodon and acceptor stem, whereas in tRNA\textsubscript{Ala}, effects are associated with the acceptor stem but not with anticodon nucleotides. Since these results are consistent with results previously published by other laboratories, and since the system is highly sensitive to elements outside of the anticodon, it should be possible to use this system to study the recognition elements of tRNA isoaccepting groups that have not yet been examined.

184. Studies of tRNA identity: The cysteine system

\textit{George A. Komatsoulis}

In \textit{Escherichia coli} there is a single cysteine transfer RNA (tRNA) isoacceptor, which recognizes two codons. The transfer RNA itself is structurally unusual, as it appears to be lacking several of the tertiary interactions postulated for the phenylalanine tRNA. Because of its apparently unusual structure, and because the synthetase has to recognize only a single species of tRNA, we felt that cysteine might present us with an interesting system to continue our lab's investigations into tRNA identity.

We are studying this problem from a variety of directions, currently using \textit{in vivo} approaches to determine
the identity set, but eventually we plan to perform in vitro experiments to complement this work. The general plan is to convert an alanine amber suppressor tRNA into a cysteine suppressor tRNA with as few changes as possible. The tRNAs are constructed from six oligonucleotides, synthesized at the Caltech Microchemical Facility. The oligonucleotides contain the tRNA gene, as well as a T7 RNA polymerase promoter and a BstNI restriction site so that the tRNAs may be transcribed easily in vitro. The tRNA genes are cloned into pGFIB-1 under the control of the lpp (lipoprotein) promoter which causes the tRNAs to be constitutively expressed in vivo. Suppression of an amber mutation in β-galactosidase allows us to determine suppression efficiency, while suppression of an amber mutation at the active site of thymidylate synthetase allows us to determine if the suppressor is being charged with any cysteine.

Determination of the amino acid(s) with which a tRNA is charged is made by sequencing dihydrofolate reductase (DHFR) produced from a strain carrying both the suppressor tRNA and a copy of the DHFR gene with an amber at position 10. Before the protein can be sequenced, however, it must first be pyridylethylated to derivatize the cysteine, which would otherwise appear as several different amino acids in the sequenator due to its different oxidation states. The product of this is pyridylethyl-cysteine (PE-Cys) which produces a single peak on the chromatograms produced by the sequenators. These protocols are performed at the Caltech Microsequencing Facility.

As a first step, the cysteine suppressor produced by J. Normanly by changing the cysteine anticodon to a CUA was subjected to study. This suppressor was found to be about 24% efficient, and to be charged with cysteine by the thymidylate synthetase assay. By DHFR sequencing it was found to be charged with 94% cysteine, 4% glutamine, 1% glutamate, and 1% other amino acids. Several mutants were then constructed to attempt to determine the minimum number of changes required to convert an alanine suppressor tRNA into a cysteine suppressor tRNA. These mutants are known as acm (Ala to Cys mutants) tRNAs. Acm1 had seven changes which converted the acceptor stem to a cysteine configuration while leaving the sequence of the remainder of the tRNA unchanged. This tRNA was less than 1% efficient and did not appear to insert cysteine by the thymidylate synthetase assay. Acm2 had changes to make its sequence that of a cysteine tRNA in the acceptor stem, D-stem and loop, extra arm, and T7C stem and loop. Its efficiency was approximately 12% and it inserted cysteine as determined by suppression of the thymidylate synthetase. DHFR sequencing indicated that it inserts principally a mixture of glutamine and cysteine. This result indicates that the anticodon stem (the only part of the tRNA that did not have the sequence of a cysteine suppressor tRNA) has important recognition elements. Acm5, acm7, and acm8 were designed to test this theory. There are three base pairs in the anticodon stem that are different between the cysteine and AlaUGC tRNAs. These mutants are cysteine suppressor tRNAs with one of the three base pairs mutated to the alanine sequence. If any one of these base pairs is important, then one tRNA will produce DHFR similar to acm2, while the other two should produce a profile similar to the cysteine suppressor. If any two of the base pairs are important, two of the genes will produce DHFR similar to acm2, while the third will give results similar to the cysteine suppressor (or one will produce data similar to acm2, one like the cysteine suppressor, and one will be in between the other two). If all three are important, none of the tRNAs will give sequencing data similar to the cysteine suppressor. The sequencing of the DHFR of these genes is in progress now.

185. The identity set of leucine tRNAs

Giuseppe D. Tocchini-Valentini

The recognition of transfer RNA (tRNA) by its cognate synthetase has a pivotal role in protein synthesis. Recognition and correct aminoacylation of a tRNA depends on whether the tRNA contains elements that result in a positive interaction with its cognate synthetase and negative interactions with the 19 other synthetases in the cell. These elements are defined as the identity set of the tRNA. I define an identity set as the minimal changes that result in the aminoacylation of a tRNA with a different amino acid.

My current studies focus on determining the changes necessary to convert the identity of a serine tRNA to that of leucine. One approach to this problem involves in vivo
studies using dihydrofolate reductase (DHFR) as a reporter gene. An amber-suppressing serine tRNA with changes that should convert their identity from serine to leucine is tested in this system. The amino acid inserted at position 10 in the DHFR indicates the identity of the tRNA.

Last year I constructed a mutant (L2) with 14 changes. This tRNA, which was a good suppressor (75% by β-galactosidase assay), was aminoacylated with leucine. The mutations were localized in two different domains, the acceptor stem and the variable pocket of the tRNA. Another mutant (L3) with the same set of changes but with the extra arm deleted was still charged with leucine and small amounts of glutamine. This excludes the involvement of the extra arm in recognition by leucyl tRNA synthetase (LRS). This is consistent with the in vivo results of J. Normanly and T. Ollick in our laboratory. Two mutants that are missing the extra arm, which present changes in the acceptor stem (L4) and the variable pocket (L5) only, have already been constructed. The purification of the respective DHFR is in progress. Thus, it is difficult to establish which of the mutated sites contributes to the positive interaction between the tRNA and the LRS. This site will be revealed by determining the in vitro kinetics of this tRNA with purified LRS. Elements that are positive for LRS but negative for other synthetases can also be revealed by in vitro aminoacylation assay using the appropriate synthetase. It results from the in vivo data that glutamyl tRNA synthetase and the seryl tRNA synthetase are the most likely competitors of the LRS.

### PUBLICATIONS


or in combination the nervous system, the skeletal muscles, the heart, the retina and the auditory apparatus, organs that have high energetic demands and thus depend heavily on the respiratory functions of mitochondria. The development of cellular models of mitochondrial diseases by transfer into human mtDNA-less ($\rho^0$) cells of mitochondria from patients affected by these disorders has provided a powerful tool for the dissection of the molecular basis of the disease. At the same time, the mutant cell lines constructed by mitochondrial transfer are extremely valuable to study basic problems concerning the replication and expression of the mitochondrial genome, the function of the enzyme complexes of the oxidative phosphorylation apparatus and nucleo-mitochondrial interactions.

The general picture that has emerged from work carried out in many laboratories around the world has indicated that mitochondrial tRNA genes are hot spots for mutations causing neuromuscular diseases. Particularly intriguing is the observation that mutations of different tRNA genes can cause different clinical syndromes. This observation may be related to the unique organization of the mammalian mitochondrial genome. In fact, in this genome, the tRNA genes have more than one function. Besides encoding the tRNAs required for mitochondrial protein synthesis, their sequences are known to function as signals for the enzymes that process the polycistronic transcripts of the heavy and light strand of mtDNA. Therefore, the mutations of these genes, which have been recently associated with mitochondrial diseases, could exert their deleterious effects by directly affecting the tRNA functions in protein synthesis, and/or by disturbing the normal mitochondrial RNA processing, and thereby reducing the levels of functional tRNAs, rRNAs or mRNAs. In the case of the tRNA$^{Lys}_{\text{UUR}}$, a third function has been shown to be associated with the tRNA sequence, in particular that of containing the recognition signal for a specific protein factor (transcription termination factor, mTERF), discovered recently in our laboratory, which is involved in producing termination of transcription at the 3' end of the 16S rRNA gene. This termination phenomenon has a crucial role in determining the great excess of rRNA over mRNA synthesis from the heavy strand promoters.

In the past year, we have applied the technology of mitochondrial transfer to the analysis of two mitochondrial diseases caused by tRNA mutations. In the first case, severe mitochondrial protein synthesis and respiration defects were transferred with mitochondria from myoblasts of patients suffering from myoclonic epilepsy and ragged red fiber (MERRF) syndrome into two genetically unrelated $\rho^0$ cell lines, pointing to an mtDNA alteration as being responsible and sufficient for causing the disease. The transfer of the defects correlated with the presence in the transferred mtDNA of a tRNA$^{Lys}$ mutation, thus establishing a direct link between this mutation and the biochemical defects. Transfer of another disease phenotype has been recently achieved with mitochondria from myoblasts of patients affected by the MELAS syndrome, a disease characterized by myopathy, encephalopathy, lactic acidosis and stroke-like episodes. In this case, the mtDNA alteration responsible for the biochemical defects has been shown to be an A to G transition within the mTERF binding domain in the tRNA$^{Leu}_{\text{UUR}}$ gene. Experiments utilizing the purified transcription termination factor and a mtDNA fragment containing the wild-type or the mutated binding domain have shown that the MELAS mutation drastically decreases the affinity of the factor for the binding site. The in vivo effects of the mutation are presently being actively investigated in $\rho^0$ cell transformants carrying the mtDNA from MELAS patients as compared to transformants carrying the mtDNA from maternally related individuals lacking the mutation.

The technology of mitochondria transfer into $\rho^0$ cells is also being utilized to investigate the role of selective pressure in the repopulation of $\rho^0$ cells with functional exogenous mitochondria, as well as the role of selection, segregation and complementation in the manifestation of the defective phenotype in cells carrying both wild-type and mutated mtDNA. These studies should provide valuable insights into the rules that govern the maintenance and transmission of mtDNA mutations in the population.

In another line of investigation directed towards developing new tools for mtDNA manipulation, intensive efforts have been made to establish an efficient system for mtDNA-mediated transformation of human cells in culture. An extensive series of experiments has been
carried out by the "biolistic approach," and a variety of parameters have been investigated. Although no success has so far been achieved, efforts are continuing using a new model of the particle delivery system which uses helium as a propellant instead of gun powder, and which should improve considerably the transformation efficiency. Success in this endeavor would have a profound impact on mitochondrial genetic research.

In the area concerning the mechanisms and control of mitochondrial gene expression in human cells, a major effort is being made towards the structural and functional characterization of mTERF. The polypeptides responsible for the footprinting activity of this factor have been identified by assays carried out after high resolution oligonucleotide affinity chromatography and by Southwestern blots of the affinity-purified factor utilizing wild-type and mutated probes. Experiments are also in progress towards the cloning of the cDNA for mTERF by screening of expression libraries. Furthermore, the identification, mentioned above, of a mitochondrial disease (MELAS) associated with a mutation in the binding domain for mTERF and the transfer of the mutation to mtDNA-less cell lines have added a new dimension to the investigation of the function of mTERF. The availability of established cell lines carrying the MELAS mutation, which affects the activity of mTERF, opens the way to the dissection in vivo of the role of this factor in mitochondrial RNA metabolism.

Another focus of our research concerns the structure and function of the first enzyme in the respiratory chain, the NADH dehydrogenase or Complex I. The extraordinary interest of this enzyme resides in the major role that mtDNA plays in its control; in fact, this DNA contributes information for seven subunits to its assembly. These subunits comprise about 60% of the mtDNA protein-coding capacity. The function of these subunits is for the most part unknown. We are following two approaches towards understanding their physiological role. The first approach aims at studying the expression of the mitochondrial genes encoding these subunits under different physiological or developmental situations. This approach has already revealed a developmental regulation of one of these subunits, ND5. In fact, this polypeptide is the only mtDNA-encoded subunit of the enzyme that appears not to be synthesized in mitochondria of isolated rat brain synaptic endings and of isolated rat muscle, pointing to the interesting possibility of a growth-dependent regulation of the synthesis of this subunit. The other approach aims at investigating the effects of mutations, either naturally occurring or produced artificially, on the assembly and function of Complex I. One class of mitochondrial diseases, typified by the Leber hereditary optic neuropathy, are caused by mutations in mitochondrial genes encoding subunits of NAD dehydrogenase, most frequently the ND4 or the ND1 gene. The ND1 subunit has been shown to bind rotenone, a specific inhibitor of the enzyme, and presumably contains or participates in the formation of the ubiquinone binding site. Transfer of these mutations to the p5 cell lines should produce permanent cell lines amenable to an in-depth analysis of the effects of these mutations on the assembly or function of Complex I. At the same time, we are trying to artificially produce mutations in the ND1 gene in established human cell lines, using resistance to rotenone as a selectable marker. Progress has also been made in the analysis of the nuclear-coded subunits of Complex I. The determination of the cDNA-derived amino acid sequence of the NADH-binding 51 kDa subunit of the bovine enzyme has unexpectedly revealed striking sequence similarities with a major portion of a subunit of a bacterial NAD+-reducing hydrogenase. This result fits in with the emerging evidence indicating that NADH dehydrogenase derives from the evolutionary association of two moieties, each contributing a distinct segment of the electron pathway, and having homologous counterparts in present-day bacterial enzymes.

186. Identification of the polypeptides associated with the mitochondrial DNA binding activity of the transcription termination factor

Andrea Daga

The mechanisms that govern transcription in mammalian mitochondria are not yet well understood. However, a noteworthy step forward has been taken with the identification of a protein factor that halts the progression of the RNA polymerase at the 3' end of the 16S rRNA gene in human mitochondrial DNA (Kruse et al., 1989). This DNA-binding protein(s) protects, in a
footprint assay, a 28 bp DNA segment immediately adjacent and downstream of the 16S rRNA/ARNALeu gene boundary. The importance of this termination factor lies in its role in maintaining, within the mitochondria, the appropriate proportion of rRNAs and messenger RNAs.

To further proceed in the analysis of the transcription termination factor, we have set out to identify the polypeptide(s) responsible for the mitochondrial DNA binding activity of the factor. For this purpose, we have utilized an oligonucleotide affinity column carrying the binding site for the protein(s) and eluted the bound proteins either with a linear KCl gradient or in a stepwise fashion, utilizing 0.3 M, 0.4 M, 0.5 M, 0.6 M, 0.8 M and 1.0 M KCl steps. It was found that the distribution of three polypeptides in the eluate, a 34 kDa doublet and a 31 kDa polypeptide, correlates well with the footprinting activity.

A more detailed analysis, using eluate fractions highly enriched in the 34 or 31 kDa polypeptides and various combinations of them, has clearly indicated that the presence of each of the two components efficiently enhances the footprinting activity of the other. Support for this conclusion has come from the result of a Southwestern blotting experiment in which affinity-purified proteins, transferred onto a nitrocellulose membrane, have been probed with a double-stranded oligonucleotide harboring the protein binding site. The results have shown that both the 34 kDa polypeptides and, less effectively, the 31 kDa polypeptide bind the DNA probe.

In light of these data, we now believe that the mitochondrial transcription termination factor is represented by the 34 kDa doublet, while the 31 kDa polypeptide may be a proteolytic product of the full-size polypeptide(s). The structural relationship between the two 34 kDa polypeptides and between these and the 31 kDa polypeptide is presently being investigated.

Reference:

188. Effect of the mitochondrial tRNA^Leu(UUR) mutation in MELAS encephalomyopathies on the DNA binding affinity of the mitochondrial transcription termination factor

Andrea Daga

Mutations in the mitochondrial genome have been linked with several human diseases. We became interested in one of these diseases, the MELAS syndrome (characterized by myopathy, encephalopathy, lactic acidosis and stroke-like episodes), because mitochondrial DNA in the affected individuals carries in most cases an A to G transition at a highly conserved position of the gene for the tRNA^Leu(UUR) (Goto et al., 1990); more importantly, this mutation lies within the binding site for the transcription termination factor (mTERF) (Kruse et al., 1989). Therefore, the idea arose that this point mutation could impair the termination process by diminishing the ability of mTERF to bind to its cognate sequence. Hence, we tested the effect of the MELAS mutation on mTERF
binding. To do so, we compared in a gel shift assay the binding of mTERF to the wild-type and the mutated (i.e., bearing the MELAS substitution) double-stranded oligonucleotide probe.

First, we carried out a competition experiment in which the purified protein(s) was incubated with the labeled wild-type probe in the presence of increasing amounts of either wild-type or mutated unlabelled double-stranded oligonucleotide. The results showed that the binding of the labeled probe to the protein was competed for 3 to 4 times more efficiently when the competitor oligonucleotide had the wild-type sequence. Corroboration of this result has come from an analysis of the stability of the complexes formed with either wild-type or mutant oligonucleotide. The labeled double-stranded oligonucleotide, wild-type or carrying the MELAS mutation, was incubated with the termination factor and then exposed to a 200-fold excess of its homologous cold species over different lengths of time. It was found that the stability of the mutant DNA-protein complex was decreased by an order of magnitude in comparison with the wild-type DNA-protein complex. Hence, we conclude that the MELAS mutation affects the binding of mTERF to the DNA, presumably impairing the termination process; this effect may be, at least in part, responsible for the pathogenetic role of the MELAS mutation.

References:

189. Effect of a single base mutation of mitochondrial DNA in MELAS encephalomyopathy on the transcription termination activity of the transcription termination factor

Vicente M. Micol

Several mutations of the mitochondrial genome have recently been shown to be associated with human diseases. These mutations are represented by deletions, duplications or single base changes. A point mutation has been demonstrated in patients affected by the MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes). This mutation results from a transition from A to G (Cambridge nucleotide number 3243) within the DNA binding region of the transcription termination factor downstream of the 16S rRNA gene (Goto et al., 1990).

In order to construct a template for in vitro transcriptional termination containing the MELAS mutation, the insert of pTER, a plasmid containing human mtDNA segments encoding the transcription initiation and termination regions (Kruse et al., 1989), was cloned in the EcoRI and HindIII sites of pBluescript II KS+, yielding the plasmid pTERBS.

Oligonucleotide-directed mutagenesis of pTERBS, using a uracil containing DNA template (Kunkel, 1985), resulted in the plasmid pTERBSM. The production of the mutation was confirmed by DNA sequencing.

Probes labeled at their 5' end or 3' end will be prepared from the wild-type pTERBS and from pTERBSM plasmids. Using these probes, we intend to test the transcription termination factor for DNA-binding properties by gel mobility shift and DNase footprinting assays. We also plan to test the effects of the mutation in the template on transcription initiation and termination in the in vitro system developed in this laboratory (Shuey and Attardi, 1985).

References:

190. cDNA-derived amino acid sequence of the NADH-binding 51 kDa subunit of the bovine respiratory NADH dehydrogenase reveals striking similarities to a bacterial NAD+-reducing hydrogenase

Salil D. Patel, Ruedi Aebersold

A λgt10 bovine brain and a λgt11 bovine heart cDNA library were screened with oligonucleotide probes corresponding to partial protein sequences directly determined from the isolated 51 kDa subunit of the bovine respiratory chain NAD dehydrogenase. Clones were isolated that encode a protein of 464 amino acids containing all the 11 partial tryptic peptide sequences determined from the 51 kDa subunit. The size and amino acid composition of this protein agree with those determined for the purified 51 kDa subunit. Furthermore,
this protein contains a putative NADH-binding domain, a possible FMN binding site and a putative binding site for an iron-sulfur cluster. The above evidence indicates that the cloned protein is the 51 kDa subunit or its precursor. A search for sequence similarity with proteins in the PIR protein database has unexpectedly revealed that the 51 kDa subunit has a 32% amino acid sequence identity with a major portion of the α subunit of the soluble NAD⁺-reducing hydrogenase from Alcaligenes eutrophus. In particular, there are three segments of high sequence similarity (70 to 88%) between the two proteins which correspond to the three ligand binding sites mentioned above.

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191. Analysis of altered complex I in human cell lines

Gotz Hofhaus

The complex I or NADH dehydrogenase is the first enzyme of the mitochondrial respiratory chain. This large membrane-bound enzyme consists of about 30 subunits. Seven of these are encoded by mitochondrial DNA (mtDNA) and form, together with some small nuclear-encoded subunits, the membrane part of complex I. Knowledge about the structure and function of these subunits is poor. The mitochondrial genome of many organisms is completely sequenced and, therefore, the primary structure of all subunits is known. However, because the complex is not present in yeast or prokaryotes, where deletion and mutagenesis experiments could be easily performed, further genetic studies of the subunits are difficult.

It looks, therefore, reasonable to search for mutants of these subunits in human cells. This approach can be especially valuable, since the transfer of mitochondria into human mtDNA-less (ρ⁰) cells allows the stabilization of any mutation found in nature (e.g., in myopathy patients) and the study of the defect in much more detail than it would be possible with the little biopitc material available from the patient.

For a start, I have focused on a specific subunit that is encoded by the ND-1 gene of the mtDNA. This subunit is known to bind rotenone, a specific inhibitor of the NADH-dehydrogenase. Screening for rotenone-resistant cells should allow the isolation of cells with a somewhat altered subunit exhibiting a lower sensitivity of their respiration to rotenone than wild-type cells. It turned out to be important to perform the screening in medium lacking pyruvate, which makes the dependence of the cells on respiration more stringent. This is best illustrated by the absolute pyruvate dependence of the ρ⁰ cells that lack a mitochondrial respiratory chain.

The sequence of the altered subunit should provide some information about the structure of this subunit. If the altered subunit can still be assembled into an intact complex, this can be compared with the wild-type complex with respect to the binding of rotenone, ubiquinone and DCCD. This may lead to some insights into the function of this subunit.

192. Differential regulation of expression of the multiple ADP/ATP translocase genes in human cells

Joël L. Lunardi, Giuseppe Attardi

The expression of the genes encoding the three isoforms of the human ADP/ATP translocase (T1, T2 and T3) has been investigated in cultured cell systems under different experimental conditions, using isoform-specific probes. In several human cell lines tested, i.e., HeLa, Hep3B, 143B, and HL60, the T3 gene is expressed as a single 1300 nt mRNA, whereas the T2 gene produces two species of mRNA, 1450 nt and 1600 nt in size. These two species, which are present in HeLa cells in approximately equivalent amounts, were shown to derive from the use of two different polyadenylation signals. The gene for the muscle-specific isoform of ADP/ATP translocase, T1, was not found to be expressed in any of the cell lines investigated. The levels of T2 and T3 mRNAs in HeLa cells are differentially affected by the growth conditions. In fact, the T2 mRNA level remains relatively constant throughout the exponential and stationary phases, whereas the T3 mRNA level decreases progressively in the second half of the exponential phase and in the stationary phase down to less than 50%. This difference in quantitative behavior of the two mRNAs must reflect changes in their rates of synthesis, since their half-lives are very similar (t_{1/2} = 5-6 h), with no significant growth-related differences. Treatment of HL60 cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) or
retinoic acid (RA), two agents that induce cessation of cell proliferation and cell differentiation, resulted in a marked decrease in both T2 and T3 mRNA levels. Exposure of HeLa cells to chloramphenicol (CAP) produced a pronounced decrease in the levels of both T2 and T3 mRNAs after 48 to 72 hr of treatment. Half-life time measurements strongly suggested that this decrease reflected a reduction in the rate of synthesis of the two transcripts. Treatment of HeLa cells with dinitrophenol (DNP) also produced a dramatic decrease in the steady state levels of both T2 and T3 mRNA, which, however, in contrast to the just mentioned situation, could be accounted for by a decrease in their metabolic stability. Control experiments indicated that the CAP- and DNP-induced changes were not a non-specific consequence of mitochondrial dysfunction. The observations reported here clearly demonstrate that the expression of the multiple ADP/ATP translocase genes in human cells is sensitive to the cell physiological conditions, responding to the varying cellular demands by changes in the rate of synthesis or stability of their mRNAs.

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193. In vitro genetic transfer of protein synthesis and respiration defects to mitochondrial DNA-less cells with myopathy-patient mitochondria

Anne Chomyn, Susan Lai, Giuseppe Attardi

A severe mitochondrial protein synthesis defect in myoblasts from a patient with mitochondrial myopathy was transferred with myoblast mitochondria into two genetically unrelated mitochondrial DNA (mtDNA)-less human cell lines, pointing to an mtDNA alteration as being responsible and sufficient for causing the disease. The transfer of the defect correlated with marked deficiencies in respiration and cytochrome c oxidase activity of the transformants and the presence in their mitochondria of mtDNA carrying a tRNA^{Lys} mutation. Shoffner et al. (1990) had found, while our work was in progress, an association between an A to G transition in the mitochondrial tRNA^{Lys} gene and the MERRF syndrome. Furthermore, apparently complete segregation of the defective genotype and phenotype was observed in the transformants derived from the heterogeneous proband myoblast population, suggesting that the mtDNA heteroplasmy in this population was to a large extent intercellular. The present work thus establishes a direct link between mtDNA alteration and a biochemical defect. (This work has been carried out in collaboration with Giovanni Meola, Nereo Bresolin, and Guglielmo Scarlato, Institute of Clinical Neurology, University of Milan.)

Reference:

194. Transformation of human mitochondrial DNA-less cells with mitochondria from MELAS patients

Andrea Martinuzzi

The fusion of human cells lacking mtDNA (p0) with cytoplasts derived from cells of patients with mitochondrial encephalomyopathies offers a good opportunity to study the mitochondria of the patients in a "neutral" nuclear background and to assess their functionality. Information could then be obtained on the pathogenetic significance of specific mtDNA mutations, and at the same time the natural mutants might provide good models to study the regulation of mammalian mtDNA replication and expression. The MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) (Pavlakis et al., 1984) is linked in most of the cases to a mtDNA A to G transition in the tRNA^{Leu} at position 3243 (Goto et al., 1990). We obtained myoblast culture from three members of a MELAS family: a severely affected boy, his mother and her sister. Molecular analysis of the muscle biopsy from which the myoblasts were derived revealed heteroplasmcy for the tRNA^{Leu} mutation with large prevalence of the mutated genotype in the affected boy (88%), while in the mother and the aunt, the mutated mtDNA was 61% and 55%, respectively. The myoblasts from the patient, his relatives, and a control were enucleated by centrifugation in the presence of cytochalasin-B. The cytoplasts were then fused with a large excess of p0 cells in the presence of polyethylene-glycol and dimethylsulphoxide. The cybrids were selected in a medium containing bromodeoxyuridine and lacking pyrimidines (for which p0 are auxotrophic). The isolated clones are presently being analyzed in their
biochemical and molecular properties (see Abstract No. 195).

References:

195. Biochemical and molecular analysis of mitochondrial transformants carrying the MELAS mtDNA mutation
Anne Chomyn, Makoto Yoneda, Susan Lai

The pathological syndrome in man called MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) has been shown to be associated, in most cases, with a single base substitution (A to G at position 3243) in the tRNALeu(UUR) gene of mtDNA (Goto et al., 1990; Kobayashi et al., 1990). The mutation occurs in a region that corresponds to the binding site for the mitochondrial transcription termination factor (Kruse et al., 1989).

Towards the end of determining the pathogenetic mechanism of the tRNALeu(UUR) mutation in this disease at the molecular level, we are analyzing transformants obtained by transferring mitochondria from myoblasts of a MELAS patient to a mtDNA-less cell line, pO206, by cytoplast fusion (see Abstract No. 194). Both the original myoblast culture and all the derived mitochondrial transformants contain predominantly mutated mtDNA. The transformants also show a marked decrease in mitochondrial protein synthesis rate and in respiration. Whether or not termination of transcription is affected in vivo in these transformants is presently under investigation. Most of the transformants made with an asymptomatic relative’s myoblasts contained exclusively wild-type mtDNA and exhibited no evidence of biochemical alteration.

References:

196. Mitochondrial gene segregation and phenotype expression in mtDNA-less human cells repopulated with a disease-associated mtDNA
Makoto Yoneda, Anne Chomyn

Recent molecular biological studies have revealed that a number of human diseases are associated with either deletions or point mutations of the mitochondrial genome (mtDNA). The presence of mixed populations of mtDNA, mutant and wild-type, in variable proportions has been observed in different individuals. The variety and severity of the clinical phenotype depends, at least in part, on the segregation of the two types of mtDNA, in variable proportions, in different tissues or cells.

To investigate the mechanism of mtDNA segregation and the relationship between genotype and phenotype expression, we introduced a disease-associated mtDNA into a mtDNA-less (pO) cell line and quantitated the functional effects of the mtDNA mutation. Myoblasts were isolated from a patient carrying an A to G transition mutation in the tRNAlys gene of mtDNA, which causes the MERRF phenotype of mitochondrial encephalomyopathy. The myoblasts were enucleated and fused with pO cells. Using polymerase chain reaction with a mismatched oligonucleotide primer, we have created a new restriction site for NaeI, which can be recognized only in the mutant type sequence; this allows rapid quantitation of the mutated mtDNA proportion in a sample. Although all transformants were heteroplasmic, with a predominant mutant genome, two of the clones showed almost normal respiratory function and mitochondrial protein synthesis pattern. Moreover, subclones derived from a single cell of each of these two cell lines also carried heteroplasmic mtDNA, indicating intracellular heteroplasmy. These results suggest either the existence of a threshold of expression of the phenotype caused by the mutant genome or the presence of genetic complementation.

197. Characterization of a disease-associated mitochondrial DNA mutation
Ian Holt

Although the last year has seen the identification of a number of deleterious mitochondrial DNA point mutations, these have mainly been located in transfer RNA genes. The single base substitution in the ATPase 6 gene described by Holt et al. (1990) is one of only three
mutations described in a protein coding gene. The other two are both associated with Leber's hereditary optic neuropathy.

Patient cells carrying the ATPase 6 mutation were enucleated and fused to two cell lines lacking mitochondrial DNA. Selection in the absence of uridine allowed only the growth of fused cells. Clones were analyzed for the presence of mutant mitochondrial DNA, which harbors an additional restriction enzyme site. The original patient cells contained both mutant and normal mitochondrial genomes, although the normal molecules represented less than 5% of the total. The clones appeared also to have some large fragments, indicative of normal mitochondrial DNA molecules; however, repeated restriction endonuclease digestion showed that the proportion was not constant and often no large fragment was discernible. This suggests incomplete digestion and, therefore, that the clones were in fact homoplasmic mutants. Analysis of clones that had been in continuous culture for up to six months revealed that the mutant population was stable.

Measurement of the total respiratory capacity of the clones indicated only a marginal reduction. Individual activities for respiratory complexes I and IV and ATP synthetase were also measured. Again, there was no significant or consistent decrease in $V_{\text{max}}$ between the transformants and controls.

Mutations in ATPase 6 have resulted when selecting for oligomycin resistance in CHO cells. We therefore investigated the sensitivity of the transformants to this drug. There was some suggestion of increased resistance in one of the transformant lines, but this was not seen in the second line examined.

In vitro $^{35}$S-methionine labeling of the mitochondrial translation products indicated a consistent increase in the labeling of ATPase 6 in the transformants. This contrasted with the result from immunoprecipitation of labeled ATP synthetase from these cells. In the holoenzyme of a transformant line, ATPase subunit 6 was reduced relative to the control. These data imply that the mutant gene is transcribed and its mRNA, translated normally; however, incorporation of the subunit is impaired, or else the subunit is unstable in the complex. Some effect on the enzyme activity would be predicted from this observation; we are therefore applying additional assays to the mutant ATP synthetase.

Reference:

198. The role of selection for respiratory competence on the repopulation of human mitochondrial DNA-less cells by exogenous mitochondrial DNA

Carlo Ausenda

Human cell derivatives lacking mitochondrial DNA (mtDNA) ($\rho^0$) and resistant to bromodeoxyuridine (BRDU) have been isolated in this laboratory. These cells can be repopulated with exogenous mtDNA by fusion with cytoplasts, i.e., enucleated cells, derived from any respiratory competent cell line, in the presence of polyethyleneglycol (PEG). The fusion products, cybrids, are exposed to a selective medium containing BRDU and free of uridine, which is necessary for the survival of $\rho^0$ cells. This medium allows the growth of only the fusion products, which are able to replicate the exogenous mtDNA and survive with aerobic energy production. The presence of mtDNA can be checked by Southern blot hybridization or by using the polymerase chain reaction.

The goal of the present project is to test the role, if any, of selective pressure for respiratory competence on the capacity of the exogenous mtDNA to replicate and repopulate the recipient cells. A prerequisite for these experiments is the definition of conditions that allow a high efficiency of cybrid production. For this purpose, a variety of approaches are presently being tested, involving different procedures for cell-cytoplast fusion.

199. Biolistic transformation of human cells with mtDNA

Andrea Martinuzzi, Michael P. King

The aim of this project was to establish an efficient transformation system for mtDNA in human cells. The biolistic approach (Sanford et al., 1987) was chosen because of the success reported with Chlamydomonas chloroplasts (Boynton et al., 1988) and yeast mitochondria (Johnston et al., 1988). We first set the conditions leading to the highest number of nuclear transformants in human cultured cells shot with tungsten
particles coated with a plasmid for G418 resistance (pNeoG418). The best conditions yielded about 5-8 x 10^-5 G418 resistant transformants utilizing as target cells both ρ^+ cells 143B.TK^- and their mtDNA-less derivative ρ^-206 (King and Attardi, 1989). The frequency obtained is actually higher than what has been reported previously in the literature (Zelenin et al., 1989), but is still quite low, considering the next step we intended to pursue: the mtDNA-mediated transformation of human cells. With an estimated 10^-3 successful mtDNA transformation for each nuclear transformation (as inferred from data obtained on yeast, Johnston et al., 1988), we were expecting an overall frequency of 10^-8 mtDNA transformants. We shot 6 x 10^8 ρ^- cells with whole human mtDNA and 4 x 10^8 ρ^+ cells with whole mtDNA from a chloramphenicol (CAP)-resistant human cell mutant or with a 7 kb construct (pmtnCAP) carrying both heavy and light strand origins of replication and the 16S rRNA gene with the mutation conferring CAP resistance. Cells were bombarded with both pNeoG418 and mtDNA (or pmtnCAP), or with the mtDNA (or pmtnCAP) alone. Several selection protocols were applied, but no mtDNA transformants were isolated. A finer tuning of the selection protocol might be important in future experiments. Furthermore, a substantial improvement should come from the use of a new model of the particle delivery system, which uses helium as propellant instead of the gunpowder presently utilized. This new system reduces the large (about 50%) cell loss during the blast and improves considerably the transformation efficiency.

References:


PUBLICATIONS


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Summary: Our laboratory is interested in the structure and function of molecules involved in cell surface recognition, particularly those molecules mediating the immune response. We are using a combined approach of x-ray crystallography to determine three-dimensional structures, molecular biological techniques to produce proteins for crystallization and to modify them, as well as biochemistry to study the properties of the proteins we make.

We are interested in understanding the atomic details of the recognition between T cells and their targets, which are either virally-infected cells or other cells recognized as "non-self" by the immune system. T cells have a complicated recognition system that includes a membrane-bound receptor (the T-cell receptor or TCR), the multi-subunit CD3 molecule, and the accessory or co-receptor molecules CD4 or CD8. A fundamental difference in recognition by T cells and by antibodies is that while antibodies bind to soluble antigens, T cells only recognize antigen when it is on the surface of a cell and "in the context of" a molecule of the major histocompatibility complex (MHC): either a class I MHC molecule in the case of cytotoxic or T-killer cells, or a class II MHC molecule in the case of T-helper cells. This latter property of T cells is termed "MHC restricted recognition," and the molecular details are beginning to be understood. Work from many different laboratories has revealed that foreign antigens are fragmented inside antigen presenting cells into short (~7-30 amino acids) peptides, which are then bound by MHC molecules as they exit to the cell surface. It is therefore a complex of an antigenic peptide plus an MHC molecule that is recognized by a TCR on the T cell.

Probably because of the complicated form of the TCR ligand, and because there is apparently no naturally occurring secreted form of a TCR, much less is known about the details of TCR recognition and binding compared to the relative wealth of knowledge of antibody-antigen interactions. The three-dimensional crystal structure of a TCR would serve as a basis for understanding how the diversity in primary sequences of TCRs is accommodated in the folded protein to create a binding site that is specific for an MHC molecule containing a bound antigenic peptide. In collaboration with Mark Davis' lab (Stanford), we have obtained microcrystals of a genetically engineered soluble T-cell receptor. Current efforts center upon improving the purity of the protein in hopes of obtaining larger crystals suitable for a crystallographic structure determination. The MHC and peptide ligand of the TCR we are crystallizing is also available in a soluble form, and we will attempt a co-crystallization of the ternary complex.

We are also interested in defining the precise molecular nature of the complex formed between an antigenic peptide and an MHC molecule. Previous work on the structure of a human class I MHC molecule revealed the peptide binding site to be a deep groove with two long α-helices forming its sides and a β-pleated sheet forming the bottom (Bjorkman et al., 1987a,b). The binding site was occupied in the crystal structure, presumably with a peptide or mixture of peptides that co-purified and co-crystallized with the molecule. A variety of evidence from other laboratories also suggests that the binding sites of class I and class II MHC molecules are occupied with endogenous peptide, and the presence of endogenous peptide may explain why conventional binding studies involving synthetic peptides and purified MHC molecules have not succeeded in producing quantitative binding (i.e., an equimolar complex). Our laboratory is concentrating upon producing forms of class I and class II MHC molecules that have empty peptide binding sites, in order to allow binding of exogenous peptide for measurements of binding properties, and formation of an equimolar complex with an antigenic peptide for crystallization. We are using a bacterial expression system to produce heterodimer class I MHC molecules and a chimeric single chain form of a class II MHC molecule. Purified protein has been denatured to remove any bacterial peptide capable of binding to an MHC molecule and then refolded in the presence and absence of the appropriate antigenic peptide. Correctly assembled heterodimeric class I protein can be renatured in the presence or absence of peptide and purified on a conformationally sensitive monoclonal antibody affinity column. We are now beginning to assay the peptide binding properties of the bacterially produced protein.

We have recently become interested in a heterodimeric Fc receptor that, for completely unknown reasons, appears to resemble class I MHC molecules. This receptor is expressed in the intestine of newborn mammals and is
responsible for transferring immunity from mother to infant by binding the Fc portion of immunoglobulin found in milk. The intriguing structural similarity between this Fc receptor and histocompatibility antigens, molecules with different functions, raises interesting questions which we wish to address using biochemical and structural methods. Some of these questions are: Given that the Fc receptor contains two domains similar to those implicated in peptide binding by class I MHC molecules, is it transported to the cell surface with endogenous peptide (as is the case for class I molecules), or is the "peptide binding site" in the Fc receptor the place where Fc binds? If there is a resident peptide in the Fc receptor molecule, what is its nature? Is β2-microglobulin (the light chain of both class I MHC molecules and this Fc receptor) implicated in Fc binding, and how does its association with Fc receptor heavy chain compare with its association with the class I MHC heavy chain? We have expressed a soluble form of this molecule in Chinese hamster ovary cells and crystallized it, so we will begin to address some of these questions in a crystallographic structure analysis.

We would like to determine the three-dimensional structures of other molecules mediating cell surface recognition, many of which are members of the immunoglobulin gene superfamily. A number of the molecules found to contain immunoglobulin homology units mediate cell-cell interactions in the nervous system as well as the immune system. Since most of these molecules are not available in milligram quantities, and the bacterial expression system we use for the MHC platforms may not be appropriate for more complicated molecules, we have collaborated with Prof. Kai Zinn to set up a eukaryotic expression system capable of producing milligram amounts of a fasciclin I, a cell surface adhesion protein found on insect axons. Having this quantity of pure protein has allowed the crystallization of this molecule, and should also allow biochemical experiments to find its natural ligand (perhaps by coupling the molecule to a column and passing over cell lysates). In collaboration with Profs. Henry Lester and Norman Davidson, we also plan to use this expression system to produce milligram amounts of the extracellular portion of the acetylcholine receptor for crystallographic and biochemical studies.

References:


200. The crystallization of a soluble form of a murine T-cell receptor

Roland K. Strong

We are collaborating with Mark Davis' laboratory at Stanford to crystallize a soluble T-cell receptor (TCR). They have produced a soluble form of a murine TCR by replacing the membrane spanning region with a signal that directs the cell to attach a phosphatidyl inositol moiety to the protein, so that it is attached to the cell membrane with a lipid linkage (Lin et al., 1990). Lipid-linked TCRs have been expressed in Chinese hamster ovary cells, and can be quantitatively released in a soluble form by treatment of the cells with phosphatidyl inositol-specific phospholipase C (PI-PLC). Milligram amounts of soluble 2B4, a well-characterized TCR with specificity for the class II molecule IEk complexed with a peptide of pigeon cytochrome c, can be produced and have been purified to homogeneity (Lin et al., 1990). The IEk substrate for the 2B4 TCR has also been produced in a soluble form using the same strategy (Wettstein and Davis, submitted). We are attempting to crystallize the soluble 2B4 TCR and the IEk molecule, as well as a complex between the two.

In the first step of the purification protocol established in the Davis laboratory to purify α/β heterodimer from homodimer and monomer, the supernatant obtained after PI-PLC treatment is passed over both anti-β and anti-α monoclonal antibody affinity columns sequentially. A low pH "shock" (-3.5-4.0) is used to elute the protein from these columns. Subsequent purification in our laboratory involves using a Pharmacia FPLC Mono Q anion exchange column to resolve two forms of the TCR that differ in the degree to which oligosaccharides have been processed posttranslationally (Forms I and II). 2B4 TCR heterodimer purified in this manner can be concentrated to >20 mg/ml. The last six-week production run at Stanford yielded 5.8 mg of Form I and 8.1 mg of Form II α/β heterodimer after concentration to 20 mg/ml. Form I has a lower apparent Mr both in SDS-PAGE and gel filtration experiments. It is also insensitive to neuraminidase
treatment, as shown by native isoelectric focusing gel electrophoresis. Form II shows altered mobility on SDS-PAGE after treatment with neuraminidase, Endo H or Endo F; neuraminidase treatment also raises the observed pI and reduces the heterogeneity of the material. Crystals have been obtained with the Form I heterodimer from polyethylene glycol. Crystals appear either as segments of regular octahedra or as regular hexagonal rods after approximately ten days. The largest crystals to date are approximately 250 µ in size. Diffraction patterns of a hexagonal crystal show lattice constants of 113.5 ± 0.3Å by 50.2 ± 0.2Å and diffract to ~6Å resolution using a conventional rotating anode source.

Our current efforts are proceeding along two lines: 1) to try to establish methods to collect data from the current crystals; and 2) to improve the quality of the current crystals. To improve the quality of the data obtained from the current crystals, we are exploring the use of synchrotron radiation and cryopreservation, two well-established methods for improving data quality. Second, in an attempt to purify to homogeneity a molecular species of 2B4 conducive to crystallization, we are exploring preparative treatment of 2B4 protein with various glycosidases followed by subsequent purification.

Reference:

201. Amplification of T-cell receptor expression in Chinese hamster ovary cells
David M. Penny, Roland K. Strong

As a means of increasing the amount of soluble T-cell receptor (TCR) produced by the Chinese hamster ovary (CHO) cells, we are in the process of co-transfecting a vector carrying the glutamine synthetase (GS) gene, a dominant selectable marker that promotes DNA amplification, with the expression vector that contains the TCR genes (the GS vector was obtained from Celltech, Inc.). With the GS gene present, the transfected DNA is amplified when recipient cells are cultured in media lacking glutamine but containing methionine sulfoximine, a potent inhibitor of GS function. The GS selection and amplification system has been used to produce large quantities of fasciclin I from CHO cells grown on the CellPharm (~1 mg of fasciclin I per daily harvest with phosphatidyl inositol-specific phospholipase C (see Abstract No. 205), and ~10 mg per daily harvest of soluble Fc receptor (see Abstract No. 204). We hope to be able to increase the level of TCR expression to a level similar to these by selecting clones in which amplification has been achieved using a TCR-specific monoclonal antibody and fluorescence-activated cell sorting.

202. Bacterial expression and renaturation of murine MHC class I proteins: A reappraisal
Margaret L. Fahnestock, George Liu, Ilana Tamir

One of the major goals of our laboratory is to examine the interaction of antigenic peptides with the major histocompatibility complex (MHC) class I proteins which present them for immune recognition by the T-cell receptor, in order to study the determinants of specificity and the molecular requirements for presentation. Towards that end we have expressed derivatives of the extracellular portions of murine H-2 heavy chains (Kd and Ld) and β2-microglobulin light chain to high levels in a bacterial system. The expressed polypeptides, purified as inclusion bodies, are denatured in 6 M guanidine hydrochloride, completely reduced with DTT and further purified by size exclusion chromatography to remove low molecular weight contaminants (e.g., peptides). The material is then dialyzed into 8 M urea and diluted from or dialyzed out of the denaturant to allow renaturation and disulfide bond formation. Diagnosis of proper folding is currently based on recognition of the renatured product by conformation-specific monoclonal antibodies, expected molecular weight by size exclusion chromatography, and resolution on native isoelectric focusing gels (denatured heavy chain will not enter a native gel).

Our initial hope was that the heavy chain's peptide binding domain ("platform") would prove to be an independent structural unit that could be produced and renatured independently of the rest of the heterodimer. Although a small amount of material of approximately the expected size can be sifted out of platform renaturations, problems of aggregation and precipitation even in the presence of detergents have made this approach impractical. To circumvent these problems, we are currently experimenting with platforms in which three hydrophobic amino acids that contact β2-microglobulin in the native
molecule have been changed to hydrophilic residues by site-directed mutagenesis. It is possible that the side chains of these three residues form a "hydrophobic pocket" that induces aggregation through its incompatibility with the aqueous environment. Experiments are now in progress to test this hypothesis.

Results with renaturation of the extracellular portion of the heavy chain in combination with light chain have been much more promising. Using antibody affinity chromatography as both final purification step and criterion of renaturation, we have obtained Kd heterodimers in both the presence and absence of a Kd-restricted peptide. The yield is low (1-5%), but one strength of the bacterial expression system is that starting materials and production expenses are not limiting. Further characterization of the renatured products (e.g., amino-terminal sequencing to determine constituent stoichiometry) will indicate whether this approach can be used to generate mouse MHC class I proteins for crystallography. As insurance, the mouse \( H-2 \) Kd and Ld and \( \beta_2 \)-microglobulin cDNAs are also being cloned into vectors suitable for expression in mammalian and insect cells with the expectation that the resulting products, having been glycosylated and constrained for proper disulfide bond formation in vivo will be more amenable to manipulation in vitro.

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203. The expression of mouse class II MHC molecule peptide binding platforms in bacteria

Andrew H. Huber

Although the three-dimensional structures of several class I major histocompatibility complex (MHC) molecules are known at atomic resolution, the detailed three-dimensional structure of a class II MHC molecule has not yet been determined. However, it is known that class II molecules consist of two distinct transmembrane polypeptides called \( \alpha \) and \( \beta \), each composed of two extracellular domains. Class I MHC molecules also have a total of four extracellular domains. Class I heterodimers are composed of a single transmembrane polypeptide containing three extracellular domains (\( \alpha_1, \alpha_2, \alpha_3 \)) that are noncovalently associated with the single domain of \( \beta_2 \)-microglobulin. The peptide binding site of a class I molecule is formed by the interaction of the \( \alpha_1 \) and \( \alpha_2 \) domains. It has been proposed that the four extracellular domains of the class II MHC molecule are analogous to those of the class I MHC molecule and that class II molecules have a three-dimensional structure similar to that of class I molecules (Brown et al., 1988). This model assumes that the \( \alpha_1 \) and \( \beta_1 \) domains of the class II heterodimer are structurally analogous to the \( \alpha_1 \) and \( \alpha_2 \) domains that form the class I binding site.

Since the bacterial expression of a monomeric protein is usually more straightforward than the expression of a heterodimer, I have used polymerase chain reaction to modify the \( \alpha_1 \) and \( \beta_1 \) domains of murine class II MHC such that they can be joined to each other via a small hinge region. The hinge sequence was chosen by aligning a three-dimensional model of a class II mouse MHC molecule to the known structure of the class I MHC molecule HLA-A2. The amino acids that formed the hinge between the \( \alpha_1 \) and \( \alpha_2 \) domains in HLA-A2 were used to join the \( \alpha_1 \) and \( \beta_1 \) domains of the class II MHC molecule at residue positions analogous to those joined by the hinge in the class I MHC molecule. This hinge should allow the two class II MHC molecule domains to interact to form the antigen binding site as proposed to occur in vivo. This class II chimeric protein has been expressed in large quantities in bacteria. The protein is sequestered in inclusion bodies and is therefore harvested in an insoluble and denatured form, followed by solubilization with a protein denaturant such as guanidine hydrochloride.

I am currently determining the optimal conditions for the renaturation of the expressed protein. Promising results have been obtained by using a gel filtration column to rapidly remove the protein denaturant. The remaining soluble protein is then passed through an affinity column that uses a peptide ligand that is known to be bound by the class II heterodimer. The peptide is bound to the column matrix in a fashion that should allow elution of the peptide/MHC platform complex. The protein eluted from this affinity column is believed to be correctly folded. My long-range goal is to crystallize the peptide binding domains of a class II MHC molecule complexed with a defined peptide. This complex should allow the determination of the manner in which the peptide is bound as well as the structure of the MHC molecule.
Specifically at receptor. The transfected cells bind rat IgG or rat Fe with specific antibodies against the two chains of Fe receptors have been extensively characterized by their light chain and a truncated FcRn heavy chain. After amplification, a cell line producing large amounts of the molecule was selected and used to seed a Cell Pharm (a hollow-fiber tissue culture system that allows growth of ~10^11 cells in a 300 ml vessel). Over 50 mg of soluble Fc receptor have been purified by affinity chromatography on a Sepharose column containing 70 mg of immobilized rat IgG. The FcRn molecule appears pure in one step of purification, as assayed by SDS-PAGE, gel filtration and N-terminal sequencing. Purified FcRn has been crystallized in PEG 8000 and ammonium sulfate. Several crystalline habits have been observed, including large thin plates, hexagonal rods, and cubes. The cube-like crystals diffract beyond 3.5 Å, and are space group C222_1, with unit cell dimensions of a = 126 Å, b = 190 Å, and c = 148 Å. We are currently exploring methods of crystallographic data collection, as well as biochemical experiments to characterize the molecule and its binding site for Fc. We will also try to co-crystallize the FcRn with its natural ligand, rat Fc.

Reference:

205. Expression and structural studies of grasshopper fasciclin I

Wen-Ching Wang, Kai Zinn

Fasciclin I (f1) is a membrane-associated protein that is expressed on certain subsets of neurons and fasciculating axons in the developing central nervous system of grasshopper and Drosophila. The analysis of its regional expression, primary structure and adhesion activity suggests that f1 may mediate axon pathfinding via a homophilic interaction between cells. An analysis of the cloned genes from grasshopper and Drosophila suggests that the protein consists of four homologous domains of 150 amino acids each, which may define a new structural motif for cell surface proteins, since they are not related to other sequences in the current databases.

We have developed an expression system to produce large quantities of soluble f1 for crystallization and biochemical analysis. A stable Chinese hamster ovary
(CHO) cell line was constructed by transfecting a vector containing a strong promoter (SRα) and the glutamine synthetase gene, a dominant selectable marker that allows a one-step amplification of the cloned genes. Since f1 is linked to the membrane with a glycosyl-phosphatidylinositol moiety, the protein can be cleaved from the cell surface by treatment with phosphatidylinositol (PI)-specific phospholipase C (PI-PLC). Up to 1 mg of f1 protein can be purified on an immunofinity column per daily harvest from the cell line grown on the Cell Pharm hollow-fiber tissue culture system that allows growth of ~10^{11} cells in a 300 ml vessel. Single crystals (~0.5 mm^3) of grasshopper f1 have been grown in ammonium sulfate or Na,K phosphate precipitants. The molecule crystallizes in space group C2221, with unit cell dimensions 156 Å x 352 Å x 168 Å. The crystals diffract anisotropically, with diffraction to 3.5 Å resolution in one direction, and diffraction in the other two directions to only 5 Å resolution.

Our current efforts are aimed at modifying the expressed f1 protein in an attempt to grow crystals that diffract to higher resolution. The f1 molecule expressed in CHO cells has a molecular weight ~90 kDa, as compared to 75 kDa when expressed in insect cells. This increase in molecular weight is presumably due to extra glycosylation in mammalian cells, which may interfere with crystallization. The part of the PI tail that remains attached to soluble f1 after treatment with PI-PLC may also interfere with crystallization. Our strategies to improve the crystal quality include the following: remove extra carbohydrates by deglycosidases, remove the remainder of the PI tail by genetic engineering, and/or express f1 in Drosophila cells where extra glycosylation will not occur. A modified f1 gene coding for a secreted protein has been transfected into CHO cells and Drosophila cells. In both cases, we observe soluble f1 protein in the supernatants and are beginning to purify the secreted protein. Biochemical experiments with the purified proteins will be done to analyze the oligomeric state of the protein, which is relevant to an understanding of the homophilic adhesion mechanism. Finally, we will examine whether different forms of soluble grasshopper f1 can perturb pathway decisions made by identified growth cones in grasshopper embryo culture.

206. Expression and structural studies of a truncated soluble murine CD4 fragment

Wen-Ching Wang, Pamela J. Bjorkman

CD4 is found on T-helper cells and is a co-receptor along with the αβ T-cell receptor heterodimer that recognizes antigenic peptides bound to major histocompatibility complex (MHC) class II molecules, presumably by binding to a monomorphic determinant on the class II molecules. The CD4/class II MHC interaction is also critical for a key stage in thymocyte differentiation and maturation. In addition, CD4 is the receptor for human immunodeficiency virus (HIV).

The extracellular portion of the CD4 molecule consists of four tandem immunoglobulin (Ig) variable-like domains called V_1, V_2, V_3, and V_4. The crystal structure of an N-terminal fragment of human CD4 consisting of two Ig-like domains, V_1V_2, was recently determined (Ryu et al., 1990; Wang et al., 1990). As predicted, both domains are similar to Ig variable domains, but V_2 has a truncated β barrel and an unusual intrasheet disulfide bond. The sites of interaction with the HIV gp120 protein were mapped on the structure, but the sites of interaction with MHC class II proteins remain obscure.

Because the interaction of murine CD4 with murine class II MHC molecules has been well studied, we plan to work on an x-ray analysis of the murine CD4 molecule in an attempt to elucidate a clearer relationship of the structure and function of CD4. In addition, since murine CD4 does not bind HIV gp120, we can eventually compare the murine CD4 structure to the human V_1V_2 structure to gain a molecular understanding of which parts of the CD4 molecule are important in its role as the HIV receptor. As a first step towards these goals, we have produced a Chinese hamster ovary cell line that expresses high levels of a truncated murine CD4 molecule consisting of the V_1 and V_2 domains, using a similar expression system as described in Abstract No. 205 for the expression of fasciclin I.

Pilot amounts of secreted murine V_1V_2 have been purified using a monoclonal antibody affinity column. We are currently scaling up production of this protein and hope to crystallize it using similar conditions to those successful for human V_1V_2 (Chamov et al., 1990).
References:

207. Expression of a β2-microglobulin binding protein from human cytomegalovirus
Margaret L. Fahnstock

Human cytomegalovirus (CMV), a member of the herpes virus family, is a ubiquitous pathogen. Although normally associated with asymptomatic infection or a mononucleosis-like syndrome, CMV can cause severe disease in immunocompromised individuals and the developing fetus. It has been known for some time that CMV particles can adsorb β2-microglobulin, the light chain of the antigen-presenting major histocompatibility complex (MHC) class I heterodimer, but the nature and results of this association have not been characterized in detail. Recent reports have identified a viral gene whose predicted protein product bears striking resemblance to eukaryotic MHC class I heavy chains (Beck and Barrell, 1988). This viral protein has been shown to bind β2-microglobulin when co-expressed with it in a mammalian expression system, a phenomenon that is accompanied by the loss of MHC class I expression at the cell surface. This suggests that one role for the viral protein in the infected cell is to deplete the intracellular pool of β2-microglobulin upon which class I heavy chains are dependent for proper assembly and translocation to the cell surface, effectively blocking presentation and immune recognition of viral antigens.

In keeping with our interest in form and function of proteins of the immune system, we have begun the process of introducing the class I heavy chain cognate gene (UL18) of HCMV strain AD169 into mammalian expression vectors for large-scale production of protein in mammalian cells. At least two forms of the gene will be used. Polymerase chain reaction (PCR) has been used to generate a full-length copy. PCR plus site-directed mutagenesis are being used to manufacture a truncated form composed of the portion corresponding to the MHC class I extracellular domain fused through a four amino acid Factor Xa cleavage site to the decay accelerating factor (DAF) sequence which mediates attachment of proteins to the cell membrane via a phosphatidyl inositol linkage. The latter form should be recoverable from the surface of the expressing cell by digestion with phospholipase C followed by removal of foreign amino acid sequences by specific proteolytic cleavage at the Factor Xa site. Our goal is to produce and purify sufficient protein for physical characterization and crystallization of this interesting viral product to determine the nature of its interaction with β2-microglobulin and its functional characteristics with respect to peptide binding and presentation.

Reference:

208. The crystallographic analysis of secretory component
Roland K. Strong

Secretory component (SC) is involved in the transcytosis of immunoglobulin (IgA) multimers from the maternal blood supply into the milk. A membrane-anchored form of SC, called the poly-Ig receptor, binds circulating IgA. This complex is then transported to the lumen where it is released through hydrolysis. Receptors in the intestinal tract of a nursing neonate can then absorb these secreted maternal antibodies for utilization by the neonate's fledgling immune system. The human protein has been shown to comprise five immunoglobulin variable-like domains on the basis of the primary sequence. SC, purified from pooled human milk, was crystallized from (NH₄)₂SO₄. Crystals grow over a period of six to eight weeks at room temperature, and have the appearance of square plates, or stacks of plates, with rounded corners that grow up to 500 μ in size. These crystals diffract to ~6Å resolution, but are quite disordered. Treatment with neuraminidase, while greatly reducing the pl heterogeneity, does not improve the quality of the crystals. Due to the difficulty in obtaining human milk, pooled or otherwise, and the extreme heterogeneity of the protein on native isoelectric focusing gels, we are attempting to purify and crystallize the bovine analog of this protein. We are currently in the process of developing...
a purification protocol for this protein.

209. Expression of the extracellular ligand binding domain of the neuronal AChR α7
Daniel Vaughn, Norman Davidson, Henry A. Lester

We are developing an expression system to produce large amounts of the chick neuronal nicotinic acetylcholine receptor α7 (described in Abstract No. 290) for a crystallographic structure determination. We will stably express in host Chinese hamster ovary (CHO) cells the approximately 700 bases of the cDNA encoding the signal sequence, the 208 amino acids comprising the putative extracellular domain, and genetically engineered stop codon. Supernatants from transfected CHO cells will be assayed for secreted protein corresponding to the extracellular domain of the ion channel by binding of acetylcholine and/or α-bungarotoxin. Secreted protein will be purified by α-bungarotoxin affinity chromatography and assayed for resistance to proteases as a verification of correct folding. Crystallization trials will be attempted with acetylcholine-bound, α-bungarotoxin-bound, and empty receptor.

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necessary for DNA replication, origins of replication, called ARSs in yeast, and the proteins that interact with them. ARSs have at least three important regions, a core consensus, an adjacent A/T-rich region that binds protein, and the recognition site for an abundant transcription factor that is also important for initiation or DNA replication. We have purified the two latter proteins and studied them both biochemically and genetically. Interestingly, as yet, no one has identified a protein that binds to the third element, the consensus sequence found at all ARSs.

In yeast, commitment to DNA synthesis occurs at the step controlled by the CDC28 protein kinase (homolog of cdc2/MPF) called START. DNA synthesis does not ensue immediately, however. Both the induction of genes encoding proteins required for DNA synthesis and posttranslational modification of replication proteins are part of the events following START. In addition to our studies of specific gene induction, we have been defining the elements of a protein kinase cascade that links the CDC28 step and the actual onset of DNA synthesis by defining intermediate regulatory steps and the actual replication proteins that respond to the kinases. Thus, our studies span both the regulation of DNA replication within S phase and the regulation of the entry into S phase itself.

210. Role of yeast CDC7 gene in initiation of DNA replication

Hye-Joo Yoon, Seng H. Loo, Judith L. Campbell

The yeast Saccharomyces cerevisiae Cdc7 protein appears to have a myriad of functions in cellular physiology, including roles in the control of initiation of DNA synthesis during mitosis, synaptonemal complex formation during meiosis, and DNA repair. Research in the Campbell group is directed towards elucidating the mechanism(s) by which the Cdc7 protein carries out its functions, particularly its role in regulating initiation of chromosomal DNA replication. During the G1/S transition, Cdc7 functions following START, an event that commits cells to DNA synthesis, and immediately preceding DNA replication. START itself is mediated by the CDC28 gene product, a yeast analog of the protein kinase subunit of the human Cdc2/MPF. Although cells become committed at START, DNA synthesis only occurs after completion of additional events, including the Cdc7 step. Central to the goal of unraveling the mechanism of Cdc7 action would be the identification of the substrates and/or proteins with which Cdc7 protein interacts. An important clue as to how Cdc7 acts was recently obtained when the Cdc7 protein was shown to be a phosphoprotein with protein kinase activity (Yoon and Campbell, 1991). Therefore, Cdc7 may regulate the various metabolic pathways by phosphorylating one or more target substrates.

Currently, the laboratory is actively engaged in exploring the hypothesis that Cdc7 is involved in a phosphorylation cascade during the G1/S transition, leading to the initiation of DNA replication. More specifically, we found that CDC28 immune complexes can phosphorylate Cdc7 in vitro, and that Cdc7 immune complexes can phosphorylate the p36 subunit of yeast replication protein A (RP-A), a single-stranded DNA binding protein that may be involved in DNA replication (Kenny et al., 1990). The in vitro interaction between Cdc7 and CDC28 is supported by in vivo work that shows that the activity and level of phosphorylation of Cdc7 kinase is dependent, either directly or indirectly, on CDC28 function. Similarly, p36 RP-A phosphorylation seems to be dependent on the Cdc7 step. These biochemical studies suggest that Cdc7 may serve as a molecular link between the regulatory apparatus active at START and the catalytic apparatus involved in the initiation of DNA replication. We plan to investigate whether Cdc7 is a true physiological substrate for the CDC28 kinase by determining the sites of phosphorylation in the Cdc7 protein in vivo and comparing them to the sites phosphorylated by CDC28 in vitro. By site-directed mutagenesis, we will alter the phosphorylation sites and determine if the engineered mutations have effects on any of the metabolic processes in which Cdc7 is involved. Similar kinds of mutagenic studies have also been planned for the yeast RP-A protein.

In addition to the studies described above, we are also attempting to purify active Cdc7 from yeast, using an epitope tagging technique. When Cdc7 was expressed in Escherichia coli, it was found to be inactive as a kinase, indicating that either covalent modifications or interaction with other yeast cellular proteins is essential for Cdc7 kinase activity. Our approach to studying this is to purify
the active kinase from yeast and characterize it with respect to posttranslational modifications and subunit structure. A Cdc7-hemagglutinin (HA) epitope fusion gene was constructed that could complement cdc7 temperature-sensitive and disrupted strains. Immunoprecipitations and Western blots have been successfully carried out from both E. coli and yeast cells expressing the Cdc7 fusion protein using a monoclonal antibody that recognizes the HA epitope. An immunoaffinity column has also been prepared for the large-scale purification of active Cdc7 kinase from yeast. Therefore, we now have the reagents necessary for Cdc7 purification, and once this is accomplished, we can proceed with a more detailed biochemical characterization of the Cdc7 kinase.

References:

211. Cell cycle regulation of DNA polymerase α and other yeast DNA replication genes
Rati Verma

In Saccharomyces cerevisiae, the genes encoding at least 10 enzymes involved in DNA replication are periodically expressed in the late G1 and S phases of the cell cycle. All of these genes have one copy or more of the sequence AGCCGT, which conforms to the recognition site for the Mlul restriction endonuclease. For the CDC21 (McIntosh et al., 1991), CDC9 (Lowndes et al., 1991), and POL1 genes (Gordon and Campbell, 1991), which encode thymidylate synthase, ligase and DNA polymerase α, respectively, the Mlul site has been shown to be absolutely required for periodic transcription.

Using nuclear extracts fractionated by conventional and oligonucleotide affinity chromatography, we have purified a 17 kDa protein that recognizes the Mlul motif. Synthetic oligonucleotides containing mutated Mlul sites do not bind the protein. In contrast, synthetic oligonucleotides derived from the CDC2, CDC6, and CDC21 genes, which are expressed with the same timing as POL1, bind purified protein efficiently.

Polyclonal sera has been raised against the purified protein (designated MCBF) permitting biochemical studies aimed at understanding the mechanism by which MCBF confers POL1 periodicity. MCBF could be synthesized at a discrete stage in the cell cycle or it could be post-translationally modified, for example, by CDC28 protein kinase. The serum is also being used to screen a λgt11 expression library. Cloning of the gene and its subsequent mutational analysis should help define the cascade of events set in motion in G1 and completed during the S phase of the cell cycle.

References:

212. The roles of ABF1 in DNA replication and transcriptional activation

Peter R. Rhode, Judith L. Campbell

Autonomously-replicating sequences, or ARSs, act as origins of replication in the yeast Saccharomyces cerevisiae. Based on prokaryotic and viral systems, it is reasonable to assume that proteins that bind to specific DNA sequences in ARSs are required for initiation or regulation of DNA replication. We are using a combined genetic and biochemical approach to characterize such proteins and their roles in yeast DNA replication.

In Sweder et al. (1988), we described two ARS binding proteins, ABFI and ABFIII. We have focused on ABF1 since its binding site is important for ARS function, while the ABFIII binding site is dispensable. Although ABF1 was first recognized as an ARS binding protein, it has recently become clear that this abundant, high affinity DNA binding protein specifically recognizes the motif RTCRYBNNA.CG at many sites in the yeast genome including promoter elements. Mutational analysis of these sites suggests that ABF1 regulates basal and carbon-source inducible transcriptional activation, transcriptional silencing and ARS activity. The fact that disruption and overexpression of the ABF1 gene results in loss of cell viability provides further evidence for the critical role of ABF1 in cell growth (Rhode et al., 1989). Since ABF1 is an essential gene, before carrying out genetic analysis to better define the roles of ABF1, we have isolated conditional lethal mutations in the ABF1 gene that confer temperature-sensitive (ts) growth. Three of the ts mutants
isolated show rapid growth arrest at the nonpermissive temperature. The abfl-ts mutants show distinct variations in ABFI-DNA binding activity. Marker rescue and DNA sequence analysis reveal that two of the missense mutations (abfl-1 and abfl-4) responsible for these ts phenotypes fall within the bipartite DNA binding domain of ABF1 and the third mutation (abfl-5) changes a proline residue located between the binding domains.

The phenotypes of the mutants with respect to DNA replication and transcriptional activation have been analyzed in detail. Flow cytometric analysis of abfl-5 mutants synchronized with α-factor and then released at the nonpermissive temperature shows that cells fail to progress efficiently from G1 through S phase, indicating a cell cycle defect in this mutant. In addition, metabolic labeling reveals that DNA and RNA synthesis are inhibited 75% and 70%, respectively, compared to cells released at the permissive temperature, while protein synthesis is normal for several hours. To examine the effects of ABF1 on DNA replication and transcription in more detail, we measured ARS activity and ABF1 binding site-specific UAS activity in the abfl-ts mutants. Loss of ARS1-21-CEN3 plasmids in the abfl-1 and abfl-5 mutants grown at the semipermissive temperature was fivefold greater than in the same mutants grown at the permissive temperature and increased over 25 fold relative to an isogenic ABFI strain, verifying the importance of ABF1 in ARS activity. Together with the cell cycle defects, these results provide evidence for a direct role for ABF1 in the initiation of DNA replication. Transcriptional defects were also detected, as transcriptional activation of a heterologous promoter by the ABF1 binding site decreased twofold in the abfl-1 mutant at the semipermissive temperature.

References:

213. Characterization of the helicase and ATPase activities of OBFA

Christopher C. Byrd, Rati Verma, Judith L. Campbell

The yeast protein OBFA was originally identified as a potential replication protein based on its affinity for ARS-1 DNA. Subsequent study revealed that although OBFA did not appear to bind to a single conserved site in ARS-1, it nevertheless possessed three associated activities that are characteristic of proteins involved in DNA replication: an ATP-dependent helicase activity, a DNA-dependent ATPase activity, and a DNA unwinding activity. The first two of these three activities were the subject of our recent investigations.

Studies of the ATP requirements of the helicase activity using the nonhydrolyzable ATP analog ATP [γS] have confirmed that ATP hydrolysis is required for activity, while studies of the directionality of the helicase have revealed that it translocates along the DNA in the 5'-3' direction. Investigation of the nucleotide and DNA cofactor preferences of the associated DNA-dependent ATPase activity have established that ATP and dATP are the preferred nucleotide substrates and that poly(dT) is the preferred DNA cofactor.

Current investigation is aimed at localizing the helicase and ATPase activities to specific polypeptides within our OBFA preparations. Highly purified preparations of OBFA contain two polypeptides, an 18 kDa protein which is responsible for the ARS binding activity and a 43 kDa protein of unknown function. Using affinity purified antibodies specific for each of these polypeptides, we hope to perform antibody inhibition assays to determine in which polypeptide the helicase and ATPase activities reside.

214. Analysis of the dna154 gene, a gene involved in DNA replication

Martin Budd

A mutant, dna154, is defective in DNA replication both in vitro and in vivo at 36°C (Kuo et al., 1983). When dna154 cells are incubated at 36°C for 3 hr, they exhibit a terminal phenotype analogous to DNA pola(ts), pol8(ts) strains, in which the nucleus has migrated to the isthmus but has not entered the daughter cell. The DNA154 gene has been cloned by complementation from a YCp50 library and by deletion analysis has been localized to a 4.5 kb fragment. The clone was shown to code for the Dna154 protein by use of the marker rescue technique using a fragment containing only the DNA154 gene. The message size is 5 kb and exists in the same abundance as DNA polymerase α message. We are now sequencing the gene.

Reference:
215. Construction of temperature-sensitive mutants of DNA polymerase ε

Martin Budd

We have previously purified DNA polymerase ε and have generated polyclonal antibody to the catalytic subunit (Budd et al., 1989). The enzyme is highly processive on a poly(A) or oligo(dT) template and is essential for cell viability (Morrison et al., 1990) which makes it a candidate along with pol8 (Saccharomyces cerevisiae cdc2 gene product) for a leading strand polymerase. To test the hypothesis that polε is a leading strand polymerase, we are constructing temperature-sensitive mutants of DNA polymerase ε using the plasmid shuffle technique previously worked out in the laboratory. A clone containing the DNA polymerase ε is inserted into a pSEY18, a ura3 containing plasmid, and YCp19B, a trpl containing plasmid. A pol2-3[LEU2]-ura3-52 trpl-289 pSEY18(POL2) strain is transformed with a mutagenized YCp19Bpol2 plasmid, replica plated onto 5-fluoro-orotic acid, and the resulting transformants tested for a resulting temperature-sensitive phenotype.

References:

216. DNA polymerases from yeast

William Clay Brown

Three nuclear DNA polymerases, α, δ and ε, have been characterized and shown to be essential in Saccharomyces cerevisiae. Previous work (Sitney et al., 1989) had shown that DNA polymerase δ (formerly III) was encoded by CDC2. In an attempt to overexpress polymerase δ in bacteria, the CDC2 gene was cloned into the expression vector pKK223-3. This construct and several others employing T7 vectors were found to be quite unstable and readily underwent insertions and deletions of CDC2. Overlapping fragments of the gene could be stably cloned when out of frame, suggesting that uncontrolled expression is responsible for the instability; thus, the protein product of CDC2 must be toxic to Escherichia coli.

To suppress lethality by controlling readthrough expression of CDC2, a special vector was constructed from pT7-7. An 800 base fragment of pKK223-3 containing the promoter region of the β-lactamase gene and the rho-independent transcription terminators T1 and T2 from the rm-B operon was cloned into two sites upstream of the T7 promoter. These were oriented so that each promoter was directed away from the cloning region and was followed by the terminator sequences. This fragment was also inserted into the last restriction site of the polylinker, again such that the promoter was directed away from the cloning region. This plasmid yielded stable constructs containing either the entire CDC2 sequence or progressively smaller restriction fragments lacking specific conserved regions. Experiments are currently under way to express these polypeptides and compare them for biochemical activities such as polymerization, exonuclease, DNA binding and template preference. A full-length copy of the CDC2 gene has also been cloned into the yeast expression vector pSEYC68gal. This construct complements cdc2 temperature-sensitive mutants at the nonpermissive temperature and overexpresses polymerase activity by at least fivefold.

Work has also been done on obtaining monoclonal antibodies to DNA polymerase ε (II). A 170 kDa polypeptide which displays polymerase activity in trap assays and is recognized by polyclonal antibody in Western blots was excised from a polyacrylamide gel and used for immunizations which were done by the Caltech Monoclonal Antibody Facility. After fusions, cell lines were screened for activity by immunoprecipitations of partially purified polymerase ε. Positives were subcloned and these are currently being screened.

Reference:

217. Molecular genetic study of DNA polymerase ε stimulatory factor one

Jean Smiley

Yeast DNA polymerase ε (pol ε) is the product of a gene essential for yeast viability that has an unknown function in the cell. It may be active in DNA replication, recombination, and/or repair. Pol ε has been purified to homogeneity, and in the process, two stimulatory factors were identified. One of these factors, stimulatory factor I (SFI), has been purified. It contains three polypeptides of 66, 37, and 13.5 kDa. Single-stranded DNA binding
activity has been assigned to the large polypeptide. To further understand the function of SFI, its genes are being cloned and characterized. The gene corresponding to the 66 kDa polypeptide, stimulatory factor one (SFO), has been cloned by screening a yeast genomic λgt11 library with a polyclonal antibody specific for p66. Three positive clones were identified and shown to be overlapping by restriction enzyme analysis. These clones were demonstrated to be of the p66 gene and not of genes to proteins that crossreact with the p66 polyclonal antibody by hybridizing oligonucleotides that had sequences derived from p66 trypic peptides to the positive clones. The SFO gene was disrupted by transposon mutagenesis. This demonstrated that SFO is essential for yeast viability. To further characterize this gene, it is being sequenced and strains containing conditional alleles will be constructed. It will be determined if they have a defect in DNA replication, recombination, or repair. By genetically studying this gene, a better understanding of the possible in vivo functions of pol ε will be obtained.

218. Functional domains of yeast DNA polymerase α
Suzanne Elsasser

Yeast DNA polymerase α, a vital protein required for DNA replication in yeast, shares six regions of striking homology with human DNA polymerase α, and several eukaryotic and bacterial virus polymerases. The function of these conserved regions has largely been approached by assessing viral mutations. Analysis of mutations in Herpes simplex virus DNA polymerase that confer altered sensitivity to nucleotide mimics suggests that the active site of HSV DNA polymerase, and homologous polymerases, is comprised by the conserved domains II, III, and V.

In order to investigate the assignment of the functional active site in yeast DNA polymerase α, site-directed mutants in domains II and III have been constructed and can be driven by an inducible promoter. These mutant proteins will be overexpressed and purified by immuno-affinity chromatography. A monoclonal column has been constructed that allows the rapid and quantitative purification of highly active DNA polymerase α. Tyrosine 843 falls within domain II and is conserved among yeast, HSV, and a number of other sources. Mutation of this tyrosine to histidine in HSV DNA polymerase confers aphidicolin resistance and phosphonoacetic acid hypersensitivity. Purified yeast DNA polymerase α with the analogous mutation will be analyzed for altered sensitivity to these compounds. Similarly, asparagine 948, found in domain III, is conserved over many polymerases. Mutation of this asparagine to serine in HSV DNA polymerase confers resistance to acyclovir, and characterization of yeast DNA polymerase α with the analogous mutation will clarify the functional similarity.

219. Mechanisms underlying amplification of the DFR1 gene in Saccharomyces cerevisiae
L. Elizabeth Bertani

Gene amplification is a basic genetic phenomenon of fundamental importance in evolution, development, adaptation and the origin of cancer. High levels of amplification of the dihydrofolate reductase gene can be obtained in cultured eukaryotic cells by selecting gradually for resistance to the drug methotrexate (MTX). We are studying amplification in yeast where it should be easier to elucidate the mechanisms—such as recombination and replication—that are thought to be involved.

Starting from a standard, genetically-marked strain (SS-111), we have selected derivatives that are resistant to increased levels (up to 1 mM) of MTX. The chromosomes of these strains were separated by pulsed-field gel electrophoresis and probed simultaneously for the single copy gene UR3 (on chromosome V) and the gene that codes for dihydrofolate reductase (DFR1) (on chromosome XV). About 25% of the strains examined had extra copies (up to eight) of the DFR1 gene. However, these strains are also amplified for other genes (such as ADH1 and HIS3) on chromosome XV, leading to the conclusion that they are polysomic, i.e., have extra copies of the whole chromosome. Thus, low frequency, mitotic nondisjunction may be the most common mechanism for acquiring resistance to these levels of MTX.

We are also examining the effect of chromosomal structures that might stimulate recombination or replication. In the first trial, a plasmid containing the cloned DFR1 gene and a yeast origin of replication (ARS1) was
integrated into the chromosomal DFRI gene to produce a construct consisting of two direct repeats of the DFRI gene with the plasmid and ARSI sequences in between. Six subisolates of one strain were subjected to increasing levels of MTX, up to 1 mM. We expected that these strains would prove—like the first ones—to be polysomic. Instead, in all six cases the resultant strains were found to contain about 30 extrachromosomal copies of the original plasmid. Although the analysis is not complete, it seems most likely that this result is the consequence of a process of excision via the direct repeats, which occurs with higher frequency than non-disjunction. The presence of the ARSI sequence would allow the excised plasmid to replicate further.

We intend to continue these studies by examining the effect of direct repeats without an ARSI sequence present, since in this case excision would not result in MTX resistance. In addition, we will evaluate constructs that have an ARSI sequence without a direct repeat or with inverted repeats.

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Summary: Our laboratory works with two groups of human pathogens, the alphaviruses and the flaviviruses. Much of the molecular characterization has been done with the type alphavirus, Sindbis, which is nonpathogenic for man, and with the 17D vaccine strain of the type flavivirus, yellow fever, but as discussed below and described in the individual reports, we have also studied a number of other alphaviruses and flaviviruses as well. We wish to understand on a molecular level many aspects of alphavirus and flavivirus replication, including the interactions of the proteins with one another during virus assembly and for RNA replication, the nature and location of determinants important for pathogenesis, and the interactions of the viruses with their hosts. Furthermore, we are interested in determining the relationships between current viruses and in understanding RNA virus evolution.

Sindbis virus has a single-stranded RNA genome of 11,703 nucleotides. The genomic RNA is an mRNA encoding the four nonstructural proteins of the virus, which are responsible for RNA replication. The structural polypeptides (a capsid protein C, which encapsidates the RNA in an icosahedral nucleocapsid, and two integral membrane proteins, E2 and E1, which form the spikes of the lipoprotein envelope that surrounds the capsid) are encoded in a subgenomic 26S mRNA. Both the nonstructural and structural proteins are translated as
polyprotein precursors, which are proteolytically processed to the final products.

The alphaviruses are vectored by mosquitoes and possess the ability to replicate both in mosquitoes as well as in a wide range of vertebrates. We are making a major effort to identify the cellular receptors to which Sindbis binds when it invades a cell. We would like to determine the distribution of receptors on various cell types, relate this to tissue tropism and disease symptomology of alphaviruses, and determine whether the receptors used to infect phylogenetically diverse hosts are common or closely related molecules. Another major effort is aimed at understanding in detail the nature of the two virus-encoded proteinases that process the nonstructural and structural polyproteins. The nonstructural proteinase is located in nsP2, and we have determined that certain polyproteins containing nsP2 have preferences for particular cleavages in the processing pathway. In vivo, the sequence of cleavages forms a defined developmental pathway to temporally regulate RNA synthesis.

We have a cDNA clone of the entire Sindbis genome from which infectious RNA can be transcribed in vitro. Using this clone, it is possible to perform site-specific oligonucleotide-directed mutagenesis, or to substitute restriction fragments in the clone with the corresponding fragment of cDNA from previously characterized mutants, and then examine the resulting phenotype in viruses rescued by RNA transfection. Studies of this kind include mapping of the nonstructural protease, mutagenesis of conserved nucleotide domains at the 3' and 5' regions of the genome, mutagenesis of the cleavage sites between proteins, and dissection of the contributions of certain amino acid substitutions to neurovirulence in mice. Many of the domains selected for mutagenesis were identified originally by comparative sequencing of different strains of Sindbis virus, including Ockelbo virus and the causative agent of Karelian fever, and of different alphaviruses such as Middelburg, Western equine encephalitis, Ross River, and O'Nyong-nyong. In addition, we now have a complete cDNA clone of Ross River virus, and have constructed a number of viable hybrids between Ross River and Sindbis, which are currently being characterized.

The family Flaviviridae contains about 70 members, many of which are important human pathogens. Like alphaviruses, flaviviruses are enveloped viruses with a single RNA molecule of about 10,700 nucleotides of plus polarity as their genome. Unlike alphaviruses, the entire genomic RNA serves as the only mRNA. It contains a single long open reading frame which encodes the structural proteins at the 5' end followed by a series of nonstructural polypeptides whose functions have not been defined. The resultant translation product is a polyprotein that is processed by both cellular and viral proteinase activities. We have constructed expression plasmids with portions of the dengue genome which exhibit normal processing when transcribed and translated in vitro. From these constructs we could establish that one of the flaviviral proteinases is located in protein NS3. We are also producing monospecific antibodies to each final polypeptide and identifying antigenic epitopes on the viral proteins important for immunity to dengue. Finally, we are constructing a full-length and hopefully infectious clone of a flavivirus. Once an infectious clone can be obtained, parallel experiments to those outlined above for alphaviruses can be performed to determine the functions of particular regions of the genome, to map virulence determinants, and ultimately to engineer safe and efficacious flavivirus vaccines.

220. Molecular characterization of the Sindbis virus receptor

Kangsheng Wang, Richard J. Kuhn, Ellen G. Strauss, Susan Ou, Chen-Wen Yao, James H. Strauss

To look for monoclonal antibodies (mAb) reactive with the cellular receptor for Sindbis virus on baby hamster kidney (BHK) cells, two BALB/c mice were immunized with whole BHK cells and one mouse was immunized with 200 µg BHK cell membranes per injection. Approximately 3,200 hybridoma clones were screened by plaque reduction assay. One IgM mAb from a mouse immunized with whole BHK cells (mAb 1C3) inhibited Sindbis virus AR339 attachment to BHK cells by 80% at 20 µg/ml and immunoprecipitated a 68 kDa membrane protein from BHK cells. mAb 1C3 also inhibited Sindbis virus attachment to Vero cells (monkey) and SW13 cells (human) and immunoprecipitated a 68 kDa protein from Vero cells, SW13 cells, and mouse N18 cells. The antibody did not block virus attachment to
chicken cells but did immunoprecipitate a 71 kDa protein from chicken cells. Fifteen reactive phages were found by screening $10^6$ plaques from a λgt11 cDNA library from BHK cells with mAb 1C3. Six out of the 15 clones were shown by sequence analysis to react with overlapping regions of a protein that was identical in sequence to the mouse high affinity laminin receptor and had 99% amino acid sequence identity to the human laminin receptor. A full-length laminin receptor cDNA was cloned into a pcDNA1/neo vector in both the plus and minus orientation. BHK cell lines stably transfected with vectors expressing the plus sense BHK laminin receptor cDNA were three to fivefold more susceptible to the virus as measured by plaque assay, and overexpress receptor protein on the cell surface as measured by flow cytometry analysis. Cells expressing antisense RNA were only 60% as susceptible to the virus as were nontransfected cells, and less laminin receptor is present on the surface of these transfecants as measured by flow cytometry.

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221. Analysis of the proteins bound to the 5' nontranslated region of the Sindbis genome

Nathalie Pardigon

The 5' nontranslated region of the Sindbis virus RNA has been proposed as one possible promoter for initiation of transcription/replication of the viral genome. We are currently examining the ability of this region to bind protein(s) from cytoplasmic extracts, both infected and uninfected, using minus polarity transcripts (produced and labeled in vitro) of the first 125 bases of the 5' region of Sindbis virus genome and of a mutagenized derivative, SNTd5, that is identical to the wild-type probe except for the deletion of nucleotide 5. The probes and cellular extracts were mixed in the presence of poly(dI-dC) in order to saturate nonspecific binding sites, and the complexes were analyzed by acrylamide gel electrophoresis and quantitated by scanning. We observed a major shifted band that was specific to Sindbis transcripts and migrated identically, using either the wild-type or mutant probe. The calculated binding constants were 0.22 x $10^{-10}$ M and 0.29 x $10^{-10}$ M for the wild-type and the mutant probe, respectively. Time course competition experiments using unlabeled probes allowed us to calculate a dissociation constant of $2.2 \times 10^{-3} \text{sec}^{-1}$ for the wild-type probe and $7.2 \times 10^{-3} \text{sec}^{-1}$ for the mutant probe corresponding to half lives of 5.5 and 16 min, respectively. The deletion of nucleotide 5 in the SNTd5 variant is lethal in vivo. Our results indicate that nucleotide 5 plays a major role in the binding we observed, and that the mutant sequence apparently binds more tightly than the wild-type sequence. The slower kinetics of the complex dissociation might be responsible for the death of the virus because it could prevent other change(s) in the RNA structure necessary for further interactions (with viral proteins, for example) necessary to form the replicase complex.

We also showed that a shorter wild-type probe representing only the 5' nontranslated region (5'NTR) of Sindbis virus genome allowed formation of complexes equivalent to those obtained using the 125-base probe, demonstrating that the 5'NTR contains a binding site for cellular proteins. Using UV crosslinking experiments, we showed that three cellular proteins of molecular weights 42, 44 and 52 kDa were specifically bound to each probe. These proteins may be cellular cofactors required for Sindbis virus replication.

222. Analysis of Sindbis virus variants mutagenized in the 5' nontranslated region and their revertants

Nathalie Pardigon, Swathi Ganaraj

In order to study the role of the 5' nontranslated region of the Sindbis genome, H. G. M. Niesters constructed a number of thermosensitive mutants by site-directed mutagenesis in this region (Niesters and Strauss, 1990). By passaging these mutants at the nonpermissive temperature, we were able to select revertant viruses that were no longer thermosensitive. These revertants were shown by sequence analysis to have retained the original mutation, indicating that the functional reversion was due to a second site mutation. Assayed on secondary chicken cell cultures, the revertant viruses, as well as their mutant parents, showed reduced plaque size compared to the plaque size of the wild-type virus. Moreover, RNA synthesis by both mutant and revertant viruses is altered at both the permissive and nonpermissive temperatures, although RNA synthesis of the revertants is less dramatically affected.
We are currently mapping the location of the mutation(s) responsible for the reversion. The strategy we have chosen is to amplify specific fragments of the revertant viral genome by first strand cDNA synthesis followed by amplification by polymerase chain reaction. DNA fragments representing portions of the revertant viral genomes are then substituted into the corresponding full-length mutant clone background, and the phenotype of the recombinant viruses assayed on secondary chicken cell cultures. The fragment found to be responsible for the revertant phenotype will be sequenced to determine the nucleotide change(s). We have started with fragments from the nonstructural region. Replacing a fragment containing nucleotides 2712 to 7334 in certain mutants led to viruses that are still thermosensitive, suggesting that the second site mutation is not located within either nonstructural protein nsP4 or nsP3.

Reference:

223. Degradation of the Sindbis virus nsP4 protein by the ubiquitin proteolytic pathway follows the N-end rule
Raoul J. de Groot1, Tillmann H. Rümenapf, Richard J. Kuhn, Ellen G. Strauss, James H. Strauss

Upon infection of cells by Sindbis virus, four nonstructural proteins, termed nsP1-4 in order from 5' to 3' in the genome, are produced by posttranslational cleavage of a polyprotein. Sindbis virus downregulates the production of nsP4, which is believed to function as the viral RNA polymerase, as compared to the other nonstructural proteins by readthrough of an opal termination codon. Furthermore, the concentration of nsP4 is temporally regulated in that nsP4 is found very early in infection, whereas later in infection P34 (a polyprotein containing the sequences of nsP3 and nsP4) predominates. Free nsP4 from both Sindbis virus and the related alphavirus Semliki Forest virus is unstable in infected cells. nsP4 translated in vitro in reticulocyte lysates is degraded by the N-end rule pathway of ubiquitin-dependent proteolysis. When the N-terminal residue of nsP4 (which is normally Tyr, a destabilizing residue) is changed by mutagenesis, the half lives of the mutant nsP4s follow the N-end rule, with the half life of N-terminal Met>Ala>Tyr≥Phe>Arg.

Addition of dipeptides Tyr-Ala, Trp-Ala, and Phe-Ala to the translation mixture inhibits degradation of N-terminal Tyr-nsP4 and Phe-nsP4, but not Arg-nsP4. Conversely, dipeptides His-Ala, Arg-Ala, and Lys-Ala protect N-terminal Arg-nsP4 but not Tyr-nsP4 or Phe-nsP4. We have mutagenized two conserved lysine residues at positions 16 and 17 from the N terminus without effect on stability. There appears to be no requirement for a Lys residue in the first 43 residues of nsP4 for degradation, indicating that a more distal residue functions as the ubiquitin acceptor. Strict control of nsP4 concentration appears to be important for the development of the virus life cycle, since the concentration of nsP4 in infected cells is regulated at three different levels: initial translation by readthrough, differential processing by the virus-encoded proteinase, and degradation by the cellular ubiquitin-dependent pathway. Although the ubiquitin pathway is found in many cell types, examples of native substrates are rare, and it may be that this pathway normally degrades a small subset of proteins, mainly regulatory in nature.

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224. Further characterization of the active site of the nsP2 nonstructural proteinase of Sindbis virus
Ellen G. Strauss

We have been interested in localizing the active site residues of the nsP2 nonstructural proteinase of Sindbis virus. This virally encoded activity had been mapped to the carboxy-terminal half of the nsP2 polypeptide by deletion analysis. Furthermore, this molecule showed limited sequence similarity with members of the papain family of cysteine proteinases which have been shown to have an active site composed of a Cys residue, a His residue, and in some cases an Asp residue. When the amino acid sequences of nsP2 of five alphaviruses (Sindbis, Semliki Forest, Ross River, Venezuelan equine encephalitis and O'nyong-nyong) are aligned, there are two conserved Cys residues, a conserved Ser, five conserved His, and four conserved Asn's within the proteinase domain. Each of these amino acids has been changed by site-specific mutagenesis of the full-length cDNA clone of Sindbis virus. RNA transcribed from
these clones was translated in vitro in rabbit reticulocyte lysates to determine the effects on proteolytic activity. Cys-481 was determined to be the catalytic Cys, since substitutions of this amino acid by either Ser, Arg, or Gly abolished proteolysis. Substitution of the second conserved Cys (Cys-525) by Ser gave normal activity, although Arg-525 abolished proteolysis, presumably due to alteration of the overall conformation of the enzyme.

All five of the conserved histidines have been mutagenized to Ala and all gave normal processing except His-558. When His-558 was substituted by Ala, Gln or Tyr, there was no proteolytic activity. His-709 gave normal processing when replaced with Ala or Tyr, but no proteolysis when replaced with Arg, illustrating again that substitution with Arg can seriously disrupt the enzyme. His-558 is followed by Trp-559. When Trp-559 is changed to Ala, aberrant processing takes place. It has been suggested that the planar rings of the His and Trp may interact to stabilize the active site.

After mutagenizing all four of the conserved Asn residues to both Ser and Asp, we have concluded that no Asn residue is essential for the proper functioning of the nsP2 proteinase, although different independent constructs in which Asn-693 (the most distant Asn from the other catalytic residues) is replaced with either Ser or Asp give somewhat variable cleavage patterns.

225. Biological characterization of nonstructural protein cleavage-site mutants of Sindbis virus

Yukio Shirako

Previously, we tested the effect of amino acid substitutions at the nsP1/nsP2 and nsP2/nsP3 cleavage sites upon proteolytic processing in vitro and viability in chicken embryo fibroblast (CEF) cells, using RNA transcripts from pToto full-length cDNA clones. The results indicated that cleavage at the nsP1/nsP2 site is required for processing at the nsP2/nsP3 site, and that the nsP1/nsP2 cleavage-deficient virus grew 10^{-5} as efficiently as the wild type. The abolishment of cleavage at the nsP2/nsP3 site did not block processing at the nsP1/nsP2 site and the mutant grew 10\% as well as the wild type. Thus, the replication functions normally performed by mature nsP1, nsP2 and possibly nsP3 are still expressed at reduced levels in polyproteins P123 and P23. In this study, we also included amino acid substitutions at the nsP3/nsP4 cleavage site. Abolishment of processing at the nsP3/nsP4 site by a Gly-to-Val substitution at the P2 position did not block the cleavage at either the nsP1/nsP2 or the nsP2/nsP3 sites and the in vitro translation profile was identical to that of wild type except for the absence of mature nsP4. However, the mutation appeared to be lethal in CEF cells, indicating that mature nsP4 is an essential component required for virus replication and cannot be substituted by any nsP4-containing polyprotein. Further in vivo analysis of protein and RNA synthesis of these mutants is in progress.

226. In vitro analysis of enzyme/substrate relationships in proteolytic processing of Sindbis virus nonstructural polyproteins

Yukio Shirako

The four species of nonstructural proteins of Sindbis virus, nsP1, nsP2, nsP3 and nsP4, are produced from two precursor polyproteins, P123 and P1234, by a proteolytic activity residing in the carboxyl-terminal half of nsP2. It has been found that the nsP3/nsP4 site is cleaved rapidly and efficiently after translation of P1234, whereas cleavage at the nsP1/nsP2 site proceeds rather slowly. However, this cleavage is required to release P23 or P234, which then can cleave the nsP2/nsP3 site. Thus, the various nsP2-containing polyproteins have differing specificities for particular cleavage sites. These specificities may play significant roles in viral replication such as the temporal regulation of the nsP4/P34 ratio early in infection (de Groot et al., 1990). To systematically clarify these processing events, two series of cDNA constructs for in vitro transcription/translation of polyproteins were prepared based on the set of enzyme constructs made by de Groot. Each member of each series has only one of the three cleavage sites accessible for proteolysis. To simplify interpretation of the in vitro translation products, both series have Ser instead of an opal termination codon near the end of nsP3 and a Tyr-to-Ala substitution at the amino terminus of nsP4; the former avoids nsP3 production by the opal codon and the latter prevents ubiquitin degradation of nsP4. One series has a Cys-to-Gly substitution at position 481 of nsP2 to inactivate endogenous protease activity, so that the translation products are substrates for cotranslational and post-
translational trans-cleavage assays. The second series, containing a wild-type proteinase domain, is used in studying the effects of dilution of input RNA transcripts on the cis-cleavage activity in reticulocyte lysates.

Reference:

227. Molecular genetics of virulence in Ross River virus
Richard J. Kuhn, Ronald C. Weir

Geographic isolates of Ross River virus (RRV) from different regions of Australia differ markedly in virulence for mice. The type virus (T48) isolated in the northeast is highly virulent, whereas strain NB5092 from a more southern region is virtually avirulent. Furthermore, by passaging NB5092 in neonatal mice, clonally-related variants of high virulence have been isolated (Meek et al., 1989).

We are constructing a full-length cDNA clone of the mouse avirulent strain (NB5092) of RRV as a basis for identifying the molecular genetic determinants of mouse virulence for this virus. As a full-length clone for T48 already exists, this aim is being furthered initially by generating a series of intra-typic genetic constructs between the virulent strain (T48) and NB5092. Several hybrid viruses have been recovered and their virulence will be assayed in young mice. Subsequently, when the full-length NB5092 has been assembled, mutations identified in passage NB5092 will be more rigorously tested for their effect on virulence by recombination into the defined genotype.

Reference:

228. Replication of Sindbis/Ross River chimeric viruses
Richard J. Kuhn, Zhang Hong

Utilizing cDNA clones of two distinct members of the alphavirus genus, Sindbis virus and Ross River virus, we have constructed a series of hybrid clones which have been used to generate chimeric viruses. These chimeric viruses have been used to probe the functions of both coding and noncoding domains in the alphavirus genome. The chimeric viruses are of two types: those that exchange only the 5' and 3' nontranslated regions of Sindbis and Ross River viruses and those that exchange coding regions.

Chimeric viruses which exchange the 3' nontranslated regions (524 nucleotides in Ross River, 322 nucleotides in Sindbis) replicated as well as the parent viruses in both Vero and C6/36 mosquito cells. The ability to exchange these sequences, with little effect on virus replication, strongly implicates host proteins in the synthesis of virus minus-strand RNA. Exchange of the 5' untranslated regions produced chimeric viruses that were replication deficient in Vero and C6/36 cells. Interestingly, the chimeric virus containing the 5' untranslated region of Ross River and the coding sequence of Sindbis produced small plaques in Vero cells and failed to grow in mosquito cells. Large plaque variants of this virus have been isolated and the nature of the change is under investigation.

Exchanges involving the structural and nonstructural regions of Sindbis and Ross River have produced two viruses, whose replication properties differ widely. The differences in replication efficiencies suggest an interaction between structural and nonstructural components. We are currently constructing a set of viruses that will exchange the nonstructural proteins and the capsid protein of one virus with the envelope glycoprotein of the other virus. Such chimeras will investigate the interactions between the capsid and the E2 glycoprotein.

229. Expression of trpE fusion proteins in Escherichia coli
Zhang Hong, Kangsheng Wang, Richard J. Kuhn

cDNAs encoding both the structural and the nonstructural proteins of Ross River virus (RRV) have been used to generate trpE fusion proteins using the trpE-E. coli expression system. Using this system, we have constructed a series of plasmids that collectively express all of the RRV proteins. Following expression, these fusion proteins have been purified and used to immunize rabbits for the production of monospecific polyclonal antibodies. A panel of antibodies that have specificities for each of the proteins produced during RRV infection has now been generated. These reagents will allow us to study the RRV replication cycle in greater detail than was previously possible.

In addition to producing fusion protein specific for
RRV, we have recently constructed clones that produce trpE fusions with the high affinity laminin receptor from baby hamster kidney (BHK) cells. The BHK laminin receptor molecule has been proposed to serve as a receptor for Sindbis virus (see Abstract No. 220). We have constructed two fusion plasmids: the first construction contains trpE fused to the carboxy-terminal 83 amino acids of the laminin receptor (this region has been implicated in virus binding); the second construction contains trpE fused to the entire 295 amino acids of the laminin receptor. Antibodies, which are currently being produced in rabbits, will be tested for their ability to immunoprecipitate the laminin receptor molecule. The antisera will also be tested for its ability to block virus binding to BHK and other cell types. The availability of such antisera will be invaluable in our study of alphavirus receptors.

230. Mutagenesis studies of the E2 glycoprotein of Ross River virus
Richard J. Kuhn, Ronald C. Weir

Alphaviruses contain two envelope glycoproteins (E1 and E2) which together form spikes found on the exterior of the virion. These "spike" proteins perform a number of functions essential for virus propagation. The majority of the neutralizing monoclonal antibodies that have been raised against alphaviruses react with the E2 glycoprotein, suggesting that it may play a role in receptor binding. In order to investigate this and other potential functions of E2, we have undertaken a molecular genetic analysis of this protein using the Ross River virus (RRV) cDNA clone.

We are examining two distinct regions of the E2 glycoprotein. The first region surrounds amino acid 55. In Sindbis, this amino acid has been shown to be an important determinant for neurovirulence. Interestingly, in RRV, a naturally occurring variant of the virulent T48 strain has a deletion of 7 amino acids centering on Sindbis amino acid 55 in the aligned proteins. This variant virus dE2 is attenuated in mice, but shows increased levels of RNA synthesis in tissue culture. Using our cDNA clone of RRV, we have constructed and recovered a virus that has a deletion of the 7 amino acids. This rescued virus has the phenotype of the naturally occurring dE2 virus, providing formal proof that the deletion in E2 is solely responsible for the attenuated phenotype. The mechanism by which RNA synthesis is increased by a deletion in the structural glycoprotein E2 of the virus is being investigated.

RRV replicates extremely poorly in chicken embryo fibroblasts. Using chimeric viruses, we have determined that the replication block maps to the structural proteins. Less than 5% of chicken fibroblasts can be infected with RRV as determined by immunofluorescence. However, infected cells produce normal amounts of virus. Taken together, these observations suggest that virus entry is limited into the majority of cells. Passaging RRV in chick cells selects variants with amino acid substitutions at position 218 of E2 (R. Weir, L. Dalgarno and P. Kerr, unpublished data). We have therefore introduced a series of amino acid substitutions at position 218 using the cDNA clone and are now testing the growth properties of the resulting viruses. We believe this region of the glycoprotein is involved in the initial contact between the virus and its cellular receptor.

231. Processing of the Ross River virus nonstructural proteins
Richard J. Kuhn, Jean-Paul Kovalik

To understand the replication cycle of Ross River virus, a detailed knowledge of the processing of the nonstructural polyprotein is required. We have begun a molecular genetic analysis of polyprotein processing by constructing mutants that fall into two functional groups: the first group examines the nature of the protease and its enzymatic activities; the second group examines the processing events themselves and the temporal arrangement of polyprotein processing and its impact on viral RNA synthesis. Many details of polyprotein processing have been worked out previously on Sindbis by members of this laboratory. In the present study, we wish to expand our knowledge on processing and use the information acquired in both Sindbis and Ross River studies to set forth fundamental principles of alphavirus polyprotein processing and replication.

To analyze the nature of the protease, we have introduced mutations into the putative active site Cys491 of Ross River nsP2. These mutations inactivate the protease as determined by in vitro translation and produce a Ross
River polyprotein which is a substrate for \textit{trans}-only cleavages. Using both Sindbis and Ross River nsP2-containing enzymes, we can analyze the substrate binding requirements for the nsP2 protease by performing \textit{trans}-cleavages on protease-inactivated polyprotein substrates. Additionally, we have altered the nsP2-nsP3 cleavage site and are currently studying the cleavage of these mutants in an \textit{in vitro} translation system.

232. Molecular evolution of Sindbis virus: Comparison of the nsP3 and nsP4 genes of three geographical isolates

\textit{Yukio Shirako, Edith M. Lenches}

The nucleotide sequences of the nsP3-nsP4 region (3.5 kb) of two geographical isolates of Sindbis virus, A1036 isolated in India in 1953 and MRM18520 isolated in Australia in 1975, were determined from cloned cDNAs. The deduced amino acid sequences were compared with that of the prototype AR339 strain isolated in Egypt in 1952 to study the evolutionary relationships among them. This region was chosen because nsP4 is the most highly conserved domain in the various alphaviruses, and the C-terminal portion of nsP3 is the most highly divergent. Furthermore, the evolutionary divergence of the nonstructural proteins is less influenced by interaction with the host immune system than the structural proteins.

The amino acid sequences of the Indian strain and the Australian strain diffused by 3.4%, 12.9%, and 2.2% in the N-terminal half of nsP3 (nsP3-N), the C-terminal half of nsP3 (nsP3-C), and in nsP4, respectively. In these same three domains, the amino acid sequences between AR339 and the Indian strain differed by 9.9%, 41.6% and 7.5%, respectively, and AR339 and the Australian strain differed by 10.2%, 43.5% and 8.0%. Using these measures of divergence and the dates of isolation, we could construct an evolutionary tree. Assuming that the three isolates had a common ancestor and have evolved at the same rate, nsP3-C, nsP3-N, and nsP4 are evolving at less than 0.023%, 0.086% and 0.014% per year, respectively. For the Indian and Australian isolates, nsP3-N is diverging 1.4 times as fast as nsP4, and nsP3-C is diverging 5.6 times as fast as nsP4. From this we can estimate that the Indian and Australian isolates were separated from the Egyptian strain about 400 years ago and that the Indian and Australian isolates separated about 150 years ago. We are currently extending this study by determining the sequences of strains from other regions of the world and strains with different dates of isolation.

233. Studies on Semliki Forest virus nsP4: Generation of Semliki Forest virus infectious cRNA

\textit{Tillman H. Rümeneraff}

Semliki Forest virus (SFV) and Sindbis virus are the two most intensively studied alphaviruses. Both are Old World alphaviruses, and they share 40-70% amino acid sequence identity depending on the protein. Importantly, however, these viruses differ in their translation strategies. Sindbis virus (and most other alphaviruses) terminate translation of a nonstructural polyprotein with an opal codon at the COOH end of nsP3, so that nsP4 is produced only by readthrough translation. SFV has an Arg codon at this position and could theoretically produce higher levels of nsP4. To examine the amounts of nsP4 produced in SFV infection and the stability of this protein, antibodies were raised against a synthetic peptide representing amino acids 2238-2256 of the Sindbis polyprotein. This region is conserved among all alphaviruses sequenced so far and the antibody should precipitate nsP4 from both viruses and allow quantitative comparisons of the nsP4 levels produced by Sindbis virus and SFV.

As a genetic approach toward understanding the processing of SFV proteins, we are constructing a SFV cDNA clone capable of serving as a template for generation of infectious RNA. We have cloned the entire genome and will use a synthetic oligonucleotide containing the missing 5' end as well as a promoter for SP6 RNA polymerase to make a full-length cDNA that can be transcribed \textit{in vitro} with SP6 RNA polymerase.

234. Molecular characterization of Aura virus

\textit{Tillman H. Rümeneraff}

The evolution of alphaviruses became a topic of major interest three years ago when Western equine encephalitis (WEE) virus was characterized as a recombinant virus containing sequences from two different alphaviruses. The envelope glycoproteins are closely related to those of the Old World alphavirus Sindbis virus, while the nonstructural proteins (as far as determined), the capsid
protein, and the 3'-terminal sequence are closely related to Eastern equine encephalitis virus, a New World alphavirus spread over both American continents.

To investigate whether recombination is a common event in alphavirus evolution, we have been examining Aura virus. By serology, Aura virus is grouped with WEE into the Sindbis-related group of alphaviruses. Most interestingly, the virus was isolated from a mosquito in Brazil, where New World alphaviruses are widespread. Sequence analysis will reveal whether Aura virus is a New World Sindbis virus or a recombinant (similar to or different from WEE).

For sequence analysis, we have cloned almost the entire genome (only anking the 5' end) of Aura virus into λ ZAP phages/Bluescript plasmids and are currently preparing templates for automated sequencing. Preliminary sequence data suggest that Aura virus differs considerably from WEE as well as from Sindbis virus. We expect that the results will contribute toward our understanding of alphavirus evolution.

235. In vitro and in vivo processing of dengue type 2 virus nonstructural proteins NS4A and NS4B

Frank Preugschat

Positive-stranded RNA virus replication occurs in close association with intracellular membranes. Many RNA viruses encoded multiple, small hydrophobic nonstructural proteins which are proposed to play a role in the establishment of replication complexes. It is of general interest to establish the precise contributions of viral and cellular proteins in regulating the assembly of functional replication complexes.

The flaviviral nonstructural proteins NS2A, NS2B, NS4A and NS4B are small, hydrophobic proteins which are translated in association with host cell membranes. The amino termini of NS2A and NS4B are believed to be generated by host cell signalases, whereas the amino termini of NS2B and NS4A are generated by the nonstructural proteinase NS3. NS2B plays an active role in modulating the proteinase activity of NS3, but specific biochemical roles for NS2A, NS4A and NS4B have not been elucidated. To begin to assess the functions of NS4A and NS4B, we have undertaken in vitro and in vivo expression experiments to explore the processing and association of these hydrophobic proteins with cell membranes.

A rabbit monospecific antiserum directed against NS4B was used to study processing of nascent NS4B in dengue-infected cells. A protein of approximately 29 kDa molecular weight was immunoprecipitated from infected, but not mock infected, cell extracts under denaturing and nondenaturing conditions when brief labeling pulses were used. The 29 kDa NS4B is then posttranslationally processed by an unknown mechanism into a 27 kDa protein. Hamster and monkey kidney cells process the 29 kDa protein into a stable 27 kDa protein with a halftime of 1.5 to 2 hr. In mosquito cells, the 29 kDa protein is processed with an estimated halftime of 4 hr and the 27 kDa protein is degraded. The nature of the posttranslational modification and the role of the 29 and 27 kDa proteins in the viral life cycle are not known at this time.

When membrane-associated and soluble fractions from NS4A-programmed in vitro translations were analyzed by SDS-PAGE, it was determined that the majority of NS4A is membrane associated. Protease protection experiments indicate that the membrane-associated NS4A is partially, but not completely protected from digestion. These data suggest that NS4A is located within the plane of the microsomal membranes and is probably not translocated into the lumen of the ER. NS4A may prove to be a useful marker for analyzing the metabolism of intracellular integral membrane proteins during viral infection.

236. An investigation of the interplay between proteases and substrates

Frank Preugschat

Flaviviruses encode their structural and nonstructural proteins as a single long polyprotein that is cotranslationally and posttranslationally processed by both cellular enzymes and a viral-encoded proteinase. The nonstructural proteinase of dengue virus type 2 (DEN2) is functionally contained within the first 184 amino acids of NS3. In vitro the proteinase has been shown to process both the 2A/2B and 2B/3 cleavage sites in cis, with the initial cleavage occurring between NS2A and NS2B, followed by cleavage of the 2B/3 site. In contrast, similar experiments in vitro with the yellow fever virus (YF) proteinase (which also acts primarily in cis) have shown
that for this virus the initial cleavage occurs between NS2B and NS3. We constructed chimeric DEN2/YF polyproteins as a model system for studying the differences in enzyme-substrate interaction between these two related proteinases. Chimeras made within the protease domains themselves and site-directed mutagenesis allowed mapping of regions involved in substrate recognition. Site-directed mutagenesis of cleavage sites revealed that sequences at the 2B/3 cleavage site limited the activity of chimeric proteinases which expressed heterologous substrate binding pockets. Mutagenesis of cleavage sites modulated processing efficiency but did not change the apparent order of cleavage. In contrast, site-directed mutagenesis of the substrate binding pocket modulated both processing efficiency and order. These studies provide a basis for our understanding of how the substrate binding pocket and cleavage sites interact and identified some of the critical amino acid residues within the substrate binding pocket and cleavage sites that are responsible for determining the efficiency and order of polyprotein cleavage.

237. Molecular biology of plant RNA viruses
Yukio Shirako

Unlike bacterial and animal viruses, the majority of plant viruses (about 80%) possess a positive-stranded RNA genome. Many of them can be classified into either the alphavirus or picornavirus superfamily based on their genome organization, terminal structure and, particularly, amino acid homology in nonstructural proteins, implying that certain plant and animal RNA viruses have evolved from common ancestors. On the other hand, plant RNA viruses have unique features compared to animal counterparts: 1) most of them have multipartite genomes which are separately encapsidated; 2) viruses within each superfamily show significant divergence in genome structures as well as in particle morphology; and 3) a cell-to-cell transport protein is one of the essential viral gene products. In addition, plant viruses are subjected to daily and seasonal temperature shifts under natural conditions, the number of virus replication cycles per period and per area may vastly exceed that of an animal virus due to availability of abundant host materials in nature, co-infection of taxonomically unrelated viruses in the same host plant can occur frequently, and plants do not have an immune system. These circumstances would enable plant RNA viruses to evolve more rapidly and form recombinants more frequently than animal RNA viruses.

I have been studying soil-borne wheat mosaic virus (SBWMV), the type member of the furovirus group with a bipartite RNA genome, RNA I (7.1 kb) and RNA II (3.6 kb), and the plasmodiophoraceous fungus vector, Polymyxa graminis. This virus is of particular interest because it is transmitted only by soil-inhabiting fungi and the fungal resting spores serve as the virus reservoir in the infested field. Once a field has been infested, the virus can infect wheat plants every year and so evolve continually as long as host wheat plants are grown in that field. Also, this virus tends to undergo frequent deletion mutations in the RNA II sequence; these deletion mutants produce more severe symptoms than the wild-type virus. Thus, SBWMV is an ideal system for studying mutation, adaptation and evolution of an RNA virus genome. We have determined the nucleotide sequences of the RNA IIs of the Nebraskan and Illinois isolates of SBWMV. Both RNAs are 3593 nucleotides in length; the overall nucleotide divergence is 9.7% and the amino acid divergence is 3.2%. However, the 5' and 3' nontranslated regions showed only 4.5-4.6% divergence, whereas the capsid protein coding region has 12.7% nucleotide difference but the amino acid sequences were completely identical, indicating that the exact amino acid sequence of capsid protein is essential. Considering that wheat cultivation was introduced to these areas about two hundred years ago, all of these mutations have apparently accumulated within the last two centuries. It would be of great interest to determine nucleotide sequences of other geographical isolates in the U.S., as well as those from Europe, Asia and South America, to explore the origin and evolution of SBWMV in a case study of RNA virus evolution.

In addition, construction of full-length cDNA clones from which biologically-active RNA can be transcribed is in progress to facilitate investigation of structure/function relationships in the furovirus genome.


gene regulation problems in cardiac muscle, which presents interesting similarities as well as major differences relative to skeletal muscle.

At the cellular and molecular levels, it is clear that negative regulators of skeletal myogenesis are probably just as important in properly regulating the outcome as are the positive MyoD family regulators. It is the interaction between the two sets of regulators that is of particular interest to us. The negative regulators of skeletal muscle differentiation that we find especially interesting and relevant are those that are naturally expressed in proliferating muscle precursors, and it is postulated that these are crucial for maintaining precursors in an undifferentiated state and in regulating their proliferation. Specific regulators in this class are myc, Max/myn and Id. Myc is well known as a proto-oncogene, but in this context is interesting for its ability to both direct cell cycle progression and inhibit muscle differentiation. Sean Tavtigian, Jeff Miner and Lisa Neuhold are studying different aspects of this problem.

What regulates the regulators? This is the inevitable and important question when an important level of regulation is discovered. In this case, the specific problem is to discover what turns on and then maintains expression of MyoD family members during development. Ardem Patapoutian and Jeff Miner are studying this problem with special attention to herculin. Studies of other regulators that appear important in the mesodermal lineages before expression of MyoD family members have focused on mouse twist, recently cloned and characterized in the laboratory. Sam Murray, Carlotta Glackin, Kyuson Yun and Roger Wagner have looked at different aspects of its activity. Once a MyoD family regulator is expressed, a further substantial problem is understanding the influences of the host cell environment on its activity. Previously, the influence of cellular context and the regulatory range of MyoD class regulators have mainly been studied in model cell lines in culture. In the past year, Jeff Miner has made considerable strides in examining MyoD in the developing animal by making transgenic mice.

Finally, we are continuing a very interesting series of collaborative studies with Prof. Peter Dervan of the Division of Chemistry and Chemical Engineering. Joe Hacia and Jim Maher (members of both the Dervan and Wold groups) have studied different aspects of triple helical nucleic acid structures under conditions that increasingly closely imitate those in the living cell. These projects are aimed at determining whether intermolecular triple helices can form in living cells and what the chemical and regulatory consequences of such interactions might be.

238. A partial skeletal muscle phenotype initiated by ectopic MyoD in the hearts of transgenic mice

Jeffrey H. Miner, Jeffrey B. Miller

MyoD is a member of the basic helix-loop-helix family of myogenic regulatory, sequence-specific DNA binding proteins, which are expressed solely in skeletal muscle cells. Extensive studies in cultured cells have shown that MyoD can, upon transfection, cause diverse cell types to express a skeletal muscle phenotype. It is not known, however, if MyoD expression can activate all or part of the skeletal muscle program during embryogenesis. To determine whether and in what manner ectopic MyoD can alter the fundamental characteristics of a non-skeletal muscle tissue in the animal, we have produced transgenic mice that carry the MyoD protein coding sequence under the control of the mouse muscle creatine kinase enhancer/promoter. This promoter directed overexpression of MyoD in skeletal muscle and ectopic expression in cardiac muscle; cardiac muscle is structurally, functionally, and developmentally distinct from skeletal muscle and normally expresses none of the MyoD family regulators. Transgenic hearts exhibit morphological abnormalities, and the fetuses die at 16-18.5 days of gestation. Of the four skeletal muscle regulators, transgenic hearts also express myogenin but not Myf-5, MRF4/herculin, or the endogenous MyoD gene. These hearts express sarcomere genes normally specific to skeletal muscle, such as skeletal α-actin, embryonic and perinatal myosin heavy chain isoforms and nebulin. However, complete conversion to fused skeletal myotubes was not detected. We conclude that a partial induction of the skeletal muscle phenotype by MyoD is initiated in the presence of the fetal cardiac program. Thus, earlier steps of the skeletal muscle lineage seem not to be required for MyoD activity and heart cells contain whatever molecular machinery is needed for
MyoD to activate genes normally destined to be silent in the heart.

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239. c-myc inhibition of MyoD- and myogenin-initiated myogenic differentiation

Jeffrey H. Miner

In vertebrate development, a prominent feature of several cell lineages is the coupling of cell cycle regulation with terminal differentiation. We have investigated the basis of this relationship in the skeletal muscle lineage by studying the effects of the proliferation-associated regulator, c-myc, on the differentiation of MyoD-initiated myoblasts. Transient cotransfection assays in NIH 3T3 cells using MyoD and c-myc expression vectors demonstrated c-myc suppression of MyoD-initiated differentiation. A stable cell system was also developed in which MyoD expression was constitutive, while myc levels could be elevated conditionally. Induction of this conditional c-myc suppressed myogenesis effectively, even in the presence of MyoD. c-myc suppression also prevented up-regulation of a relative of MyoD, myogenin, which is normally expressed at the onset of differentiation in all muscle cell lines examined and may be essential for differentiation. Additional experiments tested whether failure to differentiate in the presence of myc could be overcome by providing myogenin ectopically. Cotransfection of c-myc together with myogenin, MyoD, or a mixture of myogenin and MyoD showed that neither myogenin alone nor myogenin plus MyoD together could bypass the c-myc block. The effects of c-myc were further dissected by showing that c-myc can inhibit differentiation independently of Id, a negative regulator of muscle differentiation. These results led us to propose that c-myc and Id constitute independent negative regulators of muscle differentiation while myogenin, together with any of the other three related myogenic factors (MyoD, Myf-5 and MRF4/herculin/Myf-6), act as positive regulators.

240. Molecular cloning of mid-G1 regulated genes and myc target genes

Sean V. Tavtigian

For some time, the three major hypotheses for the function of c-myc have been a transcriptional or posttranscriptional role in mRNA synthesis or a role in the initiation DNA replication. Although these are not mutually exclusive, several lines of evidence point to a prominent transcriptional function. The temporal pattern of c-myc expression following serum stimulation of quiescent cells suggests that it plays an important role in G1 following emergence from growth arrest but preceding entry into S-phase. Our work and conceptually similar studies from other labs have shown that acute overproduction of c-myc is sufficient to stimulate a significant subset of quiescent cells to progress through G1 and enter S-phase, confirming its functional importance during G1. Elucidation in other proteins of the functional roles of some of the structural motifs present in c-myc suggests that it should possess protein multimerization, DNA binding, and transcriptional regulatory activities. Finally, both the isolation of a pairing partner of c-myc, max/myn protein, and demonstration of sequence-specific DNA binding in vitro, strengthen the analogy between its function and that of other basic helix-loop-helix motif transcription factors.

These observations led to the hypothesis that the activity of c-myc is responsible for regulating an identifiable subset of the genes induced or repressed in G1 following emergence from growth arrest. Accordingly, we have prepared libraries from growth arrested and mid G1 cells, differentially screened those libraries with corresponding cDNA probes, and obtained a panel of cDNA clones subject to the expected temporal regulation following serum stimulation of quiescent cells. In previous work, we had constructed cell lines that both overproduce c-myc in response to treatment with zinc and, following growth arrest, progress through G1 in response to c-myc overproduction. These cell lines now serve as a source of RNA, obtained under physiological conditions where we know that c-myc overproduction stimulated progression through S-phase, against which the panel of serum-regulated clones is being examined for their potential response to c-myc. In addition, cell lines that inducibly co-express c-myc and max/myn are in preparation.

For any individual clone, there is no a priori way of knowing how many regulatory steps occur between c-myc overproduction and an observed accumulation or decay of
transcript abundance. However, we have already isolated a large enough number of "c-myc regulated" clones to provide some confidence that several of the possible regulatory pathways are represented. As some sequence information is obtained from each clone during the analysis, increasing the number of identified clones subject to regulation by c-myc will also help clarify the phenotypic consequences of c-myc misregulation \textit{in vivo}. While the cloning aspect of this work continues as an ongoing project, my own effort will now focus on defining the levels (transcriptional versus post-transcriptional) at which the observed regulation occurs.

241. \textit{In vivo} pairing of HLH class regulators of gene expression in myogenesis

\textit{Lisa A. Neuhold}

We wish to understand how an undifferentiated myoblast gives rise to a differentiated myocyte by studying the regulatory proteins that interact and stimulate myogenic gene expression. To date, a family of four positive-acting myogenic determination/differentiation genes have been identified and cloned. These genes encode MyoD, myogenin, Myf-5, and MRF-4/herculin. When DNA encoding these regulators is transfected into a non-myogenic fibroblast cell line, withdrawal from the cell cycle and differentiation into muscle cells is observed after reduction of serum. The biological activities of these regulators depend on their function as sequence-specific DNA-binding ligands, which, in turn depends on their association, via protein:protein interactions, with other regulatory proteins. These prospective partners share a related basic region helix-loop-helix (B-HLH) motif, essential for either homo- or heterodimer formation. Dimerization subsequently stimulates muscle-specific gene expression. Members of this B-HLH superfamily include the immunoglobulin regulators E12 and E47. MyoD has been shown to dimerize with both E12 and E47 in an \textit{in vitro} binding assay, although the partners with which the myogenic regulators dimerize \textit{in vivo} are still not known. The overall goal of this project is to determine which of these B-HLH proteins dimerize in undifferentiated myoblast and in differentiated myocytes and to test \textit{in vivo} the activities of particular pairs.

To approach this problem, I have developed an experimental strategy to test the function of specific heterodimers and evaluate the manner in which dimerization of HLH MyoD-class regulators is affected by HLH inhibitors of MyoD activity such as Id and myc.

It is conceivable that the myogenic regulators may dimerize with as yet unknown partners or form higher order oligomers. To begin to address these questions, DNA encoding a foreign epitope sequence has been inserted into the nonconserved regions of MyoD and myogenin plasmids. The use of this epitope tag will be helpful in retrieving specific family member proteins and their partners from cell lysates. As new potential HLH partners are revealed, their \textit{in vivo} activities will be tested using the covalent linkage approach in both myoblasts and myocytes.

242. Analysis of the regulatory region of the herculin gene

\textit{Jeffrey H. Miner, Ardem Patapoutian}

Herculin, also known as MRF4 and Myf-6, is a member of the MyoD family of myogenic regulatory genes. The four members of this family are thought to be important in the determination and differentiation of the skeletal muscle lineage, since forced expression of any of them in multipotential mesodermal stem cells converts these cells into muscle. We are interested in learning how the herculin gene is activated in muscle during embryogenesis. To begin to address this question, we have cloned a 6 kb piece of DNA upstream of the herculin coding region, because this DNA is likely to contain herculin regulatory sequences. In place of the herculin coding region, we have inserted two reporter genes, bacterial $\beta$-galactosidase and chloramphenicol acetyltransferase. Transfection of these and other constructs into muscle cells that express the endogenous herculin gene should allow us to determine which regions of the herculin 5' flank are important for proper regulation. In a different but complementary approach, we have produced transgenic mice that carry the $\beta$-galactosidase construct. These are currently being bred and will be analyzed by staining tissues for $\beta$-galactosidase activity.
243. **Isolation, characterization and expression of mouse twist**

*Samuel S. Murray, Katherine A. Winters, Carlotta A. Glackin*

In this project, we are studying the expression and function of the helix-loop-helix (HLH) family of genes in vertebrates. Products of some of these HLH genes are known to be involved in the regulation of cell proliferation and differentiation in many different cell types. The prototype genes from this family are c-myc and MyoD1. The shared feature of this large family of genes is a conserved motif that consists of two amphipathic helices separated by an intervening loop. This motif mediates protein:protein interactions that result in dimer formation. Dimerization is necessary for DNA binding functions that result either in positive or negative regulation of target genes. There is also an adjacent basic region in many of the molecules that is required for DNA binding. At this time, the best understood family members regulate skeletal muscle differentiation. Those are of the MyoD subfamily, and their expression is detected only in differentiated skeletal muscle and its immediate precursors which are called myoblasts.

We have isolated and characterized mouse twist, a new mammalian member of the HLH gene family. This was accomplished by using available sequence data from *Xenopus* and *Drosophila* twist to make appropriate degenerate polymerase chain reaction (PCR) primers. These primers were then annealed to both mouse and *Xenopus* (control) chromosomal DNA. PCR products giving rise to the correct size fragment for both mouse and *Xenopus* were isolated, cloned and sequenced. Once the sequence had been verified for *Xenopus* from published data, the mouse PCR (HLH) clone was used to screen cDNA libraries. Mouse twist cDNA and genomic subclones were sequenced. The start site of transcription was determined by RNase protection using two different riboprobes and by primer extension. The genomic sequence revealed a 650 bp intron in its 3'-untranslated region when compared to the cDNA sequence data. Also, 1 kb of the 5'-flanking region was sequenced for future promoter/enhancer studies. Protein homology searches revealed a very high homology to the other twist genes from *Drosophila* and *Xenopus* as well as lower similarity to other members of the HLH gene family, i.e., MyoD, herculin, myogenin. A series of Northern blots and RNase protection experiments was performed in order to study the patterns of expression. Mouse twist is expressed at relatively high levels in bone and fat among a large set of adult tissues examined. Further expression studies showed that twist is expressed at high levels peaking around day 10 in mouse embryonic development and that its expression decreases during later fetal development. Studies of cultured cell lines showed high levels of expression in some mouse fibroblast lines (e.g., BALB/c and NIH 3T3) and no expression in others (e.g., L cells). Primary cells derived from fat, bone and heart tissues had the highest levels of twist expression, as did the immortal fat and bone cell lines. We are now proceeding with studies involving the biological function of twist in bone and fat tissues using mouse twist antibodies. We are also setting up stable bone and fat cell line transfection experiments using twist and some of the other HLH genes to look for changes in phenotype.

244. **Protein:DNA interactions at the muscle creatine kinase enhancer in cardiac myocytes**

*Katherine A. Winters*

Several molecules have been found to induce many cell types to differentiate into skeletal myocytes when mitogens are subsequently removed from culture medium. MyoD is the prototype member of this family of myogenic DNA-binding proteins which also includes myogenin, Myf-5, and Mrf-4/herculin. MyoD has been shown to bind in vitro to a functionally essential site in the transcriptional enhancer of muscle creatine kinase (MCK) in skeletal myocytes. However, none of these regulatory molecules are expressed in cardiac cells. Nevertheless, cardiac myocytes actively express MCK, a terminal differentiation gene expressed at high levels in both skeletal and cardiac muscle. Moreover, it has been shown that the same MCK enhancer element functions in both of these muscle tissues. This raises the question of how this MCK enhancer is activated in the cardiac myocyte, given that all known MyoD family members are absent.

To begin to address this question, I have initiated a series of experiments to determine the sites of protein:DNA interactions at the MCK enhancer in cardiac myocytes. My initial goal is to compare the *in vivo*
cardiac DMS footprint at this enhancer to the previously determined skeletal myocyte footprint at the same enhancer. Preliminary studies show intriguing similarities and differences between the location and nature of the protected regions at this enhancer.

245. In vivo footprinting analysis of chromatin structure in testis-specific and muscle-specific genes

Paul A. Garrity

We are currently investigating the regulation of gene expression at the transcriptional level during the processes of spermatogenesis and skeletal muscle differentiation. Our goal is to find the ways in which enhancer and promoter regions are selectively utilized by characterizing their configuration in the actively transcribing, living cell. This means finding out, for example, where and when gene-specific trans-acting factors are bound, whether the DNA is associated with nucleosomes, and whether the DNA is bent or melted. Once assembled, this information can both point to and rule out possible mechanisms in the activation or repression of a gene's expression.

In order to obtain this information, we have expanded our repertoire of in vivo footprinting reagents and refined our ligation-mediated polymerase chain reaction technique. Previously, through the use of dimethyl sulfate, we had only been able to detect the binding of gene-specific trans-acting factors. Now, using DNase I and micrococcal nuclease, among other reagents, we detect additional trans-acting factor interactions and can see alterations in chromatin configuration that dimethyl sulfate, which easily penetrates nucleosomes, is insensitive to.

In our study of gene expression during spermatogenesis, we (in collaboration with Murray Robinson and Prof. Mel Simon) have been examining the mouse metallothionen gene, which is ubiquitously expressed but shows an unusual transcription initiation pattern in the meiotic germ cells of the testis and the mouse PGK-2 gene, which is expressed only in the meiotic germ cells of the testis. We have been performing in vivo footprints of these genes in partially purified meiotic germ cells to gain an understanding of the way in which a single promoter can be utilized in entirely different ways in different cell types and the way in which a testis-specific promoter is regulated.

In our study of gene expression during skeletal muscle differentiation, we have taken advantage of cell lines that undergo myogenesis in culture. We have focused on the muscle-specific enhancer of the muscle creatine kinase gene. Previous experiments using dimethyl sulfate found factors associated with this enhancer only in differentiated muscle cells, despite the presence of potential binding factors in extracts from undifferentiated cells. We are re-examining this enhancer with additional footprinting reagents. We hope to resolve questions about what role overall chromatin configuration (e.g., sequestration by nucleosomes) may play in muscle creatine kinase expression (and regulation of tissue-specific gene expression in general) as well as probe the nucleoprotein complex that constitutes the functioning enhancer.

246. Twist: A helix-loop-helix DNA binding protein

Kyuson Yun

Twist is a member of a growing family of DNA binding proteins sharing the helix-loop-helix structure. There is increasing evidence that these proteins regulate transcription of various developmentally important genes as hetero- or homodimers. Twist is of special interest, as it is one of the first marker genes expressed after the establishment of the mesodermal germ layer in Drosophila and Xenopus. I am interested in studying the interaction between twist and its partner(s). How are the temporal and positional expression patterns of these molecules coordinated such that proper partners are present together? How do different hetero- or homodimers recognize different target genes to regulate their expression patterns? Currently, I am taking in vitro approaches to identify the candidate partner(s) of twist and its possible DNA target site sequence preferences.

247. Characterization of mouse twist protein expression

Roger A. Wagner

Two peptides representing conserved regions of the twist protein were used to immunize rabbits and produce polyclonal sera containing immunoglobulins specific for the mouse twist protein. These sera have been used on Western blots to localize and quantitate the expression of the twist protein in various mouse tissues and tissue
culture lines, including undifferentiated and differentiated mesodermally-derived cell lines. The Western blot procedure has also been used to quantitate the expression of the *twist* protein throughout mouse embryonic development.

The localization and temporal sequence of expression of *twist* protein during embryonic development is being studied using *in situ* antibody binding to fixed sections of mouse embryos. These studies may provide hints about what role *twist* plays in the commitment and differentiation of various cell populations in the developing embryo.

248. Analysis of promoter-specific repression by triple-helical DNA complexes in a eukaryotic cell-free transcription system

*L. James Maher, III*

Pyrimidine oligonucleotides bind to homopurine sequences in double-helical DNA by Hoogsteen hydrogen bonding in the DNA major groove, forming a local triple-helical complex. Previous studies in this collaborative project between the Wold and Dervan laboratories have shown that site-specific triple-helical DNA complexes can inhibit DNA recognition by eukaryotic transcription factor Sp1. To examine the functional consequences of such inhibition, homopurine target sequences for oligonucleotide-directed triple-helix formation were inserted in various configurations relative to Sp1 transcription activator binding sites, upstream of the TATA element of recombinant eukaryotic promoters. The resulting promoters were tested for activity in the presence or absence of recombinant human Sp1 in a *Drosophila in vitro* transcription system lacking endogenous Sp1. When triple-helical complexes were assembled on the promoters by incubation with specific oligodeoxyribonucleotides, promoter-specific repression of basal transcription was observed in the absence of Sp1. The degree of basal repression was a function of the number and proximity of triple-helical complexes relative to the basal promoter complex. Repression did not result from triple-helix-induced template degradation. Addition of recombinant Sp1 did not cause derepression. These results suggest that triple-helical complexes can repress transcription primarily by blocking promoter DNA assembly into initiation complexes rather than by occluding Sp1 binding. One of several plausible mechanisms for triple-helix-induced repression involves changes in DNA flexibility. Evidence in favor of this model is provided by a permutation-dependent gel mobility assay in which formation of site-specific gel mobility assay in which formation of site-specific triple-helical complexes is shown to stiffen double-helical DNA. These results enable us to begin evaluating the prospects for regulation of gene expression by oligonucleotide-directed DNA triple-helix formation *in vivo*.

249. Site-specific recognition of DNA by oligoribonucleotides

*Joseph G. Hacia*

My interests include examining the ability of RNA oligomers to bind to specific DNA target sites. We have utilized oligonucleotide-directed triple-helical recognition of DNA to form stable three-stranded complexes composed of a DNA target site and an RNA oligomer. These triple-helical complexes were used to prevent restriction endonuclease binding in order to measure affinities of different RNA and RNA analogs for a specific DNA target. Currently, triple-helical complexes are most readily formed at homopurine runs of greater than 15 base pairs. New modes of RNA-directed recognition of DNA are being investigated through application of in vitro selection protocols. Briefly, large populations of random RNA oligomers are screened through affinity chromatography for DNA recognition properties. The selected molecules are converted to cDNAs which are subsequently amplified and used as templates to generate populations of RNA molecules with an enhanced affinity for the target sequence. Multiple rounds of selection and amplification lead to the identification of RNA molecules with the desired recognition properties.

In addition, we are currently investigating the possibility of expressing RNA molecules in a bacterial setting in order to bind to specific target sites inside a cell. The endogenous expression of RNA molecules circumvents difficulties in transporting triple-helix-forming oligonucleotides across membranes to access chromosomal DNA. The characterization of RNA-directed triple-helical complexes inside bacterial cells will provide important information for designing similar systems in eukaryotes such as yeast and in mammalian cell culture.


CELLULAR, MOLECULAR AND
DEVELOPMENTAL NEUROBIOLOGY

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Summary: A major problem in developmental neurobiology concerns the origin of neuronal diversity. That is, how do all of the different cell types in the nervous system form, in the right numbers at the right time and in the right place? We have chosen to address this question in the mammalian peripheral nervous system, which derives from the neural crest. Different studies in the laboratory are approaching various aspects of this problem at the cellular level, or at the molecular level. A major focus of investigation is a sublineage of the neural crest called the sympathoadrenal lineage. Among the differentiated derivatives of this sublineage are the chromaffin cells of the adrenal medulla (which are endocrine cells) and the neurons of the sympathetic ganglia. These two cell types have been shown to derive embryologically from a common bipotential progenitor cell whose choice of fates is determined by signals in its local environment. This developmental "decision" is of particular interest because, unlike those in most other cell lineages, it is not irreversible: mature chromaffin cells retain the ability to convert to sympathetic neurons in response to environmental signals such as nerve growth factor (NGF). This raises the additional interesting question of the molecular basis of such phenotypic plasticity.

Cellular studies center on two major issues. First, what are the environmental signals that control the differentiation of the bipotential sympathoadrenal progenitor, and how does the responsiveness of differentiating cells to these signals change with time? Second, when does this progenitor first become committed to the sympathoadrenal sublineage, and how is this commitment event controlled? Experiments addressing the first question have been facilitated by the fact that committed progenitors can be isolated from developing rat embryos using specific cell-surface monoclonal antibodies and fluorescence-activated cell sorting. These cells can then be cultured under various conditions and their behavior analyzed. To facilitate obtaining large quantities of these cells for biochemical experiments, we have immortalized them by introducing the v-myc oncogene using a recombinant retrovirus. Fibroblast growth factor (FGF), but not NGF, is able to trigger neuronal differentiation in these cells. During this process, the cells become dependent upon NGF. Thus, development along the neuronal pathway appears to be controlled, at least in part, by a "two factor relay," in which one factor initiates neuronal differentiation and also primes the cells to respond to a second factor, which controls later maturation and survival. Similarly-derived cell lines, as well as primary cultures, are currently being used to investigate the developmentally-earlier question of how neural crest cells first commit to the sympathoadrenal lineage.

Molecular studies of development in this system are aimed at understanding how the developmental history of a progenitor cell programs it to respond to a given environmental signal in a specific way. This challenging question is being tackled from two different and complementary directions. First, genes that are specifically expressed during neuronal or chromaffin differentiation have been cloned and the mechanisms controlling their expression are being studied, using both cell transfection and transgenic mice as assay systems. The ultimate aim is to identify important proteins that interact with the regulatory regions of these genes, and then to understand how they in turn are regulated during neural crest cell lineage diversification. Second, the cell lines we have developed are being used to isolate candidate regulatory genes that may control development in this system. Such candidates are being selected based on their homology to regulatory genes controlling analogous developmental
processes in organisms such as *Drosophila*. For example, we have isolated several mammalian homologs of the *achaete-scute* genes, which are required for neuronal determination in *Drosophila*. At least one of these homologs, MASH-1, is specifically expressed in the developing vertebrate nervous system. The function of such putative regulatory genes can ultimately be investigated by perturbing their expression, both in cultured cells and in transgenic mice.

250. Generation of polyclonal and monoclonal antibodies specific for MASH-1

**Liching Lo, Jane E. Johnson**

Antibodies to MASH-1 protein would facilitate the studies of MASH-1 expression in developing embryos. As a first attempt, we tried to raise rabbit polyclonal antibodies to two peptides that lie outside the basic helix-loop-helix region and are unique to this protein. Both rabbit antisera generated against the KLH-conjugated peptides reacted with *in vitro* translated MASH-1 protein. The antisera were further purified by BSA-conjugated peptide affinity chromatography. By eluting with 4 M MgCl₂, 80% of the antibody reactivity was recovered, while almost all of the reactivity to KLH was removed. However, these affinity-purified antibodies have not shown any specific nuclear staining on cells or embryo sections. The reasons for this failure may be the low titer of the specific antibodies, or some nonspecific antibodies, which obscure the staining pattern. Another possibility would be that the specific antibodies are directed against epitopes not preserved by the fixation methods so far tried. We next tried to generate monoclonal antibodies against a recombinant MASH-1 protein (see Abstract No. 254). A MASH-1 cDNA was cloned into the vector pET by site-directed mutagenesis. Upon induction with IPTG, MASH-1 protein accumulated in the inclusion bodies fraction. We gel-purified the polypeptide and used it as immunogen. Initial screening of hybridoma supernatants was done by dot blot. Positive clones were immediately rescreened on PC12 and RAT1 fibroblast cells. All positive clones were then confirmed by staining on E13.5 rat embryo sections. Out of 803 hybridomas screened, 44 were positive by dot blot, and only four showed specific nuclear staining on PC12 and E13.5 neural tube but not RAT1 fibroblasts. The clones were immediately subcloned and Western blotting confirmed that one of these monoclonal antibodies, MASH-1A, detected a closely spaced 34 kDa doublet in MAH cells and E13.5 brain. No specific band was detected in RAT1 fibroblast or E13.5 liver. The antibody fails to recognize either MASH-2 or E12 and therefore appears specific to MASH-1.

251. Expression of MASH-1 during embryonic development

**Liching Lo**

Rat embryos staged from embryonic day 7.5 to E13.5 were serially sectioned and stained with anti-MASH-1 monoclonal antibodies. Our staining data reveal that MASH-1 expression, like that of its *Drosophila* counterparts, the *achaete-scute* genes, is specific to the nervous system and is detected in both the central and peripheral nervous systems (CNS and PNS). In the developing CNS, MASH-1 first appears at E10.5. Clusters of MASH-1-positive nuclei can be seen in the roof of mesencephalon and telencephalon. In the neural tube, a few positive cells are observed flanking the floor plate. By E11.5, the MASH-1-positive nuclei can be seen in the dorsal part of the spinal cord. The stained nuclei flanking the floor plate occupied a different region than SCG10-positive motor neurons. By E13.5, the MASH-1-positive nuclei occupy primarily the ventricular zone of the spinal cord containing mitotic neuronal precursor cells; by contrast, cells expressing terminal differentiation markers such as SCG10 and NF are present only in the marginal zone. In the E11-12 forebrain, well defined bands of labeled nuclei can be seen in the diencephalon and telencephalon. In the telencephalon, staining is confined to the basal region. Expression of MASH-1 in the developing PNS seems to be restricted to the sympathetic/enteric lineage. At E11.5, the MASH-1-positive nuclei occupy a region adjacent to the dorsal aorta, in the sympathetic primordium. At this stage, tyrosine hydroxylase (TH) is not yet expressed. MASH-1-positive cells are also found ventral to the sympathetic ganglia, in the gut. At E12.5, by using double-labeling techniques, TH expression was seen to overlap the MASH-1-positive cell population in the sympathetic ganglia. By E13.5, MASH-1 expression in the sympathetic ganglia was
almost undetectable. These data suggest that neural crest cells induce expression of MASH-1 when they arrive in the sympathetic primordia, then extinguish MASH-1 as they activate expression of TH and other lineage-specific genes. MASH-1 staining was not detected at any stage in migrating neural crest cells or in dorsal root ganglia. Taken together, these data suggest that MASH-1 expression is confined to positionally restricted subsets of neuroepithelial and neural crest cells and precedes, but is extinguished upon, neuronal differentiation. This pattern of expression supports the idea that MASH-1 is a vertebrate neuronal determination gene.

252. Induction and repression of MASH genes during neuronal differentiation of P19 embryonic carcinoma cells

Jane E. Johnson, Kathryn Zimmerman

The mammalian achaete-scute homologs, MASH-1 and MASH-2, are related members of the basic helix-loop-helix (bHLH) family of transcription factors. Although structurally similar, the expression patterns of these two genes are quite different. We have examined the regulation of MASH-1 and MASH-2 during neuronal differentiation of murine P19 embryonic carcinoma cells. MASH-1 mRNA accumulates to high levels during retinoic acid-induced neuronal differentiation, but is not expressed in undifferentiated P19 cells. In contrast, MASH-2 RNA is expressed in undifferentiated P19 cells and is repressed during neuronal differentiation. These reciprocal expression patterns suggest different roles for MASH-1 and MASH-2. Further examination of MASH-1 protein expression in these cells suggests that 1) the appearance of MASH-1 protein precedes but later overlaps with expression of two neuronal markers, and 2) MASH-1 expression may be transient. These features of MASH-1 expression in the P19 culture system are consistent with MASH-1 expression during embryonic development (see Abstract No. 251). These correlations suggest that P19 cells provide a model system for studying the regulation and function of MASH-1 during neuronal differentiation. To test whether MASH-1 was capable of inducing neuronal differentiation in the absence of retinoic acid, the effects of forced expression of an exogenous MASH-1 gene in undifferentiated P19 cells was examined. Our findings indicate that, although MASH-1 expression is correlated with neuronal differentiation, MASH-1 is not sufficient for inducing neuronal differentiation from undifferentiated P19 cells. Experiments to address the question of whether expression of MASH-1 is required for neuronal differentiation are in progress.

253. DNA binding activity and transcriptional activation function of the MASH genes

Jane E. Johnson, Susan J. Birren

The MASH genes are members of the helix-loop-helix (HLH) family of transcription factors. This family includes MyoD, which is required for muscle determination, and the achaete-scute complex (AS-C) genes, which play a role in neuronal determination in Drosophila. All of these genes encode proteins that share related DNA binding and dimerization domains. Both MyoD and AS-C gene products are known to bind DNA when complexed to another HLH protein, El2. A comparison of predicted amino acid sequence suggested that the MASH genes should behave in an analogous manner. We therefore undertook to examine the DNA binding properties of the MASH gene products. Using a coupled transcription-translation system and gel retention assays, we assayed the binding of the MASH proteins to the MEF1 sequence of the muscle creatine kinase (MCK) promoter. This sequence is a target for the MyoD/El2 heterodimer in vitro. In the absence of a known target sequence for the MASH proteins, we chose the MCK enhancer in the hope that a highly homologous DNA binding region would provide a site for the binding of these proteins. We found that both MASH genes bind this DNA sequence and that this activity is dependent on El2. Similar results were obtained with bacterially-expressed MASH-1 (see Abstract No. 254).

To examine the function of the MASH proteins in vivo, we tested whether they could effect transcription of a model reporter gene. Expression constructs containing MASH cDNAs driven by the Rous sarcoma virus LTR were cotransfected into 10T1/2 fibroblast cells with a reporter gene. The reporter contains the CAT gene driven by a promoter containing the MEF-1 sequence used in the binding studies. In these cotransfections, the MASH genes clearly activated transcription from the reporter gene. Thus, the MASH proteins not only bind DNA but
function as transcriptional activators. Moreover, the MASH genes are able to activate expression of endogenous MCK in transiently-transfected 10T1/2 cells. However, they are unable to activate full expression of the myogenic differentiation program. Domain switching experiments in which the basic region of MASH-1 is replaced with the basic region of MyoD, and vice versa, suggest that the MyoD basic region alone is insufficient to confer full myogenic activity upon a heterologous HLH protein. This implies that regions outside the basic HLH region of MyoD are necessary for its biological activity.

254. Bacterial expression of E12 and MASH-1 proteins

Tetsuichiro Saito, Jane E. Johnson

E12 and MASH-1 proteins are members of the helix-loop-helix (HLH) protein family. E12 is a ubiquitously expressed member of this family and MASH-1 is expressed specifically in the developing nervous system. E12 and MASH-1 proteins translated in vitro form a heterodimer and bind a DNA sequence motif, called the E2-box. To obtain sufficient amounts of the proteins for biochemical analysis, the coding sequences from the E12 and MASH-1 genes were cloned downstream of a T7 promoter and expressed in Escherichia coli carrying T7 RNA polymerase. The bacterially-expressed E12 (bacE12) and MASH-1 (bacMASH-1) proteins were recovered from insoluble inclusion bodies. The inclusion bodies were solubilized in 6 M urea and the proteins were renatured using dialysis with gradually decreasing concentrations of urea. At high concentrations, the bacE12 protein binds the E2-box, as was seen with the other bacterially-expressed HLH proteins, MyoD and myogenin. In contrast, the bacMASH-1 protein will bind the E2-box only when complexed with bacE12 protein. This suggests either that the E2-box is not the real target of the MASH-1 protein, or that homodimerization of MASH-1 protein is less efficient than that of E12, MyoD, or myogenin. It would not be surprising if the E2-box is not the real recognition sequence for the neural-specific MASH-1 protein, since the E2-box was identified in the promoter of the muscle creatine kinase gene. The bacMASH-1 and bacE12 proteins will be useful for identification of the actual target genes for MASH-1 protein.

255. E-box binding activity in neuronal determination

Tetsuichiro Saito

One group of DNA-binding proteins is characterized by the helix-loop-helix (HLH) domain preceded by a basic amino acid-rich region. Several proteins in this group can bind a DNA sequence motif (E-box) by dimerizing with the other HLH proteins (E12 or E47) and are involved in transcription related to differentiation of some cell types. The MASH-1 protein, a member of this group, can also bind E-box in vitro in the presence of E12 protein. This expression pattern of MASH-1 protein suggests that this protein is involved in neuronal determination (see Abstract No. 251). E-box binding activity may be regulated in the process of neuronal differentiation. To address this possibility, the activity was examined by gel-shift analysis using nuclear extracts from various cell lines. The activity was detected in MAH cells (sympathoadrenal precursor cells) but not in NCMI cells (peripheral glial progenitor cells), suggesting that the activity is related to neuronal determination. Anti-MASH-1 antibodies supershifted a significant amount of the activity in the MAH extract, indicating that MASH-1 protein is expressed at high levels in MAH cells. In P19 embryonal carcinoma cells, retinoic-acid treatment induces the activity just before a neural phenotype is observed, but the activity supershifted by the anti-MASH-1 antibodies was much smaller than that of the MAH extract. The E-box binding activity was also detected by mixing untreated P19 extract with bacterially-expressed MASH-1 protein. These observations suggest that an E12-like protein is already expressed in the absence of retinoic acid and that E-box binding proteins other than MASH-1 protein are induced by the retinoic-acid treatment.

256. Characterization of the murine MASH-1 gene

Benton N. Yoshida

MASH-1 expression is spatially restricted to regions of the developing forebrain and to neural crest precursors of the peripheral nervous system. This expression is transient and precedes the appearance of differentiated markers. Jane E. Johnson has demonstrated that P19 embryonal carcinoma cells induced to become neurons by
retinoic acid treatment also transiently express MASH-1. In order to study the molecular basis of this regulation, a mouse cosmid clone containing the MASH-1 gene was isolated. Partial sequence data confirm the identity of the clone. LacZ constructs will be made and tested in the P19 cell system for regulatory elements necessary for the observed pattern of expression. If faithful regulation is achieved in cell culture, the constructs will be tested for correct regulation in transgenic mice. The identification of regulatory regions in the MASH-1 gene should also be useful for determining the fate(s) of the neural precursor cells that express MASH-1, and for expressing dominant-negative mutant genes designed to interfere with MASH-1 function in MASH-1-expressing cells.

257. Characterization of MASH-2 expression

Kathryn Zimmerman

The MASH-1 and MASH-2 genes are mammalian homologs of the Drosophila achaete-scute complex of neural determination genes. To further understand the role of the MASH-2 gene in mammalian development, we have characterized MASH-2 RNA expression in numerous cell lines as well as during rat embryonic development. MASH-2 expression in cell lines is largely restricted to those of neural or neuroendocrine origin. MASH-2 is expressed at low levels in the myc-immortalized, sympathoadrenal progenitor (MAH) cell line from which it was cloned. However, the MASH-2 gene is expressed at much higher levels in two myc-immortalized cell lines that represent an earlier stage in sympathoadrenal differentiation than the MAH cells, MNC-5 and 2D10. In these cell lines, MASH-2 expression is induced by FGF in parallel with induction of the catecholamine synthetic enzyme, tyrosine hydroxylase. The expression pattern of MASH-2 suggests that it may function at an earlier time-point in the development of the sympathoadrenal lineage than MASH-1, which is expressed at high levels in MAH cells and is not expressed in either MNC-5 or 2D10. The pattern of MASH-2 expression in embryos contrasts with the neural specificity of expression observed in cell lines. MASH-2 is expressed at low levels in all tissues of embryonic day 16 (E16) and postnatal day 2 (P2) rats. High levels of MASH-2 expression are not observed in a timecourse of E11 through P2 rat brain. In fact, in E9.5 rat embryos, highest levels of MASH-2 expression have been localized by in situ hybridization to the ectoplacental cone, extra-embryonic cells that contribute to the placenta. In situ hybridization of E12.5 embryos reveals no increased MASH-2 expression in spinal chord, sensory ganglia or sympathetic ganglia. At present, it is difficult to correlate the in vivo pattern of MASH-2 RNA expression with the neural specificity of expression observed in cell lines. However, the structural similarity of the MASH-2 gene and the Drosophila achaete-scute genes suggests that the neural-specific expression of MASH-2 in cell lines is not without meaning and that a more detailed analysis of MASH-2 RNA or protein during embryonic development might reveal additional important sites of MASH-2 expression.

258. The search for additional mammalian achaete-scute homologs

Kathryn Zimmerman

The Drosophila achaete-scute complex consists of four homologous genes. We have previously isolated two mammalian homologs of these genes, MASH-1 and MASH-2, from the sympathoadrenal progenitor MAH cell line by degenerate oligonucleotide-primed polymerase chain reaction (PCR). The degenerate primers used to isolate MASH-1 and MASH-2 represented regions of the basic helix-loop-helix (bHLH) domain completely conserved in all members of the Drosophila achaete-scute family as well as partially conserved in other bHLH family members. As the Drosophila genome contains four achaete-scute genes, we reasoned that additional mammalian genes might also exist. We therefore attempted to isolate additional mammalian achaete-scute homologs from rat genomic DNA, as well as from cDNA prepared from various sources, by PCR using multiple degenerate oligonucleotide primers covering the conserved bHLH region. In all cases, MASH-1 and MASH-2 were the only bHLH-containing genes isolated even though all four Drosophila genes could be isolated under the same conditions. Similarly, DNA probes made from the bHLH region of either MASH-1 or MASH-2 cDNA cross-hybridized to both the MASH-1 and MASH-2 genes on genomic Southern blots but did not hybridize to any other genes. Our results do not preclude the possibility that
additional bHLH genes are important for neuronal differentiation. However, our findings suggest that such genes would be less related to MASH-1 and MASH-2 than these two genes are to one another. We are currently examining expression cloning systems as a means of isolating these more distantly related but potentially important genes.

259. Homeo and POU domain genes in the neural crest

Christopher Schoenherr

Previous in situ hybridization studies have shown that a large number of murine homeodomain genes, the so-called Hox genes, are expressed in at least some derivatives of the neural crest during embryonic development. This is also true for the related POU-domain gene family. From their considerable homology to genetically characterized genes from *Drosophila* and *Caenorhabditis elegans*, it is believed that these gene families play an important role in mammalian development. It is with this in mind that we examined the expression of these gene families in two derivatives of the neural crest, the MAH and NCM-1 cell lines. The MAH line is derived from the sympathoadrenal bipotential precursor cell that can become either a sympathetic neuron or an adrenal chromaffin cell. The NCM-1 line appears to represent a Schwann progenitor cell.

In order to examine concurrently the expression of such large gene families, we designed degenerate oligonucleotide primers, which could be used in the polymerase chain reaction (PCR), specific to two separate regions shared by all family members. These primers allowed us to clone a large number of known genes, as well as a few new genes. Northern blot analysis and RNase protection assays were performed on a number of these clones to confirm and examine their patterns of expression in relevant cell lines and tissues. The results of these studies suggest that the MAH cells express only one Hox gene, called H2. H2, however, is expressed in a number of crest-derived lines and, thus, is not sympathoadrenal specific. NCM-1 cells, on the other hand, express up to 10 different Hox genes. One clone, H20, is present at high levels but is present at considerably lower levels in a cell line derived from NCM-1 cells. This line no longer expresses glial markers. H20 is also not expressed in postnatal sciatic nerve, a rich source of Schwann cells. These results suggest that H20 warrants further study to elucidate its role in Schwann cell development. The POU gene family gave less interesting results. While a number of new genes were identified, they were either ubiquitously expressed or had extremely low levels of expression.

In the course of these experiments, we devised a more rapid method of determining the complement of each gene family expressed. Instead of cloning the PCR product and sequencing a number of clones, the PCR product was radiolabeled and used to probe a clone blot containing the large number of distinct clones generated during the initial experiments. PCR products derived from genomic DNA, in which all relevant genes are equally represented, show that the primers are not strongly biased. This technique enables us to determine quickly which genes should be examined more thoroughly. And, due to the sensitivity of PCR, it will allow us to assay small amounts of tissue.

260. Analysis of the SCG10 silencer

Nozomu Mori, Christopher Schoenherr

A well-studied aspect of differentiation in the neural crest is the onset of expression of various cell type-specific genes. For example, SCG10, a neuron-specific gene, appears in crest-derived neuronal precursors at E11.5 in the rat and, subsequently, is up-regulated as neurites are extended. A less well-studied aspect of differentiation in the neural crest is the combination of intrinsic regulatory factors governing cell fate. A reasonable approach to address cell fate questions, given the considerable knowledge of cell-specific gene expression, is to determine the intrinsic factors that regulate these genes. With this in mind, the promoter of SCG10 was examined in transient transfections and in transgenic mice. These studies separated the promoter into two major regulatory domains: a proximal region that is active in many cell types and tissues, but preferentially in neural tissue, and a silencer region (at about 1.5 kb upstream) that is preferentially active in non-neuronal tissues and cell lines. Thus, SCG10's tissue-specific regulation appears to be a combination of neuronal-preferred enhancement and non-neuronal silencing.
Further work was focused on the silencer region. Using 5' promoter deletions and transient transfections in HeLa cells, the silencer region was narrowed down to 60 base pairs. The region was further narrowed down to 36 base pairs by assaying the silencing activity of oligonucleotides on the constitutively active proximal construct. Competition experiments with multimerized binding sites and a gapped deletion mutant also strongly implicate this 36 base pair region as containing a silencer element. This information allowed us to perform gel shift and footprint experiments using HeLa cell nuclear extracts. These studies, however, have not been successful in identifying proteins that interact with the SCG10 silencer. To address this problem, a DNA-affinity column will be made from the silencer region in an attempt to concentrate any activity that might be present in the extract. If the column is successful, it will allow us to optimize gel shift and footprinting conditions, as well as the extraction procedure. In order to clone the silencer gene, a lambda expression library will be screened with radiolabeled concatamerized binding sites. Also, a yeast expression library will be screened. These methods could allow us to clone the gene encoding the silencer in the absence of a biochemical assay for this regulatory factor.

261. Control of growth factor-responsiveness during neuronal differentiation of the sympathoadrenal progenitor cell

Susan J. Birren, Adela S. Augsburger

The rat peripheral nervous system is derived from the multipotent cells of the neural crest. During development, these cells become restricted in their developmental potential. In the sympathoadrenal lineage, it is possible to isolate a bipotential precursor cell that can give rise to either a sympathetic neuron or an adrenal chromaffin cell. We are interested in studying the factors that cause this progenitor cell to undergo neuronal differentiation. We have therefore isolated immortalized cell lines in this lineage which, under appropriate conditions, will differentiate down the neuronal pathway. Using these cell lines as a model system, we have been able to make several predictions as to how the sympathoadrenal bipotential precursor cell may behave in vivo and in primary culture. First, although mature sympathetic neurons are dependent upon nerve growth factor (NGF) for survival, we predicted that the undifferentiated precursor cell would not respond to NGF. Second, we predicted that this cell would undergo neuronal differentiation in response to fibroblast growth factor (FGF), despite the fact that FGF is not a survival factor for the mature neurons. And lastly, we predicted that FGF would induce an NGF responsiveness in the bipotential precursor cell.

To test these predictions, we isolated bipotential precursor cells from the developing adrenal glands of embryonic day 14.5 rat embryos using specific cell surface antibody markers and a fluorescence-activated cell sorter. In short term assays, we examined the effects of FGF and NGF on process outgrowth (neuronal differentiation) and proliferation of these cells in primary culture. In accordance with our predictions, we found that the bipotential precursor cells do not respond to NGF, but have a strong FGF response. Although we cannot yet say that FGF treatment leads to NGF responsiveness, we have demonstrated that FGF induces expression of the low affinity NGF receptor on these cells. These results are consistent with a model in which a factor such as FGF plays a role in triggering a precursor cell to become NGF responsive, and eventually dependent upon NGF for survival.

We are also interested in other factors that may play roles in the differentiation or survival of sympathetic neurons. We are therefore examining the effects of known neurotrophic factors in our system. CNTF, BDNF, and NT-3 have been included in this survey. While NT-3 stimulates proliferation of the precursors in a manner similar to NGF, it is not a long-term survival factor for these cells. Likewise, BDNF does not lead to increased survival of mature neurons. CNTF, in contrast, acts as an inhibitor of MAH cell and normal progenitor proliferation. These results are consistent with those obtained previously in other laboratories, using chick sympathetic neuroblasts. The results of this survey thus suggest that FGF and NGF play unique roles in sympathetic neuronal development.

262. A biphasic response to steroid hormones controls chromaffin cell differentiation

Arie M. Michelsohn

Adrenal chromaffin cells and sympathetic neurons
both derive from a common embryonic progenitor but can easily be distinguished on the basis of their different morphologies (sympathetic neurons [SN] are process-bearing while chromaffin cells [CC] are not) and transmitter phenotype (SN are noradrenergic while CC are adrenergic). PNMT, the enzyme responsible for the conversion of noradrenaline into adrenaline, appears late (E17) in adrenal development relative to the time at which chromaffin precursors are inhibited from developing a neuronal phenotype (by E14.5). Previous work has shown that glucocorticoids (GCs) both inhibit neuronal differentiation and stimulate PNMT expression in cultured chromaffin cells. The delay in PNMT expression has been previously attributed to a delay in the timing of appearance of GC, or of its receptor. However, both of these explanations would preclude a role for GCs in the early inhibition of neuronal differentiation. We have shown that in vitro, purified chromaffin cell precursors isolated from E14.5 adrenal glands are inhibited from extending neurites by GC within 24 hours of culture. However, GCs are incapable of inducing PNMT in most such cells until 1-2 days later (although particularly potent GCs such as dexamethesone can induce “precocious” expression in some cells). Both effects of steroid in this system are likely to be mediated via type II GC receptors, but the pharmacological properties of the two effects are different. The delayed expression of PNMT does not simply reflect the kinetics of protein accumulation. Rather, it appears that cells are initially not competent to respond to GCs by induction of PNMT, but are capable of responding to GCs by inhibition of process outgrowth. The acquisition of competence to express PNMT seems to reflect the operation of an intrinsic cellular “clock” that runs in the absence of GCs but which is extinguished when cells differentiate into neurons. Thus, by inhibiting cells from undergoing neuronal differentiation during early times in development, GCs can effectively increase the pool of chromaffin cell precursors. The requirement that developing chromaffin cells first “decide” not to become neurons, and then later express PNMT, may explain how PNMT expression is restricted to the adrenal gland.

263. Generation of committed Schwann cell progenitors from a multipotent neural crest cell with stem-like properties

Derek L. Stemple

The neural crest is a vertebrate innovation that forms a diverse array of derivative cell types after undergoing extensive migrations. These cell types include, among others, the neurons and glia of the peripheral nervous system, bone and cartilage of the face, and melanocytes. Studies of individual early neural crest cells both in vivo and in vitro have shown them to be multipotent. Specification of their ultimate phenotype appears to be modulated by the environment to which they are exposed. Moreover, lineage restrictions may also contribute to the determination of crest cell fate. Current evidence suggests that such restrictions may also occur progressively, in a manner analogous to hematopoiesis.

One feature of hematopoiesis is the existence of a multipotent stem cell. Such a cell is capable of producing several committed progenitor cells but also has the ability to regenerate itself. To test whether neural crest cells undergo a progressive restriction from a multipotent stem-like cell to committed progenitor cells, I am using embryonic day 10.5 rat neural crest cells in a clonal culture system. A monoclonal antibody to the rat low affinity nerve growth factor receptor (LNGFR) recognizes a subset of neural crest cells, as well as Schwann cells, and allows for their identification in a non-destructive way. When neural crest cells are cloned after 24 hours and assayed after an 8-day culture period, the majority of clones that contain Schwann cells also contain non-Schwann cells. However, when crest cells are cloned after 8 days and assayed 6 days later, most of the Schwann cell-containing clones are pure, that is, they contain no other cell type. When clones of mixed phenotype are subcloned at day 8, I find that three types of subclones arise after an additional 6 days in culture: 1) subclones that are purely Schwann cells, 2) subclones that are purely non-Schwann cells and, 3) subclones that, like the founder clone, are comprised of both Schwann and non-Schwann cells. These data suggest that some crest cells can divide asymmetrically to produce both Schwann progenitors, or non-Schwann cells, and can self-renew. Once generated, Schwann cell progenitors divide symmetrically, like a blast cell. The limits of self-renewal
of the LNGFR+ neural crest cells are not clear, but can be assayed by serial subcloning. These studies provide the first evidence that neural crest cells can exhibit self-renewal properties, and undergo commitment to a particular sublineage, in vitro. This system now provides the opportunity to examine the environmental signals that control self-renewal and lineage commitment, in the neural crest.

264. Gene expression in the embryonic telencephalon

John Montgomery

I am continuing a search for genes that may be involved in patterning the embryonic telencephalon of the rat. Recently, the basic helix-loop-helix gene, MASH-1, isolated in our laboratory by Jane Johnson, has been shown to be expressed in positionally-restricted subsets of neuroepithelial cells in both the peripheral and central nervous systems of the embryonic rat (Abstract No. 251). In the E12.5 telencephalon, the MASH-1 protein is strikingly localized to the ventral, anterior region of the neuroepithelium. This novel expression pattern is intriguing in light of recent observations arising from both chimeric mouse and retroviral lineage-tracing experiments, suggesting the existence of antero-posterior developmental "compartments" in the embryonic telencephalon. In the chick and mouse hindbrain, the differential expression of various homeobox- and zinc finger-containing genes appears to delineate compartmental boundaries defined by the eight metameric rhombomeres.

Using a directional, E12.5 telencephalon cDNA library constructed in the lambda-EXLX vector developed by Palazzolo et al., I have performed a series of differential cDNA screens between various tissues, such as E12.5 telencephalon versus E12.5 mesencephalon, as well as the anterior third versus the posterior third of the E12.5 telencephalon. As yet, clones that have been isolated and re-screened have not shown sufficiently dissimilar abundance levels in these tissues to warrant further study. Libraries cloned in an orientation opposite to that of the telencephalon cDNA, such as one constructed using E12.5 mesencephalon cDNA, will be used for the more sensitive method of subtractive hybridization in an effort to isolate additional genes that are asymmetrically expressed within the telencephalon.

PUBLICATIONS


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Summary: In the development of the nervous system, one can define several stages, including the proliferation of precursor cells, their determination to become neurons, their differentiation into specific neuronal types, the formation of precise networks, and the maintenance of the functional integrity of the system. The research of our group on *Drosophila* addresses such questions at various levels, using mutants. For instance, in the *eyes absent* mutant, specifically the compound eye precursor cells undergo programmed cell death, while other structures derived from the same imaginal disc develop normally. In other mutants, the photoreceptor cells develop, but do not become properly organized into the normally very precise arrangement. In some, the axons go astray in finding their proper targets in the optic lobe. In some mutants, genetic defects are manifest with late onset, as in the mutant *dropdead*, where the adult brain degenerates.

Many genes and proteins show homology between *Drosophila* and vertebrates. These include developmental genes such as the homeobox-containing genes involved in pattern formation and segmentation, cytoskeletal proteins, receptors, oncogenes, and growth factors. Retinal molecules known to be conserved between humans and *Drosophila* include the opsins, the arrestins, protein kinase C, G proteins, and phospholipase C. It is likely that a common ancestor of humans and *Drosophila* possessed a simple photoreceptive system, and that the complex dipteran and primate retinas arose by separate, but in many ways parallel, elaboration.

Both *Drosophila* and humans suffer from many genetic developmental abnormalities. For instance, in retinitis pigmentosa, a major cause of blindness in humans, the photoreceptor cells degenerate. The cause has been shown, in certain cases, to be due to a mutation in the structural gene for rhodopsin. The same effect occurs for mutations in the structural gene for *Drosophila* rhodopsin. Humans and flies also display a variety of other genetic defects affecting the eye or brain.

The methods of gene mapping and cloning available in *Drosophila* enable one to proceed from a mutant gene to cloning of the gene and identification of its protein product. With monoclonal antibodies (MAbs), one can approach these problems in the opposite direction. A MAb that identifies an antigen specific to certain neurons makes it possible to purify the antigen, which can then be sequenced, leading to the isolation of corresponding gene clones, followed by mapping of the gene and mutating it to observe the consequent phenotype. A third way to proceed is to begin with cDNA clones which are readily isolated and screened for expression in tissues of interest, e.g., the eye. Given such a clone, one can readily determine the map position of the corresponding gene and identify its transcript.

A set of previously isolated eye-specific *Drosophila* cDNAs is being used to isolate potential functional human retinal cDNA homologs. The *Drosophila* cDNAs include several with known sequence homology to human eye genes, such as arrestin and rhodopsin, as well as others of novel sequence. Mutations will be induced in the fly genes corresponding to the *Drosophila* eye cDNAs, and the resultant phenotypes characterized for anatomical and functional defects in the retina and visual transduction pathways.

To test whether a putative human homolog expresses a similar function in the fly, we are attempting to rescue fly mutant phenotypes with human cDNA. This is done by constructing hybrid genes in which *Drosophila* promoter sequences are linked to the protein-encoding regions of the human homologs. These constructs are tested for gene and protein expression in *Drosophila* tissue culture cells, then introduced into the *Drosophila* germline by P element-mediated transformation, and analyzed for expression in the fly. Partial or full rescue of the phenotype will be evidence for conservation of function.

Memory loss in humans is associated with degeneration of specific neuronal cells in the brain, as in
Alzheimer's disease and other neurological disorders. We are studying related degenerative phenomena in *Drosophila*. In both the *Drosophila* and human central nervous systems, neuronal cell bodies and axons are associated with glial cell processes. In the *Drosophila* mutant *drop-dead*, it appears that the glia suffer from a genetic defect that may be the cause of brain degeneration. Genetic disruption of glial cell function may therefore provide a powerful tool in which to evaluate neuronal and glial cell interactions.

265. To be or not to be: A *Drosophila* mutation that influences cell death

*Nancy M. Bonini, William M. Leiserson, Seymour Benzer*

Regulation of cell number, growth, and differentiation by elimination of cells through cell death is common during normal development, yet the molecular mechanisms are largely unknown. In the *Drosophila eyes absent* mutant, precursor cells for the adult eye undergo massive programmed cell death just prior to the time when the first overt differentiation events in eye morphogenesis ordinarily occur. In normal flies, only a small amount of cell death is seen at this stage. This suggests that the *eyes absent* gene may influence a critical decision in the cell: to undergo differentiation events toward neurogenesis, or to be eliminated through cell death. The molecular mechanisms that underlie regulation of this process may be fundamental to the control of many types of normal and abnormal differentiation pathways. We are cloning and molecularly characterizing the *eyes absent* gene to determine its biological function in the cell degeneration/differentiation process during normal development, and to understand how loss of function of the *eyes absent* gene leads to abnormal developmental events.

A 25 kb segment of genomic DNA has been cloned and identified as the *eyes absent* region by cytological and molecular analysis of alleles with chromosomal breaks. Cloned segments of this DNA have been used to identify transcripts in the region and to isolate candidate cDNAs. Probing of poly(A)+ RNA Northern blots of fly heads and bodies indicates two head-specific transcripts of approximately 7.0 and 3.5 kb that splice through the region and share regions of homology. Both transcripts appear to be disrupted by all six breakpoint alleles. In the original *eyes absent* allele, both transcripts are reduced, and a probe from the 7.0 kb transcript shows altered sizes. Another of the non-breakpoint alleles examined so far also shows polymorphisms in these transcripts. The 3.5 kb transcript is expressed in embryos and in third-instar larval eye discs. Therefore, we have so far focused most work on the 3.5 kb transcript.

By tissue *in situ* hybridization to eye discs, the 3.5 kb transcript was located in a band just ahead of the morphogenetic furrow in the undifferentiated region of the eye disc. It was less intense at the furrow, and was again expressed posterior to the furrow, in the differentiated region of the disc. Interestingly, the expression in the disc ahead of the furrow corresponds in location to the region where a small amount of cell death normally occurs in eye development, and where cell death in *eyes absent* mutant alleles is greatly increased. In completely eyeless alleles, expression in the disc is greatly reduced or absent. Expression in the eye disc is consistent with the results from somatic mosaic analysis, in which we have shown that the gene action is cell-autonomous and cell-lethal in the disc. During embryogenesis, the transcript shows an elaborate expression pattern, starting at blastoderm in the acron of the embryo, spreading through various regions in the head and coming on segmentally, and finally resolving into expression in clusters throughout the brain and along the ventral nerve cord. Analysis of homozygous lethal alleles shows that the transcript is missing from some of these alleles, again consistent with this transcript being associated with the *eyes absent* gene.

We are currently performing transformation rescue with cDNAs encoding transcripts of the region in an attempt to provide definitive evidence that we have identified the proper cDNAs, and to determine whether the two transcripts encode different or overlapping functions.

266. Using mutant alleles to probe the function of the *eyes absent* gene product

*William M. Leiserson, Nancy M. Bonini, Seymour Benzer*

The *eyes absent* (*eya*) gene is indispensable in eye development; it controls early events of whether eye precursor cells will undergo overt differentiation or die (see Abstract No. 265). Understanding the function of the *eyes absent* gene product(s) may shed light on the
regulation of differentiation processes. We are analyzing mutant alleles to determine the nature of the mutations and their genetic and molecular consequences. Such studies may provide evidence for functional domains of the gene product(s), may suggest whether the molecule acts as a mono- or multimer, and may indicate what role eyes absent plays in development.

We have generated 40 additional alleles beyond the original eyeless eyes absent allele (Sved, 1986). Most of these are homozygous lethal, indicating that the eyes absent gene has a vital function in addition to its role in eye development. We are exploring the biological nature of the vital function, and the genetic analysis of the mutant alleles suggests that the locus is complex. To study the function in eye development, the mutant phenotypes have been studied over the original eyeless allele, which complements the lethality. These phenotypes range from eyeless to mildly reduced eyes, with many alleles producing intermediate phenotypes.

Preliminary results indicate that the eyes absent locus exhibits a complex complementation pattern. While most alleles are semi-lethal, the eye phenotype can be scored in escapers. We have found that a severe eye phenotype does not necessarily correlate with a severe lethal phenotype, as measured by the number of homozygous escapers. Furthermore, it appears that the alleles cannot be ordered according to severity of eye phenotype. For example, the eya1 homozygote is eyeless and the eya19A-3 homozygote has a severely reduced eye, yet the heteroallelic combination eya1/eya19A-3 gives a less severe phenotype than either homozygote, suggesting that the two alleles partially complement. Several examples of such complex complementation have been found, suggesting that the eyes absent gene product may be a multimer.

Reference:

267. Neurogenetic analysis of brain degeneration in the Drosophila drop-dead mutant
Robert L. Buchanan, Seymour Benzer

Cellular mechanisms involved in late-onset central nervous system (CNS) degeneration are largely unknown. To better understand the genetic and cellular basis of brain degeneration in Drosophila, we have examined the CNS of the drop-dead (drd) mutant. This mutant carries an X-linked recessive mutation that causes severe behavioral defects and brain degeneration in adult mutant flies, beginning several days after emergence and culminating in death. Evidence from genetically constructed mosaic files indicates that the brain is the focus of the drd mutant phenotype (Benzer, 1971; Hotta and Benzer, 1972).

Our ultrastructural analysis of the newly emerged adult brain has identified degenerate glia with attenuated cell processes throughout the drd mutant brain. In addition, a fraction of the mutant brain neurons display abnormal cytoplasmic bodies indicative of the earliest stages of neurodegeneration. Ultrastructural studies on earlier stages of development suggest that the drd mutation may influence the appropriate development of CNS glia; the presence of defective glia in the adult drd mutant brain may cause progressive neuronal damage, which likely contributes to brain degeneration and death. Understanding how the drd gene product might influence glial cell development or function will require molecular cloning of the gene, which is currently in progress.

We are also interested in understanding the mechanism by which wild-type tissue can prevent neurodegeneration in genetically mosaic brains containing drd mutant tissue, since mosaic analysis indicated that drd gene function, while mapping to the brain, required both sides of the brain to be mutant; a normal half-brain appeared to present degeneration of a mutant half, suggesting a putative brain-specific factor (Hotta and Benzer, 1972). An alternative interpretation is that cell migration during development may "seed" the mutant side of the mosaic with wild-type glia, thereby rescuing the entire brain from neurodegeneration. Distinguishing between these models has recently become feasible by the development of transgenic fly strains containing inserted β-galactosidase genes that are expressed in glial cells (Liu et al., 1988). Mosaic brain tissue containing marked glia of known genotype will be produced by standard genetic methods. Histological examination of mosaic brain cell boundaries may reveal whether or not cell migration is responsible for preventing neurodegeneration in drd brain tissue.

References:
268. Innervation-dependent gene expression in the optic ganglion of the developing larval brain of *Drosophila*

Brian A. Mozer, Seymour Benzer

In the adult fly visual system, photoreceptor cell axons synapse in a precise array with specific neurons in the lamina and medulla, the second and third optic ganglia of the brain. Axons from the outer photoreceptor cells, R1-6, of each ommatidium terminate in the lamina, while axons from the inner photoreceptor cells, R7 and R8, project to the medulla. These neuronal connections form during development in the third larval instar. The development of the lamina and most of the medulla is dependent on the establishment and/or maintenance of these connections; the optic lobes in eyeless mutants are severely reduced. To study the activation of postsynaptic targets of the photoreceptor cells and their role in development of the visual system, a collection of "enhancer trap" insertion lines was screened for β-galactosidase staining of the larval brain and eye disc. Two third-chromosome insertion lines were identified with patterns of β-galactosidase expression in the lamina precursor cells.

One line, A72, is a homozygous viable third-chromosome insertion that maps cytogenetically to band 77B. β-galactosidase expression is first detected in the early second instar larva in a pair of cells located in central positions within each hemisphere of the brain. In later second instar, a cluster of 8-12 cells expresses β-galactosidase; they are positioned close to the dorsal surface of each brain hemisphere, at a site adjacent to the point of attachment of the eye stalk, through which the axons of the photoreceptors innervate the brain. In the third instar larval brain, a crescent-shaped cluster of cells expresses β-galactosidase. This pattern was compared with the pattern of staining of the monoclonal antibody 24B10, which recognizes the antigen chaoptin that is specific to photoreceptor cells and their axons, and highlights the axon terminations in the optic lobes. Double-labeling experiments with anti-β-galactosidase and Mab 24B10 revealed that the β-galactosidase-positive cells are brain cells contacted by the photoreceptor cell axons.

β-galactosidase expression from the A72 insertion was examined in two mutants lacking eyes. No staining was detected in larval brains of the *eyes absent (eya)* mutant. In the *sine oculis (so)* mutant, the majority of brains exhibited no expression; a small number showed partial staining, consistent with the partial penetrance of ommatidial formation of this mutant. The A72 gene/enhancer may thus require innervation for development of the target cells, or for activation of expression of the gene. Experiments are in progress to try to discriminate between these two models.

To identify the gene corresponding to this expression pattern, DNA flanking the insertion was cloned by plasmid rescue and used to isolate 50 kb of overlapping genomic DNA clones from a cosmid library. DNA fragments surrounding the site of the P element were used as probes for whole mount *in situ* hybridization of larval brains. A 7 kb restriction fragment containing highly repetitive sequences, immediately adjacent to the insertion site, gave a hybridization pattern identical to the β-galactosidase staining pattern in the larval brain. In *eyes absent* mutant brains, the crescent-shaped pattern of hybridization was missing. Experiments are in progress to more precisely define and characterize this gene and its enhancer.

269. A gene expressed in retina and brain of *Drosophila* codes for an unusual cysteine motif

Konrad E. Zinsmaier, Kai K. H. Eberle, Heike Duerr, Seymour Benzer

A monoclonal antibody (Mab ab49) from a hybridoma library (Buchner et al., 1988) stains synaptic neuropil regions of the *Drosophila* central nervous system as well as synaptic terminals on muscle. On Western blots of dissected fly brains, it detects a characteristic protein triplet of sizes from 32 to 36 kDa. This antibody led to the cloning and characterization of two neuron-specific expressed cDNAs which were shown to derive from a single gene localized at 79E1-2 on the third chromosome (Zinsmaier et al., 1990). Both cDNAs encode very similar proteins (csp32 and csp29) which differ only in their C terminals and a medial deletion of 21 amino acids in csp29. Their most striking common feature is a contiguous string of 11 cysteines, flanked by another pair of cysteines on each side. In accordance with this motif, the proteins are called cysteine-string proteins.

By immunizing mice with purified fusion protein of bacterially expressed csp32 protein, polyclonal sera were
obtained showing staining patterns on brain sections and on Western blots identical to Mab ab49. In addition, new monoclonal antibodies were isolated and screened for their ability to specifically detect csp32, which contains a unique region of 21 amino acids. Preliminary results with this new antibody (K1G12) indicate different expressions of the two isoforms. csp32 seems to be expressed predominantly in the neuropil of the lamina and, at lower levels, in the central neuropil of the brain. In contrast, antibodies reactive against both isoforms stain all neuropils with the same intensity. The csp proteins become detectable in 11- to 13-hour-old embryos, continuing to the adult stage.

Mab K1G12 on Western blots of *Drosophila* head homogenates detects specific proteins within the native protein triplet. This makes it very likely that the cDNA-derived fusion protein corresponds to the same protein triplet detected by ab49. The native csp proteins have now been purified to homogeneity by a three-step purification scheme and are ready for sequencing to test this hypothesis.

The genomic region corresponding to the csp cDNA has been cloned and characterized by Southern blots. Sequencing provided insight into the alternatively spliced exon/intron structure of the gene, which spans a genomic region of approximately 7 kb.

The species-specificity of the csp gene is being investigated. Immunohistochemical data obtained with ab49 imply the presence of csp antigen in all subspecies of *Drosophila* and in less related diptera such as *Musca* and *Calliphora*. In more remotely related orders, such as *Hymenoptera* and *Saltatoria*, the antigen seems to be absent. Using low stringency Southern blotting, homologous sequences could be detected in all of the investigated fly species as well as in higher vertebrates. To identify functional domains of the proteins which could be highly conserved from insects to vertebrates, the cloning of the csp gene in different species is under way. Functional aspects are to be investigated by inducing mutations into the cloned gene.

References:


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270. Expression of the human tumor suppressor gene *RBI* in *Drosophila*

James M. Dunn, Seymour Benzer

The focus of our work is to study homologies between human and *Drosophila* genes and to use these homologies to better understand their functions. One gene we are studying is the human tumor suppressor gene *RBI*. This involves two approaches: low stringency screening of *Drosophila* DNA and RNA with the human *RBI* gene, and ectopic expression of the human *RBI* gene in *Drosophila* tissue culture cells and transgenic animals. To date, no cross hybridizing species has been detected in either *Drosophila* DNA or RNA. Human *RBI* can thus be studied as an ectopic gene in transgenic flies. We have constructed several lines of transformed *Drosophila* that express human *RBI*. These lines express *RBI* under the control of the *Drosophila* Rh1 promoter, the expression of which is restricted to the photoreceptors R1 through R6, late in development. These experiments have shown that the human *RBI* cDNA can be transcribed and translated in *Drosophila*. Future experiments will look for the cellular localization and cell cycle-regulated phosphorylation of the gene product as well as its capacity to bind the viral antigen SV40 large T. The Rh1 promoter was chosen since its expression pattern involves only cells that have terminally differentiated, to minimize detrimental effects of *RBI* expression during development. Interestingly, some of the transgenic flies appear to have eye defects which may be due to interactions between the human *RBI* and *Drosophila* cellular proteins. Preliminary *Drosophila* tissue culture experiments using the inducible heat shock promoter, hsp70, to express *RBI* produced stable transformed cell lines with constructs containing the *RBI* cDNA in the anti-sense orientation, but not with the sense orientation. These data suggest that the human *RBI* gene may function in *Drosophila* cells. If this hypothesis is correct, we will be able to use the powerful genetic tools of *Drosophila* to study the interactions of *RBI* and its cellular targets, thus elucidating some aspects of this gene's function.
271. Expression of the human rhodopsin gene in Drosophila
Kirk Mecklenburg, Seymour Benzer

To determine the extent to which DNA sequence similarities between visual system-specific molecules in humans and Drosophila represent true homologies, we are attempting to express human eye genes in the Drosophila eye to see whether they can function similarly.

In preliminary experiments, we have introduced human rhodopsin into Drosophila. We synthesized a construct containing the human rhodopsin cDNA driven by the Drosophila Rhl promoter, and used it to transform flies. A ninaE strain, deleted for the Drosophila rhodopsin, was chosen as recipient, to test whether the human gene could rescue the mutant phenotype. Two independent lines were established and are being characterized. Both strains have integrated the entire construct intact. In the first line examined, the human rhodopsin gene is expressed; transcripts containing the human cDNA are detected in poly(A)+ RNA extracted from heads. Anatomical and electrophysiological experiments aimed at determining whether the protein is translated and rescues the phenotype are currently under way.

272. Isolation of a gene involved in the formation of pigment cell lattices in the Drosophila eye
Kwang-Wook Choi, Brian A. Mozer, Seymour Benzer

The fly eye is an ideal system for genetic analysis because of its repetitive structural organization. Although several genes involved in the development of the eye have been extensively studied, many questions remain as to the mechanisms underlying formation of the pattern. In an attempt to identify additional relevant genes, we have generated single gene mutations by using P element-mediated enhancer trap techniques (O'Kane and Gehring, 1987; Bier et al., 1989).

We have focused on one of the lines, Fl-76a, which shows interesting eye defects. Eyes of the homozygous adults have abnormal shape, slightly elongated in the dorso-ventral direction, and irregular arrays of ommatidia. These phenotypes are recessive and fully penetrant. The mutant line shows complex β-galactosidase expression patterns during development. In the third instar larva, staining with X-gal is observed in the photoreceptor cell clusters behind the morphogenetic furrow, in neuroblasts of the optic anlagen in the brain, and in neuroblasts of the ventral ganglion. During pupal stages, β-galactosidase expression is also seen in additional cell types in the eye, including pigment and bristle cells.

The eye phenotype was revertible when the P element was induced to excise from the insertion site, demonstrating that the mutant phenotype was caused by the insertion. Additional alleles were also generated by imprecise excision events. About 20 of them are homozygous lethal and show eye phenotypes similar to the Fl-76a homozygotes as trans-heterozygotes with the original P allele. Preliminary complementation tests suggest that there are at least two lethal complementation groups in the region; the complementing trans-heterozygotes show eye phenotypes similar to the Fl-76a homozygotes. These genetic data suggest that the mutant phenotype is a null phenotype.

Tangential sections of the mutant eyes show that each ommatidium is changed from the normal hexagonal to a quadrilateral form; often two or more ommatidia are fused. Electron microscopy of tangential sections shows that most ommatidia have the normal number of photoreceptor cells, but 5-10% of the ommatidia either lack R7, or are severely reduced in the size of the R7 rhadomere.

The hexagonal lattice of the normal ommatidium is established during the pupal stage by precise localization of pattern elements, including pigment cells of three different types, which surround the photoreceptor cell clusters. It has been suggested that this involves cell-cell interaction and elimination of excessive cells that were not recruited into the lattice (Cagan and Ready, 1989). Electron microscopy of the mutant eyes suggests that they lack tertiary pigment cells, which normally occupy three vertices of an hexagonal lattice. Instead, they have super-numerary secondary pigment cells, apparently leading to impairment of the lattice formation.

We have cloned genomic DNA flanking the P element insertion site by plasmid rescue. Using the rescued plasmid as probe, cosmids covering about 50 kb of the genomic region have been mapped. From adult head libraries, a set of cDNA clones that hybridize to regions nearby the P element insertion site was isolated. Sequence analysis of a 2.6 kb cDNA revealed an open reading frame...
that shows extensive amino acid sequence homology with the catalytic domain of a family of serine/threonine protein kinases. This cDNA detects multiple transcripts on Northern blots of poly(A)+ RNA from pupae and adult heads. We have identified three related classes of cDNAs that are differentially spliced. Sequence analysis of the P element insertion region accurately localized the insertion site in an intron near the 5' end of a cDNA sequence.

References:

273. Drosophila visual system arrestins
Kirk Mecklenburg, Seymour Benzer

We are interested in molecular homologies between Drosophila and humans, in order to identify the functions of human genes using Drosophila as a model system. A set of eye-specific Drosophila cDNAs was isolated (Hyde et al., 1990). We, and others (Hyde et al., 1990; Smith et al., 1990), discovered a Drosophila photoreceptor-specific molecule with over 40% DNA sequence identity to human arrestin. Arrestin in humans is thought to bind photoactivated rhodopsin and quench its activity. An autoimmune disease in humans, uveitis, which results in blindness, is the result of antibody recognition of arrestin.

We also discovered an additional eye-specific arrestin, also identified by Yamada et al. (1990) and LeVine et al. (1991). The arrestins were designated Arr1 and Arr2. The existence of at least two eye-specific arrestins in Drosophila suggests that humans may also have multiple forms. Because arrestin interacts with rhodopsin, and Drosophila, like humans, have multiple forms of rhodopsin, we investigated whether the arrestins might be specific to different rhodopsins. The mutation sevenless removes the photoreceptor class that expresses rhodopsins Rh3 and Rh4; in the mutant, the Rh3 and Rh4 transcripts are drastically reduced. Probes specific to Arr1 and Arr2 were used to probe Northern blots of the mutant and wild type. Neither arrestin transcript was missing in the mutant. These data suggest that neither arrestin is specific to Rh3 or Rh4.

DNA sequence comparisons between human arrestin and Drosophila Arr1 and Arr2 have identified two regions that are highly homologous. We have synthesized primers and are currently attempting to PCR amplify human retina cDNA in order to isolate additional human arrestins.

References:

274. 7F10: A Drosophila CDP-diglyceride synthetase?
Erich M. Schwarz, Seymour Benzer

The 7F10 gene is one of 20 novel genes expressed preferentially in the adult Drosophila retina (Hyde et al., 1990). We find that our longest 7F10 cDNA (2344 bp) encodes a protein of 493 amino acids with 29% identity to the CDP-diglyceride synthetase (CdsA) of Escherichia coli. No eukaryotic homolog of CdsA has previously been cloned, although extensive biochemical studies of CDP-diglyceride synthetase in Saccharomyces cerevisiae have been done.

Much of the identity between 7F10 and CdsA is concentrated in two blocks of 27 and 30 amino acids (each 67% identical). Both the Drosophila and E. coli conceptual proteins have six putative transmembrane helices; the blocks of strong identity are separated by two helices in both 7F10 and CdsA. Unlike CdsA, 7F10 encodes two regions of hydrophilic amino acids that precede and follow the six helices; these regions encode 10 potential substrates for protein kinase A, protein kinase C, or casein kinase II.

29% amino acid identity, while statistically significant, could be due either to 7F10 being Drosophila's CdsA, or due to its being a paralog of CdsA with a divergent function. We have detected no Drosophila genomic DNA, other than that of 7F10 itself, that cross-hybridizes with 7F10 at low stringency. Thus, we suspect that 7F10 is, in fact, Drosophila CdsA. Elevated expression of this gene in the Drosophila retina could be required to maintain the retina's elaborate photosensitive microvilli.
We find that blots of adult head and body poly(A)+ RNAs show a 2.7 kb 7F10 RNA in both tissues, while all of 7F10 appears to reside in 5.5 kb of cloned genomic DNA. We are preparing to sequence 7F10's genomic DNA and to map the 7F10 promoter by RNA-polymerase chain reaction (PCR).

Our first approach to establishing functional homology has been to test 7F10 for CdsA+ activity in E. coli. Preliminary data indicate that 7F10 cannot complement the conditional lethality of cdsA- E. coli. This could be due either to the inability of E. coli to subsist on eukaryotic CdsA activity, or to the existence of a true Drosophila CdsA homolog, distinct from 7F10, that cross-hybridizes poorly with it. We intend to deal with both possibilities by using degenerate PCR to isolate genes from Drosophila and Saccharomyces containing the two blocks of strong identity between 7F10 and CdsA. This should allow us either to isolate a genuine, non-7F10 Drosophila CdsA, or to demonstrate that 7F10 can functionally replace the endogenous CDP-diglyceride synthetase of budding yeast.

Reference:

275. An antibody screen for genes regulating brain development
Claytus A. Davis, Seymour Benzer

Screening for regional expression patterns is one approach to isolating genes involved in regulating brain development. In one variation of this strategy, monoclonal antibodies are raised against whole tissue homogenates and screened by immunohistochemistry for those showing informative binding patterns. However, the subsequent isolation of the corresponding genes is complicated by the occurrence of antibodies whose binding is dependent on posttranslational modifications. To circumvent this difficulty, we will use, as antigen, pooled peptides encoded by a cDNA expression library. For monoclonal antibodies showing interesting patterns of binding, the corresponding cDNAs should be easily isolated from the library. This approach presents the problem that, in cDNA expression libraries, most of the cloned cDNAs are in the incorrect reading frame and will generate irrelevant peptides.

To address this, we have engineered a cDNA expression vector that enriches for cDNAs cloned in the correct reading frame. The vector contains the glutathione-binding region and the thrombin cleavage sequence of the pGEX-2T cloning vector (Smith and Johnson, 1988), which permits the easy isolation of large quantities of cDNA-encoded peptides. Immediately downstream of the cloning site, a poly-stop/start sequence followed by the Kanamycin-resistance coding sequence has been inserted. The vector is designed such that the Kan^r sequence will be translated only if the sequence immediately 5' to the stop/start region is translated, as would usually occur only if the cDNA were cloned in the correct reading frame. Initial tests suggest that the vector is functioning as designed and more definitive tests are under way prior to constructing the library.

Reference:

276. A novel nuclear protein expressed in adult Drosophila and human retina
J. Martin¹, M. Nawrot¹, A. Mohit¹, Seymour Benzer, Carol A. Miller

Monoclonal antibodies have identified immunochemical cross-reactions between cells in the human and Drosophila nervous systems (Miller and Benzer, 1983). Monoclonal antibody (MAb) 23E9 reacts with both the Drosophila and human retinae and with human brain. Electron microscopy localizes the protein to cell nuclei in both organisms. Western blots identify a 200 kDa band in Drosophila head homogenates and a 130 kDa band in preparations of cell nuclei isolated from human grey matter. A cDNA clone of 2840 bp has been isolated from a Drosophila expression library. It includes an open reading frame of 2585 bp encoding an 87 kDa protein. The nucleotide and deduced amino acid sequence reveal no similarities with GENBANK or NBRF protein databases, respectively. Northern blots show a 3.1 kb mRNA expressed in adult Drosophila head but not body. In situ hybridization with the cDNA probe confirms localization to the adult Drosophila retina with a distribution similar to the immunochemical pattern.

Using the same MAb, a cDNA clone of 1100 bp was isolated from a human retina expression library. It too
shows no similarity to sequences in the databases. However, the human clone has 27% amino acid sequence identity and 42% similarity to the Drosophila clone. Our data indicate the 23E9 antigen is a novel protein expressed only in the cell nuclei of the Drosophila retina and may have a human homolog. The nuclear localization of this protein in fly and human, together with the similarity in the sequences, suggest that it may serve similar functions in both organisms.

Reference:

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PUBLICATIONS


Summary: Despite extraordinary advances in molecular biology in recent years, the genetic, molecular and cellular mechanisms that carry out the choreography of embryogenesis remain a mystery. Perhaps the most remarkable of these developmental events is the formation of the brain and the nervous system. Almost half a century ago the Caltech neuroscientist Roger Sperry exploited the properties of the developing visual system to show that the axons of neurons born in the retina of young embryos appear to be individually programmed to seek their correct targets in the brain some distance away. The cells that form the retina are generated in a developmental pattern that creates a dish shape of retinal ganglion cells. More than one million such cells are formed, and as they are born they send out long processes tipped by ameboï­d-like growth cones. Somehow the genetic programs that generate a cell in a particular position in the retina also program the expression of specific cell-surface proteins and complex carbohydrates that are used by the growth cone as it seeks its correct address in the brain.

The primary focus of our research is to identify those genes and proteins responsible for encoding these neural addresses. We have developed special techniques for isolating and fractionating neural cell-surface molecules and preparing monoclonal antibodies (mAbs) directed against them. Since address molecules must be expressed in different patterns, we have selected several hundred mAbs that stain the developing embryonic nervous system in interesting patterns. The mAbs are then used to purify the individual proteins for protein chemical studies, to study the function of these proteins, and to screen...
expression libraries to clone the genes coding for molecules of particular interest (see Abstract No. 277).

Our first work in this field was a collaborative study with Melitta Schackner's group in Germany. David Teplow, then a postdoctoral fellow in this laboratory, focused his effort on a protein referred to as L1 that is expressed on essentially all axons that grow from the developing retina to the embryonic brain. The sequence of the cDNA coding for this protein is now published and shows that the molecule is made up of a number of different functional domains, each of which is believed to play a role in cell-cell interaction during embryogenesis of the brain (Moos et al., 1988). For example, there are six immunoglobulin-related domains. In addition, the L1 protein has three fibronectin repeating units, two RGD (arginine, glycine, aspartic acid) sequences, and complex carbohydrate side chains. Each of these is also implicated in cell-surface recognition processes in evolutionarily related proteins found in other systems. We examined our library to seek mAbs that react with proteins with properties similar to L1, and have already found 12 such mAbs that react in different patterns. We believe that these proteins may also represent part of the code used by growth cones to assemble the embryonic nervous system. The sequence of the first of these to be analyzed by us reveals a homology to L1, but it is expressed in a different pattern (see Abstract No. 278).

A second protein is expressed on only a subset of retinal axons both in the retina and in the optic nerve; hence it may help distinguish this subset of neurons during embryogenesis. Jost Vielmetter has cloned the gene that codes for this cell-surface protein and has obtained provocative partial sequence information (see Abstract No. 279).

Janet Roman has isolated a clone for a gene that codes for a second protein that is expressed on a subset of retinal ganglion cell axons, although sequencing has just been initiated (see Abstract No. 280).

It will be particularly interesting to determine whether these proteins are members of families of proteins expressed in different patterns in the developing nervous system. The database of known sequence information is now so large that it is usually possible to learn a great deal about possible functions for newly discovered proteins by comparison with evolutionarily-related molecules. We plan to supplement that type of information with appropriate biological assays.

This is a new project in the laboratory, although it has already begun to generate extremely interesting new information. It also seems likely that the methods used and the large library of mAbs can be used to detect many more proteins that play a role in coding the assembly of the nervous system.

Selected Publications:


277. The generation of monoclonal antibodies reactive with cell-surface molecules of the developing chicken retinotectal system

Jon Faiz Kayyem, Janet M. Roman, Uli Schwarz1, William J. Dreyer

Our approach to obtaining monoclonal antibodies (mAbs) against new and possibly scarce cell-surface molecules seeks to increase the relative abundance of rare cell-surface molecules in a particular immunogen fraction so that the immune response to more abundant proteins does not severely diminish or completely mask the response to rarer molecules. First, we purified cell-surface molecules using a biotin-avidin system. Selective biotinylation of only the surfaces of cells in intact tecta
allowed avidin-affinity purification of the very small fraction of protein molecules expressed on the cell-surface (or easily soluble from the extracellular matrix) during the period of time retinal axons are seeking their targets in the tectum.

Second, we further reduced the number of competing antigens in a particular fraction by separating these molecules on the basis of their molecular weights. By example of other well-studied systems like the immune system, it seems reasonable to assume that evolutionary pressures would have resulted in many cell-surface molecules involved in neurogenesis belonging to highly polymorphic families with some sequence and functional variability among the members. That such complex families exist among cell-surface molecules of the nervous system is supported by recent results showing that many of the proteins involved in cell-cell and cell-environment interactions during neural development are related to each other, either as members of the immunoglobulin superfamily or the integrin family. If large gene families of closely related cell-surface molecules exist in the developing retinotectal system, then we suspect our molecular weight fractionation would tend to group them together into the same or closely related fractions.

To increase the probability of inducing antibody production against rare and possibly more highly conserved molecules, we coupled these cell-surface, size-fractionated antigens to a small hapten and cross-linked them to large carrier proteins. Long-term immunization was designed to ensure selection for high affinity IgG antibodies.

Our preliminary results suggest that these methods were quite successful. Several thousand hybridomas were produced against cell-surface molecules, and nearly all supernates reacted with the developing chick nervous system. From the 120-150 kDa size range alone, over 1,000 hybridomas were generated. Many of these mAbs reacted in spatially restricted patterns, and these were organized into 14 distinct groups of "Pattern" mAbs. Perhaps suggestive of the power of this method, it is interesting to note that over 100 mAbs were generated against the well characterized neural cell-surface molecule G4/NgCAM, while few, or in some cases just one, mAbs were generated against other Pattern antigens. This suggests that without the extensive enrichment for rare cell-surface molecules, and removal of more abundant proteins, many of the Pattern antibodies we found might have gone undetected. Therefore, we believe these procedures should prove useful in other applications where families of related cell-surface molecules are thought to play important roles.

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278. "Bravo," a cell-surface antigen identified by our new method, is a novel immunoglobulin superfamily member
Jon Faiz Kayyem, David B. Teplow, Janet M. Roman

We have analyzed (to greater and lesser degrees) several of the antigens identified by our method. Remarkably, all of these molecules so far studied are members of the immunoglobulin (Ig) superfamily of cell-surface molecules. Some of these antigens have been previously described in chickens, such as N-CAM, Contactin and Neurofascin. In at least one other case, it appears that we have identified a cell-surface receptor similar to the lymphocyte antigen-related (LAR) tyrosine phosphatase of Drosophila and mouse (see Abstract No. 279).

In addition, we have identified and characterized to some degree two other Ig-superfamily members with likely roles in cell adhesion and pathfinding. The first, Alpha, turns out to be the previously identified G4 or NgCAM molecule. Recently sequenced, NgCAM is a membrane-bound cell-adhesion molecule that includes six Ig domains and five fibronectin type III repeats, displays the HNK-1 carbohydrate epitope (which is associated with neural cell adhesion molecules) and shares clear and strong sequence homology with the mouse molecule L1.

We have also identified, cloned, and sequenced a novel molecule, Bravo, which is closely related to NgCAM (same number of Ig domains, fibronectin repeats, HNK-1 epitope, transmembrane domain and highly conserved cytoplasmic region).

The close relationship of Bravo to known cell-adhesion molecules NgCAM and L1 strongly suggests that Bravo functions in promoting adhesion of axons carrying this molecule. But unlike NgCAM and L1 (which appear by most indications to be functionally very
similar proteins), Bravo is detectable on optic fibers only on their way to the optic tectum, and not on that part of the fibers that has reached the tectum (de la Rosa et al., 1990).

This topological restriction of a cell-adhesion molecule to the proximal and not the distal axon is a novel feature of Bravo and suggests functional roles which we are currently investigating. A particularly appealing hypothesis derived from the data is that Bravo promotes local adhesion of axons to each other and possibly to cellular or extracellular substrate while axons are leaving the retina for the tectum. As these axons reach the tectum, Bravo antigen is reduced, which reduces the affinity that optic fibers have for each other, and thus allows optic fibers to more readily interact with their guidance cues in the tectum. This hypothesis, while pure conjecture at this point, is consistent with the staining pattern of anti-Bravo antibodies and with the implications of Bravo's close relationship to L1 and NgCAM, both known to have homophilic adhesion properties. Furthermore, the reduction in Bravo staining on optic fibers coincides roughly with the reduction in fasciculation (bundling) of those same fibers, as would be expected from a molecule thought to be involved in promoting dense fasciculation of axons.

Analyses of Bravo's function, or (more likely) functions, are likely to take advantage of our ongoing collaboration with Uli Schwarz and his colleagues at the Max Planck Institute in Tübingen, Germany. The migration of a postdoctoral fellow (J.G.V.) from the MPI to our laboratory will more easily allow us to perform in vitro analyses of fiber/fiber and fiber/substrate interactions; this is expected to shed some light on Bravo's roles in development.

In addition, more molecular analyses of Bravo will be emphasized. For example, investigations of Bravo genomic sequences (two genomic clones have been identified) may reveal control regions involved in the hallmark disappearance of Bravo from the distal axon once fibers pass through the optic chiasm. Also, we have already identified three alternatively spliced forms of Bravo. The identification of still other alternative forms of Bravo and the distribution of these different forms to different regions in time and space is an area of study we hope will provide clues as to how the retinotectal system generates the enormously high degree of specificity for which it is famous.

Reference:

279. A retinal cell-surface protein unequally distributed on optic fibers
Jost G. Vielmietter, Jon Faiz Kayyem, Robert P. Lane, Janet M. Roman

The proper establishment of projections in the nervous system may be achieved by an address system of cell-surface proteins. These address molecules are expected to be distributed unevenly temporally and spatially. One candidate for such a molecule was detected with the monoclonal antibody 10-22A8 produced as described in Abstract No. 277. In the chick retina at embryonic stages E7 to E8, 10-22A8 stains the optic fibers of the nasal half of the retina much more intensely than the fibers of the temporal half. This might be due to the fact that the differentiation of the temporal retina is more advanced and therefore has already downregulated the expression of this molecule. Alternatively, the unequal distribution of this protein could reflect the unequal distribution of an address molecule marking nasal fibers differently than temporal fibers. Sequencing of a cDNA clone containing a portion of the gene coding for this protein has revealed that it has homologies to the fibronectin type III domains of LAR (leukocyte antigen related, human) and DLAR (neuro-specific, Drosophila), two members of the family of membrane-bound tyrosine phosphatases. Cloning and sequencing of the entire coding sequence will reveal whether this is in fact a novel member of the tyrosine phosphatase family and will help in generating epitope-specific antibodies to investigate the function of this molecule.

280. Attempts to clone the 'NAVY' pattern molecule
Janet M. Roman, Jon Faiz Kayyem

Using the monoclonal antibodies generated as described in Abstract No. 277, we have identified a molecule found on a highly restricted subset of retinal ganglion cell axons. This molecule, which we call NAVY
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(after its partial amino acid sequence), is restricted to a specific bundle of fibers connecting the retina and tectum.

NAVY has an apparent relative molecular weight of 100 kDa, and the sequence of the first 38 amino acids has been determined. This sequence is unrelated to anything currently in the sequence data banks. Using this sequence, we have constructed four non-overlapping degenerate probes containing small percentages of inosines. Using the polymerase chain reaction (PCR) procedure, we are searching cDNA libraries constructed from chick retinal RNA populations. Initial experiments produced multiple PCR bands, and after further PCR analyses, these will eventually be cloned and sequenced and used as probes to extract the appropriate gene from the libraries.

281. Differentiation of embryonal carcinoma cells in culture
Frank Miskevich, Robert P. Lane

We have recently begun research into the differentiation of P-19 and NT2/D1 embryonal carcinoma cells after addition of retinoic acid to these cells in culture. We wish to elucidate some of the molecular mechanisms involved in this process. We are examining the expression of RAG-1 and RAG-2 in the induced P-19 culture system as recently reported by Chun et al. (1991) in relation to the sequential activation of the HOX 2 homeobox cluster in NT2/D1 cells after induction (Simeone et al., 1990). Our goal is to repeat the results and to determine whether these events are linked in any way, either to each other or to the process of neuronal differentiation in general.

References:

Summary: The Kennedy lab studies molecular mechanisms of synaptic regulation in the central nervous system. Regulation of synaptic efficacy is important for information processing and storage in the brain. For example, 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. Our understanding of the signal transduction mechanisms that are important for synaptic regulation is still far from complete. Neurons communicate with each other primarily through chemical synapses that transmit information by releasing neurotransmitters that open ion channels and alter the electrical potential across the membranes of their target neurons. Many of these transmitters also initiate biochemical changes in the signaling machinery of postsynaptic cells. We are studying the molecular organization of effector systems that produce these biochemical changes.

In one major project, we are studying a calcium-dependent protein kinase (type II CaM kinase) that is at the center of an important neural protein phosphorylation pathway. Recent studies in our lab and others suggest that this protein kinase may play an important role in producing changes in synaptic strength that underlie memory formation in the mammalian brain (Kennedy, 1989). In the past year, we have used organotypic hippocampal cultures to study regulation of type II CaM kinase in situ. We have also mapped and characterized a gene

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encoding a type II CaM kinase in *Drosophila melanogaster* in preparation for genetic studies.

In a second major project, we are studying several protein components of a prominent synaptic organelle in the central nervous system called the postsynaptic density. This organelle is rich in type II CaM kinase and seems likely to be an important site at which the CaM kinase regulates synaptic transmission. An understanding of the molecular structure of this organelle is necessary to understand its function, as well as the postsynaptic functions of the CaM kinase.

**Reference:**

### 282. Regulation of CaM kinase activity by autophosphorylation in hippocampal neurons

**Sean S. Molloy**

To learn about the functions of type II CaM kinase *in situ*, we have studied regulation of the kinase in intact hippocampal neurons. Previous annual reports described the roller tube cultures of rat hippocampal slices that we have used for these studies. We found that approximately one-third of the kinase in the hippocampal neurons is autophosphorylated and in a Ca\(^{2+}\)-independent state even when the cultures are electrically inactive. Pharmacological agents known to block synaptic activity in hippocampal neurons had no effect on the resting level of Ca\(^{2+}\)-independent CaM kinase in the cultures. Furthermore, measurements by Drs. Wade Regehr and Dave Tank with FURA-2 showed that the cultured hippocampal neurons have normal low concentrations of free Ca\(^{2+}\) under resting conditions (31 ± 2 nM). We found that homogenates of adult hippocampus also had substantial levels of autophosphorylated CaM kinase.

Our recent experiments suggest that the high level of Ca\(^{2+}\)-independent kinase at basal Ca\(^{2+}\) concentrations reflects a balance between continued Ca\(^{2+}\)-dependent autophosphorylation and dephosphorylation by endogenous phosphatases. For example, the proportion of kinase in the Ca\(^{2+}\)-independent state is reduced by exposure to low external Ca\(^{2+}\) concentrations. To establish a time course for this reduction, cultures were transferred to salt solution containing 200 mM EGTA for various times, then homogenized and assayed for Ca\(^{2+}\)-independent CaM kinase activity. The removal of external Ca\(^{2+}\) produced an 80-90% decrease in the resting level of Ca\(^{2+}\)-independent kinase within 30 minutes (t\(_{1/2}\) of 5-7 minutes). In addition, application of the membrane permanent kinase inhibitors H7 and W7, which block ATP and calmodulin binding, respectively, produced substantial reductions in the proportion of Ca\(^{2+}\)-independent kinase. Conversely, exposure to okadaic acid, a specific inhibitor of phosphatases 1 and 2A, resulted in an increase in the level of Ca\(^{2+}\)-independent activity. These findings indicate that continuous Ca\(^{2+}\)-dependent autophosphorylation is required to maintain the high resting level of Ca\(^{2+}\)-independent kinase. They also show that the proportion of kinase in the Ca\(^{2+}\)-independent state reflects a dynamic steady state between the rates of autophosphorylation and dephosphorylation.

Together, these observations suggest that the autophosphorylation mechanism acts to maintain a relatively high level of constitutively active CaM kinase at basal Ca\(^{2+}\) concentrations. This finding is in contrast to previous predictions about CaM kinase regulation in hippocampal neurons and may help clarify the function of the kinase in these cells.

### 283. Antibodies specific for the autophosphorylated type II CaM kinase

**Bruce L. Patton**

The activity of the type II CaM kinase is regulated by autophosphorylation of a specific threonine residue, which generates a Ca\(^{2+}\)/calmodulin-independent activity *in vitro*. Recently, we showed that about 30% of the kinase is autophosphorylated *in situ* in organotypically cultured neurons (Molloy and Kennedy, 1991). To study the distribution and level of autophosphorylation at the cellular and subcellular levels, we have produced antibodies that bind specifically to the autophosphorylated CaM kinase subunits.

Monoclonal antibodies specific for phosphorylated kinase were produced by immunization with thiophosphorylated synthetic peptides corresponding in sequence to the autophosphorylation site at threonine-286 on the \(\alpha\) subunit of the rat CaM kinase. Peptides were enzymatically thiophosphorylated by purified CaM kinase with ATP\(_7\)S as substrate. Peptides were thiophosphorylated, rather than phosphorylated, since thio-
phosphorylated peptides are resistant to dephosphorylation by protein phosphatases. Thiophosphorylated peptides were purified from nonphosphorylated peptides by reverse phase HPLC, and then coupled to keyhole limpet hemocyanin for use as immunogen. Mice were immunized by repeated injection with a mixture of free and conjugated peptide. Hybridomas were produced and culture medium was screened by ELISA against nonphosphorylated and autophosphorylated CaM kinase. The screen yielded 24 monoclonal antibodies that bound more than tenfold more avidly to the autophosphorylated CaM kinase than to the nonphosphorylated kinase. This represents 2% of the total number of hybridomas tested. Further characterization demonstrated that one monoclonal antibody, 22B1, was highly specific for phosphorylated CaM kinase on Western blots of total rat brain homogenate. A kinetic correlation between antibody binding and autophosphorylation of threonine-286 indicated that the primary epitope recognized by 22B1 contains this phosphorylated residue. 22B1 also reacts with autophosphorylated type II CaM kinase in fixed sections of rat brain.

A monoclonal antibody that binds specifically to the autophosphorylated β subunit of the rat type II CaM kinase was produced by a second immunization method, in which intact autothiophosphorylated kinase was the immunogen. To enhance the phosphokinase-specific response, the immune response to nonphosphorylated kinase was suppressed with cyclophosphamide before immunization with thiophosphorylated kinase (Matthew and Patterson, 1983; Patton et al., 1991). Hybridomas produced from two immunized mice were screened by ELISA as described above. Only one clone secreted antibodies that were specific for the autophosphorylated CaM kinase. Twenty-three clones produced antibodies with greater than threefold selectivity for the autophosphorylated kinase (Patton et al., 1991).

In this study, immunization with thiophosphorylated peptides generated a more robust phosphoprotein-specific immune response than the immunosuppression method. Thus, thiophosphorylated peptides may be more generally useful in preparing antibodies specific for phosphorylated proteins. We are now interested in using the phosphokinase-specific antibodies to study the role of CaM kinase autophosphorylation in neuronal function.

References:

284. Antibodies specific for nonphosphorylated type II CaM kinase

Bruce L. Patton

We produced monoclonal antibodies that bind specifically to the autophosphorylated type II CaM kinase in order to study CaM kinase autophosphorylation at the cellular and subcellular level. (see Abstract No. 283). We have also produced polyclonal antisera specific for nonphosphorylated CaM kinase by immunization with nonphosphorylated peptide. The use of antibodies specific for nonphosphorylated kinase, together with those specific for the phosphorylated kinase, will allow sensitive detection of variable levels of kinase autophosphorylation. A change in kinase phosphorylation will produce an increase in binding of one antibody and a decrease in binding of the other. A change in the ratio of binding can be measured more sensitively than a change in the binding of either antibody alone.

Polyclonal antibodies specific for nonphosphorylated kinase were produced by immunization with nonphosphorylated synthetic peptides corresponding in sequence to the threonine-286 autophosphorylation site on the α subunit of the rat CaM kinase. Peptides were purified by reverse phase HPLC and coupled to keyhole limpet hemocyanin for use as immunogen. Rabbits were immunized by repeated injection with a mixture of free and conjugated peptide. Of four immunized rabbits, one produced antibodies that specifically recognized purified, nonphosphorylated type II CaM kinase on Western blots. Autophosphorylation of the CaM kinase for 5 seconds blocked antibody binding to the CaM kinase, indicating that the polyclonal antibodies bound primarily to the threonine-286 autophosphorylation site. Because antiserum from this rabbit cross-reacted with several other rat brain homogenate proteins, the antibodies specific for the type II CaM kinase were purified by affinity chromatography on a peptide-conjugated resin.
285. Characterization of the CaM kinase II a subunit in Drosophila melanogaster

Kyung-Ok Cho, Shelley A. Mueller

Type II CaM kinase is a key enzyme involved in a major neural protein phosphorylation pathway. Biochemical mechanisms for the regulation of type II CaM kinase activity and the structure of the rat brain enzyme have been extensively studied in recent years. However, little is known about the biological functions of the kinase. To study its biological functions, we chose to examine the CaM kinase in Drosophila melanogaster in order to exploit the possibility for genetic analysis.

We have isolated fly cDNAs using a probe generated by the polymerase chain reaction (PCR) method. The sequence obtained from one cDNA contained a 1470 bp open reading frame encoding a 490 amino acid protein of molecular weight 55,419. The overall identity at the amino acid level between the fly subunit and the rat subunits is 77%. The CaM binding site and adjacent inhibitory domain are nearly identical in the two species, and the two autophosphorylation sites important for the regulation of kinase activity are also conserved in the fly subunit. The domain structure of the deduced amino acid sequence most resembles the rat a subunit. Furthermore, the mRNA of the fly gene is expressed at much higher levels in the head than in the body. This resembles the expression pattern of the rat a subunit which is neuron-specific. Therefore, we conclude that the cDNA encodes a fly a subunit of CaM kinase.

We know from previous biochemical studies of the fly kinase that at least three CaM kinase subunits are present. Thus, as in rat, there may be other Drosophila genes encoding additional CaM kinase subunits. It is also possible that the other fly subunits are encoded by alternatively spliced products of the gene encoding the fly a subunit. The gene encoding the a cDNA is located at the boundary between 102E and 102F on the fourth chromosome. We are currently trying to obtain a fly mutant defective in this gene to study the biological functions of the CaM kinase.

286. Expression of CaM kinase II mRNA in Drosophila melanogaster

Masamichi Ito

To study the level of CaM kinase II mRNA during development of Drosophila, primer extension analysis was performed with total RNAs isolated from various developmental stages. An end-labeled primer that specifically hybridized to the 5'-noncoding region of Drosophila CaM kinase a-subunit mRNA was used as a probe. The experiment showed that CaM kinase a-subunit mRNA is not expressed until later stages of embryogenesis. Its expression increases dramatically at the larval stage, and then decreases at the pupal stage and remains constant in the adult.

To localize the CaM kinase II message during development in Drosophila, in situ hybridization was performed with whole mount embryos and a digoxigenin-labeled, random-primed probe that was synthesized from the entire 2.7 kb Drosophila CaM kinase II a-subunit cDNA. It showed that the a-subunit mRNA is only expressed in mature central nervous system neurons of late stage embryos. The mRNA is not detected in undifferentiated neuroblasts or mature peripheral nervous system neurons. In addition, in situ hybridization in cryostat-sections of adult head showed that all neurons in the brain express CaM kinase II mRNA including the mushroom bodies and optic lobes. However, the message is not detected in the retina.

The control of CaM kinase a-subunit expression in the fly is remarkably similar to its control in the rat where its expression is confined to neurons, and high levels of expression begin 10 days to two weeks after birth.

287. Monoclonal antibodies against postsynaptic density proteins

Michelle Apperson, Kyung-Ok Cho

We can use monoclonal antibodies to study the molecular composition of the postsynaptic density (PSD) proteins by following the subcellular distribution of PSD-associated antigens. Antibodies were raised in two mice by injecting with either heat-denatured or native PSD fractions. Heat denaturation may have exposed some hidden epitopes since the two sera reacted with different sets of PSD proteins monitored by Western blots. With the help of the Biology Division's Monoclonal Antibody Facility, over 1,000 hybridomas prepared from these mice were screened using Western blots. Several ascites were made from clones that reacted strongly with single PSD proteins.
We have used these monoclonal antibodies to follow specific antigens through the steps in PSD purification. Equal amounts of forebrain homogenate, synaptosomes and PSD fractions were prepared for Western blotting. Five monoclonal antibodies specifically reacted with antigens only in the PSD fraction. 2A7 recognized two bands of apparent molecular weight 95K in the PSD fraction and also reacted with the protein expressed from the cDNA encoding this PSD protein (see Abstract No. 288). 3H2 reacted with a different band of about 93 kDa and did not react with the peptide expressed from the cDNA for the 95K protein. 2D8 recognized a 195 kDa antigen which was also concentrated in the PSD fraction. Two different antibodies, 1A1 and 1C1, both had strong reactions with a 350 kDa protein in the PSD. Using a mixture of these two antibodies, it was possible to immunoprecipitate the 350 kDa protein from solubilized fractions of PSD. This protein was visualized with Coomassie stain and appears to be phosphorylated by endogenous Type II CaM kinase in the PSD.

The PSD proteins recognized by these monoclonal antibodies were differentially solubilized upon detergent extraction. After 1% n-octylglucoside or 1% CHAPS treatment, the 2A7, 3H2 and 2D8 antigens were not solubilized. The 350 kDa protein recognized by 1A1 and 1C1 ascites was solubilized by CHAPS but not by n-octylglucoside. All of these proteins were completely solubilized in 3% n-lauroyl sarcosinate and partially solubilized in 4 M urea. The monoclonal antibodies and the differential solubilities can be used to follow PSD antigens through purification steps.

We hope to use these antibodies for further characterization of their antigens, including immuno-cytochemistry, to determine their distributions in the brain. We may also use the monoclonal antibodies to clone the genes for these PSD proteins by affinity purifying antigens for protein sequencing or by screening expression libraries for genes encoding the less abundant PSD proteins.

288. Molecular characterization of an 80 kDa protein in the postsynaptic density fraction from rat brain

Kyung-Ok Cho

The postsynaptic density (PSD) is a specialized submembranous cytoskeleton that adheres to the postsynaptic membrane, opposite presynaptic terminals. It has been postulated to have a role in regulating postsynaptic neurotransmission, but its precise function is unknown. Biochemical studies have identified several proteins that associate with PSDs, including two subunits of type II CaM kinase, calmodulin, and cytoskeletal proteins such as tubulin, actin, and fodrin. However, there are at least 20 unknown proteins present in PSD fractions. Identification of these unknown proteins will be important for understanding the functions of PSDs during synaptic transmission.

One major protein in PSD fractions prepared by the established biochemical method has an apparent molecular weight of 95K. It appears to be a core component of PSDs because it remains associated with the PSD structure after several detergent extractions. We have obtained the sequences of several tryptic peptides from the 95 kDa protein. This sequence information was used to generate oligonucleotide probes and subsequently to obtain cDNA clones encoding the protein. The cDNA clones encode a protein of 724 amino acids with a calculated molecular weight of about 80K. Northern blot analysis suggests that the mRNA for the protein is specifically expressed in rat brain but not in other tissues of rat.

We expressed this cDNA in Escherichia coli with the T7 expression system. The expressed protein co-migrates with the PSD protein with an apparent molecular weight of 95K. It was used to generate rabbit antisera that recognize the PSD protein. Currently, we are using the antibodies to localize the protein in rat brain by immuno-cytochemical methods.

PUBLICATIONS


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Summary: We are interested in the molecular basis of membrane excitability. The twin tactics of a) cDNA cloning and b) heterologous expression allow one to make hypotheses about structure, function, and other aspects of the proteins that underlie membrane excitability; to express these proteins in foreign cells; and to test these hypotheses with the appropriate functional measurements such as electrophysiology, ligand binding, and second messenger biochemistry.

Over the past year, our group has continued to employ cDNA cloning, heterologous expression, and site-directed mutagenesis in our studies on nicotinic acetylcholine receptors, 7-helix receptors coupled to G proteins in collaboration with both the Simon and Zinn groups, and electrically excitable channels for sodium, calcium, and potassium ions, and Na-coupled transporters. We are exploiting the newly developed vaccinia virus system, which permits the expression of ion channels and receptors in almost any mammalian cell with high efficiency, both in terms of percentage of cells expressing and of expression per cell. These powerful new techniques have allowed us to construct an excitable cell, starting from one that has no channels at all.

Structure-function studies have now progressed to the point where we think that we understand ion flux through the acetylcholine receptor channel: permeating ions interact with charges and with hydroxyl side groups in the M2 helices. Studies in other laboratories are yielding clear
The data about the so-called "inactivation gates" of voltage-dependent channels: these seem to be flaps on the internal surface. Major unsolved questions now concern the mechanisms of activation for ligand-gated, voltage-gated and G protein-gated channels. These will be active areas of research for us.

We have begun several new projects in the past year. First, it is time to obtain three-dimensional structures for these channels. The vaccinia overexpression system is being investigated for this purpose. Second, we envision applying these expression techniques to the brain itself. We are developing methods to modify the signal-processing functions of brain cells by introducing new channels and receptors.

Jerome Pine, Professor of Physics, is also associated with the group. He and his coworkers are primarily interested in studies of cultured neural networks. The development and application of techniques for noninvasively determining the synaptic connectivity of small networks is the major focus of their work. By using such techniques, it is hoped that patterns of connectivity which evolve in developing networks will be revealed. Furthermore, by selective long-term stimulation, the effects of activity on synaptic connectivity will be studied. The technology involves both electrical and optical extracellular recordings and the development of a family of microdevices for two-way electrical communication with individual neurons. One outgrowth of this work is a project to develop a "neuroprobe," a communication link to a host central nervous system based on cultured neurons captive in a silicon structure. A second effort, related to the study of synapses in cultured networks, is the imaging of whole cells at suboptical resolution with a newly developed scanning transmission x-ray microscope.

289. Ion-selectivity of mutated and wild-type nicotinic ACh channels
Bruce N. Cohen, Cesar Labarca

We investigated the effects of site-directed mutations in the MII region and changes in subunit composition on the ion selectivity of nicotinic acetylcholine receptor (nAChR) channels cloned from BC3H-1 cells (mouse) and expressed in Xenopus oocytes. MII was defined as the 19-residue segment beginning with α:M243, β:M254, γ:C252, or δ:T257 (position 1). Oocytes were injected with 2-19 ng of mRNA per subunit and ACh-induced currents were recorded 2-5 days later using a two-electrode voltage clamp. The permeability of the expressed nAChRs to a variety of monovalent cations relative to Na⁺ was estimated from the shift in the bi-ionic reversal of the ACh-induced current after substituting the cation of interest for Na⁺. Wild-type (WT) mouse nAChRs expressed heterologously in oocytes were relatively nonselective among the monovalent metal cations, Na⁺, Li⁺, K⁺ and Cs⁺, as are nAChRs at the frog neuromuscular junction, but were significantly more permeant to a number of large organic cations than frog nAChRs. None of the mutations had a significant effect on the relative permeabilities of the monovalent metal cations except the position 2, α:T to A and δ:S to A (α:T2A and δ:S2A), hybrid which reduced the relative Cs⁺ permeability by 19%. However, several had dramatic effects on the relative permeabilities of the nAChR to organic cations. Deletion of the mRNA for the δ subunit from the injection mixture reduced relative TRIS⁺ permeability by 86%. δ-less channels are thought to have two γ subunits instead of one γ and one δ subunit. One notable difference between γ and δ in MII is the substitution of an uncharged glutamine for a negatively-charged glutamate at position -1. The position 2 mutant hybrids, (1) δ:S2F, (2) α:T2A and δ:S2A, and (3) α:T2A, β:G2S, and δ:S2A, reduced the relative permeability to TRIS⁺ by 58-67%. The relative permeability of δ:S2F to several other large organic cations was also significantly smaller than the WT. Deletion of the γ subunit from the injection mixture, substitution for ε for γ, and mutations at other positions in MII had no significant effect on the relative permeability to TRIS⁺. The results support the hypothesis that MII lines the ion pore of nAChR and are consistent with models of the nAChR which suggest that position -1 is the narrowest portion of the channel pore.

290. Ligand binding and gating of the neuronal nAChR
Daniel Vaughn, Pamela J. Bjorkman

The nicotinic acetylcholine receptor, nAChR, is located in the postsynaptic membrane at synapses. Its function is to bind the neurotransmitter acetylcholine and upon this binding to form a pore through the membrane permeable to most atomic cations. One recently cloned
variety, the α7 of chick, forms homomeric receptors that bind α-bungatoxin with high affinity. Recently, great strides have been made towards understanding the cation permeation pathway formed by the receptor. On the other hand, very little is known about transmitter binding or gating (how the receptor couples the acetylcholine binding signal to an opening pore). It is thought that ligand binding and the initial phase(s) of gating are localized in the extracellular portion of the protein formed by the N terminal ~200 amino acids.

The strategy for expression of this protein is given in Abstract No. 209. In addition to the crystallization trials, biochemical studies will be carried out on the expressed protein. Of particular interest are competitive binding experiments to study the relative affinities of a number of competitive agonists and antagonists. Also of interest is the role of glycosylation in agonist/antagonist binding.

291. Heterologous expression of the neuronal acetylcholine receptors in mammalian cells
Antonio Figl, Cesar Labarca

The acetylcholine receptor is the most extensively studied ligand-gated channel. Unfortunately, it still remains poorly understood at the level of molecular structure. Crystals of this protein have not diffracted at high enough resolution. We hope that this project will in some way bridge this structural gap. To overproduce the receptor we shall employ a vaccinia virus system, which is rather effective for the expression of foreign genes. The development of the eclectic plasmid pTM1, with sequences from picornaviruses (the encephalomyocarditis virus), bacteriophages (T7) and orthopoxviruses (the group to which vaccinia belongs) may enable the use of vaccinia as a protein-producing factory. This system has been employed in the production of the intracellular chloramphenicol acetyltransferase gene where it comprised 10% of the total cell protein. More recently, this approach has been extended to membrane proteins such as the cystic fibrosis transmembrane regulator.

We are employing vaccinia in the overproduction of the neuronal acetylcholine receptors cloned in the last few years. These include the α2, α3, α4 and β2 subunits. In addition, we are extending our methodology to include the newly cloned α7 subunit, which possesses the unique features of forming acetylcholine-gated channels without the need of further subunit. The ultimate goal of the project is to test whether the vaccinia overexpression will provide sufficient protein needed for purification studies. It is our hope that the amount of pure receptors will be high enough to invest time in a crystallization effort. Structural studies will be carried out in collaboration with other groups at Caltech.

292. Does a flap in the nAChR α subunit block ion access to the channel?
Stefan I. McDonough, Bruce N. Cohen

We will investigate whether the opening of the muscle nicotinic acetylcholine receptor (nAChR) is due to the motion of a portion of the α subunit which acts as a flap, that is, whether the protein has a polypeptide sequence that normally physically blocks ion access to the channel and that moves aside like a hinge to allow cations to permeate the channel in response to agonist binding. Such a conformational change has been found in other proteins by structural studies and computer modeling.

We will apply different segments of the α subunit (obtained from B. Conti-Tronconi) to BC3H-1 cells which have nAChRs. With the whole-cell patch clamp technique, we will look for peptides that block the response more strongly than others. If a particular polypeptide sequence does block ACh response strongly, it will indicate that this sequence could be a flap that moved aside in response to ACh binding and that blocked the channel when perfused onto the open channel again. Zagotta et al. (1990) used this technique successfully to study inactivation of the Shaker potassium channel. We studied the blocking effects of d-tubocurarine as a test of the experimental system. As controls, we found that polylysine, polyarginine, lysine and arginine blocked the response, and that serine, glutamate and pyroglutamate did not. If further investigation with the α-subunit peptides is warranted, we will test for competitive and voltage-dependent effects at the whole-cell and single-channel level. We will also test nAChRs expressed through mRNA injection in Xenopus oocytes.

Reference:
293. Modulation of a cloned potassium channel

Jeffrey H. Hoger, Alfred E. Walter, Dolly Vance1, Lei Yu2

Modulation of ion channels is an important mechanism for regulating neuronal excitability. We studied the mechanism of one type of modulation for a cloned voltage-dependent K+ channel expressed in oocytes. When the K+ channel was coexpressed with a cloned mouse brain serotonin receptor (5HT1C) in oocytes, activation of the 5HT1C receptor by a brief application of serotonin resulted in a suppression of the I<sub>K</sub> amplitude over the next 20 min. I<sub>K</sub> could also be suppressed by activation of G proteins. Suppression was also caused by intracellular Ca<sup>2+</sup> injections and was blocked by intracellular injection of EGTA. Calmodulin antagonists block the I<sub>K</sub> suppression, but a known protein kinase inhibitor did not block suppression. The 5HT1C suppression was reversible; recovery from suppression was blocked by the protein kinase inhibitor H-7. These data suggest that the I<sub>K</sub> suppression occurs through a novel mechanism independent of A- or C-type protein kinases; suppression is best explained as being due to the action of a Ca<sup>2+</sup>/calmodulin-activated phosphatase; recovery from suppression is due to the action of a protein kinase.

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294. Cell-specific posttranslational events affect functional expression at the plasma membrane but not tetrodotoxin sensitivity of the rat brain IIA sodium channel α-subunit expressed in mammalian cells

Xian-cheng Yang, Cesar Labarca, Joel Nargeot1
Begonia Y. Ho, Orna Elroy-Stein2, Bernard Moss2

The rat brain IIA Na+ channel α-subunit was expressed and studied in mammalian cells. Cells were infected with a recombinant vaccinia virus (VV) carrying the bacteriophage T7 RNA polymerase gene and were transfected with cDNA encoding the IIA Na+ channel α-subunit under control of a T7 promoter. Whole-cell patch-clamp recording showed that functional IIA channels were expressed efficiently (~10 channels/µm<sup>2</sup> in ~60% of cells) in Chinese hamster ovary (CHO) cells and in neonatal rat ventricular myocytes, but were expressed poorly in undifferentiated BC3H1 cells and failed to express in Ltk- cells. However, voltage-dependent Drosophila Shaker H4 K+ channels and Escherichia coli β-galactosidase were expressed efficiently in all four cell types with VV vectors. Because RNA synthesis probably occurs without major differences in the cytoplasm of all infected cell types under the control of the T7 promoter and T7 polymerase, we conclude that cell type-specific expression of the Na+ channel probably reflects differences at posttranslational steps. The gating properties of the IIA Na+ currents expressed in cardiac myocytes differed from those expressed in CHO cells; most noticeably, the IIA Na+ currents displayed more rapid microscopic inactivation when expressed in cardiac myocytes. These differences also suggest cell-specific posttranslational modifications. IIA channels were blocked by ~90% by 90 nM tetrodotoxin (TTX) when expressed either in CHO cells or in cardiac myocytes; the latter also continued to display endogenous TTX-resistant Na+ currents. Therefore, the TTX binding site of the channel is not affected by cell-specific modifications and is encoded by the primary amino acid sequence.

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295. Why build artificial neurons?

Hsiaolan Hsu, Xian-cheng Yang, Andreas Karschin, Cesar Labarca, Antonio Figl

The electrical and chemical excitability of neurons is controlled by a rich variety of membrane proteins including ion channels activated by changes in potential, channels activated by extracellular and intracellular ligands, 7-helix receptors coupled to G proteins, and transporters. As a result, neurons generate a series of nerve impulses (action potentials) whose temporal pattern reflects various stimuli and modulatory influences. At present, two major approaches are available to analyze the contribution of a particular channel, receptor, or transporter to impulse firing patterns: 1) pharmacological activation and elimination, and 2) numerical modelling based on channel gating kinetics. We have recently added an additional approach based on heterologous expression of foreign excitability proteins. We constructed simple artificial neurons by co-expressing two voltage-gated channels, the rat brain IIA Na channel and the Shaker H4/ShB. As a first step, we verified two predictions: 1) that neuron-like action potentials could be produced by
expressing only these two channel types in a cell that otherwise expressed no voltage-dependent channels, and 2) that a transient K current could produce repetitive firing.

The surprising result was that repetitive firing frequencies ranged between 1 and 40 Hz in an individual cell (roughly linear with applied current). The simple artificial neurons thus display one hallmark of a real neuron: the ability to encode inputs by varying the impulse frequency over a wide range in response to sustained inputs. As we were conducting these experiments, other laboratories were reporting that Shaker ShB/H4 actually displays at least two inactivation time constants (−10 ms and −1 s) when expressed in oocytes. This observation explains in qualitative terms how a cell with H4 as its dominant or only K+ channel should fire repetitively over a wide frequency range. We are now pursuing quantitative simulations. We contend that extensions of this approach will produce more surprises, aid in hypothesis testing and, incidentally, enable the construction of entirely new classes of excitable cells.

296. Expression of the serotonin 1A receptor (5-HT1AR) with a vaccinia virus system in mammalian cells
Begonia Y. Ho, Andreas Karschin

Expression of the human 5-HT1AR was achieved with a vaccinia virus (VV) system using the highly efficient bacteriophage T7 RNA polymerase for transcription. The T7 RNA polymerase promoter and the 5-HT1AR gene were inserted into vaccinia virus by homologous recombination to form a recombinant VV (VV:5-HT1A). When monkey kidney BSC-40 cells, COS-7 cells, or rat pituitary tumor GH3 cells were co-infected with VV-5-HT1A and a helper VV carrying the T7 RNA polymerase gene, expression of the 5-HT1AR was detected with 12 hr postinfection when assayed with receptor binding to [3H]serotonin and [3H]8-OH-DPAT, a highly-selective 5-HT1AR agonist. Expression peaked at about 24 hr and declined after 72 hr. The Kd values for both ligands were approximately 1 nM which is consistent with reported values for brain 5-HT1AR. Approximately 3 pmol/mg protein 5-HT1AR was expressed, corresponding to 3 x 10^5 receptors/cell or 60 receptors/µM^2. Expression of the 5-HT1AR (approximately 1 pmol/mg protein) was also observed when COS-7 cells were infected with the helper VV and transfected with plasmids containing the T7 RNA polymerase promoter and the 5-HT1AR gene. The activation of GTPase by serotonin and 8-OH-DPAT in co-infected COS-7 cells further supports the expression of 5-HT1AR, and suggests a functional coupling of the expressed receptor of endogenous G proteins.

297. Analysis of G protein-gated K+ currents activated by 7-helix receptors
Andreas Karschin, Begonia Y. Ho

We have described vaccinia virus-mediated high-efficiency expression of human 5-HT1A receptors in primary cultures of neonatal rat atrial cells. The expressed receptors coupled to endogenous G protein-gated K+ channels normally activated by mACh receptors (Karschin et al., 1990). Here we report about the kinetic analysis of the currents activated by ACh and 5-HT and suggest a model for direct K+ channel activation by G protein subunits.

The inwardly rectifying K+ current I_K[ACh] evoked by ACh (5 µM) activated with a time constant of \( \tau_{act} = 317 \pm 12 \text{ ms (mean ± SEM; } n = 32) \) and deactivated with \( \tau_{deact} = 965 \pm 75 \text{ ms ( } n = 23 \) ) upon removal of the agonist. Responses mediated by the expressed 5-HT1ARs (5 µM 5-HT) showed similar deactivation with \( \tau_{deact} = 924 \pm 39 \text{ ms ( } n = 16 \) ), but slower activation time constants \( \tau_{act} = 1682 \pm 180 \text{ ms ( } n = 12 \) ) and smaller current amplitudes. Internal perfusion with GTPyS, performed to investigate this difference, caused the persistent activation of I_K[ACh] within several minutes. In addition, GTPyS stimulated an increase in the rate of current activation and a decrease in deactivation rate, and, especially for 5-HT, an increase in current amplitude. Thus, irreversible G protein activation by GTPyS enhances the efficiency of transduction by a subsequent pulse of agonist, possibly changing the number or nature of rate-limiting steps for activation/deactivation. These effects could be interpreted in a straightforward fashion if the channel was much more likely to be activated by several G protein α subunits than by a single one.

Reference:
298. Analysis of receptor and effector specificities of Gα proteins in Xenopus oocytes

Yasuhito Uezono, Jonathan Bradley, Kai Zinn

G proteins, composed of α, β and γ subunits, are transducers that couple membrane receptors to effector proteins. The study of the mechanism of G protein function has been directed toward defining the functional domains of the Gα subunit because the Gα subunit determines the specificity of the interaction with receptors and effector proteins.

We wish to identify the epitopes in a Gα subunit that determine its receptor and effector specificities. To investigate this, in collaboration with Kai Zinn, we are making cDNAs for a set of chimeric molecules containing segments of the rat Gαo subunit joined to segments of either Gαx or Gαolf (the olfactory Gαx-like subunit). These subunits are expressed in oocytes and tested for activation by 5-HT1c receptors (5HT-1CR) and β-adrenergic receptors (βAdR), which normally activate the phospholipase C (PLC) pathway via Gαo and adenylyl cyclase via Gαx, respectively. The increase in PLC activity is assayed electrophysiologically by measuring Ca2+-activated Cl− current; PLC activates IP3 formation which in turn releases Ca2+ from intracellular stores. Adenylyl cyclase activity is assayed by measuring the cAMP content in oocytes.

We have found that coinjection of rat Gαo transcript with 5HT1c transcript into oocytes increases the Cl− current response to 5HT over that for oocytes injected with 5HT1cR alone. Similarly, coinjection of rat Gαx transcript and βAdR transcript causes an increase in cAMP response to isoproterenol over that for oocytes injected with βAdR alone.

To date, we have made and assayed a number of different chimeras. According to Masters et al. (1988), the C-terminal 40% of Gαx subunit contains the structural element required for specific interaction with receptor and effector. In making chimeras, therefore, we focused on the C-terminal region. In one set of experiments, we made three Gαo/Gαolf chimeras: Gαo(326)/Gαolf(28), Gαo/Gαolf(69) and Gαo/Gαolf(125). (The notation Gαk(m)/Gαj(n) or Gαk/Gαj(n) means a chimera containing m N-terminal amino acids of Gαk joined to n C-terminal amino acids of Gαj.) The first of these behaved like Gαo in its Cl− current response to the 5HT1cR; the other two produced much less Cl− current increase than Gαo. Two other chimeras, Gαx/Gαo(147) and Gαo/Gαolf(121)/Gαolf(28), also behaved like Gαo. These data argue that the receptor and effector activities of Gαo are encoded by sequences between amino acid 206 and 326. In other experiments, we have obtained evidence that a Gαo(206)/Gαx(160) chimera behaves like Gαx in its cAMP response to βAdR stimulation. Gαolf was not able to augment the endogenous cAMP response to βAdR stimulation, suggesting that it couples less efficiently to the receptor. We are currently making other chimeras between Gαo and Gαx with junctions within the C-terminal region in order to localize the essential sequences for each of the proteins.

Reference:

299. Functional expression of the G protein Gq in Xenopus oocytes

Michael W. Quick, Anna Aragay, Melvin I. Simon

A variety of cellular functions are mediated through the interaction of membrane-bound receptors and intracellular second messenger systems. One such family of receptor proteins, having seven membrane spanning regions, interacts with these second messenger systems through the activity of guanine nucleotide binding (G) proteins. We have begun to study the role of one cloned G protein, Gq, whose effect activates a phospholipase C pathway. Gq RNA was produced through in vitro transcription. It was expressed, along with RNA encoding the mouse 5HT1c receptor in Xenopus oocytes. The functional effect on Ca2+-activated Cl− conductance in the presence of serotonin was examined in these oocytes using the two-electrode voltage clamp.

Results have shown that the co-expression of Gq significantly enhances the serotonin-induced chloride current in a dose-dependent manner. There is a threefold increase in peak response to 1 micromolar 5HT when 1 ng Gq is co-expressed with .01 ng of 5HT1c, as compared to the response elicited to .01 ng 5HT1c expressed alone. The peak response is sixfold increased with the co-expression of 5 ng Gq. These data indicate that Gq is
functional in oocytes and that it can interact with the membrane-bound serotonin receptor to enhance cellular signaling.

300. Heterologous expression of rat brain Ca\textsuperscript{2+} channels and their modulation by G proteins

Nancy F. Lim, Cesar Labarca, Terry P. Snutch

Calcium influx through voltage-dependent channels in the plasma membrane triggers a wide range of cellular responses, including muscle contraction, regulated secretion, and differentiation. Therefore, it is not surprising that calcium channels may be regulated physiologically by neurotransmitters and hormones. These modulators do not bind directly to Ca\textsuperscript{2+} channels but rather to specific membrane receptors that are linked through G protein-activated pathways to Ca\textsuperscript{2+} channels. Our goal is to understand the molecular mechanism by which G proteins can increase or decrease Ca\textsuperscript{2+} currents.

In order to define more precisely G protein effects on Ca\textsuperscript{2+} currents and to manipulate components of the receptor/G protein/Ca\textsuperscript{2+} channel pathway, we plan to express these proteins in foreign cells suitable for functional assays. We are currently focusing on the development of methods to express the various rat brain Ca\textsuperscript{2+} channel genes (Snutch et al., 1990) in mammalian cells. The skeletal muscle Ca\textsuperscript{2+} channel is a pentamer, composed of \(a_1\), \(a_2\), \(\beta\), \(\gamma\), and \(\delta\) subunits, where the \(a_1\) subunit is the dihydropyridine-binding pore forming subunit. Recent reports indicate that successful expression of the Ca\textsuperscript{2+} channel \(a_1\) subunit may require auxiliary Ca\textsuperscript{2+} channel subunits to be coexpressed or endogenously available (Tanabe et al., 1988; Mori et al., 1991). We are constructing several plasmids containing the coding region of different rat brain Ca\textsuperscript{2+} channel \(a_1\) subunits as well as the \(a_2\) and \(\beta\) subunits, for use with the vaccinia virus expression system. Cells with endogenous Ca\textsuperscript{2+} currents will be transfected with \(a_1\) cDNA only; cells lacking endogenous Ca\textsuperscript{2+} currents (e.g., Chinese hamster ovary cells) will be transfected with \(a_1\), \(a_2\), and \(\beta\) subunit cDNA. Determination of the biophysical and pharmacological properties of the expressed currents will enable a functional definition to be assigned to the various Ca\textsuperscript{2+} channel \(a_1\) subunits as well as provide important insights into structure-function relationships. Subsequently, the extent of Ca\textsuperscript{2+} channel/G protein interactions will be explored by testing which brain Ca\textsuperscript{2+} channel types are regulated by G proteins, and which G protein types are effective modulators. Also, the molecular interaction between Ca\textsuperscript{2+} channels and G proteins will be studied by determining 1) whether G proteins modify neuronal Ca\textsuperscript{2+} channels directly or through second messenger-mediated pathways, 2) the biophysical properties of the channel that are altered by activated G proteins, and 3) the region(s) of the Ca\textsuperscript{2+} channel primary sequence involved in G protein binding and specificity.

References:


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301. Expression of ion channels and receptors in Xenopus oocytes using vaccinia virus

Xian-cheng Yang, Andreas Karschin, Cesar Labarca, Orna Elroy-Stein, Bernard Moss

The cytoplasmic injection of mRNA synthesized in vitro into Xenopus oocytes is widely used for heterologous expression of ion channels and neurotransmitter receptors. We report two new methods for expression of ion channels and receptors in oocytes using vaccinia virus (VV). 1) A recombinant VV carrying the Shaker H4 K\textsuperscript{+} channel cDNA driven by the VV P7.5 early promoter was injected into oocytes. 2) A recombinant VV containing the bacteriophage T7 RNA polymerase driven by the P7.5 promoter was coinjected along with plasmids containing a T7 promoter and cDNAs for channels and receptors. The functionally expressed proteins include a) voltage-gated ion channels: the Shaker H4 K\textsuperscript{+} channel and the rat brain IIA Na\textsuperscript{+} channel, b) a ligand-gated ion channel: the mouse muscle nicotinic acetylcholine receptor (AChR), and c) a G protein-coupled receptor: the rat brain 5HT\textsubscript{1C} receptor. After virus/cDNA injection with oocytes, these channels and receptors generally showed characteristics and expression levels similar to those
observed in mRNA-injected oocytes. However, the AChR expressed at lower levels in virus/cDNA-injected oocytes than in mRNA-injected oocytes. Because our methods bypass mRNA synthesis, they are more rapid and convenient than the mRNA injection method. Potential applications to structure-function studies and expression cloning are discussed (Yang et al., 1991)

Reference:

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302. Molecular studies of the CF transmembrane conductance regulator

Nael A. McCarty, Cesar Labarca, Bruce N. Cohen

Cystic fibrosis (CF) is the most common lethal genetic disorder among Caucasians, affecting approximately 1 in 2,000 live births. The disease is manifested primarily in epithelia, where a defect in the cAMP-dependent control of cell membrane Cl⁻ permeability leads to inappropriate secretion and absorption of water. Hence, secretion in the lung and pancreas and reabsorption in the sweat gland, for example, are impaired.

The gene encoding the protein that is defective in CF was cloned from human cDNA libraries in 1989 (Riordan et al., 1989). The predicted protein product has 12 putative transmembrane segments (reminiscent of several ion channels), two putative nucleotide-binding folds (reminiscent of p-glycoprotein and several other ATP-dependent transporters), and a unique putative regulatory domain. Since the function of this protein is unclear, it was dubbed the cystic fibrosis transmembrane conductance regulator (CFTR). Four hypotheses for its function dominate: a) CFTR is itself a Cl⁻ channel; b) CFTR is a pump which extrudes or accumulates a regulator of Cl⁻ channels; c) CFTR controls the insertion of Cl⁻ channels into the plasma membrane; and d) CFTR is the controlling subunit of CFTR-Cl⁻ channel complex. The most recent experimental evidence, relying on heterologous expression studies, favors the first hypothesis.

Our objective is to study the properties of the CFTR on the molecular level. Specific goals are as follows. 1) We are expressing CFTR in Xenopus oocytes in order to perform detailed electrophysiological analyses of the functional effects of site-directed mutagenesis. These experiments will identify essential domains in the protein that determine its function. 2) In an effort to characterize the channel pore, we are performing an analysis of the molecular basis of channel blockade using anion-channel blockers. 3) CFTR is being subcloned into the transfer vector for the vaccinia virus expression system, in order to express and purify large quantities of the protein. This will allow investigations into three-dimensional structure. 4) We are also attempting the isolation of CFTR-related molecules from cDNA libraries of other tissues; currently, these include rat choroid plexus and Drosophila libraries. Obtaining related but nonidentical clones will allow us to make chimera molecules between the two species. This approach has been successfully applied, in the past, to define structural determinants of function in ion channels.

Reference:

303. Molecular biology of neurotransmitter transporters

John G. Guastella

Many cells in the nervous system possess ion-coupled high affinity transporters for neurotransmitters. These neurotransmitter transporters are thought to terminate synaptic activity by clearing the synaptic cleft of neurotransmitter, and may also play a variety of other roles in nervous system physiology. We recently cloned and characterized a cDNA for a rat brain GABA transporter (GAT-1) (Guastella et al., 1990). The deduced amino acid sequence for this clone predicted a very hydrophobic protein containing 12 or more transmembrane domains, a feature common to other transporters. More recently, a group at Yale has cloned a norepinephrine transporter from neuroblastoma cells whose predicted amino acid sequence is almost 50% identical to GAT-1 (almost 70% when conservative amino acid substitutions are taken into account) (Pacholczyk et al., 1991). This degree of similarity is remarkable considering that these two transporters have very different pharmacological properties. These results suggest that the GABA and norepinephrine
transporters are members of a family of proteins that probably includes transporters for other neurotransmitters, and might also include transporters for non-neurotransmitter substrates. Based on this assumption, we are using a polymerase chain reaction-based approach to isolate additional transporter cDNA clones in an effort to further define the extent of this protein family.

References:

304. Determination of the membrane topology for the GABA transporter
Janis L. Corey

The GABA transporter (GAT-1) has recently been cloned and sequenced in this laboratory and used to express GABA uptake activity in Xenopus oocytes. From the predicted amino acid sequence and hydropathy profile, the membrane orientation of the transporter was proposed, locating both the amino and carboxy termini of the transporter protein intracellularly. This predicted topology will be tested by generating a fusion protein consisting of the GAT-1 protein and an epitope from the hemagglutinin (HA) protein. Using polymerase chain reaction (PCR), the HA epitope is added to either the N or C terminus of the transporter protein and the PCR product is used to generate mRNA by in vitro transcription. The resulting mRNA is microinjected into Xenopus oocytes and the GAT-1/HA fusion protein is expressed. Using a monoclonal antibody specific for the HA epitope, the location of the amino and carboxy termini can be identified. The antibody will be applied directly to intact oocytes expressing the GAT-1 fusion protein or microinjected into the oocyte. The ability of the antibody to bind the epitope in each case will be determined. Another method will be to digest intact oocytes with protease followed by addition of antibody. This will indicate whether the epitope is protected by an intracellular location, or lost upon protease digestion, suggesting an extracellular position.

305. A cultured neuron probe
Jerome Pine, Svetlana Tatć,1 Yu-Chong Tai1, Wendy Markson

We are developing a silicon microstructure in the form of a thin pointed probe, which can be inserted into rat brain tissue. The probe will contain 16 wells, into which dissociated neurons will be placed and grown for a short time in culture. Then the probe will be implanted, but two-way communication will be maintained with the neurons. Each well will have an electrode in the bottom, in intimate contact with the cell body, for stimulation and recording. Electrical leads to the electrodes will be part of the probe structure, and will be connected via fine wires to a percutaneous skull plug.

The initial purpose of this effort is to establish the feasibility of such a neuron probe as a means of selective two-way communication with a host nervous system. It remains to be seen whether the implanted neurons will survive, and if so whether they will integrate synaptically with the host. We are encouraged by the success of neural grafts, which have been shown to survive for long periods and to become synaptically integrated. If the probe proves to be successful, it offers a possibility for highly specific long-term two-way communication, between specific types of probe neurons and their natural pre- and postsynaptic partners. This has important potential uses in system neurobiology experiments and for neural prostheses.

During the past year, we have developed a successful culture system for hippocampal neurons, which we will implant in the probes. A method has also been developed for picking up individual newly-dissociated cells and moving them so they can be inserted into probe wells. The electronic interface between probe and computer, and the software for controlling it, have been designed and built. Probes have been constructed that are designed for implantation in rat hippocampus. They have 4 mm shafts, which are 15 microns thick and 100 microns wide in the tip region where the neuron wells are located. During the coming year, we expect to complete the probe structures, to grow cells in vitro to establish their viability, and then to make initial tests in rats, collaborating with Yuri Busaki at Rutgers University.

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306. Studies of cultured neural networks with voltage-sensitive dyes

Chi-Bin Chien, Wendy Markson, Jerome Pine

Our dye recording system, with 256 photodetectors, was used to record from small networks of rat superior cervical ganglion (SCG) neurons. The recording system performed very well and permitted the observation of subthreshold postsynaptic potentials. It was found that signal averaging for observing monosynaptic connections worked well, so that the subthreshold signals could be cleanly observed. The cultures contained a great mass of axons, which made extracellular stimulation of a single neuron difficult, and which contaminated dye signals detected from cell bodies with those from close axons. Nonetheless, preliminary experiments mapping the synaptic connections of small cultures were performed.

For future work, hippocampal pyramidal cell cultures will be used because they exhibit interesting plasticity in vivo and in vitro. Cultures will be prepared that have the cell bodies in a limited region, for dye recording and disk-electrode stimulation, but provide a large area for dendritic arborization and axon growth. In this way, we hope to minimize some of the difficulties with SCG cultures. Mass hippocampal pyramidal cell cultures are now being grown, which will be used for screening fluorescent voltage-sensitive dyes to find the best ones.

307. Soft x-ray absorption imaging of whole wet tissue culture cells

John R. Gilbert, Jerome Pine

Soft x-ray microscopy, utilizing x-rays at 2.3 nm < λ < 4.5 nm, the "water window," offers the possibility of making high resolution images of wet biological samples. In the last year, we have succeeded in taking 100 nm resolution images of whole wet unfixed tissue culture cells, and near 50 nm resolution images of whole wet fixed cells.

The microscope we have been working with is the Stony Brook/NSLS Scanning Transmission X-Ray Microscope (STXM) at the National Synchrotron Light Source. The STXM operates by focussing x-rays to a spot (about 50 nm FWHM) and scanning a sample through that spot. Images are formed by counting the transmitted photons at each position "pixel" in the sample.

At Caltech, we have built a miniature controlled-environment culture chamber, the "wet cell," for mounting wet whole tissue culture cells on the STXM scanning stage. The Caltech wet cell has been used for imaging whole chromosomes as well as tissue culture cells. Our laboratory has been working with dissociated primary cultures of neurons and fibroblasts. Cells are grown using conventional tissue culture techniques on silicon nitride substrates 120 nm thick and 3 x 3 mm across. The wet cell is composed of a stainless steel body with a 1 mm aperture which contains the plumbing for liquid control. An exit window and an entrance window (the cell substrate) are mounted on the downstream and upstream ends of the chamber. The windows are mounted vertically, perpendicular to the x-ray beam. The wet cell allows us to freely cycle the environment of cultures mounted within it between culture medium and humid air. General x-ray losses in the wet cell itself are about 50% at λ = 3.6 nm.

We have also used the STXM to produce several images of the same cell at differing wavelengths. In principle, this will allow us to generate elemental densities of C, N and O if the image signal-to-noise is good enough. We are now working to analyze those data.

308. The effect of electrical stimulation and growth-cone-calcium on neurite outgrowth in rat synaptic neurons

Tina Kramer Garyantes, Wade G. Regehr1, Jerome Pine

Suprantheshold electrical stimulation at 4 Hz has been shown to halt neurite outgrowth in Helisoma neurons. Growth-cone-calcium concentration ([Ca]_{gc}) increases with this electrical activity, and has been proposed as the causative agent in the halting of neurite outgrowth. In order to learn if electrical stimulation generally stops outgrowth of neurites, we investigated the effect of electrical activity on the rate of neurite growth of cultured neonatal rat superior cervical ganglion (SCG) neurons. Stimulation of SCGs at 10 Hz for 1 hr was found to have little effect upon their growth rate.

During sustained electrical stimulation, [Ca]_{gc} behaved similarly in SCGs and Helisoma neurons. Immediately following the onset of stimulation, [Ca]_{gc} (measured using fura-2) transiently increased from about 100 nM to greater than 500 nM in SCGs, versus from 100 nM to between 300-400 nM in Helisoma neurons, but sustained [Ca]_{gc} remained below 250 nM in SCGs, versus between 225
and 325 nM in Helisoma neurons. Thus, unlike Helisoma neurons, SCG neurons do not seem to stop growing when [Ca]_o is increased. To see if SCG neurons would stop growing if their [Ca]_o was increased to significantly higher levels, we grew the neurons in media with the calcium ionophore bromo-A23187. Unfortunately, SCG neurons did not increase their steady-state calcium levels, but rather cycled their calcium concentrations.

These findings indicate that SCG outgrowth, unlike Helisoma outgrowth, is unlikely to be greatly influenced by electrical activity at frequencies as high as 10 Hz, or by [Ca]_o in the range studied. Differences in calcium buffering systems, density or type of voltage-dependent calcium channels, or sensitivity of outgrowth to [Ca]_o between rat SCGs and Helisoma neurons may explain their different responses to electrical stimulation.

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PUBLICATIONS


Hsu, H., Yang, X.-C., Karschin, A., Labarca, C., Figl, A., Elroy-Stein, O., Moss, B., Davidson N. and Lester, H. A. Neuronlike action potentials in mammalian cells after heterologous expression of voltage-gated channels. Submitted for publication.


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Summary: The interests of this laboratory concern two aspects of the development of the nervous system: factors that control the choice of neuronal phenotype, and molecules involved in axonal growth and the formation of distinctive patterns of synaptic connections. Much of our effort in the first area has centered on sympathetic neurons developing in cell culture. These neurons are remarkably plastic in their choice of phenotype, and their decisions as to which differentiation pathway to follow can be controlled by a group of proteins termed the neuronal differentiation factors. These factors instruct the neurons as to patterns of gene expression without affecting growth or survival. We cloned the cDNA for one of these factors, CDF/LIF, and a polymerase chain reaction (PCR) assay has been developed to identify more of these factors by expression cloning. The tissue distribution of CDF/LIF mRNA is being determined using a reverse transcription PCR assay. We are also perturbing development in vivo by local application of recombinant differentiation factors and antibodies against them. The latter project is being carried out in collaboration with Dr. Story Landis at Case Western Reserve University.

Studies of the role of hormones and growth factors in other phenotypic choices in this lineage demonstrated the interconversion of adrenal chromaffin cells, SIF (small intensely fluorescent) cells and neurons in culture. The relationships of these cells during normal development in vivo is also under study, and we recently generated a set of monoclonal antibodies (mAbs) that allowed the isolation of the probable precursors of all of the cells of this lineage by FACS. Thus, this area of work involves the delineation of commitment vs. plasticity at various stages of neural crest differentiation. This necessitates the identification, purification and study of the molecular action of cues in the embryonic environment that influence
phenotypic choices. We are also making use of the ability to control phenotypic choices in order to produce catecholaminergic and cholinergic neurons from adrenal chromaffin cells for neural grafting experiments involving rat models of Parkinson's and Alzheimer's diseases. The latter experiments are being carried out in collaboration with Dr. Fred Gage at the UCSD Medical School.

The second area of investigation involves the control of neurite outgrowth and regeneration, and the molecules that underlie the specificity of connections. We have identified a number of neuronal macromolecules, surface-bound and secreted, that mediate axonal growth, and several of these are being studied in antibody perturbation experiments and with mutant cell lines. The neuronal surface glycoprotein, Thy-1, was originally found on lymphocytes. Since it shares sequence homology with immunoglobulins, Thy-1 has been postulated to play a role in adhesion or recognition in the nervous system. We have used antibody, peptide, and phospholipase perturbations, as well as Thy-1-deficient mutant cells, to obtain evidence that Thy-1 plays a role in neurite outgrowth or sprouting. As for neuronal secreted molecules, we previously determined that plasminogen activator and its naturally occurring inhibitor can control the rate of axonal outgrowth in culture. Using highly specific monoclonal and polyclonal antibodies, an in vivo project is aimed at determining the role of these and other proteins in nerve regeneration in the adult and initial outgrowth in the embryonic animal.

A novel method for producing mAbs against rare antigens is being used to investigate the molecular basis for the distinctive patterns of synaptic connections found in the mammalian nervous system. In a search for surface molecules that may mediate the rostrocaudal positional bias of neuronal connections, mAbs were generated that bind preferentially to membranes from rostral nerves and ganglia. A protein antigen for one of these mAbs (ROCA1) has been isolated and is being sequenced. ROCA1 binds to glial cells in rostral nerves, and its binding declines in a gradient in caudal nerves. Curiously, ROCA1 binds to neuronal progenitors in embryonic ganglia, and switches its binding to glia as development proceeds. The control of this switch will be studied in cell culture.

309. Comparison of cholinergic factors in vitro and in vivo
Mahendra Rao, Story Landis1, Paul H. Patterson

A minority population of neurons in sympathetic ganglia use acetylcholine as their transmitter, and they innervate particular target organs such as the sweat glands of the rat footpad. These neurons switch their transmitter phenotype from noradrenergic to cholinergic after their axons innervate the sweat glands during the second postnatal week. We find that footpad extracts contain two factors that can induce cholinergic differentiation in cultured sympathetic neurons. The major cholinergic activity is biochemically and immunologically related to ciliary neurotrophic factor (CNTF). Preliminary data indicate that the other factor can be blocked by antibodies against the cholinergic differentiation factor (CDF/LIF). The latter finding is consistent with the presence of CDF/LIF mRNA in the footpad during development (see Abstract No. 311).

We find that recombinant CNTF and CDF/LIF each induce the same pattern of changes in neurotransmitter and neuropeptide gene expression in cultured sympathetic neurons. The action of the two factors can, however, be distinguished by the differential effects of depolarizing the neurons. Depolarization in the presence of calcium blocks the action of CDF/LIF, but has no effect on the neuronal response to CNTF. This result suggests that these factors control the same set of genes through different mechanisms. In addition, while CDF/LIF can alter the expression of a series of neuropeptides in cultured dorsal root ganglion sensory neurons, CNTF has no detectable effect on these genes.

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310. Control of neuronal immediate early genes by CDF/LIF
Tetsuo Yamamori

To elucidate the initial events whereby the neuronal differentiation factor CDF/LIF controls the phenotype of cultured sympathetic neurons, the expression of several immediate early genes was examined. c-fos and jun-B were induced within 30 minutes after the addition of CDF/LIF. In contrast, no effect on the expression of c-myc, fra-1, v-jun or actin mRNA was detected at this time point. Thus, the early actions of CDF/LIF may
involve alterations in the levels of specific immediate early genes, and these may have downstream effects on the positive and negative regulation of neurotransmitter and neuropeptide gene expression elicited by CDF/LIF.

311. Localization of CDF/LIF mRNA in the rat brain and peripheral tissues

Tetsuo Yamamori, Xiao-Yi Xie, Paul H. Patterson

CDF/LIF is a cytokine that controls cell proliferation and gene expression in many tissues, from the earliest embryo through adulthood. To understand the role of this factor in normal development, it is essential to determine where it is produced in vivo. We employed a semi-quantitative, reverse transcription polymerase chain reaction (RT-PCR) method. Actin and tubulin mRNA were used as internal controls, and two different sets of CDF/LIF primers were compared. In postnatal rat peripheral tissues, CDF/LIF mRNA is selectively localized in the target tissue of developing, sympathetic cholinergic neurons; the mRNA is not detected in the targets of sympathetic noradrenergic neurons. This finding supports the hypothesis that CDF/LIF is a target-derived, neuronal differentiation factor. In postnatal rat brain, CDF/LIF is localized selectively in two parts of the visual system, visual cortex and superior colliculus. Thus, CDF/LIF may play a role in this system as well, and these neurons could display a previously unsuspected phenotypic plasticity.

Initial experiments on embryonic tissues reveal the presence of CDF/LIF mRNA in heart but not liver. Since the mRNA is not detected in adult heart or liver, this gene is developmentally regulated in select tissues. It will be important to determine whether regulation in the heart is important for the development of that muscle and/or for the neurons that innervate it. These findings also underline the importance of localizing CDF/LIF in the embryonic brain.

312. CDF/LIF regulates sympathetic neuron phenotype by alterations in both the amounts and size of neuropeptide mRNAs

Hiroyuki Nawa, Shigetada Nakanishi, Paul H. Patterson

CDF/LIF can dramatically alter neurotransmitter production as well as levels of several neuropeptides in cultured sympathetic neurons. We find that these changes are likely to be caused by alterations in the mRNAs for these proteins and peptides. Growth in recombinant CDF/LIF induces mRNA for choline acetyltransferase (ChAT), somatostatin (SOM), substance P, and vasoactive intestinal polypeptide, while lowering mRNA levels for neuropeptide Y (NPY) and tyrosine hydroxylase. In addition, the sizes of the mRNAs for ChAT, SOM and NPY are larger after CDF/LIF treatment. Since it is likely that the smaller form of the ChAT mRNA corresponds to an embryonic form of the enzyme, we propose that CDF/LIF specifically induces the adult form of the enzyme.

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313. Search for CDF/LIF homologs in distant species

Lindee Goh, Ming-Ji Fann

The deduced amino acid sequences for CDF/LIF from human, mouse and rat cDNAs display 80-90% identity. By sequencing cDNAs from more distant species, chicken and zebra fish, we hope to identify the most conserved regions of this protein. Such information could be useful in functional studies, as well as for identifying related neuronal differentiation factors. Primers from the rat sequence were used in the polymerase chain reaction method to produce candidates from chicken and fish genomic DNA. These are currently being sequenced.

314. Identification of neuronal differentiation factors by expression cloning

Ming-Ji Fann

This laboratory has characterized several instructive factors from heart cell conditioned medium that control neuronal phenotype. In an effort to facilitate the identification of these and other factors, I am employing an expression cloning approach. First, the reverse transcription polymerase chain reaction (RT-PCR) method was established for the analysis of mRNA levels of neuropeptides and neurotransmitter biosynthetic enzymes from microcultures of sympathetic neurons. Thus far, mRNAs for 12 different phenotypic markers can be simultaneously measured from approximately 3000 neurons. Several cytokines have been tested for their effects on neuronal gene expression, CDF/LIF having the expected effects, and TNFα inducing somatostatin mRNA. Second, expression libraries from rat brain and cultured primary heart cells are being constructed with
Kahan Leong (AmGen). Expressed proteins secreted by transfected COS cells will then be tested for neuronal differentiation activity in the RT-PCR assay.

315. The progenitors of the sympathoadrenal lineage
Josette F. Carnahan, David J. Anderson, Paul H. Patterson

Several lines of evidence indicate that the primary derivatives of the sympathoadrenal lineage, sympathetic neurons and adrenal medullary chromaffin cells, are derived from a common progenitor cell. Using the cyclophosphamide immunosuppression technique, we produced six monoclonal antibodies (SA1-6) that stain cells of early embryonic sympathetic ganglia and adrenal medullae in situ. The antibodies were used to separate the SA1+ cells by FACS. That these cells are bipotential is shown by growing them in either glucocorticoid, whereupon they acquire chromaffin cell characteristics, or in basic fibroblast growth factor, insulin and NGF, whereupon they express neuronal markers. The SA1+ cells are also observed to undergo the transition from the progenitor state to the neuronal phenotype in vivo by double-labeling studies using neuron- and chromaffin cell-specific markers.

SA1 also stains neuroblasts in the early embryonic gut. These cells express several markers in common with the sympathoadrenal progenitors, and the same transitions to the neuronal phenotype are observed in the gut as were found for sympathetic ganglia. These findings suggest that the sympathoadrenal progenitor may also give rise to the enteric nervous system.

316. Culture-derived neurons for brain grafts in disease models
Kenji Niijima, Ming-Ji Fann, Fred Gage1, Paul H. Patterson

Parkinson's disease is a degenerative disease that results in severe motor disability. Although there are drugs that can temporarily ameliorate the symptoms of the disease, there is an inexorable progression to a drug-resistant state. This has stimulated efforts to examine the feasibility of neural grafting as a potential therapy. Recent clinical studies using autographs of adrenal chromaffin cells into the brain have yielded controversial results. Since we have previously shown that chromaffin cells can be converted into catecholaminergic neurons in culture, we are examining the possibility of using autographs of adrenal-derived neurons in a rat model of Parkinson's disease.

Another major degenerative neurologic disease is Alzheimer's disease. One of the key deficits in this disorder is a loss of cholinergic neurons in the basal forebrain. Since we have shown that the appropriate environmental signals can convert adrenal chromaffin cells into cholinergic neurons, we are using autographs of these adrenal-derived neurons in attempts to ameliorate cholinergic deficits in the rat brain induced by surgery or by normal aging.

317. A protein that defines rostrocaudal position in the mammalian nervous system
Zaven Kaprielian, Jr.

Transplantation experiments have shown that spinal cord neurons can distinguish rostral versus caudal sympathetic ganglia as targets for synapse formation. Spinal neurons can also distinguish between rostral and caudal intercostal muscles, when such novel targets are transplanted in place of the ganglia. These observations suggested the existence of a positional information system that is shared between neurons and muscles. To examine the molecular basis for such a system, the cyclophosphamide immunosuppression technique was used to produce monoclonal antibodies that bind selectively along the rostrocaudal axis of the neonatal rat peripheral nervous system. ROCA1 (for ROstroCAudal) binds in a rostro(high)-to-caudal(low) gradient in both sympathetic ganglia and in the nerves that innervate the intercostal muscles. Surprisingly, ROCA1 binds to glial cell surfaces (satellite cells in ganglia and Schwann cells in nerves) rather than to axons or neuronal cell bodies. We used ROCA1 in an immunoblot analysis to identify a 60 kDa protein present in membrane fractions of newborn and adult rat sympathetic ganglia and intercostal nerves. Quantification of the rostrocaudal distribution of this protein in blots of intercostal nerves demonstrates a gradient similar to that seen with immunohistochemistry.

Two-dimensional electrophoresis of adult rat peripheral nerve membranes, in combination with
immunoblotting, demonstrated that the 60 kDa antigen can be resolved as a single spot with an approximate pI of 5.5. Staining for total protein revealed that this antigen is clearly separated from contaminating proteins. In an effort to obtain internal amino acid sequence data, pooled spots of the 60 kDa protein are digested with trypsin, and the resulting peptides separated by HPLC. The Biology Division’s Microsequencing Facility is currently sequencing these peptides.

318. Distribution of the ROCA1 antigen in the embryonic nervous system
Shubha Tole

To determine if the distribution of the ROCA1 antigen is consistent with a role in the initial innervation of sympathetic ganglia, sections from rat embryos are stained with the ROCA1 antibody. ROCA1 binds embryonic sympathetic ganglia, staining early neurons, as identified by labeling with the B2 and TH antibodies. ROCA1 also binds the surfaces of dissociated, living cells from embryonic dorsal root sensory ganglia. With further development, ROCA1 staining is lost from neurons but is maintained on all peripheral glia. Although the embryonic antigen has not yet been identified, these data suggest that ROCA1 binding may define membership in the embryonic peripheral nervous system, as well as designate rostrocaudal position.

319. Thy-1 and neurite outgrowth
Nagesh Mahanthappa

Thy-1, the smallest member of the immunoglobulin gene superfamily, is a major surface protein on axons with long projections. We found that treating cultured neurons with anti-Thy-1 antibodies causes the release of surface Thy-1 and induces neurite outgrowth. Release of Thy-1 by phospholipase C treatment also induces outgrowth. In a third perturbation paradigm, Thy-1-deficient PC12 cells were found to sprout neurites spontaneously. Thus, the presence of high levels of surface Thy-1 appears to stabilize membranes so as to inhibit sprouting.

We have also found that Thy-1 is capable of homophilic interactions of extremely high affinity, and that this binding may contribute to the physiological role of the protein. Gel filtration and PAGE methods demonstrate that two-thirds of Thy-1 in cultured sympathetic neurons is in the form of dimers and hexamers. Furthermore, the monomer/multimer ratio increases when neurons or PC12 cells grow neurites in response to NGF. Additional evidence that Thy-1 multimerization influences neurite outgrowth comes from experiments utilizing synthetic peptides corresponding to parts of the Thy-1 molecule predicted to be involved in homophilic binding. We recently found that one such peptide specifically promotes sprouting and also increases the Thy-1 monomer/multimer ratio. Thus, multimerization of Thy-1 is correlated with its postulated inhibitory action on neurite outgrowth.

320. The distribution of Thy-1 in the developing chick nervous system
Lisa Dahm

The temporospatial distribution of Thy-1 in the embryonic chick lumbosacral spinal cord and hindlimb was determined and compared to the immunohistochemical distribution of three other members of the immunoglobulin superfamily (IgSF), TAG-1, L1 and NCAM. Immunoblot analysis confirmed that the anti-Thy-1 monoclonal antibody recognizes the same protein in E10 spinal cord as that found in P1 forebrain, the source from which avian Thy-1 was cloned (Dowsing et al., 1990). Both peripheral and central chick neurons express Thy-1 during the period of axon elongation and synapse formation, suggesting a role for this protein in these events. Thy-1 is spatially regulated along the neuronal surface membrane, and its distribution differs between various types of neurons. In motorneurons, Thy-1 is expressed all along the length of the growing axon, while in another set of spinal neurons, the commisural neurons, its expression is restricted to the growth cones and distal axons. Dorsal root ganglion neurons exhibit the most extreme spatial restriction. Each sensory neuron in these ganglia sends an axon peripherally to innervate skin and muscle, and centrally into the spinal cord. Thy-1 is expressed all along the peripheral axon while its expression on the central axon is restricted to the growth cone and distal process. Each of these patterns of Thy-1 expression is distinct from those of the other IgSF family members studied. The distribution and timing of Thy-1 expression is consistent with a role in axon growth in the spinal cord and in the formation of muscle nerve branching patterns.
Elicited by injection of the plasmin inhibitor Aprotinin. These results support the culture findings and further indicate a role for protease/inhibitor systems in neurite outgrowth.

PUBLICATIONS


Summary: Our laboratory is primarily interested in the mechanisms by which specific synaptic connections are formed during development and regeneration. The segmental ganglia of the embryonic insect central nervous system (CNS) provide a good system in which to study these processes, because they contain a relatively small number of neurons that make genetically defined connections. This pattern of synapses is largely determined by the pathway choices made by neuronal growth cones during early embryonic development. Each of the early neurons in the ganglion has a unique identity and makes a programmed series of pathway choices. The basic ganglion architecture and many of these specific pathway decisions are conserved between a species with large neurons suitable for cell biology studies (the grasshopper Schistocerca) and a species with well-developed genetics (Drosophila).

Studies on grasshopper embryos have suggested that individual axons or axon bundles in the ganglia are differentially labeled by surface recognition molecules used for growth cone guidance. Several cell-surface glycoproteins, known as fasciclins, have been identified that have patterns of expression restricted to particular axon subsets, suggesting that they could function as molecular cues for specific pathway decisions. Mutations eliminating expression of fasciclins I and III have been isolated in Drosophila. These mutations alone do not cause visible alterations of the embryonic CNS. However, a combination of the fasciclin I mutation together with a mutation in the gene encoding the fly homolog of the c-abl tyrosine kinase causes an embryonic phenotype in which the commissural pathways in the CNS are absent or reduced. This is likely to be due to a defective interaction between the commissural growth cones (which express fasciclin I and abl) and the specialized midline cells of the CNS. This interaction may be mediated through two partially redundant signal transduction pathways, one involving fasciclin I and the other the abl tyrosine kinase. We are searching for other mutations that interact with the fasciclin I mutation in order to identify components of these pathways.

Although it will be instructive to analyze molecules interacting with known fasciclins, a better understanding of CNS development will require the identification of new surface molecules that mediate pathway decisions. Only three fasciclins have been identified by the monoclonal antibody approach, and these molecules could only account for specific labeling of 5-10% of the axons in the embryo. To identify new surface molecules potentially involved in growth cone guidance, we are taking two approaches. In the first approach, an antibody against a carbohydrate epitope has been used to purify a large group of fly neuronal surface proteins ("HRP proteins"). We are using sequence information from these proteins to isolate the genes encoding them.

The second approach to the identification of new surface/signal transduction molecules uses polymerase chain reaction (PCR) techniques to isolate receptor-linked protein tyrosine phosphatase (PTPase) cDNAs from Drosophila embryos. Receptor-linked PTPases are a recently defined class of molecules that have extracellular domains related to those of neuronal cell adhesion molecules such as fasciclin II, N-CAM and L1. Because the PTPase cytoplasmic domain of these molecules is highly conserved, it is possible to use it as a "handle" to isolate new cell adhesion/recognition molecules, which has not been possible thus far using extracellular domain sequences. PTPases are also of interest in the fly CNS.
because the fasciclin II/abl double mutant phenotype mentioned above shows that tyrosine phosphorylation is important for correct axon guidance. We have identified and characterized three distinct receptor-linked PTPase cDNAs whose expression is restricted to subsets of neurons in the embryonic CNS. Each of these PTPases is restricted in its expression to CNS axons. We are beginning to screen for mutations in these genes. For one of the PTPases, this will be done by developing novel methods for identifying and characterizing local P element transposition events.

In collaboration with the Lester/N. Davidson laboratory, we are also studying the mechanism of olfactory signal transduction in mammals. We are developing methods that will allow us to assay odorant receptor function in *Xenopus* oocytes, and constructing a pool of putative odorant receptor clones.

### 323. Isolation of genes encoding neuronal surface glycoproteins in *Drosophila*

**Chand J. Desai, Jian Liang**

In order to study axonal guidance, we have taken the approach of purifying and characterizing glycoproteins expressed on neuronal surfaces during axogenesis. To this end, we are using antibodies raised against horse radish peroxidase (HRP) to immunoaffinity purify a subset of such proteins. Anti-HRP binds to all insect neurons and axons. This binding is due to the expression of one or more of a group of 15-20 glycoproteins (HRP proteins) that bear a neuron-specific carbohydrate moiety. If a given HRP protein is to serve as a guidance cue, it must be restricted to a subset of the surfaces available to an elongating axon. In fact, both fasciclin I and II are HRP proteins and have such discrete patterns of expression. It seems likely that other HRP proteins may also be potential axonal guidance cues.

During the last year, we have succeeded in purifying and concentrating sufficient amounts of HRP proteins for sequence analysis. After separation by SDS-PAGE, the proteins are transferred to nitrocellulose and visualized using Ponceau S dye. Individual bands are excised, minced and digested with trypsin. The Biology Division’s Protein Sequencing Facility then separates each tryptic digest using HPLC and sequences individual peptides by Edman degradation. We were able to obtain useful information from seven protein bands. Four of these bands were non HRP-proteins: heat shock protein (HSP) 82, HSP 70, rabbit IgG heavy chain (which had leached from the anti-HRP immunoaffinity column), and yolk protein 3 (YP3). The remaining three bands were previously characterized neuronal surface proteins: fasciclin I, neurotactin and a *Drosophila* protein tyrosine phosphatase (DPTP). Neurotactin was recently cloned by several groups using monoclonal antibodies and expression libraries. It is the most abundant HRP protein. DPTP was also recently cloned by another group.

Although somewhat successful, our methods suffer from contaminating proteins and low sensitivity, as only the most abundant HRP-proteins were identified. To alleviate these problems, we now use Concanavalin A (ConA)-Sepharose as a secondary purification step. ConA binds only to glycoproteins, so many contaminating proteins (e.g., HSP 70, HSP 82 and YP3) are removed. The ConA purification is also a concentrating step, so we are able to process more material. We are currently scaling up the purification and hope to be able to generate sufficient material to sequence several of the less abundant HRP protein species.

### 324. Identification of mutations with fasciclin I-dependent phenotypes

**Chand J. Desai, Winston Chamberlin**

In addition to the biochemical approach, we are also trying to identify genes involved in axonal guidance using genetics. Several molecules expressed on subsets of neurons during axogenesis have been isolated and characterized, including fasciclin I, fasciclin II, neuroglian, amalgam and neurotactin. Although mutants that lack such molecules develop apparently normal central nervous systems (CNS), flies that lack both fasciclin I and abl (the *Drosophila* homolog of the abl proto-oncogene) die as embryos and display severe CNS defects. This result suggests that other molecules can compensate for the loss of these putative axonal guidance molecules. To identify additional interacting genes, we are screening chromosome III for loci that are lethal only in the absence of fasciclin I. The screen for chromosome III is outlined below:
The genotypes of the F3 generation are \textit{pfasl}; (th \textit{st} \textit{cu fasl})*/Bal, \textit{pfasl}; (th \textit{st} \textit{cu fasl})* and \textit{pfasl}; TM3/TM6 males and C(1)DX; (th \textit{st} \textit{cu fasl})*/Bal, C(1)DX; (th \textit{st} \textit{cu fasl})* and C(1)DX; TM3/ TM6 females.

\textit{th} homozygotes have threadlike antennae; \textit{st} homozygotes have scarlet eyes; \textit{cu} homozygotes have curly wings; \textit{fasl} homozygotes lack fasciclin I; \textit{pfas} is an insertion of the \textit{fasl} gene on the X chromosome; Bal represents the chromosome III recombination suppressors TM3 and TM6 that are lethal when homozygous; * represents the mutagenized chromosome; C(1)DX is a covalently linked pair of X chromosomes such that female progeny receive the maternal X double chromosome while male progeny receive the paternal X chromosome.

The F3 generation of this cross will be screened for cases where the only scarlet-eyed curly-winged flies are males. This result would suggest that a mutation has been induced on chromosome III that kills flies only when they lack fasciclin I. Although scarlet-eyed curly-winged males are homozygous for \textit{fasl}, they carry an insertion of the \textit{fasl} gene on their X chromosome. Scarlet-eyed curly-winged females, however, completely lack fasciclin I and would be missing in a strain with an induced fasciclin I-dependent lethal mutation on chromosome III. If a normal lethal mutation is induced on the chromosome III, then no scarlet-eyed curly-winged F3 progeny will be observed. If an irrelevant mutation is induced on chromosome III, then both male and female scarlet-eyed curly-winged F3 progeny will be observed. Similar screens are designed for chromosome I (X) and II.

Any isolated \textit{fasl}-dependent lethal mutation would be characterized in the standard genetic manner, including mapping and dosage studies. In addition, the CNS of flies bearing such mutations would be examined for defects.

325. Identification and characterization of genes encoding receptor-linked protein tyrosine phosphatases expressed in the embryonic \textit{Drosophila} central nervous system

Shin-Shay Tian, Pantelis Tsoufas

The fasciclin I/abl double mutant central nervous system (CNS) phenotype indicates that signal transduction via tyrosine phosphorylation is important for the establishment of axon pathways. The identification of receptor-linked protein tyrosine phosphatases (PTPases) containing immunoglobulin-like (Ig) and fibronectin-like (FN) domains homologous to those of neural adhesion molecules like N-CAM, L1, fasciclin II, and neuroglian suggested that these molecules may function in cell adhesion/recognition events in the nervous system. Two such PTPases, DLAR and DPTP, have already been identified in \textit{Drosophila} (Streuli et al., 1989). We used polymerase chain reaction (PCR) techniques to identify five novel PTPase cDNAs expressed in \textit{Drosophila} embryos 178, 268, 11-1, 153 and 260. Since we were mainly interested in PTPases involved in nervous system development, we performed \textit{in situ} hybridization experiments to identify PTPase mRNAs expressed in the CNS during the period of axon outgrowth. We found that two of the novel PTPases, 178 and 268, as well as the previously identified DLAR, are only expressed in subsets of CNS neurons. Two other PTPases, 11-1 and DPTP, are expressed in the CNS and elsewhere.

Our subsequent experiments have focused on 178 and 268. The longest 178 cDNA clone is 6 kbp, and has a 3.9 kb open reading frame. The extracellular domain has three fibronectin type III (FN) domains, followed by a hydrophobic transmembrane domain linked to two PTPase domains. Several 268 cDNAs were sequenced, and they define a 4.8 kb open reading frame encoding multiple FN repeats linked to a single PTPase domain. We then made antisera against bacterial fusion proteins for 178, 268, and DLAR, and found that all three PTPases are expressed only on CNS axons. 268 is expressed primarily in the anterior commissure and the longitudinal tracts, while the other two molecules appear to be expressed uniformly in all axon tracts.

We have expressed 178 in \textit{Drosophila} S2 tissue culture cells. Cell aggregation experiments will be performed on these lines to determine whether 178 can
mediate cell adhesion. We are also attempting to isolate mutations in the genes encoding 268 and 178.


326. Isolation of a zebrafish LAR protein tyrosine phosphatase (PTPase) cDNA
Arash Bashirullah

The expression of a Drosophila PTPase, DLAR, is restricted to a subset of central nervous system (CNS) neurons (see Abstract No. 325). DLAR is very closely related to a human PTPase known as LAR. DLAR and LAR are 72% identical in amino acid sequence across their cytoplasmic PTPase domains. This suggests that the substrates for these molecules might also be conserved between vertebrates and invertebrates, and that the two molecules might be expressed in related patterns. DLAR is only expressed on CNS axons, and nothing is known about the expression of the human LAR protein. To compare vertebrate and invertebrate expression of LAR proteins, we decided to make antibodies against a zebrafish version of LAR. Zebrafish embryos are clear and can be easily examined by immunohistochemistry on whole-mount preparations. In order to find a LAR homolog in zebrafish, we used degenerate oligonucleotides corresponding to LAR-specific conserved regions within the enzymatic domains as primers for polymerase chain reactions with cDNA from 36 hr zebrafish embryos. We have cloned a fragment of the putative zebrafish LAR which is 96% and 94% identical at the amino acid level to LAR and DLAR, respectively. We are currently screening a zebrafish library from 33 hr embryos to obtain partial N-terminal sequence. We intend to generate antibodies against bacterial fusion proteins in order to localize the zebrafish-LAR protein products within the CNS.

327. Biochemical analysis of fasciclin I-mediated cell adhesion
Te-Yi Kung, Wen-Ching Wang

Fasciclin I is a neuronal surface glycoprotein that can mediate homophilic adhesion when transfected into tissue culture cell lines. To study the biochemical properties of fasciclin I, we have expressed grasshopper and Drosophila fasciclin I at high levels in Chinese hamster ovary (CHO) cells. The purified grasshopper protein has been crystallized (see Abstract No. 205). We would like to perform the same types of experiments with Drosophila fasciclin I, but the current monoclonal antibodies (MAbs) against the protein cannot be used for purification of the native protein. We are thus attempting to generate a MAb against native Drosophila fasciclin I by injecting partially purified phospholipase C-cleaved protein from a fasciclin I-expressing CHO line into mice.

We are also beginning a project to study the biochemical basis of homophilic adhesion by fasciclin I. To determine whether adhesion involves binding to the carbohydrate moiety of fasciclin I, we grew fasciclin I-expressing Drosophila cell lines in the presence of tunicamycin, which blocks glycosylation. The tunicamycin block did not prevent cell surface expression of fasciclin I, and it was complete as judged by the shift in the mobility of the protein on an SDS gel. We assayed aggregation of the tunicamycin-treated cells and found that it was unaffected. This suggests that the adhesion activity of fasciclin I does not require the carbohydrate moiety.

328. Analysis of potential chemosensory mutants
Bruce A. Hamilton, Carol A. Mayeda1, Veronica Rodrigues2

We have previously reported a molecular approach to isolating P-element insertions in cloned genes (Hamilton et al., 1991). Two of the P-element insertions identified in this screen are in genes whose products accumulate predominantly in chemosensory organs. We are pursuing molecular, genetic, and behavioral analyses of each locus.

We have generated several derivative alleles from each of the original insertions by imprecise excisions of the element, which delete sequences flanking the transposon, and by local transpositions of the element, which produces new insertions within several kb of the original site. Preliminary behavioral data from one locus suggest that homozygotes for some hypomorphic alleles have deficits in contact chemoreception of sucrose. Null alleles are homozygous lethal. Long cDNA clones for each locus have been obtained and sequence analysis is in progress.
Reference:

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329. Local transposition of P elements

Bruce A. Hamilton

For several eucaryotic transposons, transposition favors insertion sites near the initial site of the mobile element. Recent observations by several investigators have shown this to be true for the P family of elements in Drosophila. We have begun to examine this phenomenon in more detail, to determine the level and effective range of this enhancement and its sensitivity to position effects. Such information will be useful in designing efficient, directed mutagenesis strategies for Drosophila genes.

One test case for these strategies is a region near the tip of chromosome arm 3R. This region contains several cloned genes whose products are thought to be involved in signal transduction and protein phosphorylation in the central nervous system. Furthermore, this region has been difficult to investigate genetically, in part because the density of Minute and other haplo-abnormal loci in the region inhibit the recovery of simple deficiencies (Kongsuwan et al., 1986). We have obtained P-element insertions in this region from several laboratories and are now using them as starting sites for local transposition mutagenesis.

Reference:

330. Molecular analysis of olfactory signal transduction

Jonathan Bradley, Kai Zinn

The mechanisms involved in olfactory signal transduction are still poorly understood. Although animals are capable of distinguishing very large numbers of different odorants, it is not known how many receptors are involved in odorant detection. In fact, a specific odorant receptor has not been identified in any species. It is also unclear how information about odor identity is organized in the olfactory bulb. This may present an interesting problem in axon guidance, since the olfactory neurons die and are regenerated from stem cells. If there is topographic specificity to their connections to the bulb, one has to explain how the axons of the regenerated neurons can reconnect to the same sites.

There is evidence showing that adenyl cyclase (AC) activation is involved in vertebrate olfactory transduction (Pace et al., 1985). Production of the second messenger requires GTP, arguing that a G protein is involved. An olfactory-specific G protein α-subunit similar to Gαs has been identified (Jones and Reed, 1989), and this molecule is expressed in olfactory cilia, suggesting that it mediates odorant signal transduction. This past year has also seen the cloning and characterization of an olfactory-specific cyclic nucleotide-gated channel (Dhallan et al., 1990) and an olfactory-specific isoform of AC (Bakalyar and Reed, 1990). These data suggest that the olfactory receptors should be G protein-coupled receptors, which have seven transmembrane helices. These seven transmembrane segments contain several conserved sequence blocks. Recently, a very large gene family of closely related olfactory-specific 7-helix receptors has been identified using a polymerase chain reaction (PCR) probe generated by amplification of olfactory epithelial cDNA with oligonucleotides based on 7-helix receptor sequences from transmembrane regions (TM) II and VII from the angiotensin receptor and the lutropin-choriogonadotropin hormone receptor, respectively (Buck and Axel, 1991). Ten complete cDNA sequences and eight partial sequences have been published thus far. Southern blotting data with probes derived from these cDNAs provide direct evidence for 70 genes belonging to the family, and as other combinations of probes are used it is likely that still more genes will be identified. It has not been proven that any of these receptors actually interact with odorants, however.

To examine the roles of these 7-helix receptors in odorant detection, we are constructing a library of the Buck and Axel sequences in a form that can be expressed in Xenopus oocytes. To do this, we first constructed a size-selected cDNA library from rat olfactory epithelial RNA. This library has a complexity of 2.5 million clones and contains many full-length clones for Golf, the olfactory cyclic nucleotide-gated channel, and other
mRNAs. We screened our library at low stringency with PCR probes recognizing the Buck and Axel sequences. Several hundred duplicate positives were observed with these probes. We have sequenced regions of 10 of these clones and found four new 7-helix receptor sequences; the remaining six clones are members of the Buck and Axel set. We are now rescreening the duplicate positives in groups of 50 to make a pool of the phage clones. The pooled phage clones can then be used to generate synthetic receptor mRNA, which will then be injected into oocytes. In collaboration with Al Walter and Yasuhito Uezono in the Lester/N. Davidson laboratory, we are developing assays for odorant receptor function in *Xenopus* oocytes. These assays will then be used to isolate and characterize cDNA clones encoding receptors for particular odorants from among our pool of candidate clones.

References:

331. Identification of Go protein domains involved in receptor and effector interactions

*Kai Zinn, Jonathan Bradley*

In order to develop an assay system for odorant receptors expressed in *Xenopus* oocytes, we have begun a series of experiments in collaboration with Drs. Yasuhito Uezono, Norman Davidson, and Henry Lester to identify receptor and effector interaction domains in Go subunits. This will be interesting in its own right for G protein biology, and may allow us to construct a hybrid G protein that will interact with 7-helix receptors (such as the odorant receptors) that normally couple to adenylyl cyclase (AC) activation, but will instead produce an increase in inositol trisphosphate (IP3) via stimulation of phospholipase C (PLC) activity. The oocyte responds to increases in IP3 by opening Ca2+-activated Cl- channels. Measurement of this Cl- current provides a very sensitive electrophysiological assay for receptor function. Previous studies on hybrid Go proteins expressed in mammalian cells have indicated that the receptor and effector interaction regions are likely to be located in the carboxy-terminal 40% of the protein (about 130 amino acids; Masters et al., 1988). We have made a number of hybrids between Go (which interacts with the serotonin 1C receptor (5HT1C) and couples to PLC), and Gs or Golf, which interact with the β-adrenergic receptor (βAdR) and couple to AC. The hybrid junctions are made at blocks of four or more residues that are highly conserved, suggesting that they have the same secondary structure in all G proteins. We have made the hybrids by a two-step "bridge" polymerase chain reaction (PCR) protocol. Using this protocol, we can generate a hybrid sequence linked to a phage RNA polymerase promoter in two days, and no cloning steps are necessary. The PCR-generated DNA template is then transcribed and the RNA injected into oocytes together with synthetic 5HT1C and/or βAdR mRNA. After several days, receptor function is assayed by adding ligand and measuring Cl- current and/or cAMP levels. Our results thus far suggest that the receptor and effector interaction domains of Goα are located between amino acids 206 and 326. We are currently making and testing other hybrids to further localize these elements.

Reference:

**PUBLICATION**

INTEGRATIVE NEUROBIOLOGY

John M. Allman
James M. Bower
John J. Hopfield
Bela Julesz
Christof Koch
Masakazu Konishi
Gilles J. Laurent
Marianne E. Olds
Roger W. Sperry
David E. Van Essen
Summary: We have begun to study with microelectrode, magnetic resonance and optical recording techniques the organization of neocortex in Monodelphis, a South American marsupial that has retained many features of primitive mammals living 100 million years ago. Monodelphis infants are born at a very early stage before the neocortex has begun to develop (see Figure 1). Thus, we believe this animal has great potential for the study of the formation of cortical areas. We also have continued our microelectrode mapping of the visual cortex in the owl monkey, and in collaboration with Steve Zucker from McGill University have constructed a model for the functions of the cytochrome oxidase blobs in primate striate cortex.

332. Physiology and functional organization of primitive neocortex
Mark O’Dell, John M. Allman

To learn about the neocortex of primordial mammals, we have been studying the physiology and functional organization of this structure in Monodelphis domesticus, a didelphid marsupial that has retained many features of primitive mammals living 100 million years ago. This work includes microelectrode receptive field mapping, intrinsic signal optical imaging and histology. Our electrode mapping experiments have begun to show the ways in which visual, auditory and somatosensory maps are represented in Monodelphis cortex. Our imaging experiments have shown that we can carry out similar mapping studies optically. They have also shown what appear to be endogenous metabolic rhythms, which persist even in quiescent unstimulated animals (see Figure 2). These variations appear to be due to constant switching or shunting of blood flow. Although blood vessels occasionally move slightly, these effects are unrelated to such motion. This evidence appears to be indicative of ongoing modulation of energy.

333. Retinotopic organization of dorsal extrastriate cortex in the owl monkey
Martin I. Sereno, Colin T. McDonald, Mark O’Dell, John M. Allman

The dorsally located representation of the lower visual field in primate V2 is adjoined by a number of visual areas. Allman and Kaas (1975) originally described a myelinated dorsomedial area (DM) in the owl monkeys adjoining the dorsal part of the anterior V2 border that contained both upper and lower fields. The upper field representation made an incongruent border with dorsal, lower field V2 (as occurs, for example, between upper field LM and lower field VI in rats). Other unpublished studies have suggested that there were additional areas directly adjoining V2 between DM and the center of gaze representation in V2. We remapped these areas in great detail (500-600 penetrations per hemisphere; inter­penetration spacing of 200 µ) in several animals, and then stained the cortex for myelin after physically flattening it.

We confirmed the overall organization of DM originally reported (lower fields medial, upper fields lateral). There is a mushroom-shaped region of myelination (with its "root" at the V1 border corresponding to "DX" in Tootell et al., 1985) within which the electrophysiological DM lies. The dorsomedially located part of the cap of the mushroom is a retinotopic area anterior to lower field DM that contains a representation of both lower and upper fields. Anterior to the more laterally located upper field representation in DM is a retinotopically organized, lightly myelinated area containing mostly upper fields. Moving directly lateral to DM, we confirmed the existence of an upper field representation, area DI, in a lightly myelinated region that is distinct from the upper field representation in DM. More laterally, there is a transitional zone of lower fields that is not part of DI (it has the opposite inversion of the visual field). Even further laterally are the peripheral parts of the lower field.
representation of three areas: DLp, DLi and DLa. Moving anterior to the periphery of those areas, there is a small moderately myelinated, retinotopically organized area, TP, containing both lower and upper fields. It is situated just medial to DLa (which adjoins MT and has the greatest anterior extent of the three DL areas). Thus, much of the parietal cortex in the owl monkey appears to be retinotopically organized.

References:

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Figure 1. A newborn *Monodelphis* infant. The scale is in millimeters.

Figure 2. Several of an ensemble of difference images, formed by subtracting two pictures of the brain surface while the animal was quiescent and unstimulated. These images come from an eutherian mammal (primate), *Galago senegalensis*, which we tested to confirm that these effects are not unique to primitive mammals. Similar results were found in *Monodelphis*. Extremes of white and black correspond to intensity differences of ±2% of the maximum dynamic range of the camera. The region shown is a 2 x 4 mm region of visual cortex, viewed through the intact dura. Note also the changes in average intensity of the whole field (the ensemble appears to oscillate over roughly the same ±2% intensity limits). The gray region on the right is a charge readout artifact of the ccd camera.

334. Cytochrome oxidase and functional coding in primate striate cortex: A hypothesis
*John M. Allman, Steven Zucker*

In 1978, Margaret Wong-Riley stained sections of squirrel monkey striate cortex for the activity of the mitochondrial enzyme cytochrome oxidase, and noticed a periodic distribution of blobs of increased enzyme activity in layers 2 and 3. Her discovery anticipated a whole series of anatomical and physiological findings from many laboratories that correlated with the distribution of this enzyme in the striate cortex of primates, yet there has
never been a satisfactory explanation as to why the
distribution of this enzyme, crucial for aerobic energy
metabolism, would be related to the functional
organization of visual cortex.

When the striate cortex is viewed from above, the
blobs form a periodic array intercalated within a lattice of
lower cytochrome oxidase concentration. We propose that
the distinction between the blobs and the lattice is related to
two different modes for representing stimulus variables.
We submit that scalar variables related to the intensity of
the stimulus are represented in the blobs. Intensity
information is encoded explicitly over a very broad
dynamic range in which activity is proportional to the
intensity variable (e.g., contrast). This encoding strategy
requires that neurons have the energetic capacity to sustain
a broad range of activity levels, which in turn is related to
the high concentration of cytochrome oxidase. The
situation is analogous to red muscle, which also is rich in
cytochrome oxidase and which is able to maintain a
sustained level of contraction over time.

We propose that in the surrounding lattice of lower
cytochrome oxidase concentration, geometric variables are
carried by neurons with orientation preference and lead to
different representational requirements. Each stimulus
orientation is possible at every retinotopic location, and
each is represented explicitly within an orientation hyper-
column. Activity varies with how well each individual orientation matches image structure at that location.
However, there is rarely more than one orientation at any
retinotopic location, so, on average, most oriented cells in
each hypercolumn are quiet. The preference of these
neurons for higher spatial frequencies (and possibly
binocular disparities) further reduces the statistical
probability that they will be active at any particular instant
in time. The average level of neural activity over time is
thus much less in the lattice than in the blobs, which is
consistent with the lower levels of cytochrome oxidase in
the lattice. There is an analogy with white muscle, which
contains less cytochrome oxidase and typically has short
bouts of rapid contraction interspersed with longer resting
periods.

PUBLICATIONS
Cortex, Vol 8A, E. G. Jones and A. Peters (Eds.),
Plenum Publishing Corp., New York, NY, pp. 269-
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247.

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Summary: The overall research effort in the 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 which is believed to be involved in the classification of olfactory stimuli, and the cerebellar cortex which is involved in sensory/motor control. In both cases, the research effort of the laboratory involves the application of a wide range of interrelated experimental techniques.

The central focus of the laboratory's experimental approach continues to be the construction and simulation of computer models based on the known anatomical and physiological structure of these networks. We continue to work with models of the inferior olive, the olfactory bulb, and the olfactory cortex. In addition, in the last year we have also undertaken a more abstract modeling effort based loosely on the organization of the olfactory cortex in an effort to interpret new experimental data demonstrating differences in the neurohumoral modulation of different synapses in the olfactory cortex. As described in this report, the results suggest a possible connection between the process of memory storage and the regulation of synaptic function. We have also undertaken the building of a detailed model of a single cerebellar Purkinje cell.

Experimentally, we have completed several studies in the last year including a two-year study of the effects of peripheral lesions on peripheral map formation in cerebellar cortex. The results suggest that reorganization in somatosensory cortex plays an important role in the reorganization of cerebellar maps. We have also continued our behavioral studies in olfacting rats, extending our abilities to record from many neurons in the olfactory bulb simultaneously. Our behavioral studies of weakly electric fish also continue apace. Recently, we have extended our behavioral experiments to include studies of the way in which rats explore objects with their perioral structures. We anticipate that these experiments will shed light on the functional significance of the tactile maps found in the cerebellar cortex.

With respect to biotechnology, our efforts to develop silicon electrodes, multichannel amplifiers, multichannel discriminators, and data collection software for large scale multi-single-neuron recording are now nearly complete. We have also just participated in the dedication of the Intel Delta Touchstone parallel supercomputer for which our laboratory will be a major user. GENESIS is now being ported to this machine which is, at present, the most powerful non-biological computer in the world.

Finally, our effort to improve elementary school science education through PROJECT SEED has continued to grow stronger and is now receiving considerable national attention.

335. Prolonged simple spike responses to activation of the granule cell layer in the rat cerebellum

*John H. Thompson, James M. Bower*

There are two input pathways to the cerebellar cortex: the mossy fiber projection from multiple sources in the CNS, and the climbing fiber projection from the inferior olive.

For the last several years we have been using our knowledge of the detailed pattern of tactile mossy fiber projections to the granule cell layers of Crus IIA of the cerebellum to explore the physiological relationships between granule cells and Purkinje cells. Specifically, using *in vivo* extracellular recording techniques, we have monitored Purkinje cell activity in Crus IIA of the rat while applying very brief (5 ms) tactile stimuli to perioral regions known to project to underlying or nearby granule cell layer locations. Our results have shown that Purkinje cell responses to these brief peripheral stimuli do consist of a short latency component (5-8 msec) but that there can also be produced a prolonged (100-400 msec) increase in simple spike activity. By discriminating between simple and complex Purkinje cell responses, we have been able to show that climbing fibers, which originate in the inferior olive, are also activated by these stimuli at latencies of around 50 msecs (see Abstract No. 337). However, when those trials in which climbing fiber responses do occur (about 1 in 5) are separated from those that have
only simple spike responses, the prolonged simple spike response is still present. This result suggests that the mossy fiber/granule cell system is capable of generating prolonged changes in Purkinje cell activity in response to natural tactile stimulation.

We plan to explore the computational implications of this result by using more complex tactile stimuli and by recording from a number of Purkinje cells simultaneously.

336. Plateau potentials following synaptic stimulation in intracellular Purkinje cell recordings in vitro

Dieter Jaeger, James M. Bower

Single neurons are capable of integrating input information in a complex non-linear fashion. Each neural type may realize a different algorithm of input-output processing, which depends on the geometry of the dendritic tree as well as the distribution of synaptic inputs and specific ionic channels. One particular mechanism of interaction between inputs consists of one input resulting in a short period of membrane depolarization (a plateau) that alters the excitability of the neuron for subsequent inputs. In the cerebellum, the Purkinje cell integrates several input streams and provides the only source of output from the cerebellar cortex. Plateau potentials have been shown to exist in Purkinje cells following direct electrical activation of parallel fiber synapses (Campbell et al., 1983). In our own previous work, we have also demonstrated prolonged changes in simple spike firing rates following natural peripheral activation of the mossy fiber/granule cell system (Bower and Woolston, 1983).

The purpose of our present research project is to explore the mechanisms responsible for generating plateau potentials in Purkinje cells. In particular, we are interested in determining the spatial and temporal characteristics of the plateaus and their effects on subsequent inputs. To do this, we use intracellular recording techniques in the guinea pig slice preparation using 350 µm slices cut parasagitally. Intracellular recordings were obtained with micropipettes within 12 hours after slicing. Stimulating electrodes were placed in the white matter, the granule cell layer and the molecular layer to separate the synaptic effects of climbing fiber and granule cell axon activation. Recordings were obtained from Purkinje cell somata and dendrites.

In the Purkinje cell soma, plateau potentials were seen following granule cell axon activation, with or without concomitant climbing fiber activation. These plateaus last between 100-400 ms and have an amplitude that increases with increasing stimulus intensity. When a plateau was triggered at a membrane potential just below firing threshold, a brief train of somatic spikes rides on top of the plateau. This indicates that plateau potentials can serve as a mechanism to translate a single input pulse to an extended sequence of output pulses. Trains of stimulus pulses (inter-stimulus interval of 10-100 ms) resulted in a temporal summation of plateau potentials leading to a somatic activation even from a hyperpolarized membrane potential. This indicates that plateau potentials can serve as a mechanism to potentiate the effect of successive synaptic inputs. Plateau potentials are also observed in dendritic recordings and also trigger somatic spikes. This indicates that dendritic plateaus are continuous with those recorded at a somatic level. However, a local dendritic role of plateaus in coupling different inputs remains a possibility. Continuing work is intended to determine the specific site(s) of plateau generation and the underlying ionic mechanisms.

References:

337. The organization of the olivocerebellar system

Maurice Y.-T. Lee, James M. Bower

We are continuing our studies of the organization of the climbing fiber (olivocerebellar) projection in the rat, a projection that arises exclusively from the inferior olivary (IO) nucleus and terminates in the cerebellar cortex. Our emphasis is on the integrated use of computer modeling and electrophysiological mapping techniques.

Electrophysiology. Our studies of climbing fiber (CF) activity in the rat cerebellum have concentrated on aspects of the temporal and spatial organization of responses to tactile stimulation. We have found that CF responses are sparse, occurring only about one-fifth of the time for regular (1 Hz) punctate stimulation. In addition, CF responses occur at latencies longer than those of granule cell (GC) multunit responses to the same stimuli: CF
Response latencies are around 50 ms, as compared to 2-5 ms for GC responses; the CF response often involves a rebound spike at around 150 ms. These CF response latencies are on the same order as those of GC-mediated increases in Purkinje cell simple spike activity in response to tactile stimulation (see Abstract No. 335). In addition, CFs with similar tactile receptive fields seem to be arranged in a "patchy mosaic" similar to, and roughly in register with, that characteristic of the underlying map of GC receptive fields (Bower and Kassel, 1990). Thus, CFs with non-contiguous receptive fields can be located close to each other in the cerebellar cortex. However, CF receptive fields are large, typically several times larger than GC receptive fields, and bilateral, even when receptive fields in the underlying GC layer are unilateral. For example, in the center of folium Crus Ila of the ansiform lobule, GCs have receptive fields on the ipsilateral upper lip, but CFs have receptive fields covering both sides of the upper lip.

**Modeling.** We are using the neural simulation package GENESIS to develop structurally accurate models of the inferior olive and of the olivocerebellar projection, towards the goal of a better functional and computational understanding of this system. At the neuronal level, we are studying the effects of microanatomy (for example, the arrangement of spines on the dendritic tree) and of electrophysiology (for example, the kinetics and distribution of various membrane ionic conductances) on cellular dynamics. It has been proposed that active conductances exist in both somatic and dendritic locations in IO neurons, and that neighboring neurons are electronically coupled through gap junctions. To what extent can these mechanisms account for the coherent subthreshold oscillations of membrane potential and the correlated periodicity of spike activity observed in groups of these neurons (see Llinás and Yarom, 1981 and 1986 for electrophysiological data). At the network level, we are looking at the dynamics of known features of the temporal and spatial organization of the olivocerebellar projection as they influence both spontaneous activity and activity in response to input.

**References:**

This research was supported by grants from the National Institute of Neurological Disorders and Stroke (NS 10642 and NS 29067). We are using the neural simulation package GENESIS to develop structurally accurate models of the inferior olive and of the olivocerebellar projection, towards the goal of a better functional and computational understanding of this system. At the neuronal level, we are studying the effects of microanatomy (for example, the arrangement of spines on the dendritic tree) and of electrophysiology (for example, the kinetics and distribution of various membrane ionic conductances) on cellular dynamics. It has been proposed that active conductances exist in both somatic and dendritic locations in IO neurons, and that neighboring neurons are electronically coupled through gap junctions. To what extent can these mechanisms account for the coherent subthreshold oscillations of membrane potential and the correlated periodicity of spike activity observed in groups of these neurons (see Llinás and Yarom, 1981 and 1986 for electrophysiological data). At the network level, we are looking at the dynamics of known features of the temporal and spatial organization of the olivocerebellar projection as they influence both spontaneous activity and activity in response to input.

**References:**

**338. Temporal relationships between cerebral cortical and cerebellar responses to tactile stimulation in the rat**

Josée Morissette, Leila M. Posakony, Caroly A. Shumway, James M. Bower

Our laboratory has been interested for some time in the circuitry associated with the tactile regions of the cerebellar cortex. In this series of experiments, we have attempted to identify the circuits responsible for the different components of the granule cell layer responses to peripheral tactile stimulation. Cerebellar granule cell responses to brief (50 msec) tactile stimuli consist of an initial component with a short latency (8-10 msec) and a second component with a more variable and longer latency (16-23 msec). We have recently shown that the longer latency component of this response is much more resilient to neonatal peripheral lesions than is the short latency component (Morissette et al., 1990). Accordingly, we were interested in determining the pathway responsible for this longer latency cerebellar response.

Several different experimental techniques were used to demonstrate that the long latency granule cell layer response component is dependent on a pathway involving the somatosensory (SI) cortex. Lidocaine injections into SI, local SI ablations, and complete midcollicular sections all substantially interfere with the second cerebellar response without having any effect on the short latency response. Further, when tactile-evoked responses were simultaneously recorded in both the cerebellar granule cell layer and layer IV of SI cortex, the latencies of both responses were strongly correlated. Intraperitoneal injections of sodium pentobarbital during these recordings produced a highly correlated increase in latency in both the SI and the second component of the cerebellar responses. These results suggest that the topologically organized SI projection to the cerebellum (Bower et al., 1981) influences cerebellar cortex at late and variable latencies.

**References:**
339. Rat cerebellar granule and Purkinje cell activity in a behavioral context
Mitra J. Hartmann, John H. Thompson, James M. Bower

A large body of evidence from physiological, anatomical, and behavioral studies supports the notion that the cerebellum is involved in the optimization of sensory acquisition. That is, as an animal actively explores its environment, the cerebellum helps coordinate the use of sensory structures so that the highest quality sensory information is continuously obtained by the rest of the nervous system. It is thought to do this by monitoring the acquisition of sensory information and adjusting motor performance accordingly.

To test this idea and to monitor the neural activity of the cerebellum in a behavioral context, we have begun a series of experiments that will coordinate neuronal signals obtained from chronically-implanted electrodes in the tactile regions of rat cerebellar cortex with video images of a variety of behaviors involving the perioral regions. Of primary interest will be feeding and grooming sequences.

The perioral surfaces have a very large representation in the rat cerebellum. Our observations indicate that as a rat feeds, there are complex manipulations of the food in its mouth. We believe that this behavior allows the rat to discriminate foods and to determine which surface to bite. We plan to have the rat discriminate between different textured foods using exclusively tactile cues.

This work compliments the electric fish behavioral work in our laboratory as well as the in vivo acute cerebellar experiments.

340. Self-generated electric fields and electrolocation in weakly electric fish
Christopher Assad, Brian Rasnow, James M. Bower

Two different orders of teleost fish have separately evolved the ability to use self-generated electric fields to detect objects in their environment, a novel sensory modality called electrolocation. Weakly electric fish are also unusual in the animal kingdom at large because of their greatly enlarged cerebellar cortex. We are investigating the electric sensory-motor system in these fish as part of the laboratory’s research of the role of the cerebellum in exploratory behavior.

As a basis for these studies, we have measured the fields produced by the electric organ discharge (EOD) for several stationary specimens of Apteronotus leptorhynchus and Gnathonemus petersii. These field mappings have sufficient resolution in both space and time to enable the quantification of sensory input (i.e., the electroreceptive “images” that the fish “sees”). Pseudo-color maps were constructed and can now be displayed in the form of animated movies, which clearly illustrate the spatiotemporal structure of the EOD potential in the water about the fish. Other species such as Eigenmannia virescens are also being mapped for comparison purposes. Further field mappings are under way to study consequences of body position and orientation, and to quantify the effects of objects near the fish. Initial results suggest the fish’s body position has a profound effect on these images. In the brain, positional effects are likely to be compensated for either by motor control or computational strategies in the sensory system.

In order to study natural exploratory strategies, we are conducting behavioral experiments in which the fish is allowed to explore and behave freely, while its movements are recorded. Then numerical techniques (finite element and boundary element methods) are used for field simulations to reconstruct the images detected by the fish's electroreceptors, found on the skin. In order for the models to prove accurate, we are characterizing the electrical properties of the fish's body and environment, and using the field mappings to test and calibrate the simulations. The final step will be to convolve the images derived from the simulations with the transfer function of the electroreceptor array, resulting in an accurate representation of the information available to the brain.

Our goal is to quantitatively simulate various sequences of electroreceptive input being sent to the central nervous system as the result of the fish’s exploratory behavior. The use of these images as input to a model of the electroreceptive processing structures may enable us to predict and understand the response characteristics of neurons in the fish’s cerebellum. Study of the system as a whole is expected to provide insight into the fish’s active sensing of its environment and may also help illuminate the functional role of the cerebellum.
341. Fractured tactile maps retain fractured somatotopy following deafferentation at different stages of development
Leila M. Posakony, José Morissette, Caroly A. Shumway, James M. Bower

Tactile projections to the rat cerebellar hemispheres are characterized by a series of patches representing different regions of the body surface (Bower and Kassel, 1990). In the current experiments, we deprived the large central upper lip patch found in Crus Ila of its normal source of peripheral innervation by cauterizing the infraorbital branch of the trigeminal nerve. Rats were deafferentated between postnatal day 2 and 30, and the resulting reorganization of tactile inputs to the granule cell layer was determined using high density physiological mapping procedures performed 2 to 3 months later.

Results indicate that these cerebellar maps reorganize regardless of the postnatal day of the lesion. In each case, the map maintains an overall patchy organization with a representation of the upper incisor consistently invading most of the denervated region. In normal maps of Crus Ila, the upper incisor has both the smallest and most variable representation. While mapping results indicated a complete fill in of denervated regions, we did find that lesions differently affected the components of the granule cell response. In normal animals, tactile evoked responses in this layer always consist of short (5-8 ms) and long (15-20 ms) latency components. In deafferented animals, some recording sites show only the long-latency component. The number of these recording sites goes up as lesions are made later in development (3% at birth to 22% at 30 days), suggesting some differences in the ability of cerebellar projecting circuits to reorganize. In Abstract No. 338, the long latency response is shown to be associated with a circuit involving the somatosensory cortex.

Reference:

342. Mammalian olfactory processing
Upinder S. Bhalla, James M. Bower

A major effort of our laboratory involves an attempt to understand structure/function relationships in the mammalian olfactory system. Much of this work has involved efforts to model the olfactory, or piriform cortex. Recently, however, we have also begun to build models and perform experiments on the olfactory bulb which receives direct projections from peripheral olfactory receptors and then provides a major source of afferent input to the piriform cortex. Our approach to studying the olfactory bulb also involves using both experimental and theoretical techniques.

Our experimental work involves recording from multiple bulbar neurons while rats perform several well-defined olfactory discrimination tasks. This protocol gives us information about the activity of neurons in the olfactory bulb while the system is actively engaged in olfactory processing. These recordings are based on a multi-channel microdrive that we have developed over the past few years. This device weighs less than 6 grams and allows 8 stereo electrodes to be independently positioned. It also has a thermistor-based respiration monitor. Implanted rats are exposed to odors in two contexts: passive and behavioral. The passive situation consists of the rat being placed in an air stream with odorants added at defined times. The behavioral paradigm requires the rat to identify an odor and, based on the odor, push one of two paddles for a water reward.

Using these procedures, we are investigating temporal and spatial patterns in bulbar neuron responses and their relationship to the behavioral context. We have found that the most common form of response is not a simple change in firing rate depending on odor quality, but rather a complex change in the phase relationships of neuronal activity with respect to both respiration and the EEG. We are extending this study to explore the evolution of neuronal response properties as the animal learns to perform the olfactory discrimination task.

The theoretical work has several tasks, all based on detailed simulations of the olfactory bulb. These are performed using GENESIS, the neural network simulator developed in our laboratory. Simulations of the neuronal types present in the olfactory bulb have resulted in several predictions about the channel distributions, and suggest experiments to verify these hypotheses. One such prediction is that voltage clamping at the soma of mitral cells should result in the effective electrical decoupling of the dendrites. Given the channel distributions derived from our simulations, this will result in independent
spiking of each dendritic branch at holding potentials around threshold. This effect has indeed been recently observed by Wellis et al. (personal communication).

These detailed single neuron models form the basis for much larger simulations of portions of the olfactory bulb which are meant to embody the network properties of the system. The objective at this stage is to derive synaptic parameters based on the body of experimental data available for electrical stimulation of the olfactory bulb. The bulb has an unusual arrangement of extensive dendrodendritic synapses between its major cell types, which necessitates great attention to details of geometrical and electrical properties of the constituent neurons in the model. Preliminary results indicate that the presence of active channels in the dendrites, as predicted by the single neuron simulations, has profound effects on the efficacy of synaptic transmission across these dendrodendritic synapses. As measured at the soma, the difference in equivalent synaptic strength can be as much as tenfold. With the conclusion of the work on synaptic strengths, we plan to incorporate this information into a functional model of the bulb as a whole. This will provide us with a way of directly comparing our simulated results on patterns of neuronal activity with the experimental results described above.

One of the ultimate objectives of GENESIS is to provide a common base for communication within computational neurobiology. Accordingly, all of our neural models, as well as the GENESIS simulation system itself, are publicly available on the academic networks. Our intention is that they should form the foundation for "standard models" for these neuronal systems, accessible to all researchers. We believe that this will facilitate the availability, integration and interpretation of the increasingly extensive amounts of neurobiological data now available. Using GENESIS, models can be continually updated to reflect advances in the field, and can therefore serve as a basis for further experimental as well as theoretical work.

343. Further development of the GENESIS system for neuronal simulations

John Uhley, David H. Bilitch, Upinder S. Bhalla, Michael D. Speight, James M. Bower

For the last several years, our laboratory has been involved in the development of a General NEural Simulation System (called GENESIS) which is intended to provide a powerful software platform for the development of realistic simulations of biological neural circuits. Last year, GENESIS was made available to the larger research community over computer networks. As a result, there are also now more than 550 sites around the world who are licensed to use the system. Forty sites are officially members of the GENESIS users group BABEL. In addition to building the users network, over the past year we have also extended and enhanced the simulator in several ways. One of the most important is the development of improved integration methods that enhance both the accuracy and the speed of the simulations.

344. Silicon-based probes for multichannel recording in cerebellar cortex

Jasho J. Banik, John H. Thompson, James M. Bower

We have been developing a new silicon-based recording probe which is designed to record extracellular potentials from a large number of individually isolated neurons simultaneously (Bower et al., 1988). The silicon probe fabrication process uses photolithography and anisotropic etching techniques to shape the silicon substrate into a structure that supports a high density of recording sites while maintaining physical dimensions comparable to standard glass or metal electrodes. Present designs incorporate from 5 to 15 recording sites per probe, although larger numbers of sites can be supported. We have recently completed construction of a computer-controlled amplifier and window discriminator system that allows us to process data from up to 100 channels simultaneously.

For our cerebellar studies, we position the recording sites during fabrication to optimize for simultaneously recording climbing fiber responses in the molecular layer, simple spike responses in the Purkinje cell layer, and granule cell responses in the underlying granule cell layer. We are using these probes in Crus Ila of the rat cerebellar cortex to record spontaneous activity and responses to tactile stimulation of the face. This work is part of our ongoing effort to understand the processing and representation of information in the cerebellar cortex, and the possible functional significance of the observed fractured somatotopy of the mossy fiber inputs.
We are currently designing and testing CMOS circuitry for on-probe preamplification and multiplexing of neural signals. This miniaturization of the front-end electronics will make these probes ideal for chronic implantation, allowing us to record activity from multiple single neurons in awake, behaving animals. We are also developing VLSI circuitry for real-time classification of neural waveforms using a powerful template-matching algorithm that is capable of recognizing and classifying overlapping waveforms (Wong et al., 1988). This technique could conceivably double or triple the number of individual neurons that can be reliably isolated and identified using these probes.

References:

345. Porting of the GENESIS neural simulation system to a parallel computer
Michael D. Speiglt, James M. Bower
The GENESIS neural simulation system is a general purpose tool for neurobiologists interested in biologically realistic modelling. One of the major factors limiting the realism and complexity of such models is the high computational demand that such a simulation entails. In order to greatly expand the number of systems that we can accurately model, we are producing a version of the simulator that will run on a parallel supercomputer (initially the Intel Touchstone Delta). Unlike conventional computers, a parallel computer can work on different aspects of the simulation at the same time. The Delta system, for example, has more than 500 independently processing units. Each of these can work independently on a different aspect of the simulation until it needs to send or receive information to one of the other nodes. Using this simulator on the supercomputer at Caltech will allow our neurobiologists to produce models of neural systems more realistic than any produced so far.

Using this system, we intend to produce both detailed single cell models and multi-layer network simulations composed of many neurons. The detailed single cell models take account of such features as neuronal branching pattern, membrane channel kinetics and intracellular calcium dynamics. The network simulations model multi-layered neural structures composed of several different classes of neurons.

By seeing how well the computer simulations mimic the biological behavior, and what that behavior depends upon, it allows us to better understand the method of functioning used by the organism. Furthermore, predictions from such models will suggest new experiments to perform in order to distinguish between competing theories of neural functioning.

346. Elementary school science: PROJECT SEED
James M. Bower, Jerry Pine, Jennifer Yure, Leila G. Posakony
In addition to their scientific interests, various members of the laboratory continue to be involved in a large elementary school science project started five years ago by Profs. Jerry Pine and James Bower. The program has grown to include collaborators at the University of Southern California, The University of California at Los Angeles, The University of California at Santa Barbara, and TRW Corporation. Project SEED now includes a number of different efforts ranging from the implementation of a "hands-on" science instruction program in the Pasadena Unified School District, to the development of new approaches to the use of microcomputers in elementary school classrooms. In the latter category, efforts now include: the use of simulation techniques to extend student experience with experimentation beyond that practical in the classroom; the use of computers as a means of testing science process skills and as a way of replacing standard facts-based scientific testing procedures; the automation of elementary school libraries and the access of children to information; and the use of computers for language and graphics arts. The Pasadena Unified School District has declared PROJECT SEED to be the science curriculum for the district and there are now 120 local teachers who have been trained at Caltech to use these materials.
PUBLICATIONS


Roscoe G. Dickinson Professor of Chemistry and Biology: John J. Hopfield

Research Fellows: Joseph D. Bryngelson, Ron S. Meir, Hans L. Liljenström

Graduate Students: Brooke P. Anderson, Dawei Dong, David J. C. MacKay

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3Division of Physics, Mathematics and Astronomy, California Institute of Technology.

Summary: The three areas in which we work are the theory of computational neurobiology, artificial neural networks, and the physics of biological molecules. In computational neurobiology, models are constructed of systems of stylized neurons, attempting to keep the aspects essential for the biological computation done, but omitting as much irrelevant detail as possible. This permits analytical mathematics to be used as a tool for studying the system.

Simulations are also done to further understand the computational dynamics. In artificial neural networks, attempts are made to solve computational problems of the sort done well by animal brains by using networks of neuron-like elements which compute in much the same way that neurobiology is believed to compute. Such elements can also be made in silicon-based hardware. Mathematical approaches based on information theory, statistical mechanics, and Bayesean ideas are particularly useful in analysis. Research has focused on structure-function relations in the chemical dynamics of biomolecular reactions and, most recently, on using neural network approaches to predicting the folding pattern of proteins.

347. Olfactory computation and olfactory object perception

John J. Hopfield

Animals that are primarily dependent on olfaction must obtain a description of the spatial location and the individual odor quality of environmental odor sources through olfaction alone. In a natural environment, a mixture of odors from many distant sources arrives at the nose. However, the variable nature of turbulent air flow makes the relative strength of the contribution of different odor sources constantly fluctuate a function of the time. The remote sensing problem of separating the odors of multiple objects can be solved if the animal can make use of the information conveyed by the fluctuation with time of the mixture of odor sources. Behavioral evidence suggests that such analysis takes place. An adaptive network can solve the essential problem, isolating the quality and intensity of the components within a mixture of several individual unknown odor sources. The network structure is an idealization of olfactory bulb circuitry. The dynamics of synapse change is essential to the computation. The synaptic variables themselves contain information needed by higher processing centers.
The use of the same axons to convey intensity information and quality information requires time-coding of information. Covariation defines an individual odor source (object), and this may have a parallel in vision.

348. Computer model of cholinergic modulation in piriform cortex

*Michael E. Hasselmo, Brooke P. Anderson*

Hasselmo and Bower found that carbachol (an analog of acetylcholine) differentially affects synapses from the intrinsic and afferent fiber systems in the piriform cortex. The intrinsic fibers (seen in layers Ib and III) are those axons and terminal fibers that originate from excitatory cells within the cortex, while the afferent fibers (seen in layer Ia) are from excitatory cells outside the cortex. Specifically, carbachol strongly inhibits intrinsic synaptic transmission while leaving afferent synaptic transmission relatively unchanged. A simplified computer model of the piriform cortex shows that the presence of acetylcholine during learning increases the recall performance of the cortex. The model suggests that acetylcholine may be useful in modulating transitions between learning and recall.

349. Modeling the olfactory cortex and its oscillatory activity with a neural network of intermediate complexity

*Hans L. Liljenström*

There has been a revived interest in cortical oscillations since the recent reports of 40 Hz membrane potential oscillations in visual (striate) cortical areas (Gray et al., 1989). Such oscillations have long been known to exist in olfactory cortex (Freeman, 1975), but their significance has not yet been ascertained.

Apart from its well-established oscillations, olfactory cortex is an ideal model system for the study of associative memory. In particular, its structure is relatively simple in comparison with, for example, the visual cortex, and it shares many features with artificial neural networks modeling this kind of memory, also known as content addressable memory (CAM).

We have constructed a three-dimensional computer model of the olfactory cortex for the study of cortical oscillations and their biological significance. The complexity of the model is intermediate between that of models by Li and Hopfield (1989) and by Wilson and Bower (1989). The basic neural units have the same properties as in the former model, e.g., with continuous sigmoid output functions, whereas the connections to a large extent correspond to those in the latter model, which is based on physiological and anatomical data. Asymmetric connections between the neurons are both short and long range and are modeled with distance-dependent delays. The architecture of the network is based on cortical "basic circuits," connected in such a way that a middle layer of excitatory (pyramidal) cells connects to two layers of inhibitory (interneuron) cells above and below it. Intra-layer connections only exist between excitatory cells.

The response of the system to different input signals, corresponding to the output from olfactory bulb, is investigated and the results are compared with physiological data. In particular, the model is able to reproduce the response pattern associated with a continuous arbitrary input signal and with a shock pulse given to the cortex.

References:

350. A practical Bayesian framework for backprop networks

*David J. C. MacKay*

A quantitative and practical Bayesian framework is described for learning of mappings in feedforward networks. The framework makes possible: 1) objective comparisons between solutions using alternative network architectures; 2) objective stopping rules for deletion of weights; 3) objective choice of magnitude and type of weight decay terms or additive regularizers (for penalizing large weights, etc.); 4) a measure of the effective number of well-determined parameters in a model; 5) quantified estimates of the error bars on network parameters and on network output; and 6) objective comparisons with alternative learning and interpolation models such as
splines and radial basis functions. The Bayesian "evidence" automatically embodies "Occam's razor," penalizing over-flexible and over-complex architectures. The Bayesian approach helps detect poor underlying assumptions in learning models. For learning models well-matched to a problem, a good correlation between generalization ability and the Bayesian evidence is obtained.

PUBLICATIONS


Visiting Professor: Bela Julesz
Research Fellow: Jochen Braun
Research and Laboratory Staff: Peiyuan Huang

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Fairchild Scholars Foundation
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Summary: Our group is interested in the processing of information by the human visual system. Psychophysical experiments over 32 years at Bell Laboratories and in the last three years both at the Laboratory of Vision Research at Rutgers and at Caltech are conducted to study early human vision using computer-generated stimuli. Investigations cover three main topics: 1) the study of binocular depth and monocular movement perception using random-dot stereograms and cinematograms; 2) the development of a theory of preattentive texture discrimination, using textures of constrained stochastic properties; and 3) studies of focal attention.

While most of these studies are done psychophysically in our group at Caltech and at the Laboratory of Vision Research at Rutgers, some of the texture discrimination work extends to the searching for texture gradient detectors in various cortical areas of the monkey in collaboration with Prof. David Van Essen's group.

351. Nonattentive vision

Jochen Braun, Bela Julesz

Visual attention is generally thought to be spatially exclusive and the metaphor of a "spotlight of attention" is often used. This immediately raises the question as to what types of visual information are discriminable outside the aperture of visual attention, in other words, as to what can be seen "extra-" or "pre-" attentively. This question can be addressed with the aid of a suitable visual task which directs visual attention to a small part of the field of view and the performance of which consequently results in the complete or partial withdrawal of visual attention from other parts of the visual field. Using this approach, one finds (Braun and Sagi, 1990) that several types of textural borders and singularities are registered normally even while attention is directed at a distant location, in agreement with the theory (Julesz 1981, 1986) that image texture is processed preattentively.

In an attempt to further explore the range and extent of preattentive visual performance, we are investigating the discrimination of color and, separately, of texturally defined shapes while visual attention is focused at a different region of the field of view. So far, our results suggest that the withdrawal of visual attention does not materially interfere with color discrimination even when the color of multiple well-separated targets is at issue. If the sensation of color does indeed arise without the intervention of visual attention, we would have to modify our working hypothesis according to which preattentive processing makes available information about "where" but not information about "what" (Sagi and Julesz, 1985). In the
In the case of texturally defined shapes, our results so far are less clear. It appears that certain shapes cannot be discriminated while attention is directed elsewhere but that others cannot. The stimulus properties which appear to determine these two different outcomes are currently being explored.

References:

352. Absence of attention mimics lesion of V4
Jochen Braun, Bela Julesz

An interesting study of the visual deficits that ensue after lesioning V4 in macaque monkeys has recently been carried out by Schiller and Lee (1991). Functional deficits in the lesioned region of the field of view were less than total, and were described as “moderate” when the monkey was required to locate a more salient target among less salient distractors. Salience was controlled by varying either target luminance, size, chrominance, or speed of motion. In the opposite situation, when a less salient target was being sought among more salient distractors, the resulting deficits were far more severe.

Although other explanations are possible, it is tempting to surmise that the monkey could locate the most salient item in its field of view by relying on pathways other than V4, but would have needed an intact V4 to discount more salient items and to have located a less salient item among them. Assuming that an inability to discount more salient distractors is in fact the true character of the observed deficit, it is only a small additional step to interpret this deficit as an attentive dysfunction.

To test these hypotheses, we have presented stimuli similar or identical to those used by Schiller and Lee to human observers while the observers’ visual attention was held at the center of the display by a demanding search task. Similar to what was observed after lesioning V4, we found little or no reduction in visual performance as a result of withdrawal of visual attention when a more salient item had to be detected among less salient ones. We varied salience by means of item luminance, size, chrominance, and shape. In the opposite situation, i.e., when a less salient item was to be detected among more salient ones, dramatic reductions in performance were obtained.

Taken by themselves, these findings have implications for the possible functional role of visual attention and, in conjunction with the lesion results of Schiller and Lee, they strengthen the suspicion that V4 is one of several anatomical sites that host the processes that constitute visual attention.

Reference:

353. Early vision and focal attention
Bela Julesz

Thirty years after the introduction of random-dot stereograms and cinematograms into psychology (Julesz, 1960) and the technique of studying preattentive texture discrimination with controlled statistical properties (Julesz, 1962), the editors of the Review of Modern Physics asked this author to write a long review article for the physicists. This review also gives an elaborate list of readings and introductory material for the novice in psychobiology. It also discusses problems of scientific creativity, strategic and metapsychological problems, evaluates the role of AI and neural modelling of the PDP kind in visual perception. The article appeared in the July issue of Review of Modern Physics. Special attention was given to focal attention, particularly to a recent paper by Saarinen and Julesz (1991) on the speed of attentional shifts.

In another publication in the 55th anniversary volume of Cold Spring Harbor Laboratory’s The Brain, a similar review article was published for workers in psychobiology.

In a third article that has been published in Representations of Vision: Trends and Tacit Assumptions in Vision Research (1991), the author raised about 40 strategic questions in visual perception that are ripe for attack. The list is given to enhance progress in psychobiology with the hope that colleagues will add their favorite problems to the list.

In an article to appear in Behavioral Brain Science, the author comments on John Searle’s thesis that “there are no
unconscious processes in psychology," with particular emphasis on Johannes Müller's doctrine of "specific nerve energies" and on preattentive vision.

Work in collaboration with Doug Williams at Rutgers appears to have solved the asymmetry problem of texture discrimination by proposing that gaps are "filled in" by illusory contours. This work also extends the texton theory by introducing antitextons that are created by the early visual system to fill in gaps, a process believed to reside in V2.

References:

PUBLICATIONS


Assistant Professor of Biology & Engineering and Applied Science: Christof Koch
Visiting Associates: Rodney J. Douglas, Heinz G. Schuster
Research Fellows: Daniel Kammen, Bartlett W. Mel, Kenneth D. Miller, Ernst E. Niebur
Graduate Students: Wyeth D. Bair1, J. Öjvind Bernander1, Christine W. Chee1, John G. Harris1, Alan B. Heirich1, Frank A. Perez2, William Softky1,4, Adam Strassberg1, Humbert H. Suarez1
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Summary: Modern theories of perception and motor control hold that neurons are the basic units of information processing; but how complex are single nerve cells? What are the biophysical mechanisms at the cellular or subcellular level that implement the elementary neuronal operations underlying all computations? What are the elementary units of information processing, the diodes and transistors of the brain? Properties and limitations of neurons and synapses are crucial in determining the
algorithms used to perform specific computational tasks. As part of our "Biophysics of Computation" program, we carry out detailed computer simulations of the underlying biophysical mechanisms in reconstructed cortical pyramidal cells, based on electrophysiological and anatomical data, to address some of these questions.

However, understanding complex tasks, such as binocular stereo, edge detection, and optical flow, requires not only an understanding of the underlying neuronal hardware, but also 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 visual cortex. The computational analysis carried out in our laboratory, coupled with psychophysical data, is complemented by computer simulations of the appropriate neuronal circuitry, the cat and the primate visual system, including the retina, the lateral geniculate nucleus, primary striate cortex, and higher cortical areas.

We continue to develop our ideas regarding the neurobiological foundation of visual awareness, and in particular of selective visual attention and short-term memory. This work is carried out in collaboration with Dr. Francis Crick at the Salk Institute. We proposed a neurobiological framework to study one particular form of consciousness, visual awareness. Our work involves two parts. We outline a general program for trying to understand the neurobiological, as opposed to the cognitive, basis of visual awareness. In particular, we discuss which important biophysical (e.g., on the basis of iconic and short-term memory) and electrophysiological experiments (on the natural history of the 35-65 Hz neuronal oscillations) are needed to link higher level cognitive theories of consciousness to experimental neuroscience.

Because both animals and machines are faced with similar problems when perceiving their environment, strategies devised over the last 500 million years can help us in building better machines. It has been shown that most problems in early vision are equivalent to minimizing non-convex cost or "energy" functions. These optimization problems can in turn be "solved" by simple resistive networks, such that the solution is given by the stationary voltage distribution at every node of the resistive grid. These resistive networks for detecting edges, computing optical flow and hue are very robust to hardware errors, and operate in real-time. The appropriate circuits are being designed at Caltech, using analog CMOS VLSI technology, and are being fabricated via MOSIS. In order to evaluate the performance of these circuits, we are building a number of small moving vehicles (toy cars) with analog chips mounted onto the car to enable them to operate autonomously.

354. NMDA-based pattern discrimination in a modeled cortical neuron

Bartlett W. Mel

Compartmental simulations of an anatomically characterized cortical pyramidal cell were carried out to study the integrative behavior of a complex dendritic tree. Previous theoretical (e.g., Feldman and Ballard, 1982; Mel and Koch, 1990) and compartmental modeling (e.g., Koch et al., 1982; Shepherd et al., 1985) work had suggested that multiplicative interactions among groups of neighboring synapses could greatly enhance the processing power of a neuron relative to a unit with only a single global firing threshold. This issue was investigated here, with a particular focus on the role of voltage-dependent N-methyl-D-aspartate (NMDA) channels in the generation of cell responses. First, it was found that when dendritic spines contain a large proportion of NMDA channels, the pyramidal cell responds preferentially to spatially-clustered, rather than random, distributions of activated synapses. Second, based on this mechanism, the NMDA-rich neuron is shown to be capable of solving a complex pattern discrimination task. We propose that manipulation of the spatial ordering of afferent synaptic connections onto the dendritic arbor is a possible biological strategy for pattern information storage during learning.

References:
355. A connectionist model may shed light on neural mechanisms for visually guided reaching

Bartlett W. Mel

Formal principles of vision-based planning and control of arm movements are used to gain new insight into the neurobiological mechanisms that underlie this important sensory-motor behavior. The primary conceptual tool used in this work has been a neurally-inspired connectionist system called MURPHY that learns to reach for visual targets among obstacles, crudely based on the style of architecture and representations in sensory and motor areas of cerebral cortex. This system has provided a concrete implementation that demonstrates how areas of cerebral cortex could in principle interact to direct both sensory-locked and internally planned reaching movements. We use MURPHY’s simple, artificial "cortex" as a point of departure in the development of two high-level cortical models for visual limb control, involving the supplementary motor area (SMA), areas 5 and 7 of the posterior parietal lobe, and several visually-responsive areas including V2, PO, and areas TPO and STP in the upper bank of the superior temporal sulcus.

356. Oscillator phase coupling for different two-dimensional network connectivities

Ernst E. Niebur, Heinz G. Schuster, Daniel M. Kammen, Christof Koch

We investigate the dynamics of large arrays of coupled oscillators with random frequencies under a variety of coupling schemes, by computing the time-dependent cross-correlation function both numerically for a two-dimensional array consisting of 128 x 28 oscillators and in an analytically solvable model. Our analysis shows that for equal overall interaction strength, a sparse coupling scheme in which each oscillator is strongly coupled to a tiny, randomly selected subset of its neighbors leads to a more rapid and robust phase-locking than nearest-neighbor coupling or locally dense connection schemes.

357. Phase coupling in two-dimensional networks of interacting oscillators

Ernst E. Niebur, Daniel M. Kammen, Christof Koch, Daniel Ruderman1, Heinz G. Schuster

Coherent oscillatory activity in a large network of biological or artificial neural units may be a useful mechanism for coding information pertaining to a single perceptual object or for detailing regularities within a data set. We consider the dynamics of a large array of simple coupled oscillators under a variety of connection schemes. Of particular interest is the rapid and robust phase locking that results from a "sparse" scheme where each oscillator is strongly coupled to a tiny, randomly selected subset of its neighbors.

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358. Collective frequencies and metastability in networks of phase oscillators with time delay

Ernst E. Niebur, Heinz G. Schuster, Daniel M. Kammen

We analyze the dynamic behavior of large two-dimensional systems of limit cycle oscillators with random intrinsic frequencies that interact via time-delayed nearest-neighbor coupling. We find that even small delay times lead to a novel form of frequency depression where the system decays to stable states which oscillate at a delay and interaction-dependent reduced collective frequency. For greater delay or tighter coupling between oscillators, we find metastable synchronized states that we describe analytically and numerically.

359. Development of orientation selectivity in visual cortex: A biologically based, testable model

Kenneth D. Miller

Neurons in the primary visual cortex of higher mammals are tuned to respond to light stimuli of a particular orientation. I propose that the development of orientation-selective cortical receptive fields occurs through an activity-dependent competition between ON-center and OFF-center inputs, just as ocular dominance columns appear to develop in visual cortex through an activity-dependent competition between left-eye and right-eye inputs. Such a competition can lead to orientation-selective cortical cells if the dark activity of ON (or OFF)-center inputs is best correlated with that of other ON (or OFF)-center inputs at small retinotopic separations, but best correlated with that of OFF (ON)-center inputs at larger retinotopic separations. Through simulation, I show that this hypothesis can account for the development of oriented "simple cell" receptive fields, for continuous and periodic arrangements of preferred orientation across the
cortex similar to those observed in cats and in monkeys, and for other known features of cortical organization. Analysis of the equations reveals the principal eigenfunctions that contribute to this final pattern, and their determination by biologically measurable parameters. A new feature, a systematic relationship between preferred orientations and spatial phases of simple-cell receptive fields, is predicted. A number of strong experimental tests of this hypothesis are proposed.

360. The role of constraints in Hebbian learning

Kenneth D. Miller, David J. C. MacKay

Models of Hebbian and similar correlation-based synaptic plasticity rules typically are unstable. All synapses may grow until each reaches a maximum allowed strength, or alternatively all synapses may decay to zero. A common method of avoiding these outcomes is to use constraints that conserve or limit the total synaptic strength over a cell. We study the dynamical effects of such constraints. Two classes of constraints are distinguished, multiplicative and subtractive. Multiplicative constraints are achieved by multiplying each synaptic weight by a constant. Subtractive constraints are achieved by subtracting a constant from each weight. For otherwise linear learning rules, multiplicative constraints lead the dynamics to converge to the principal eigenvector of the operator determining unconstrained synaptic development. Subtractive constraints, in contrast, lead to a final state in which almost all synaptic strengths reach either the maximum or minimum allowed value, and which may be dominated by weight configurations other than the principal eigenvector of the unconstrained operator. In the context of two input projections, multiplicative constraints retard the development of a difference between the two projections (for example, ocular dominance segregation), whereas subtractive constraints do not. Certain distinctions are also drawn between constraints placed upon output cells and constraints placed upon input cells. Implications for biology and for other models are discussed.

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361. Role of NMDA-receptors in visual cortical excitatory transmission

Kenneth D. Miller, Christof Koch

The N-methyl-D-aspartate (NMDA) receptor is one of at least two subtypes of glutamate-activated receptors. It is unique in many respects among glutamate receptors: in particular, the channel that it activates has a voltage-dependent conductance under physiological conditions, and the currents that it activates have a very slow time course. Recent work has revealed that excitatory transmission in response to sensory inputs in visual cortex and other regions critically depends on NMDA receptors. We are examining the effects of this relationship on the computational properties of individual visual cortical cells and of model visual cortical networks through biophysically detailed computer modeling. Our aims are 1) to develop a limited set of candidate biophysical models for NMDA receptor-mediated synaptic activation on single cells, and 2) to combine each candidate model with existing models of visual cortical networks lacking NMDA receptors. These models will be used to test the hypothesis that visual cortical spatial and temporal response properties may depend in part on the properties of the NMDA receptor.

362. A model of direction-selectivity in visual cortex with massive excitatory feedback

Humbert H. Suarez, Christof Koch, Rodney J. Douglas

The model consists of two populations of cortical neurons. One population is excitatory and represents the pyramidal neurons, and the other is inhibitory and represents smooth cells. Each cell has two compartments: the soma, and a dendritic cylinder. The pyramidal somata contain seven ionic currents that obey Hodgkin-Huxley-like dynamics, including calcium-dependent adaptation. The synaptic connections within this network are consistent with the canonical microcircuit of Douglas and Martin: the major part of the excitatory input to cortical cells is derived from other cortical cells, and only a minor part (20%) is derived from geniculate input. Visual input from the geniculate is simulated by a detailed model of X-cells. Inhibition is supplied only by intracortical neurons. In the null direction, the weak geniculate excitation is vetoed by simultaneous cortical inhibition. However, in the preferred direction, cortical inhibition is delayed and so
the weak geniculate input is amplified by the excitatory cortico cortical connections. In this model, single cells show only small increases in input conductance (<30% relative to control) in the null direction. These changes are consistent with in vivo intracellular recordings. Despite the strong excitatory feedback connections, the neural responses are graded with stimulus contrast because adaptation restricts the discharge of the pyramidal cells.

363. Synaptic background activity determines spatial-temporal integration in single pyramidal cells

J. Öjvind Bernander, Christof Koch

The standard one-dimensional Rall cable model of nerve cells assumes that their electrotonic structure does not change in response to synaptic input. This model is used in a great number of both theoretical and anatomical-physiological structure-function studies. In particular, the membrane time constant, $\tau_m$, and the somatic input resistance, $R_{in}$, are used to characterize single cells. However, these studies do not take into account that neurons are embedded in a network of spontaneously active cells. Synapses from these cells will contribute significantly to the membrane conductance, especially if recent evidence of very high specific membrane resistance, $R_m = 100 \text{ k}\Omega \text{cm}^2$, is taken into account. We numerically simulated the electrical behavior of an anatomically reconstructed layer V cortical pyramidal cell receiving input from 4000 excitatory and 1000 inhibitory cells firing spontaneously at 0-2 Hz. We found that over this range of neuronal background activity $\tau_m$ and $R_{in}$ can change by about a factor of 10 (80-7 msec, 109-12 MW) and the electrotonic length of the cell by a factor of 3. We show that this significantly changes the response of the cell to temporal desynchronized versus temporal synchronized synaptic input distributed throughout the neuron. Thus, the global activity of the network can control how individual cells perform spatial and temporal integration.

364. A simple network showing burst synchronization without frequency-locking

Christof Koch, Heinz G. Schuster

The dynamic behavior of a network model consisting of all-to-all excitatory coupled binary neurons with global inhibition is studied analytically and numerically. It is shown that for random input signals, the output of the network consists of synchronized bursts with apparently random intermissions of noisy activity. We introduce the fraction of simultaneously firing neurons as a measure for synchrony and prove that its temporal correlation function displays, besides a delta peak at zero indicating random processes, strongly dampened oscillations. Our results suggest that synchronous bursts can be generated by a simple neuronal architecture which amplifies incoming coincident signals. This synchronization process is accompanied by dampened oscillations which, by themselves, however, do not play any constructive role in this and can therefore be considered to be an epiphenomenon.

365. Computing motion using analog VLSI vision chips: An experimental comparison among four approaches

Wyeth D. Bair, Christof Koch, Andrew J. Moore, Timothy K. Horiuchi, Brooks Bishopberger, John Lazzaro

We have designed, built and tested a number of analog CMOS VLSI circuits for computing one-dimensional (1-D) motion from the time-varying intensity values provided by an array of on-chip phototransistors. We present experimental data for three such circuits and discuss their relative performance. One circuit approximates the correlation model, one the gradient model, while a third chip uses resistive grids to compute zero-crossings to be tracked over time by a separate digital processor. All circuits integrate image acquisition with image processing functions and compute velocity in real time. Finally, for comparison, we also describe the performance of a simple motion algorithm using off-the-shelf components.

The first of our three analog approaches is the Tanner-Mead motion detector circuit (Tanner and Mead, 1986) operating in a novel mode in which it performs derivative multiplication rather than computing the ratio of temporal to spatial derivatives associated with the standard gradient model. The second analog VLSI chip contains a large array of velocity-tuned units that correlate two events in time, using pulse-coding in a delay-line structure (Horiuchi et al., 1991). The third analog chip is interfaced to a digital microprocessor. This 64-pixel, 1-D chip reports the positions of edges from thresholded zero-
crossings by using resistive networks to implement an operator similar to the difference of Gaussians (Bair and Koch, 1991). A microprocessor then computes velocity by tracking the edges reported by the analog chip. These three non-clocked analog-based approaches are then compared to a simple system built out of a 1-D CCD imager and a programmable microprocessor which implements a grey-level autocorrelation algorithm. We would like to mention that most of our circuits are directly or indirectly inspired by biological counterparts in the motion pathway of flies, rabbits or primates. In fact, it has been our experience that thinking about biological motion estimation systems leads to the design of more robust electronic circuits, while thinking about machine vision systems leads to a better understanding of the problems, such as gain-control and limitations in the precision of components, that any biological vision system must face. When testing the performance of our different motion chips, we tried to directly compare their output under the same conditions, in particular, using the same stimulus pattern, speed and light intensity, whenever the operating characteristics of the chips made this practical. Accordingly, we built a conveyor belt system so that square wave gratings of various contrasts and spatial frequencies could be moved in view of the chips, with velocities that ranged over more than an order of magnitude. In terms of overall robustness, we have ranked the methods as follows: the grey-level autocorrelation method is most robust, followed in order of decreasing robustness by the zero-crossing tracking method, the pulse correlation chip, and the derivative multiplication chip. Interestingly, this roughly corresponds to their relative use of power. The CCD imager, a conventional, off-the-shelf component that forms the front end of the system, is not nearly as noisy as the photoreceptors we use on the analog chips. This is not surprising as many hundreds of man-years of engineering have been devoted to building very accurate CCD cameras. The work reported here represents an effort over three years to build robust, analog motion sensors with on-chip photoreceptors. We have achieved a moderate amount of success in this undertaking in that we are able to compute the global velocity of a 1-D image in real-time.

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366. Segmentation circuits using constrained optimization

John G. Harris

A common goal in computer vision is to segment scenes into different objects sharing common properties such as depth, motion, or image intensity. A novel segmentation algorithm using constrained optimization techniques has been simulated and mapped into analog hardware. An absolute-value penalty term is used to create an energy functional that imposes a piece-wise constant constraint on the segmented data. Since the minimized energy is guaranteed to be convex, there are no problems with local minima and no complex annealing algorithms or continuation methods of any kind are necessary. This is a sharp contrast to previous software and hardware solutions to this problem.

The standard regularization technique for smoothing or interpolating noisy inputs, $d_i$, is to minimize an energy of the form:

$$E(u) = \sum_i (d_i - u_i)^2 + \lambda \sum_i (u_{i+1} - u_i)^2 \quad (1)$$

The first term ensures that the isolation $u_i$ will be close to the data while the second term implements a smoothness constraint. The parameter $\lambda$ controls the tradeoff between the degree of smoothness and the fidelity to the data. Since the energy given by Equation 1 oversmoothes discontinuities, numerous researchers have modified Equation 1 with line processes and successfully demonstrated piece-wise smooth segmentation of various modalities. In these methods, the resultant energy is nonconvex and complex annealing or continuation methods are required to converge to a good local minima of the energy space. Resistive fuses have been used to
implement line-process discontinuities in analog hardware, but continuation methods are still required (Harris et al., 1990).

Rather than deal with networks that have many possible stable states, a network that has a single unique stable state will be studied. Consider a network that minimizes:

$$E(u) = \frac{1}{2} \sum_i (d_i - u_i)^2 + \lambda \sum_i |u_{i+1} - u_i|$$

(2)

The absolute-value function is used for the smoothness penalty instead of the more familiar quadratic term. There are two reasons why the absolute value penalty is better than the quadratic penalty for piece-wise constant segmentation. First, for the large values of $|u_i - u_{i+1}|$, the penalty is not as severe, which means that edges will not be smoothed out as much. Second, small values of $|u_i - u_{i+1}|$ are penalized more than they are in the quadratic case, resulting in a flatter surface between edges.

The energy minimized can be interpreted as the generalized power (or co-content) of a nonlinear resistive network. In order to build this network, we need a nonlinear resistor with a tanh I-V characteristic with an extremely narrow linear region. For this reason, this element is called the tiny-tanh resistor. Simulations have shown that a resistor with a linear region on the order of millivolts is required. Mead has constructed a resistor that saturates at about 50 mV (Mead, 1989) which is much too large for our purposes. A tiny-tanh resistor had been constructed that saturates for voltage drops less 5 mV. A one-dimensional version of this network has been implemented in analog CMOS hardware and its segmentation properties are demonstrated for noisy step edges which are scanned into the chip.

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Summary: We continued to study the brain mechanisms of sound localization in the owl and the neuroanatomical and physiological substrates of birdsong learning. During the 1990-1991 academic year, one graduate student finished his doctorate and three new postdoctoral fellows joined our group. In the past, all postdoctoral fellows worked with the owl and all graduate students worked with songbirds. This year, more postdoctoral fellows are working with songbirds and more graduate students with the owl.

The songbird project is carried out at several different levels of complexity. We are still trying to characterize the antigen that occurs almost exclusively in the forebrain song control areas. The intracellular study of song nuclei in vitro has paid off handsomely. Of particular significance is the presence of NMDA receptors at one of the sites that may be involved in the regulation of plasticity in song learning. We continued the analysis of song-specific neurons that respond selectively or exclusively to the individual bird's own song. The chain of song nuclei in which these neurons occur is particularly interesting, because it may be involved in the control of song development by auditory feedback. Songbirds must hear themselves sing in order to develop normal songs. There is, however, a subgroup of songbirds that does not need auditory feedback to develop song. Interestingly, this subgroup does not appear to have brain areas that are differentiated into discrete nuclei like the song nuclei.
This comparison led us to a comparative study of brain areas involved in the control of vocalizations between different avian species.

In the owl project, we have figured out how the owl’s brain computes binaural cues for sound localization, i.e., interaural time and intensity differences. We know the computational algorithm for the derivation of these cues and its neural implementation. This neural network uses several parallel pathways including two major pathways for interaural time and intensity differences that are, in turn, further divided into multiple frequency channels. These parallel pathways converge upon one another at higher-order stations. We are interested in finding out the rules and mechanisms underlying these convergences.

367. Nonlinear response properties of auditory neurons in the zebra finch’s song system: A quantitative approach

Dukwan Lim, Allison J. Doupe, Mark Konishi

Auditory forebrain song nuclei of the male zebra finch (Poephila guttata) contain neurons sensitive to the bird’s own song. These neurons respond best or exclusively to a certain configuration of song acoustic elements (Fortune and Margoliash, 1989). The response of these neurons to the effective configuration does not appear to be a simple addition of their responses to individual elements presented in isolation, indicating the involvement of nonlinear processes. To quantify these nonlinear interactions, we used a matrix-based optimization method (Lim and Capranica, 1990). This procedure made it easier to see interactions between a neuron’s responses to various acoustic stimuli. Furthermore, it lent itself well to generating the set of most meaningful acoustic components for a particular group of neurons. Thus, the selectivity of forebrain nuclei for a given set of stimuli could be represented in terms of their nonlinear dependencies. For example, we studied responses of single HVc neurons to harmonically related tones and their combinations such as f1, f2, f1+f2, f1+f3, f1+f2+f3, etc. The results showed different degrees of inhibitory and facilitatory interactions between the neuronal responses to different tones.

References:

368. Inhibition shapes response to interaural time differences in the inferior colliculus of the barn owl

James A. Mazer, Ralph Adolphs

The barn owl, Tyto alba, uses interaural time differences (ITD) to encode sound source location in azimuth. Neurons in several nuclei below the level of the inferior colliculus are sensitive to interaural phase differences in the frequency to which they respond best. Only at the level of the external nucleus of the inferior colliculus (ICx) does one find neurons capable of responding selectively to a particular ITD without ambiguity of phase, provided that the sound’s spectrum contains more than one frequency. Neurons in the ICx are broadly tuned to frequency, suggesting that one way in which phase ambiguity is resolved at this stage is by combining the responses to ITD across several different frequency channels. We investigated the role of nucleus ventralis lemnisci lateralis, pars anterior (VLVa) in generating responses to ITD in the ICx.

VLVa provides polysynaptic input to the contralateral ICx and contains neurons that are sensitive to phase differences and sharply tuned to frequency. We microiontophoretically applied agonists (GABA, muscimol) or antagonists (bicuculline methiodide, BMI) of GABA-A receptors to VLVa neurons and found that GABA agonists decreased, and antagonists increased the evoked response rates. In subsequent experiments, we pressure injected these drugs into VLVa while recording evoked responses to ITD from single neurons in the contralateral ICx. We found that: 1) increasing neural activity in VLVa with BMI led to a decreased response to ITD in the ICx; 2) decreasing neural activity in VLVa with GABA-agonists led to an increased response to ITD in the ICx; 3) these effects were greatest when the best frequencies of the VLVa site and the ICx neuron recorded from were matched; and 4) in some experiments, VLVa injections differentially affected ICx responses at the real ITD (main peak) and at phase-ambiguous ITDs (side peaks) in response to noise stimuli. These results suggest that VLVa provides functional inhibition to ICx in response to ITD, that the projection from VLVa to the inferior colliculus may be topographically organized in the frequency domain, and that VLVa may contribute to side-peak suppression of responses in the ICx.
Birdsong is a complex learned behavior, learned in two distinct phases during development. Young songbirds must first hear and remember a tutor song during a critical period early in development. They later match their vocalizations to the memorized song or "template" using auditory feedback. Experiments with songbirds deafened after the critical period but during motor learning of song demonstrate that the ability to hear the song is essential.

Song learning is controlled by a series of distinct and interconnected brain areas. In particular, the nuclei HVC, RA, and NXIt form a descending motor pathway which is specialized for song production. There must exist a link between this motor pathway and the auditory system, because auditory feedback is used to guide vocal development during song learning. Presumably, a search for auditory neurons might also lead to the song template, since auditory input is essential both in the acquisition of the song memory and in the later use of the template in song matching. We therefore undertook to characterize further the auditory pathways related to song, and to look for neurons that might encode the template.

The avian primary auditory area, Field L, is known to project to the vicinity of HVC and RA. In addition to the specialized motor neurons, HVC contains highly complex auditory neurons: these cells respond maximally to the bird's own song (Margoliash, 1983). This selectivity, which must be experience-dependent, might be particularly useful for song matching: such neurons could provide feedback on how well the bird's vocalizations matched the memorized song. The song-selective HVC neurons do not represent the template, however, because although they have complex auditory responses in young birds, they acquire their highest selectivity only after sensory learning is already over (Volman and Konishi, 1986). We became interested in two other forebrain song nuclei, MAN and X. These areas do not appear to be required for singing in the adult, but must be intact for normal song learning. Thus, they have an important, transient role in song development. One such role could be template storage.

Our studies show that these areas do have auditory responses in the adult zebra finch. In fact, each of the nuclei in a circuit from X, then down to the thalamic nucleus DLM, and back to MAN, contain highly song-selective auditory neurons. In order to describe the functional inputs to this loop, we injected the local anesthetic lidocaine into HVC, which has anatomical projections to X, while simultaneously recording from auditory neurons in X or MAN. The reversible silencing of HVC resulted in a similarly reversible silencing of the specialized auditory neurons in MAN and X. This showed that HVC is indeed the source of auditory input to this circuit, and that HVC transmits excitatory information about song to the rest of the pathway.

The presence of four interconnected nuclei with similar song-selective properties raised the question of what function these different nuclei serve. To begin to address this, we investigated the responsiveness of X and MAN auditory neurons to a variety of simpler acoustic stimuli as well as to song. The results suggest that that MAN neurons have much narrower response properties than X neurons, and thus that there is a gradient of increasing complexity and selectivity in this auditory loop. In many neural systems, increasing stimulus selectivity is the major transformation accomplished by hierarchical circuits, and this may be one function of this series of interconnected auditory nuclei in the songbird. Furthermore, although HVC responses in young birds are not highly selective, the output of this circuit might be highly selective, and thus ultimately represent the song template.

It is interesting in this light that MAN auditory neurons synapse on RA premotor neurons, which also receive input from HVC premotor neurons (Mooney and Konishi, 1991). Thus, the highly specialized auditory pathway that we have described is uniquely situated to provide an error-correcting input to the motor pathway, through its output from MAN onto RA neurons. Clearly, the crucial next step will be to examine the auditory properties of this circuit in young birds. Nonetheless, its highly selective auditory properties in adult birds, its clear role in learning, and its output to song motor neurons all raise the possibility that this circuit functions to store the auditory memory or template, and to provide auditory feedback during sensorimotor learning. The disruptive effects of lesioning this pathway in development would then be due to the elimination of the auditory-motor matching so
crucial to song learning.

References:

370. Acetylcholinesterase staining in the auditory brainstem of the barn owl

*Ralph Adolphs*

Auditory nuclei in the brainstem of the barn owl, *Tyto alba*, stain differentially with acetylcholinesterase (AChE) histochemistry. At the level of the cochlear nuclei, nucleus angularis, which gives rise to the neural pathway subserving intensity difference processing, showed very intense staining of fibers and cell bodies. Nucleus magnocellularis, the first nucleus of a pathway for time difference processing, showed only a weak staining of cell bodies and no fiber staining. Nucleus laminaris, which receives bilateral input from n. magnocellularis, showed very faint staining of terminals surrounding cell bodies. In the lemniscal nuclei, nucleus ventralis lemnisci lateralis pars posterior (VLVP) stained more intensely than the adjacent pars anterior (VLVA); VLVP is part of the intensity pathway and receives input from n. angularis, while VLVA is part of the time pathway. In the inferior colliculus (IC), the shell of the central nucleus, which is a terminal field both of n. angularis and VLVP, stained more intensely than did the core of the central nucleus, which is a terminal field of n. laminaris. The external nucleus of IC stained intensely also, as did the optic tectum.

These staining patterns suggest that nuclei or subdivisions of nuclei that process interaural level differences (n. angularis, VLVP, the shell of IC) stain more strongly for AChE than do those nuclei that process interaural time differences (n. magnocellularis, n. laminaris, VLVA, the core of IC). Moreover, this staining pattern is complementary to that seen with immunohistochemistry to the calcium-binding proteins calbindin (Takahashi *et al.*, 1987; personal observation) and parvalbumin (personal observation). Antibodies to calbindin as well as to parvalbumin appear to stain the pathway subserving interaural time difference processing darkest.

Results from baby owls indicate that the staining pattern seen with AChE has already formed at 15 days post hatching.

AChE thus appears to be a useful anatomical marker molecule that preferentially stains the owl’s intensity-processing pathway.

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combining experimental approaches (by focusing on the thoracic nervous system of an insect, the locust) and modeling techniques (numerical simulations), to try and gain some insight into the general principles underlying information processing in local circuits. Examples of such main features are the central representation of the sensory space (e.g., the surface of the legs) in ordered neural maps, or the parallel processing of sensory signals by many neurons or populations of neurons. The main objectives of this research are to understand information processing by single neurons at a biophysical level, and to understand the key organizational features of the networks that they form (e.g., central pattern generator circuits for locomotion). We have thus continued our investigations of the membrane properties of a class of local interneurons (nonspiking local interneurons), and extended it to the study of chemical transmission between spiking and nonspiking local interneurons. We have started an immunocytochemical study of the distribution of GABA-ergic synapses onto the spiking local interneurons, and developed a culture system for locust neurons. We are thus now in a position to use patch-clamp and imaging techniques to study the biophysical properties of local interneurons in vitro. The results from these studies will be used to construct realistic models of neurons and to estimate the importance of such parameters as channel density and distribution on dendritic arbors for local computations. In parallel, we have started an electrophysiological investigation of the central pattern generating circuits responsible for walking in deafferented preparations. Finally, we are studying lateral interactions between mechanosensory afferent terminals that appear to be involved in regulating the gain of afferent connections onto their follower neurons.

371. Membrane properties of nonspiking local interneurons

Gilles Laurent

Outward currents activated by depolarization were studied in the neuropilar membrane of locust nonspiking local interneurons, using the single-electrode voltage-clamp technique in situ. Preliminary observation of these currents in 272 neurons revealed two families. The first and most commonly observed (85% of recordings) showed a large transient current followed by a slowly decaying/sustained current. The second (15% of recordings) showed an additional outward current with a slow rate of activation, a peak within 100-150 msec, and a slow rate of inactivation. Only neurons of the first type were studied further. The transient current was activated by depolarization around -60 mV, with a time to peak of ~11 ms at -50 mV, and less than 3 ms at -20 mV. This current decayed exponentially with a time constant of 8.1 ms ± 1.6 (n = 8 interneurons) at -30 mV. This time constant of inactivation did not appear to depend strongly on membrane voltage, in the range where it was studied.

A second and longer time constant of inactivation of 50-400 ms could not be assigned to either of the transient and sustained components of the outward current. The ratio of transient-to-late current varied from 1.6 to 5.4, with a mean of about 2.5. The reversal potential for the transient conductance could be shifted by 14 mV by a threefold increase in the bath K+ concentration, indicating that K+ is a charge carrier for the current. The transient current became inactivated with maintained depolarization and was half-inactivated at about -60 mV (slope factor k1/2 = 8 mV). This current was thus not fully inactivated at "resting" potential (average of -58 mV). Recovery from inactivation followed a single exponential time course, with a time constant of 50-100 ms at -80 mV. The time course of recovery from inactivation of the transient current was well correlated with that of the recovery of transient outward rectification, as measured in current-clamp recording. TEA, at a bath concentration of 10 mM, reduced the transient current by 70% and the delayed current by 60%. 4-AP, at a bath concentration of 5 mM, had a significant effect in only two of five interneurons, reducing the transient current by ~85% and the delayed current by ~15%. Quinidine at a bath concentration of 100 mM was ineffective. Although these blockers did not allow a clear pharmacological separation of the currents, they were effective in reducing the outward rectification observed in current-clamp during step depolarization. Their presence never unmasked regenerative events such as action potentials in the nonspiking local interneurons. Cobalt chloride (25 mM) had no effect on either current. These results suggest that the outward currents activated by depolarization are similar to the "A-" and "delayed-rectifier" currents, originally described in other invertebrate
neurons. Inward currents such as Ca$^{2+}$ currents involved in synaptic transmission or, in a few cases, in the genesis of active potentials, will be studied in vitro with the whole-cell patch-clamp technique.

372. Quantal analysis of synaptic transmission between local interneurons

Gilles Laurent

Quantal analysis of neurotransmission at chemical synapses, which describes the quantization of post-synaptic potentials (PSPs) or currents and their variability, has been most successful at the neuromuscular junction (Del Castillo and Katz, 1954), where it was first developed. Such analysis, however, has been more difficult to apply to connections in the central nervous system, and there is still much contention as to which statistics (e.g., Poisson, simple or compound binomial if any) best describe transmission at these synapses (Redman, 1990). Moreover, central neurons typically synapse with many other neurons, and the variations in the release properties of these central synapses made by a single neuron have apparently never been investigated. In mammals, for example, a neuron in the inferior olivary nucleus may form up to 10 climbing fibers which each contact a different Purkinje cell in the cerebellum, and a thalamic afferent may connect with several thousand cortical neurons. In invertebrates, a single proprioceptive afferent can diverge to several motor neurons and local interneurons, and a single nonspiking interneuron can diverge to several motor neurons within a pool, or in different pools. A knowledge of the relative influence that a neuron has on its many central targets and of the mechanisms by which variability might come about is thus critical to understanding integration in networks such as these.

Experiments to address this issue were designed using the synapses made from spiking to nonspiking local interneurons, which underlie the execution of tactile and proprioceptive reflexes of the legs in the locust. Spiking local interneurons receive direct inputs from leg mechanoreceptors and make inhibitory connections with other spiking local interneurons, nonspiking local interneurons, motor neurons and intersegmental interneurons. These output connections appear to be made mostly from the varicosities in a sparse field of branches in the dorsal neuropil of a segmental ganglion. The nonspiking local interneurons are premotor interneurons, and have intermingled input and output synapses. A single spiking interneuron can make divergent connections to several nonspiking interneurons. Similarly, a nonspiking interneuron generally receives convergent inputs from several spiking interneurons, making the attribution of spontaneous PSPs to any specific presynaptic neurons impossible. For this reason, simultaneous intracellular recordings were made from a presynaptic spiking local interneuron in its soma, which is distant from its output synapses, and from a postsynaptic nonspiking local interneuron in one of its dendrites, close to the input synapses, and the fluctuations of IPSP amplitudes were analyzed using statistical techniques.

The distribution of postsynaptic potential amplitudes at most synapses could be described by a simple binomial model, implying uniformity of $p$ (probability of release of one quantum) for each synapse, but both $n$ (number of available quanta) and $p$ (and their product $m$) differed between the synapses made on different postsynaptic interneurons. A single spiking local interneuron can thus make output connections with several neurons, where each synapse is characterized by specific binomial parameters. These results show that quantal content can differ between the many synapses made centrally by one interneuron, and suggest that this variability arises from differences in release probabilities between the sites associated with different synapses. We will attempt to study the biophysical mechanisms that might underlie this heterogeneity of synaptic properties.

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373. Identified insect neurons in culture

Kay J. Seymour-Laurent, Karin E. Johnson

The locust central nervous system (CNS) contains several classes of neurons whose cell bodies are often found in groups or clusters. Each group is in turn composed of several identifiable neurons. Many of these neurons are interneurons, which can be local or intersegmental. Studies on the locust CNS in vivo have already yielded some information on the integrative
properties of one class of interneurons (Laurent, 1990, 1991). They receive input from sensory afferents and other interneurons and thus integrate and may gate signals according to, for example, the position of a leg. To understand the integrative properties of the local interneurons, it is necessary to know not only the inputs they receive and the outputs they make, but also the cellular, membrane and channel properties of single cells. Such experiments are very difficult to perform in vivo because of the small size of their cell bodies, their position and the complex geometry of their dendrites. Growing these neurons in culture will allow us to investigate their properties using the technique of whole-cell voltage-clamp recording. Because this technique requires clean, isolated and small cells, we have developed a cell culture procedure that allows us to grow isolated neurons from locust embryos for up to 6 weeks.

The thoracic ganglia of 55 to 80% embryos were removed in saline and dissociated by a mixture of mechanical and enzymatic techniques. A suspension of the neurons in serum-free medium was placed in plastic culture dishes previously coated with laminin and Concanavalin-A. After 1 hr, the neurons had attached to the dish surface, and serum-containing medium was added. The neurons were incubated at 30°C in a humid environment. Cells often started to grow within 2 hr of plating and up to two-thirds of single neurons showed one or more growth processes after 3 days. Neurons could exhibit monopolar, bipolar or multipolar growth. The type of growth displayed did not seem correlated with soma size. Depending on the density of neurons plated, the cells either grew in isolation or made apparent connections with one or more other neurons, possibly forming little networks. It is not yet known if these connections are functional. We are in the process of recording from isolated neurons in culture using whole-cell voltage-clamp. We plan to identify whole cell currents and the components of these currents in identified embryonic and adult neurons.

A method for culturing neurons from adult locusts is also being developed. At present, neurons from adult nervous systems will grow processes for 5-7 days, after which they detach from the substrate. Once our technique is refined, we will be in a position to compare the cellular and membrane properties of adult neurons to those of embryonic ones.

We are particularly interested in a population of spiking local interneurons that play a role in integrating sensory information from a leg. These neurons are found in a group of 50-70 somata in each thoracic ganglion. They are well described anatomically and their main inputs and outputs are known (Siegler and Burrows, 1984). To study these identified neurons specifically, we have developed a technique in which some of the local interneurons are stained with the fluorescent membrane dye Dil. The ganglia from embryos at 80-90% of development are removed and desheathed. A crystal of Dil embedded in rubber cement is placed on the group of neurons and left for a few minutes. The neurons stained are identified from the position of the somata and the tract in which the primary neurites run. The ganglia are dissociated and the resulting suspension plated and cultured as described above. In such cultures, stained neurons grow and can be detected at least 10 days after labeling. Thus, we will be able to record from identified neurons in culture. We also hope to develop a method for growing and recording from identified nonspiking interneurons in the future.

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374. Distribution of GABA-ergic synapses on local interneurons

Beulah Leitch, Gilles Laurent

An immunocytochemical study of the distribution of GABA-ergic synapses onto spiking local interneurons was initiated. These interneurons have two main fields of branches: a ventral field, which is mainly comprised of input synapses, and a dorsal field, from which most outputs are made. One finds, however, input synapses in the dorsal field and output synapses in the ventral field, but in smaller numbers. The two fields of branches are connected by a dorso-ventral process devoid of branches, which might also possibly be the integrating segment from which the action potential is generated. This study was thus undertaken to try and determine the distribution of inhibitory synapses onto the two fields of branches to try
and gain some insight into the local biophysical events which take place on the membrane of these interneurons. In particular, we would like to know if inhibitory inputs are located mainly in the ventral field, with most other (excitatory) inputs, or whether they they might act presynaptically at the release sites located in the dorsal field. We thus stained spiking local interneurons with intracellular injection of horseradish peroxidase, and used a primary rabbit anti-GABA antiserum followed by a secondary gold-labeled goat anti-rabbit antiserum on ultrathin sections of the stained ganglia. The data from these experiments are still being analyzed, and portions of the interneurons are being reconstructed serially.

375. Central pattern generating circuits for locomotion

*Sylvie Ryckebusch, Gilles Laurent*

The aim of this project is to understand how the central nervous system interacts with peripheral sensory receptors to generate the coordinated leg movements necessary for walking. We have developed a preparation that allows us to record from the circuits underlying walking in the locust. We can evoke the centrally generated rhythm by perfusing an isolated thoracic ganglion with the muscarinic agonist pilocarpine. The activity of the CPG (henceforth referred to as the "walking" CPG) is expressed by motor neurons which innervate the different muscle groups in the leg. Suction electrodes are used to record action potentials from cut nerves, or bundles of motor axons, while glass microelectrodes are used to simultaneously record from the somata of motor neurons. Some basic features of the underlying connectivity of the circuit elements can be deduced by examining the phase relationships between the different motor neurons and their subthreshold activity.

We have observed that in the isolated ganglion, the phase relationships between motor neurons of antagonistic muscle groups are qualitatively similar to those that are observed in an intact animal. We have focused on two different joints in the locust hindleg: the coxa-trochanter joint and the femur-tibia joint. Both joints are hinge joints, controlled by antagonistic pairs of muscles. The observed rhythm appeared qualitatively similar to walking in an intact animal, as motor neurons to antagonistic muscle groups in the metathoracic ganglion were active in antiphase, whereas motor neurons to synergistic muscle groups were active in phase. Levators of the trochanter were in phase with flexors of the tibia, and depressors of the trochanter were in phase with extensors of the tibia. The levator phase of activity, which would correspond behaviorally to the "swing" phase of the leg movement during walking, is shorter and less variable than the depressor phase of activity. The frequency of the observed rhythm increased approximately linearly with concentration of pilocarpine, saturating at about 0.2 Hz, which is about 5 times slower than the frequency of walking in the intact animal. The rhythms expressed by the left and right halves of a ganglion were not necessarily of the same frequency, which suggests that there are independent pattern generators for each leg. Some lateral coupling between hemiganglia was observed, however, as depressors of the trochanter on one side were generally in phase with levators of the trochanter on the other.

The same preparation allows us to study the coupling between the pattern generator for walking and other rhythmic motor patterns, such as respiration and flight. The rhythmic pattern underlying respiration can also be induced by pilocarpine in an isolated ganglion, and preliminary results suggest that this rhythm is weakly coupled to the rhythm of the "walking" CPG. Once the activity of the isolated circuit has been characterized, our aim is to use this reliable preparation to study how peripheral sensory organs interact with the centrally generated rhythm.

376. Cooperative coding in mechanosensory neurons

*Malcolm Burrows, Gilles Laurent*

Many sensory neurons report the movements and position of the joints of a limb and their signals are integrated by interneurons of differing physiological properties so that an appropriated response is elicited from the motor neurons. It is assumed that the signals generated by the sensory neurons result from their own electrical membrane properties and from the mechanical properties of the receptors. The signal that they deliver to the central neurons could, however, also be shaped by feedback to the terminals of the sensory neurons themselves which would, for example, modify the
effectiveness of the spikes in releasing transmitter. Such an arrangement is shown to occur in the approximately 50 sensory neurons from a proprioceptive organ at the femoro-tibial joint of a locust hindleg. These sensory neurons make parallel connections with local interneurons and with motor neurons and are responsible for mediating resistance and assistance reflexes. The responses of the sensory neurons are fractionated but each movement is signaled by many. The optimal movement for a particular sensory neuron nevertheless also generates many synaptic potentials in the terminals of this sensory neuron on which the orthodromic spikes are superimposed. These potentials, which are evoked indirectly by other afferents from the same organ and with similar sensitivities, are associated with large conductance changes in the terminals and have a reversal potential that is 10 mV more positive than resting potential. Normally they appear therefore as depolarizing potentials. They can be blocked by γ-amino butyric acid or by GABA picrotoxin injected close to the terminals in the neuropil. They have the same reversal as the potentials evoked by the direct application of GABA. The net effect of these presynaptic interactions is presumably to reduce the amount of transmitter that is released by a spike. This cooperative interaction between the afferents could therefore be important in regulating the effectiveness of each sensory neuron onto its follower neurons, and in adjusting the gain of each synapse to the context in which the afferent signals are processed. Afferent spikes occurring in a context where only few afferents are excited would elicit powerful responses (high synaptic gain), whereas spikes occurring during a movement that activates many afferents would each elicit smaller responses. This would constitute a new adaptive gain control mechanism for mechanosensory signaling in the central nervous system.

PUBLICATIONS


Faculty Associate: Marianne E. Olds

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Summary: This past year we have continued to work on our long-term study aimed at understanding precisely the manner in which the neurons containing the transmitter dopamine (DA) convey their influence on motor activity and on positive reinforcement to the motor neurons for translation into locomotion, oral stereotypy and purposive behavior maintained by rewarding brain stimulation. Our interest the last few years has focused on groups of non-dopamine neurons in the substantia nigra and the ventral tegmental area, and recently has been extended to neurons in the globus pallidus. The basis for this interest is the anatomical, physiological and pharmacological evidence that these neurons receive input from the DA neurons implicated in these two motor functions, that their output is influenced by the state of excitation of the DA neurons, and that they contain the transmitter GABA, whose metabolism both influences and is influenced by dopamine. On these grounds then, it has been hypothesized that these neurons might function as DA output neurons for the motor and reinforcing functions. What is not known is how the information to be conveyed to the motor neurons is parcelled out among these three groups of putative output neurons, under what circumstances one output is selected over another, and
whether the parceling is absolute or relative. One set of experiments carried out this past year aimed to provide answers to these questions.

Our studies of the role of a subgroup of DA neurons designated as A10 in positive reinforcement, using the self-stimulation rat model in which reinforcement is produced by direct electrical stimulation of the central nervous system, have this past year been extended to include the role of the brain's serotonin neurons. These neurons innervate the brain's reinforcing sites, and early studies of the role of serotonin neurons led to the view that it modulates an aversive brain system, and indirectly, as a result, modulates the reinforcing process via reduction of tonic activity in the aversive system. Thus, whereas the DA system was shown to be behaviorally activating and positively reinforcing, the brain serotonergic system was shown to be behaviorally inhibiting and negatively reinforcing. Our current studies of positive reinforcement focus on the notion that the two systems interact in this manner, and the objective has been to obtain direct evidence by up or down regulating the two systems concurrently to determine whether the effects are consistent with the hypothesis.

The approach used in the experiments concerned with the DA output pathways is to induce in the animal (the rat) one of two specific behaviors, each capable of being induced by drugs given by the intraperitoneal or the intracerebral route, each associated with heightened DA activity in a particular structure. One of these behaviors is locomotion, and it can be induced by treating the animal with DA agonists or DA itself, injected in the nucleus accumbens, a structure in the ventral forebrain innervated by the A10 DA neurons. The other is oral stereotypy, and it can be induced by injecting DA agonists or DA in the striatum, a structure in the dorsal forebrain, continuous with accumbens, but innervated by DA neurons designated as A9. The effects of enhancing DA metabolism in these structures are then determined on the output of the putative DA output neurons while the specific drug-induced motor activity is expressed. Extracellular recording techniques from multiple probes implanted in the structures containing putative DA output neurons are used to correlate unit output with behavioral output to see how the two relate, to determine if the input to a given group is favored under certain conditions.

The approach used in the experiments concerned with the interaction between DA and serotonin (5-HT) neurons in regulating the positively reinforcing process used the rat self-stimulation model and compounds that release DA and serotonin and compounds that directly activate the DA and the serotonin receptors. The self-stimulation model uses a rat chronically implanted with bipolar stimulating electrodes aimed at the medial forebrain bundle (MFB), a large fiber pathway running ventrally and linking the midbrain to the forebrain. The animal is trained to press a lever to obtain a very mild and brief shock applied to the MFB. If the electrode is correctly placed, the animal will work at this task steadily for hours to obtain the brain reward. A comparison of this behavior with the behavior of hungry or thirsty animals to obtain food or water has shown that the behaviors are comparable except for the fact that the animals are never satiated, the brain stimulus producing both the incentive and the reward. The animals in which stimulation has been shown to be rewarding are then treated with compounds known to alter the metabolism of DA and 5-HT in known ways. The effects of the treatment on the bar-pressing behavior and on the regional levels of DA and its metabolites and 5-HT and its metabolites are then measured. One study has been completed, another is ongoing and a third, using measurements of DA and 5-HT in vivo with the microdialysis method, is in the process of being set up.

377. Interaction between dopamine and serotonin neurons in the regulation of positive reinforcement
Marianne E. Olds, Arthur Yuwiler

These experiments were undertaken to determine the validity of the notion that the serotonin system is behaviorally inhibitory and tonically inhibits the activity of the dopamine (DA) neurons whose activation is critical for the induction of the rewarding effects of brain stimulation.

The preparation in this study was the adult albino rat chronically implanted with a bipolar electrode and trained to press a lever to obtain 0.25 sec of 60 Hz sine waves applied to the medial forebrain bundle. The animal was tested for the effects of amphetamine given to induce the release of brain DA, the effects of fenfluramine given to induce the release of brain serotonin and the effects of the
two drugs given jointly to animals previously treated two times with fenfluramine to induce a permanent depletion of serotonin levels in the central nervous system via the destruction of some 5-HT terminals.

The results showed that amphetamine alone (2.0 mg/kg i.p.) facilitated the bar-pressing behavior to obtain medial forebrain bundle (MFB) stimulation. This effect was immediate and lasted about 60-90 min. The facilitation of behavior was ascribed to the availability of increased DA (released by stimulation of MFB-DA fibers ascend to the forebrain in the MFB—and by treatment with amphetamine) at synapses subserving the reinforcing function. The results also showed that fenfluramine given alone (20 mg/kg i.p.) abolished the bar-pressing behavior within the first 10 min of the treatment and that this effect lasted from 24-48 hr depending on the rate of the animal's response. Partial recovery was achieved after 5-6 days. There was no evidence of sedation at the time of treatment or of motor dysfunction or illness on subsequent days. Behavior between test sessions was normal, as was consumption of food and water. This treatment was repeated when the recovery seemed as complete as it would get (5-7 days), and again the behavior was suppressed but recovery occurred earlier but was still less complete than after the first treatment. Finally, one week after the second injection of fenfluramine, the animal was injected with fenfluramine and at the same time with amphetamine. The effect of the combined treatment was to block the bar-pressing behavior, but for only 1-2 hr instead of the 24-48 hr seen with fenfluramine alone. This was followed by a facilitation of the behavior that lasted 10-11 hr instead of the 60-90 min seen with amphetamine alone given to drug-naive animals or to animals previously treated with fenfluramine.

Regional assays of the levels of DA and serotonin from rats treated three times with fenfluramine show that the levels of serotonin were lower in the frontal cortex, neostriatum and hippocampus, but normal in the midbrain, where the cell bodies of the serotonergic neurons are found. These results confirm the findings of others that chronic fenfluramine causes the destruction of serotonergic terminals and thus accounts for the lower concentrations of serotonin in structures innervated by the 5-HT neurons. The behavioral results are interpreted to mean that the release of serotonin induced by fenfluramine (each treatment) was responsible for the suppression of the bar-pressing behavior to obtain MFB stimulation. The duration of the blockade became shorter with each treatment as less serotonin became available for release due to fewer terminals being functional. After the third treatment, the levels of serotonin available for release were soon exhausted (the blockade lasting only 1-2 hr instead of 24-48 hr), leaving the DA neurons free to exert their behavioral excitatory effect without the tonic inhibitory regulation exerted on them by the serotonin neurons. The experiments now being set up are designed to measure in vivo the release and concentration of DA and serotonin in the animals pressing for the brain stimulus and treated singly or jointly with amphetamine and fenfluramine. The objective is to verify that chronic treatment with fenfluramine results in less and less serotonin being released with each treatment, although release does occur, since the serotonergic cell bodies were not destroyed by the drug, and that the joint treatment with fenfluramine and amphetamine results in a shift in the balance of the two transmitter at the stimulation site favoring DA.

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378. The output pathways for dopaminergic-induced motor activity

Marianne E. Olds, Jeffrey D. Carpenter

The two principal output pathways from the basal ganglia are the globus pallidus and the substantia nigra, pars reticulata. In addition, it seems likely that non-dopamine neurons in the ventral tegmentum also are involved in the DA output function on the basis of the continuity existing between the substantia nigra and the ventral tegmental area, and the similarity between their organization and the properties of their neurons. What is not clear is how the output task is apportioned, that is, what type of information is related via the output neurons in each structure and how the change in activity of the output neurons relates to the specific motor activity pharmacologically induced by DA agonists. We have studied these questions by recording the output of presumed DA output neurons during the increased motor activity induced by amphetamine or apomorphine, injected by the systemic route leading to a global activation of the
DA receptor in the central nervous system or injected intracerebrally in the structures associated with increased DA-induced locomotor activity or oral stereotypy.

In the study carried out this past year, we have recorded the effects of increased DA activity in the nucleus accumbens on the output of putative DA output neurons in the substantia nigra and the ventral tegmental area. Our results show that increasing DA activity in the nucleus accumbens leads to increased motor activity lasting approximately 60-90 min, and to an increase in output from about 50% of the non-DA neurons of these two structures, the duration of the increased excitability being correlated with the duration of the increased motor activity. The data suggest a greater sensitivity of the ventral tegmental area neurons than the substantia nigra reticulata neurons to input from the nucleus accumbens. This past year we have extended this inquiry to the striatum with a view to inducing oral stereotypy by enhancing DA metabolism in the striatum with infusions of amphetamine in order to determine whether the information generated is preferentially relayed to the output neurons of the substantia nigra. The data are presently being analyzed. Preliminary results suggest that the neostriatum is functionally more complex than the accumbens, and that only sites in the ventro-lateral striatum have reliably led to increased motor activity following local infusion of DA and DA agonists. Hence, only a portion of the putative output neurons have shown changes in their firing rate during the drug-induced hypermotor activity. One of the goals is to determine whether the responsive output neurons are localized in the substantia nigra, and whether more neurons are responsive in this structure than in the ventral tegmental area, which is more intimately linked to the accumbens than the striatum. We are also extending this study to include the globus pallidus, with a view to answering the question of which of the two major output pathways for dopaminergic activity related to motor activity becomes involved when that motor activity is locomotion and when it is oral stereotypy.

PUBLICATIONS

Olds, M. E. (1991) Hyperactivity induced by enhanced dopamine activity in accumbens is correlated with increased output by non-dopamine neurons in ventral tegmentum and substantia nigra. Physiol. and Behavior, in press.

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Summary: Experiments that compare human patients and macaque monkeys, both with long-standing surgical disconnection of the cerebral hemispheres, indicate that monkeys possess a basic hemispheric specialization with human-like characteristics. The findings suggest that evolution of hemispheric cognitive specialization precedes rather than follows that for language or handedness. This raises the possibility that the hemispheric cognitive specialization is an important precursor in the evolution of human-like syntax.

Visiting scientists have collaborated on the human split-brain tests, examining macular sparing, inter-hemispheric cross-communication and integration, and lateralization of various aspects of facial, linguistic and spatial processing.

Further analysis of the cognitive (consciousness)
revolution reaffirms its basis in a changed core concept of causal determinism. Traditional "bottom-up" microdeterminism is integrated with new "top-down" macro and mental causation involving nonreductive emergent interaction. Spreading adoption of the new double way "macro-mental" form of causal explanation in other sciences and philosophy, evolutionary epistemology, hierarchy and systems theory suggests that it may be in the process of replacing reductive physicalism as a more valid paradigm for scientific explanation.

The day-to-day practice and analytic approach of science are little affected, but scientific descriptions of both human and nonhuman nature are radically revised, transforming the type of worldview and associated beliefs science leads to. Nature and all reality are no longer believed to be determined solely from below upward. The higher macro, mental, vital and social forces are given their due as well as physics and chemistry.

379. Dichotic results of children suggest increase in interhemispheric collaboration at six years of age

Polly Henninger

Nonverbal stimuli are typically processed preferentially by the right hemisphere. Most children show a left ear (right hemisphere) advantage when they identify dichotically presented hummed nursery tunes by pointing to pictures representing them (Peck, 1976). Adult commissurotomy subjects, however, show a right ear advantage for these stimuli (Biology 1986, Abstract No. 278), suggesting that once the tunes can be mediated verbally, the left hemisphere may take control of processing. This exploratory study used dichotically presented musical stimuli to investigate the validity of the "split-brain" model for understanding lateralized processing in young children and to better understand shifts in hemispheric control of cognitive processing. It tested the hypothesis that children prior to age six, the age at which children shift from a preoperational, perceptually-based mode of thought to an operational, conceptually-based mode of thought and most callosal myelination is believed to be complete, perform like "split-brain" subjects. They have difficulty attending to information from both sides of space and transferring information callosally from one side to the other, and thus exhibit a larger difference in accuracy between their ears than children after age six.

Seven 5-year-old and six 6- and 7-year-old right-handed children were given Peck's dichotic melodies test. Children were required to give a written response which is more likely than pointing to elicit increased left hemisphere processing (Pechstedt, 1989). Younger children showed a significantly larger difference in accuracy between the ears than older children (p < .02), supporting the hypothesis of greater interhemispheric collaboration after age six. The two groups did not differ in overall performance. Older children identified fewer left ear, and more right ear, stimuli than younger children but the difference between groups was not significant. If reliable, these results suggest a shift in hemispheric control around age six, at least for musical processing, involving inhibition of the right hemisphere and dominance by the left hemisphere. A longitudinal study is warranted.

References:

PUBLICATIONS

Summary: Visual images provide an immensely rich source of information about the external world. We use this information efficiently and effortlessly to carry out a wide variety of tasks that contribute to perception, motor control, and cognition. Our ability to perform these tasks accurately, rapidly, and reliably in a complex natural environment requires an extremely sophisticated and well engineered visual system. To help decipher the principles of operation of this system, our laboratory studies the organization and function of the mammalian visual cortex, particularly that of the macaque monkey. We use an interdisciplinary approach that involves a combination of anatomical, physiological, computational, and psycho-physical techniques. These are directed at questions about (i) the basic structure and connectivity of different portions of the visual system, (ii) the way in which information is represented and processed by single neurons and by networks of neurons, and (iii) the specific computational strategies used to process information about form, color, motion, and depth in our visual world.

Figure 1 shows a summary of our current understanding of the layout of different visual areas in the macaque, as seen on an unfolded map of the right cerebral hemisphere. Visual cortex, shown by the stippling, occupies more than half of the cerebral hemisphere. Thirty-two distinct visual areas associated with visual processing have been identified to date. These are interconnected by more than 300 pathways into a distributed hierarchical system for processing visual information.

We are also interested in developing and applying new approaches for managing the explosion of experimental data available about the nervous system. A long-term objective is to establish graphically-oriented databases that will provide access to vast amounts of neurobiological information more rapidly and efficiently than through the conventional literature.

On a separate front, we are continuing our efforts to understand how the specificity of neural connections is established during development. We focus on the mammalian neuromuscular junction as a model system in which a wholesale reorganization of synaptic connections occurs shortly after birth. By analyzing the organization of connections within the muscle and how these change with maturation, we aim to understand important principles that are relevant to synaptic specificity and plasticity in both the peripheral and central nervous systems.

380. Representation of surface orientation in primate visual cortex

Jack L. Gallant, Jochen Braun

Neuroscientists now know a great deal about how the visual system processes and represents simple stimuli such as bars, edges, and gratings. But we know very little about the neural representation of three-dimensional (3D) objects in the visual world. We have chosen to work first on a relatively simple aspect of the problem, namely the representation of visible 3D surfaces arrayed in our visual field. There is a great deal of behavioral and computational data on this aspect of 3D vision, and it is likely to be a general problem which the visual system has to solve no matter what the neural representation of 3D structure at higher levels (those concerned with memory and object recognition) turns out to be. We have examined the responses of cells in area V4 of primate extrastriate cortex to 3D "primitives" representing canonical pieces of 3D surfaces. The simplest of these is the 3D plane. The 3D orientation of planar surfaces can be described by two parameters, slant and tilt. Slant describes the angle between the surface normal and the
line of sight, while tilt describes the direction the surface normal points (i.e., its rotation around the line of sight). The problem of determining a cell's tuning for such surfaces is complicated by the rendering problem. When we render a specific 3D plane on a graphics monitor, several additional parameters must be specified, such as its texture density, color, and size, and each of these can affect a cell's firing rate independently of the cell's selectivity for 3D orientation. Thus, each of these factors must be controlled for and examined independently.

We have studied cells in area V4 of anesthetized, paralyzed macaque monkeys. For each cell, we attempted to collect data on its responses to simple (2D) dimensions, such as color and spatial frequency, in addition to its responses to 3D surfaces. When a cell proved to be tuned for color or spatial frequency, other stimuli were generated such that they would fall within the cell's responsive range. We have managed to collect both 2D and 3D data on almost 100 cells to date. Most cells in area V4 are tuned for color, though they often respond to a range of different colors. About half of the cells appear to be tuned for spatial frequency as well. The majority responds best to low frequencies (below 4 cycles/degree), though a few are tuned for frequencies as high as 12 cycles/degree. Many cells appear to be tuned for either the slant or tilt of surfaces in 3D space, and some cells are tuned for both slant and tilt. A large proportion of cells are tuned for both 2D spatial frequency and 3D surface orientation. More importantly, their tuning in the two domains is correlated, so that they tend to be tuned for slants that correspond to their best 2D frequency, and to tilts that correspond to their best 2D orientation. However, there is a significant subpopulation of cells that are tuned for 3D surface orientation but not for 2D spatial frequency; we do not yet know what nonlinear properties these cells possess that causes them to respond in this way.

To more thoroughly assess the relationship between spatial frequency and surface orientation selectivity, we have also obtained tuning curves for frequency-modulated (FM) gratings that simulate flat gratings oriented in 3D space. Though many cells are tuned for the slant and tilt of FM gratings, their responses once again correlate with their 2D spatial frequency tuning. These data suggest that many cells in area V4 that are tuned for particular 3D surface orientations are not explicitly selective for the 3D attributes of the stimuli, but rather are responsive to those particular 3D orientations whose spectral content overlaps with their 2D selectivity. This is not entirely unprecedented in the visual system: much the same thing appears to be happening for moving stimuli in area MST.

Future research will center on the following questions: What is the nature of the subpopulation of cells that do not appear to have any spatial frequency tuning, yet are tuned for particular 3D surface orientations? How does the inhibitory surround affect responses to 3D surfaces? And finally, if it is true that cells in this area are tuned for particular 3D orientations by virtue of their 2D tuning, what does this imply about the representation of 3D surface orientation throughout visual cortex?

381. Mechanisms of attention and pattern recognition in visual cortex
Bruno Olshausen, Charles H. Anderson, Jack L. Gallant
One theory for how our brain recognizes objects in a scene is that we have an automatic process for selectively attending to only one object at a time, and that only the information from that part of the retina being attended to, the "window of attention," is routed to areas of the visual cortex specialized for doing pattern analysis and recognition. The potential advantage of such a scheme is that variations in the retinal representation of an object, such as position, size and orientation, could be removed by bringing the object into a standard, object-based reference frame within the window of attention.

In order to study how such a scheme might be implemented in visual cortex, we are developing a model neural system that both solves the problem of forming invariant representations and also is consistent with known neurophysiology and neuroanatomy. Our goal is to then use this model to generate predictions that could be tested in neurophysiological, neuroanatomical, or psychophysical experiments.

One prediction already generated by the model is that the spatial resolution of the window of attention should be fixed for different sizes of the window. That is, a small window would be capable of capturing the fine spatial detail in a scene, whereas a large window would capture more of the overall structure. Indeed, a number of
independent psychophysical observations seem to suggest that this is the case, and moreover, that the resolution is roughly equivalent to 25 x 25 pixels. However, additional experiments are needed before it is possible to draw firm conclusions. We plan to test this hypothesis using a match-to-sample task on patterns with varying degrees of resolution (spatial frequency content) displayed at different sizes and positions within the visual field. The results of such an experiment could be of practical benefit to machine vision as well as neurophysiology and psychophysics.

382. Psychophysical studies of spatial selective attention
Jack L. Gallant

Attention is most usefully viewed as a control or routing system that mediates the efficient selective transfer of information from peripheral sensory organs to central mechanisms concerned with higher order perception. Visual selective attention is most commonly described in terms of a "window" which moves across the visual field, and many studies have attempted to characterize this system in terms of the size of this "window," the speed of "scanning," and other related parameters. Good quantitative data on the dynamics of selective attention have been lacking, however. This ongoing series of experiments is aimed at employing psychophysical techniques and recent ideas from neurophysiology and machine vision in order to obtain specific quantitative data on attentional dynamics and control.

[1] Sampling dynamics of selective attention. There have been few attempts to determine the rate at which information passes through the attentional control system; the measurements are difficult to make and interpretation usually relies on a series of broad assumptions about the nature of attention. Our procedure employs the psychophysical task of spatial frequency discrimination, which has been studied extensively apart from attention. Observers can discriminate the frequency of a test grating from a referent if the two frequencies differ by a threshold amount. The basic threshold reflects visual intrinsic noise apart from attention. To determine sampling dynamics, frequency-modulating (FM) noise is added to the test grating, forcing the observer to determine the mean of the test before comparing the test mean to the referent. Since the noise is added according to a gaussian distribution, observers may make a more accurate estimate of the mean, and reduce their thresholds, by taking more samples from the stimulus. Thus, the resulting thresholds reflect both intrinsic visual noise and attentional sampling used to compensate for the added FM noise, and we can determine the number of samples an observer must have taken to obtain a given threshold. Preliminary data suggest that sampling is an exponentially decaying function of time whose rate is dependent both on the amount of noise present in the stimulus, and the amount of information available. In addition, some observers perform best when displays contain an intermediate amount of information, their performance actually declining when more information is available for making judgments. Neither of these results is predicted by standard attentional theory, which assumes a simple linear transmission rate.

[2] Bandpass limitations of selective attention. One of the most powerful models of selective attention postulates an internal Laplacian pyramid in which peak frequency and spatial extent trade off: as the "attentional window" grows larger, the passband of the window diminishes. This model has the great advantage that the number of samples within the window is fixed, regardless of the size of the window itself. There is some indirect but no direct evidence in favor of this model. We are preparing to test this model explicitly by utilizing a procedure originally developed by C. Nothdurft to investigate texture perception. We will add bandpass noise to the stimulus field in a visual search task, and will vary the passband across trials. If visual search employs an "attentional window," which is restricted to a particular spatial frequency band, performance should be poorest when the noise passband matches the critical spatial frequency band. We suspect that the classical difference between "parallel" and "serial" search lies in the passband (and so the size of the "attentional window") required to perform visual search with different stimuli. We also suspect that "illusory conjunctions," along with many other attentional phenomena, can be accounted for within this framework.
Figure 1. A two-dimensional map of cerebral cortex in the right hemisphere of the macaque monkey. Stippling indicates cortical areas implicated in visual processing (see Felleman and Van Essen, 1991).
383. Neuronal responses to texture patterns in area V1 of the alert monkey

James J. Knierim

Over the past few years we have recorded and analyzed responses of cells in the primary visual cortex (V1) of the alert monkey to computer-generated texture patterns composed of a line segment placed within the cell's classical receptive field (CRF), presented either alone or surrounded by elements placed outside the CRF. In brief, we found that the responses of a majority of cells were suppressed by the addition of the texture surround. In addition, a large number of cells responded more strongly when there was a contrast in orientation between the center element and the surround elements than when there was no such contrast. These responses correlate with the perceptual salience of the center element and are a possible physiological substrate for the perceptual effect known as pop-out.

We were interested in how these response properties developed over the time course of the neuronal response. Specifically, were the surround effects apparent from the onset of the response, or did they take time to develop? We constructed population response histograms for our stimuli by calculating the average firing rate in each 1 msec bin for our sample of 122 cells. By comparing these histograms, we found that it took about 7 msec before the suppressive influence of the surround texture became evident in the neuronal response, and about 20 msec before the orientation contrast effect was evident. These results indicate that although the surround effects are evident very early in the population neuronal response, they do take time to develop. This latency suggests that after a cell starts responding to a stimulus, additional processing occurs before the texture surround can exert its modulatory influence on the cell. This additional processing time may reflect the contribution of intrinsic connections within V1 or feedback connections from higher visual areas.

We have also continued work on a project designed to test whether the texture surround effects are influenced by the monkey's state of attention. We will record neuronal responses to texture patterns similar to those described above under two conditions: 1) when the monkey is actively attending the stimulus within the cell's CRF, and 2) when the monkey is actively attending a stimulus in the opposite hemifield from the CRF. In the latter condition, we expect to see surround effects similar to those we have seen in our previous experiments. We will compare these results with those obtained in the former condition to determine whether the monkey's state of attention can influence the responses of cells as early in the visual pathway as V1.

384. Optical studies of visual cortex

Harry S. Orbach

Changes in absorption and fluorescence of certain membrane-bound dyes are proportional to changes in membrane potential. Based on this, optical methods can be used to measure activity in the mammalian brain. Average electrical activity in many adjacent areas can be monitored by projecting an image of the brain onto an array of photodiodes and measuring the photodetector outputs in parallel. We are currently using a 10 x 10 detector array to trace connectivity in rat visual cortex. A large expanse of striate and extrastriate cortex is stained with a potential sensitive dye and imaged onto the array. Fluorescence changes are monitored in response to short trains of electrical stimulation applied through electrodes placed in striate cortex.

We have recently been investigating some intriguing complexities in the spatial and temporal structure of the cortical excitation producing the optical signals in visual cortex. The temporal structure of the signals reveals that most of the observed cortical excitation must be the result of multisynaptic processes rather than a summation of directly stimulated or even monosynaptically-evoked neural responses. Moreover, there is considerable diversity in parameters such as latency, time-to-peak, decay time, facilitation or depression and undershoot from experiment to experiment and between traces at different distances from the site of stimulation. We are currently trying to relate these parameters, and their diversity, to underlying cortical processes.

Analyzing the spatial structure of the cortical signal
and how it evolves in time has also been rewarding. For
the most part, the region of activity was elliptical, with an
anterio-posterior major axis. However, other orientations
and shapes were seen including multiple local maxima and
irregular shapes. The time evolution of cortical excitation
included the expected increase of signal over time, with
shorter latencies nearer to the stimulation site, producing
the effect of spreading of excitation from site of
stimulation to periphery. However, a more complex
substructure was seen in some experiments, including
systematic shifts of the activity maximum with time,
systematic changes in the shape of the region of activity
with time and, in one case, the splitting of the maximum
of activity into two local maxima moving away from the
stimulation site.

Our present system is limited by its $10 \times 10$ pixel
resolution. We recently evaluated a commercial system
based on fiber optics to increase resolution to $50 \times 50$
pixels and were able to demonstrate that it could be
successfully used to record biological signals from rat
cortex. We also hope to use a design of our own to
increase the “spatial” resolution to $50 \times 50$ pixels, while
keeping the millisecond time resolution and favorable
signal noise of our current system. We expect that the
unique combination of high spatial and temporal resolution
should allow us to explore currently inaccessible niches in
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385. Use of lipophilic dyes in in vivo and post­
mortem investigations of connectivity of
macaque visual cortex
Thomas A. Coogan, David C. Van Essen

We have begun anatomical studies of the intrinsic
connections of the second cortical visual area of the
macaque (V2). We are attempting to clarify a discrepancy
in the literature on the relationship of the intrinsic
connections to the modular architecture of that area (as
visualized in the pattern of cytochrome oxidase stripes) by
applying higher resolution anatomical methods, including
more sensitive and discrete tracers, finer resolution
cytochrome oxidase staining, and powerful graphics
computers.

We are employing the brightly fluorescent, lipophilic
carbocyanine compounds Di-I and Di-O for injections into
the exposed portions of dorsal V2. There is little diffusion
of the dye once injected, and the high fluorescent signal
allows us to easily identify clusters of retrogradely-labeled
neurons in stripes neighboring the one that was injected.

Our results thus far indicate that there is an intricate pattern
of connections with a higher periodicity than the cycle of
cytochrome oxidase stripes. The three-dimensional
graphical reconstructions of these patterns should allow us
to determine whether the the closely spaced connections
are made between the islands of cytochrome oxidase
staining which make up the stripes (and found within the
pale staining interstripes), or whether they represent clear
cross-talk between the different streams of visual
processing.

The lipophilic nature of the dyes means that sections
containing the dye cannot be dried onto microscope slides.
This has prompted a drive toward what has been a long­
standing goal, that of automated scoring of neuro­
anatomical material. To rapidly collect data from sections
temporarily mounted in phosphate buffer, we take video
images of dye-labeled cells and use image processing
software (Image, by Wayne Rasband of NIH) to extract
the positions of labeled cells from the video frames. This
information is transferred to a customized anatomical
reconstruction program running on a Sillicon Graphics
computer. Video frame grabbing is also used to record the
pattern of cytochrome oxidase stripe. Sophisticated
quantitative methods of analyzing the cytochrome oxidase
patterns are important to accurately record its full
complexity, since it often happens that the human eye can
visualize patterns in the staining that simple thresholding
methods fail to document.

Preliminary experiments have been done to determine
the utility of Di-I and Di-O as postmortem tissue tracers in
fixed macaque cortical tissue. Initial results show that the
dye can diffuse in axons in the white matter at a rate of
approximately 1 mm/month. Very discrete labeling has
been found in dendritic arbors as well as fine afferent
ramifications. Injections have been placed in V2, to
expand on the results of our in vivo experiments, and to
provide a comparison to calibrate the fixed tissue method,
and into cortical areas MT and MST, for studies of
modular organization in these areas.
Principles underlying the organization of callosal connections in striate cortex of the rat

James W. Lewis, Jaime F. Olavarria

In the rat’s visual system, interhemispheric connections are concentrated at the lateral border of striate cortex, where central visual fields are represented. In addition, callosal connections extend throughout the striate cortex, including medial regions representing peripheral portions of the visual field. This more extended projection is found primarily in infragranular layers.

As in all mammals studied to date, the striate cortex of the rat has a complete representation of the contralateral visual hemifield. In addition, a central portion of each hemifield is also represented ipsilaterally, in a strip of tissue located laterally in striate cortex. Consequently, the central portion of the visual field is represented twice, once in each hemisphere. Callosal connections originate from both regions of striate cortex: 1) medial striate cortex, which represents non-overlapping peripheral portions of both visual hemifields; and 2) lateral striate cortex, which represents overlapping central portions of the hemifields. The differences in the topography of medial and lateral regions of striate cortex raise the possibility that callosal connections in these regions may subserve different functions.

We investigated this possibility by studying the topography of callosal connections. To this end, we charted the location of retrogradely-labeled cells within striate cortex of one hemisphere after combined injections of two or more fluorescent tracers into multiple sites in the contralateral striate cortex. We also labeled the entire callosal pathway with larger injections of an additional tracer in order to relate the location of cells labeled by the small injections to the overall callosal pattern within striate cortex.

We found that injections into medial striate cortex labeled cells predominantly in medial, mirror-symmetric portions of contralateral striate cortex, and only rarely in lateral portions of this area. These mirror-symmetric cortical regions represent widely disparate peripheral portions of both visual hemifields. Conversely, injections into lateral striate cortex labeled cells in lateral portions of contralateral striate cortex, with few or no labeled cells in medial portions of this area. Here, however, callosal connections link cortical regions that represent the same loci in the visual field, even though these cortical regions are not arranged as physical mirror images of each other in the two hemispheres.

Our results indicate that callosally-connected regions are organized as mirror images of each other only in regions representing non-overlapping portions of the visual field. Moreover, they also show that mirror-symmetry is broken in callosally-connected regions representing overlapping portions of the visual field. Here, callosal connections link regions of identical topography, presumably contributing to binocular interactions. These organizational differences suggest possible differences in the function of callosal pathways, which appear to be capable not only of permitting interactions between midline portions of the visual hemifields, but also of mediating interactions between widely disparate inputs from mirror-symmetric peripheral portions of the hemifields.

Anatomical studies of topography in the projection to the rabbit soleus muscle

Karina S. Cramer

In a topographically innervated muscle, neighboring motor neurons within a motor pool innervate highly overlapping regions of muscle, while distant motor neurons innervate regions with little or no overlap. Our previous studies of tension overlap have shown a lack of topography in the projection to the neonatal rabbit soleus, during a period of polyinnervation. Because synapse elimination sharpens topography in some mammalian muscles, we used anatomical methods to examine topography in older animals that have undergone synapse elimination.

We injected the retrograde tracer horseradish peroxidase conjugated to wheat germ agglutinin (HRP-WGA) into the soleus muscle to label motor neurons in the spinal cord. Rabbits from both early (polyinnervated) and late (singly innervated) age groups were included in the study. Within each rabbit, we made small, local injections in one soleus, and several injections in the contralateral soleus to label the entire motor pool for comparison. Using scored, reconstructed spinal cords and labeled muscle sections, we could assess whether local regions of
muscle were innervated by clustered groups of motor neurons, and whether or not spinal cord positions map to regions within the muscle.

We found that the spatial distribution of labeled cells following local injections did not vary with injection size, suggesting that each region of muscle receives inputs from neurons from a broad distribution within the motor pool. We also found that the position of local injections did not correlate with the mean position of labeled cells in the spinal cord. Thus, the rostrocaudal positions of motor neurons do not map to regions within the soleus muscle.

These results confirm our findings for polyinnervated soleus, and extend them to singly innervated soleus. Thus, our findings suggest that in the rabbit soleus, synapse elimination does not refine topography.

388. Development of twitch/tetanus ratios during synapse elimination in the rabbit soleus muscle

Karina S. Cramer

The study of motor unit development and diversity is essential for understanding motor function at the muscular level. Our experimental model, the rabbit soleus muscle, is polyinnervated at birth and eliminates excess synapses over the first two postnatal weeks. It contains both fast and slow motor units, which, even at early ages, are known to be distinguishable physiologically by their twitch rise times. We examine here fast and slow motor unit development using a different measure, the twitch/tetanus ratio (TTR). We find that TTRs change during synapse elimination, and that these changes are different for fast and slow motor units.

In the 4-5 day rabbit soleus, the mean TTR is .24 ± .01 (s.e.m.) for fast units and .29 ± .01 for slow units. At this age, the slow units have a significantly higher TTR than fast units (p <.01). By 13-15 days, however, we find that fast motor units have significantly higher ratios than slow motor units, with means at .35 ± .02 and .21 ± .01, respectively (p <.0001). A similar distribution has been described for 17-18 day rat soleus by the Thompson laboratory. Thus, during synapse elimination, fast motor unit TTRs increase while slow motor unit TTRs decrease.

Previous studies have suggested that the ratio of mean motor unit tensions at polyinnervated versus singly innervated ages gives a useful estimate of the overall degree of polyinnervation for fast and slow muscle fibers at the early age. However, since TTRs change with age, this ratio yields conflicting estimates depending on whether the estimates are based on twitch versus tetanic tension. While estimates using twitch tension suggest that slow muscle fibers initially receive twice as many inputs as fast muscle fibers, our estimates using tetanic tension suggest that fast fibers actually receive more inputs than slow fibers. Consequently, more direct methods will be needed in order to obtain reliable measurements of polyinnervation by fiber type.

PUBLICATIONS


Supervisor: Suzanna J. Horvath


Our work still focuses on our original goal: to provide sophisticated chemical synthesis and analysis of a large number of oligonucleotides, deoxyligoligonucleotides and peptides (protein domains) to the scientific community of Caltech by using a variety of updated, automated micro-chemical and analytical techniques. We have continued, as well as generated, many exciting collaborative research projects through intensive scientific interactions with Biology, Chemistry, Engineering and Geology faculty members.

Our routine DNA synthesis in 1990 provided 4002 deoxynucleotides, which incorporated 102,130 base linkages. This includes small (0.2 µM), regular (1 µM), and large (10 µM) scale syntheses; and the synthesis of mixed probes, probes containing deoxyinosine (I), "doped" probes, and probes containing derivatized deoxyligoligonucleotides. RNA synthesis has become a routine procedure in our facility by the successful synthesis of 31 short oligonucleotide chains. We have also used the DNA extractor to prepare 379 samples of DNA.

A novel method for deoxyligoligonucleotide-directed random mutagenesis has been introduced. We have been using a new strategy for synthesizing protein-encoding oligonucleotides with randomly incorporated codon substitutions. This method was initially devised by David Botstein and Mark Zollar of Genentech (personal communication). The standard methodology for synthesis of randomly altered oligonucleotides involves the addition, at each step, of a set proportion of "incorrect" nucleotides. The amino acid substitutions that can be obtained using such "doped" oligonucleotides are limited to those that result from single base substitutions. The current method circumvents this limitation and permits substitution of entire codons, either with random codons or with a specific defined amino acid. The basis of this novel methodology is the use of two DNA synthesis columns operating simultaneously. In the first of these columns, the normal wild-type sequence is being synthesized, while in the second, the codon for the desired amino acid substitution is mutagenized. At the beginning of the synthesis, a fraction of the support resin corresponding to the desired fraction of per position substitution is placed in this second column, while the remainder is placed in the first column. After three cycles of base addition, the resin from the two columns is mixed, divided again, and the process repeated until the end of the mutagenic region is reached. We have been using oligonucleotides synthesized in this manner to introduce cysteine residues at random into the sequence of the aspartate receptor of E. coli. Analysis of disulfide formation in the resulting mutant proteins permits us to identify residues that are in close proximity and thus to analyze structural features of this protein (A. Pakula, M. Simon, S. Horvath).

As novel nucleic acid probes, we have chemically synthesized seven 2'-O-methyloligo-ribonucleotides (Sproat et al., 1989) complementary to either an snRNA or to pre-mRNA, in order to study the role of small nuclear RNAs (snRNAs) in the splicing of precursor messenger RNA (pre-mRNA) in a yeast in vitro system. Five snRNAs (U1, U2, U4, U5 and U6) function as small ribonucleoprotein particles (snRNPs) and are components of the spliceosome, the large ribonucleoprotein particle that catalyzes splicing. The oligos are used as antisense probes to specifically inhibit a snRNP. The 2'-O-methyloligoribonucleotides are particularly useful for these studies as they are not degraded or recognized by the DNase and RNaseH activities, respectively, in splicing extracts. Thus far, we have found that when oligos complementary to either the U1, U2, U4 or U6 snRNAs are added to yeast splicing extract, splicing is inhibited. We are currently assaying the specificity and effects of the inhibition on spliceosome formation. The oligos were also synthesized with "tails" containing a modified 2'-deoxycytidine which can be biotinylated or otherwise modified for use in affinity chromatography of the snRNPs and spliceosome (S. Ruby, J. Abelson, S. Horvath).

Our routine peptide synthesis in 1990 provided 161 peptides which represent 2978 amino acid linkages. Small synthetic peptides conjugated to carrier proteins are widely used with increasing success at Caltech to generate
polyclonal and/or monoclonal antibodies with desired specificities. To further aid this effort, we have started the routine synthesis of multiple antigenic peptides (MAP) introduced by J. P. Tam (1988). The direct synthesis of a peptide-antigen matrix is carried out by the usual automated stepwise solid phase method. There is no carrier protein in this system, thus both the potential immuno­
genic response of the carrier protein and the potential modification of the peptide determinants by the carrier protein are eliminated.

We are set up to develop strategies for characterizing the modified peptides generated as side products of the solid phase t-Boc synthetic chemistry. They are not detectable by amino acid analysis and protein sequencing. Our strategies combine an array of protein chemistry, high resolution HPLC, and CZE methods with a state-of-the-art mass spectrometry method. Mass spectra are recorded on a Finnigan MAT TSQ70 (Finnigan MAT, San Jose, CA) triple quadruple mass spectrometer equipped with a 40 keV cesium ion gun and a 20 keV conversion dynode. Mass analysis is performed by adding 1ml of a 5% acetic acid solution containing the peptide product of interest at 10-50 pmol level to 1 µl of monothiglycerol in a gold plated, copper probe tip, 1 mm in diameter. Peptides are sputtered out of the liquid matrix into the gas phase by bombardment with 8-10 keV Cs+ ions generated with the cesium ion gun. Typically the mass range of m/z 400-3000 is scanned 10 times at 390 daltons/s, and the resulting mass spectra are summed together. A unique computerized approach is used in the analysis of mass spectra data (J. Yates, P. Griffin, S. Horvath, L. Hood).

Semisynthesis of horse heart cytochrome c has been used to create novel analogues to study long-range biological electron transfer. First the native protein is cleaved with cyanogen bromide into two fragments, followed by recovery of the heme-containing fragment. Reconstitution of the mature protein can then be achieved by reaction of the native heme-containing fragment with a chemically synthesized 39-mer peptide that constitutes the remainder of the protein. Using this technique, histidines have been introduced in various places in the sequence (for example, at positions 72 and 79) to create binding sites for ruthenium complexes at the surface of the protein. Unnatural amino acids that will function as chelates will also be introduced at the protein surface. These analogues of cytochrome c will be used to elucidate the factors governing the intramolecular long-range electron transfer between the redox sites. Analogues with alteration of an axial ligand (amino acid 80) have also been studied (Raphael and Gray, 1989, 1991). Changes in the axial ligand have been found to dramatically alter the redox properties of the protein. The axial ligand mutation will be used to study the effect of redox potential and axial ligand on electron transfer. These experiments are directly complementary to site directed mutagenesis techniques, as SDM requires the creation of a biologically functional mutant (i.e., nature redox potential) and precludes the use of non-naturally coded amino acids (M. Bjerrum, D. Wuttke, S. Horvath, H. Gray).

As we reported last year, we determined the three-dimensional solution structure of a synthetic peptide that corresponds to one of the two zinc finger domains (ADR1b) in the yeast transcription factor ADR1 using two-dimensional nuclear magnetic resonance spectroscopy (Klevit et al., 1990). Similar work concerning the other synthetic zinc finger domain (ADR1a) and the synthetic double zinc finger domain (ADR1c) is in progress. Several point-mutant single-finger peptides of ADR1a and ADR1b have also been chemically synthesized. These mutants contain alterations of conserved aromatic and aliphatic residues, which should allow us to determine the structural and functional significance of the hydrophobic core in the folded zinc-finger structure. Potential DNA-binding residues are found throughout the structure, with the highest concentration of such residues on the external face of the α-helix. Using genetic methods, several separate alanine point mutations have been introduced in the finger corresponding to ADR1b in the protein, and the mutant proteins' ability to bind DNA has been screened. For the first round of mutagenesis experiments, we concentrated on the α-helical region of the finger. Then we chemically synthesized three selected alanine point mutants of the ADR1b-finger which in the genetic experiment (a) showed decreased DNA-binding, (b) showed increased DNA-binding, and (c) showed DNA binding that of the wild type, respectively. The structural features of these and other alanine point mutants, hopefully, will led us to an understanding of how the zinc fingers are interacting.
with DNA (R. Kievit, S. Horvath, L. Hood).

References:

CELL SORTING FACILITY

Supervisor: Ellen Rothenberg
Staff: Rochelle A. Diamond, Patrick F. Koen

The Biology Division Cell Sorting Facility provides sophisticated instrumentation and expert assistance to researchers wishing to separate and analyze cells. We currently have three research-grade sorting flow cytometers which allow cells to be sorted at a high rate of speed (>2,000 cells/second) based on the multiparameter analysis of their characteristics (i.e., size, density and fluorescence intensity in up to four colors).

This year we have purchased a Becton-Dickinson FACSscan analyzer which will enable researchers to access routine flow analyses 24 hr/day. The unit was tested in two on-site trials during the summer and winter of 1990. It demonstrated ease of operation even for casual users, with high quality results. Those interested in using this machine may contact the facility for a training appointment.

In addition, Phoenix Flow Systems has been contracted to upgrade our Ortho Cytofluorograph to eight parameter, four color fluorescence. The Ortho data will be routed to a DOS environment for analysis and integration with a network. This will fully bring the facility up to the state of the art.

In January, Rochelle Diamond presented a Division-wide seminar entitled "Flow Cytometry: A Power Tool for Biological Studies" to enhance the Division's understanding of the different uses and techniques of flow cytometry available to researchers. This has generated much interest in multfluorescence analysis.

This past year, the facility has collaborated and consulted with nine Biology and Chemistry laboratories. David Anderson's laboratory has been sorting committed progenitors from rat embryo sympathetic neurons and chromaffin cells. They have used antibody markers to identify the timing of commitment to neuronal differentiation from the neural crest to the sympathoadrenal lineage. They have also analyzed transfected cultures for their behavior under the influence of different exogenous factors (A. Michaelsohn, S. Birren, D. Stemple, A. Augsberger, J. Johnson, L.-C. Lo, J. Montgomery, D. Vandenbergh).

Leroy Hood's laboratories have been analyzing the development of tolerance in the thymus of transgenic mice expressing myelin basic protein-specific T-cell receptor genes. Panels of monoclonal antibodies were used to phenotype the transgenic thymus, spleen, and lymph node cells (D. Zaller, J. Goverman, H. Lee). FACS has also been used to characterize the cells used to make a precursor-T-cell minus mature-T-cell subtractive cDNA library (S. Orr). Analysis, sorting and phospholipase C assays monitoring Qa-2 and TLA expression were performed on T-cell clones (M. Nishimura, N. Ulker, K. Lewis, and P. Tabaczewski). The alloreactive T-cell receptors on T-cell clones were analyzed with monoclonal antibody panels (G. Osman, K. C. Cheng).

The Strauss laboratory has been analyzing Sindbis virus receptors in chicken cells with anti-idiotypic antibodies. They have also been successful in screening for Sindbis virus receptors on BHK cells using a monoclonal antibody directed against BHK cells (K.-S. Wang).

The Bjorkman group has been characterizing two stable Chinese hamster ovary cell lines expressing large amounts of two different membrane-bound forms of Fe receptors which they have constructed (L. Gastinel).

The Attardi group has used the sorter with propidium iodide to isolate and purify cell cycle-staged populations of HeLa cells for further DNA isolation (G. Paoletti, C. Novitsky).

Judy Campbell's group has been analyzing the cell cycle of Saccharomyces cerevisiae mutants with propidium iodide (P. Rhode).
Ellen Rothenberg's laboratory has been using three-color analysis to characterize intrathymically injected bone marrow derived T-cell progenitor cells (P. White). They also use multicolor phenotyping to characterize and isolate subpopulations of thymus from normal and scid mice (R. Diamond, E. Rothenberg, D. Chen, J. Yang-Snyder, L. Scherer) and screen transgenic animals for expression of haplotypic and transgene-encoded markers (S. Shen).

LABORATORY OF MACROMOLECULAR STRUCTURE ANALYSIS (LMSA)

Supervisor: David B. Teplow
Research Associate: Jane Z. Sanders
Staff: Christine A. Acklin, Tamara K. Bauer, John Rac

The LMSA helps researchers develop strategies for characterizing the primary and secondary structures of proteins and peptides. It implements these strategies using an extensive armamentarium of analytical instruments, including two protein sequencers, an amino acid analyzer, a capillary zone electrophoresis (CZE) unit, a plasma desorption time-of-flight mass spectrometer (PDMS), and high resolution HPLCs. Sample purity and enzyme kinetics are studied at femtomole levels using CZE. PDMS provides the means to identify and characterize post-translational modifications of peptides and proteins. PDMS is also being used to obtain protein sequence information from peptides and as a quality control tool in peptide synthesis. Representative abstracts from recent work are presented below.

Generation of DM-20 splice site in myelin proteolipid protein gene: An hypothesis based on analysis of the amphibian protein
Jeevajothie Karthigasan1,2, Tamara K. Bauer, David B. Teplow, Raul A. Saavedra3,4, Daniel A. Kirschner1,2

The DM-20 isoform of proteolipid protein (PLP) of central nervous system myelin is undetectable in amphibia, but readily detectable in reptiles, birds and mammals. To explain the phylogenetic origin of DM-20, it has been proposed that a donor-acceptor RNA splice site was generated by the introduction of a single base change in the codon encoding amino acid 116 of PLP. We tested this hypothesis by isolating and sequencing peptides from bullfrog PLP. One of the peptides corresponded to mammalian residues 110-122, which contain the N-terminal boundary region for the domain excluded from DM-20 in higher vertebrates. We found that the Thr115-Val116 sequence is conserved between frog and mammal. Therefore, we propose that a single base change in the third position of the codon for Thr115 would account for the appearance of the new donor-acceptor splice site. Three amino acids elsewhere in this 13-residue peptide were found to differ between bullfrog and mammal, which could account for the previously reported weak recognition of amphibian PLP by an antiserum specific for peptide 109-128. A second peptide from bullfrog PLP had a sequence identical to that of residues 45-52 in mammalian PLP. Our findings explain how a specific change in the myelin PLP gene could generate a new form of the PLP protein.

Domain of Flp recombinase that recognizes Flp-target site
Jing-Wen Chen5, Barbara R. Evans5, Sang-Hua Yang5, David B. Teplow, Makkuni Jayaram5

Binding of a partial V8 digest of Flp recombinase from Saccharomyces cerevisiae to its target site gives rise to DNA-protein complexes that are smaller than those produced by the full-sized protein. The smallest of these complexes (occupancy of one peptide monomer per site) contains either one of two polypeptides (32 kDa and 28 kDa) of the V8 digestion mixture. The amino termini of both polypeptides map to Ser-129 of Flp, corresponding to V8 cleavage at Glu-128. The relative mobilities of the complexes formed by the V8 peptides indicate that they lack the sharp substrate bend that is characteristic of Flp-derived complexes. A hybrid protein consisting of the amino-terminal one-third of the R recombinase (from Zygosaccharomyces rouxii) and the carboxy-terminal two-thirds of Flp recognizes the Flp target site.

Pseudomycins, a family of novel peptides from Pseudomonas syringae, possessing broad spectrum antifungal activity
Leslie Harrison6, David B. Teplow, Michael Rinaldi7, Gary Strobe6

A family of peptide antimycotics, termed pseudomycins, has been isolated from liquid cultures of
Pseudomonas syringae, a plant-associated bacterium. These compounds were purified using Amberlite XAD-2 and reverse phase liquid chromatography. Pseudomycin A, the predominant peptide in a family of four, was active against a wide spectrum of pathogenic plant fungi, expressed selective phytotoxicity, and showed impressive activity against the human pathogen Candida albicans. Amino acid, mass spectroscopic, and comparative electrophoretic and chromatographic analyses revealed that the pseudomycins are different from previously described antimycotics from \textit{P. syringae}, including syringomycin, syringotoxin and syringostatins. Pseudomycins A-C contain hydroxyaspartic acid, aspartic acid, serine, arginine, lysine and diaminobutyric acid. The molecular weights of pseudomycins A-C, as determined by plasma desorption mass spectroscopy, are 1224, 1208 and 1252, respectively.

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\textsuperscript{4}Department of Orthopaedic Surgery, Harvard Medical School, Boston, MA 02115.  
\textsuperscript{5}Department of Microbiology, University of Texas, Austin, TX 78712.  
\textsuperscript{6}Department of Plant Pathology, Montana State University, Bozeman, MT 59717.  
\textsuperscript{7}Audie L. Murphy Veterans Memorial Hospital, San Antonio, TX 78284.

\section*{MONOCOCLAL ANTIBODY FACILITY}

\textbf{Supervisor:} Paul H. Patterson  
\textbf{Member of the Professional Staff:} Susan Ker-hwa Ou  
\textbf{Staff:} Julianna Horn-Krist, Lauren Gohara

In addition to its service function, the Facility conducts research on immunological methods. In an effort to enhance the probability of producing monoclonal antibodies (mAbs) against rare target antigens in complex mixtures, we are comparing several techniques. In collaboration with Colin McDonald (Washington University Medical School), we compared the cyclophosphamide immunosuppression (Matthew and Patterson, 1983; Matthew and Sandrock, 1987) and neonatal tolerization (Hockfield, 1987) methods. In collaboration with Arlene Chiu (City of Hope), we compared these two methods with a polyclonal antiserum method (Barclay and Smith, 1986). The results obtained thus far indicate that the cyclophosphamide method is superior to the tolerization approach, and the polyclonal method looks very promising.

During the past year, the Facility generated mAbs for several groups. Li Ching Lo and David Anderson obtained a mAb against the coding region of MASH-1 (mammalian achaete-scute homologue). Shin-Shay Tien and Kai Zinn produced mAbs against three putative receptor-linked protein tyrosine phosphatases from \textit{Drosophila}. Pierre Jurdic and Elias Lazarides generated mAbs against surface antigens of chicken osteoclasts by suppression with chicken macrophages; macrophage markers were obtained by suppression with osteoclasts. Konrad Zinsmaier and Seymour Benzer produced mAbs against two isoforms of cysteine strain proteins in the \textit{Drosophila} brain, as well as mAbs against a fusion protein containing glutathione-S-transferase. Carl Parker produced mAbs against a recombinant \textit{Drosophila} heat shock factor. Clay Brown and Judy Campbell generated mAbs that precipitate DNA polymerase II. In addition, we are currently working with Mike Muhick from Carl Parker's lab, Ted-Yi and Chand Desai from Kai Zinn's lab, Justin Garvey from Lee Hood's lab, Brian Mozer from Seymour Benzer's lab, Art Arnold (UCLA) and Michael Lai (USC Medical School).

\textbf{References:}

\section*{PUBLICATION}
NUCLEIC ACID AND PROTEIN SEQUENCE ANALYSIS COMPUTING FACILITY

Supervisor: Howard D. Lipshitz
Staff: Leland G. Kozar

This new Facility was established this year to serve the needs of the Division. It comprises a VaxStation 2000 (GenVax), a VaxStation 3100/38 (SeqVax), a SparcStation (SeqSparc), associated internal and external hard disks and other peripherals, including a laser printer. In addition, a Macintosh IIcx was purchased to allow users to run microcomputer-based programs.

The Wisconsin Genetics Computer Group (GCG Version 7.0) package of programs constitute the major set of available software on the Vax-cluster. Additional software has been added as it has become available. This includes XQS from NBRF, TreeAlign, MuFold [Mac], LoopViewer [Mac], PSQ, CLUSTAL, RDF2, MacMolecule [Mac], GM [Sun], PlotA [Mac] and DNAstack. Direct network connections to GenBank and the NCBI were established (the latter to enable users to run the BLAST program).

A sequence analysis user manual (Sequence Analysis Learning Guide) was compiled to assist users and a Bio23 course (Computational Methods in Molecular Biology) was taught to a class of 20-30 postdoctoral fellows and graduate students.

Usership grew at a rapid pace over the past year. At the time that the Facility was established in May 1990, there were 53 users from 12 laboratories (11 in Biology and one in Chemistry), and an average of 200 log-ins per month using 25 hours of processor time. By May 1991, there were 221 users from 21 laboratories (17 in Biology, four in Chemistry and Chemical Engineering), and an average of over 700 log-ins per month using 170 hours of processor time.

TAF CIT: The Transgenic Animal Facility

Supervisor: Carol Readhead
Steering Committee: Melvin Simon, Lee Hood, Barbara Wold and Carol Readhead
Staff: Eef Goedemans, Anita Ackerman, Jorge Mata, Hector Forero

Only recently has it become possible to change the genetic make-up of mammals in a specified manner rather than by random mutagenesis or selected breeding. This is due to two techniques, namely, the introduction of novel genes into the mammalian genome by microinjection of DNA into fertilized eggs (Gordon et al., 1980) or by the inactivation of a target gene by homologous recombination with a disrupting vector in pluripotent embryonic stem cells (Doetchman et al., 1988; Mansour et al., 1988). These two techniques are complementary to one another; the transgenic technique being important for the study of spatial and temporal gene expression during development of the animal, while targeted disruption of a single gene leads to important insights into how gene products participate in the fabric of the whole organism.

The transgenic technique was established at Caltech by Carol Readhead in 1984 and has continued to flourish ever since. It is now important to provide a core facility for gene targeting in embryonic stem cells. For this technique to work it is of primary importance that the mice be free of pathogens. TAF CIT mice are free of all pathogens except murine herpes virus (MHV) which is endemic in nearly all mouse colonies. TAF CIT is now in the process of being converted to a specific pathogen-free (SPF) facility. This involves considerable time and expense, including the purchase of filter-top cages and the hiring of a full-time "professional" manager. It is hoped that we will be able to generate directed mutagenesis in pathogen-free mice within the next academic year.

TAF CIT serves the Caltech faculty. Members wishing to use the facility submit proposals to the Steering Committee who allots them a training period, time on the microscope and space for keeping animals. Principal investigators are charged according to the number of cages they are maintaining, as well as a fraction of "common stock" cages. The charge for the cages is not a reflection of the true cost but a subsidized cost. The subsidy is obtained.
from a number of core grants. Embryos from transgenic lines are frozen down, thus saving both money and space.

Seven principal investigators and their postdoctoral fellows or graduate students are presently using the facility: David Anderson (Jane Johnson and Kathryn Zimmerman), Lee Hood (Joan Goverman/Lisa Larson, Carol Readhead/Peter Mathisen, Niels Jensen and Kristine Zandvliet), Melvin Simon (Janis Lem, Murray Robinson and Thomas Wilkie), Barbara Wold (Jeffrey Miner) and Seymour Benzer (see individual reports).

References:
GRADUATES

Thirty-eight students in Biology were awarded the B.S., M.S. or Ph.D. degree in June 1991. Names, degrees conferred and titles of doctoral theses are as follows:

Bachelor of Science

Keith Takumi Akama, B.S.
Helen Hsin-I Cheng, B.S. with honor
Ajay Dinesh Chheda, B.S.
Lindsey Norman Dubb, B.S.
Milind Mukund Ganga!, B.S.
Laura Juliet Hernandez, B.S. with honor
Gary Roger Holt, B.S. with honor
Hermant Vasant Keny, B.S.

Melinda Ann Knox, B.S.
Ngocdiep Thi Le, B.S. with honor
Ralph Shih-Ying Lin, B.S. with honor
Eugene Stephen Lit, B.S. with honor
Steven Andrew McLaughlin, B.S. with honor
Mark Andrew Schmidt, B.S.
Samantha Andrews Seaward, B.S. with honor
David Marc Taub, B.S.

Master of Science

Susan Apostolaki, M.S.
Laura Lynn Brockman, M.S.

Doctor of Philosophy

Elliot Charles Altman, Ph.D. (Biology), Characterization of the SecB protein, a chaperone that facilitates protein secretion in Escherichia coli.

John Lincoln Bowman, Ph.D. (Biology), Molecular genetics of flower development in Arabidopsis thaliana.

Stephen Paul DeWeerth, Ph.D. (Computation and Neural Systems), Analog VLSI circuits for sensorimotor feedback.

Michael Roy Emerling, Ph.D. (Biology), Enzymatic hydrolysis of the amide bond: Mutagenic studies of the mechanisms of α-lytic protease and β-lactamase.

John Gregory Harris, Ph.D. (Computation and Neural Systems), Analog models for early vision.

Paul Kenneth Herman, Ph.D. (Molecular Biology and Biochemistry), Protein sorting in the eukaryotic secretory pathway: An essential role for a novel yeast protein kinase.

Jan Häkan Hoh, Ph.D. (Cellular Biology and Biophysics), Studies on the structure and molecular diversity of the gap junction.

James Julius Knierim, Ph.D. (Neurobiology), Neural responses to texture patterns in area V1 of the alert monkey.

Michael Alan Lochrie, Ph.D. (Biology), Molecular biology of G protein alpha subunits from bovine photoreceptors and the nematode Caenorhabditis elegans.

Nagesh Kalyana Mahanthappa, Ph.D. (Neurobiology), Functional and biochemical studies on neuronal Thy-1.


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Richard Daniel Mooney, Ph.D. (Biology), The development of connectivity and the nature of synaptic transmission between avian song control nuclei.

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Cellular pedigrees of mitochondrial transformants obtained by sequential transfer of mitochondria from a neuromuscular disease patient into mitochondrial DNA-less (ρ0) human cell lines. Myoblasts from the patient, which either contained or lacked a deleterious tRNA<sup>lys</sup> mutation in their mitochondrial DNA, were used as mitochondrial donors; the transformants that contained were used as donors for a second cycle mitochondrial transfer. The open symbols represent different ρ0 cell lines used as recipients of exogenous mitochondria; the solid symbols represent myoblasts or transformants with defective genotype and phenotype; the hatched symbols represent myoblasts or transformants with normal genotype and phenotype. See Chomyn, L., et al., Abstract No. 193.