FRONT COVER

Eliot Bush, Elwyn Simons, David Dubowitz, Miriam Scading, Marvin Nelson, and John Altman

Surfaces of the skull and endocranial cavity of *Parapithecus grangeri* based on CT scans performed at Children's Hospital Los Angeles. *Parapithecus* is an early anthropoid from the Fayum deposit in Egypt around 33 million years ago. It is a primitive monkey. This specimen was found by Elwyn Simons and is the most complete early anthropoid skull known. The external surface of the skull has been rendered transparent to reveal the endocranial cavity, which closely matches the external surface of the brain, thus making it possible to reconstruct the outer surface of the brain of this fossil monkey. Dark blue represents the olfactory bulbs and light blue represents the optic nerves. CT scans reveal that *Parapithecus* had a small brain for its body weight, and a small optic nerve compared to living anthropoids. These observations suggest that high acuity vision developed much later than was previously thought, and holds out the possibility that the development of high acuity may have been associated with the development of large brains in the living anthropoids.

BACK COVER

A knock-in mouse carrying a GABA transporter fused with green fluorescent protein (GAT1-GFP) shows plasma membrane GFP expression in GABAergic interneurons of the cerebellum. The brightest structures, pinecones, are aggregations of many astrocytes from basket cells that form inhibitory synapses with the axon hillock of Purkinje cells. Presynaptic terminals also form baskets around the Purkinje cell bodies. Terminals and processes of stellate and basket cells express the transporter-GFP fusion in the molecular layer, above the Purkinje cell layer. The fusion protein is expressed less strongly in the granule cell layer, below the Purkinje cells. Scale bar: 50 μm. See Lester abstracts starting on page 211.

Protein co-factors that regulate aggregation and nuclear localization of huntingtin protein (Htt), the etiological agent in Huntington disease (HD), are of great interest in understanding the pathology of this fatal condition. While Htt is normally found in various locations in the cytoplasm, it aggregates in the nucleus in HD. We have discovered that expression of IKKγ, a regulatory component of IKK signalosome, promotes aggregation and nuclear localization of hTTR exon-1 in 293 cells. Shown here is a confocal examination of cells co-transfected with wild-type hTTR exon-1 and a full-length IKKγ plasmid. Htt is fused to EGFP (upper left panel), IKKγ is stained with anti-HA antibody (upper right), and TOTO-3 is used for nuclear staining (lower left). The lower right panel presents the three colors merged. Further information on this line of experiments can be found in abstract 348.
Research Reports

Biological research summarized in this report covers the time period from June, 1999, through July, 2000. The annual report is not intended to serve as an official forum, since some portions of the research listed in this report have not yet been published. When referring to an individual abstract(s), special permission must be obtained from the investigator.

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Introduction
50 and 25 Years Ago

From the 1951 Division of Biology Annual Report

"The James G. Boswell Foundation Fund for Virus Research has made possible a development at the Institute of especial interest in the field of animal virology. This involves an attempt by Dulbecco and associates to develop a new type of animal virus assay fashioned after the now classical plaque count assay technique widely used in bacterial virology."

"The air pollution in the Los Angeles area is characterized by a decrease in visibility, crop damage, eye irritation, objectionable odor, and rubber deterioration. Haagen-Smit and coworkers have shown that these effects are closely related and are due to the oxidation of unsaturated hydrocarbons. The nitrogen oxides released in combustion processes function as oxidants in these reactions… The oxidant formed closely resembles ozone…"

"Beginning with the 'Able' bomb test at Bikini in 1946, samples of dry maize seed have been exposed to high-energy radiations… it was possible to measure the biological effect of an atom bomb exposure… The pollen semi-sterility is due to gross chromosome alterations…" From the description of the work of E.G. Anderson

"It is becoming more and more evident that chromosomes often contain small clusters of genes which are so close together and so closely related in their functioning that they mimic the behavior of a single gene. An attempt is being made by Lewis to learn as much as possible about one such cluster of genes…technically called the bithorax pseudoallelic series…” From the description of the work of E.B. Lewis

From the 1976 Division of Biology Annual Report

"After 42 years on the faculty, Professor Cornelis Wiersma has retired, marking the end of a very distinguished career of neurophysiological research."

"A symposium on the subject of 'Molecular Biology of the Mammalian Genetic Apparatus--Its Relationship to Cancer, Aging and Medical Genetics' was held at Caltech …to mark Professor James Bonner’s 65th year. The symposium drew over 450 attendees."

"By the time this report appears, two Viking spacecraft will have landed on Mars--safely, we hope--and will be engaged in historic operations on the Martian surface. Mars is the most plausible habitat of extraterrestrial life in solar system, and whether the search for Martian life gives positive or negative results, it will inevitably alter our view of the world. We hope to report on those results in this space next year, with particular attention to our own experiment, the Carbon Assimilation--also known as the Pyrolytic Release--experiment.” From the report of Norman H. Horowitz
Honors and Awards

The Lawrence L. and Audrey W. Ferguson Prize
Wenying Shou has been elected as this year's winner of the Ferguson Prize. Her thesis elucidated mechanisms by which the mitotic exit network (MEN) controls exit from mitosis in budding yeast. Mitotic exit is an essential step of the cell cycle in all eukaryotes. Wenying showed that the MEN works by controlling release of a key phosphatase, Cdc14, from an anchor protein located within the nucleolus. Her thesis also defines unexpected links between the chromatin structure of the nucleolar rDNA and the control of the cell cycle. To quote from Ray Deshaies nomination letter: "She entered one of the last uncolonized outposts of cell cycle control, and with a few deft brush stokes crafted a picture of dazzling beauty and clarity." [This phase has itself been nominated for the Lord Byron award for poetry in science].

Graduate Deans' Award for Outstanding Community Service
Keith Brown received the 2001 award which is awarded to a Ph.D. candidate who, throughout their graduate years at the Institute, has made great contributions to graduate life and whose qualities of leadership and responsibility have been outstanding.

Professorial Awards
Grace C. Steele Professor of Molecular Biology Giuseppi Attardi received the Ellison Foundation Senior Scholar Award for research on aging; he was also awarded the 2001 Special Achievement Award from Nature Biotechnology and Biacore, at the Miami Winter Symposia.

James G. Boswell Professor of Neuroscience Seymour Benzer received the following honors and awards during the past year: International Prize for Biology, Japan, 2000; Passano Award, 2001; Award in the Neurosciences, National Academy of Sciences, 2001; Elected Foreign Member, French Academy of Sciences; and was Elected Foreign Member, Genetics Society of Japan. (Photo on following page.)

Executive Officer and Professor of Biology Pamela Bjorkman was elected to the National Academy of Sciences in May, 2001.

Assistant Professor of Biology David C. Chan, has been selected as Rita Allen Foundation Scholar. This award is designed to provide financial support for researchers in the early stages of their careers who show promise of becoming leaders in one of the areas of special interest to the foundation. Chan specializes in research on mitochondria, components of the cell important in energy metabolism and also in programmed cell death. Specifically, he investigates the manner in which cells coordinate mitochondrial functions with the development of tissues and organs.

Bing Professor of Neurobiology Masakazu (Mark) Konishi was awarded the 2001 Kresge Hearing Research Award.

Thomas Hunt Morgan Professor of Biology (Emeritus-Active) Edward B. Lewis, was elected honorary member of the Genetical Society of Japan.
Chairman and Professor of Biology Elliot Meyerowitz was awarded the Wilbur Lucius Cross Medal of the Yale University Graduate School Alumni Association at the Yale graduation in May, 2001. Meyerowitz was also the Visiting A.F. Wood Professor at the University of Florida, Gainesville, in September 2000; gave the Sonneborn Lecture at Indiana University in October, 2000; gave the Steenbock Lectures at the University of Wisconsin in October, 2000; delivered a University Lecture at the University of Texas Southwestern Medical Center in January, 2001; and gave a Harvey Lecture to the Harvey Society in New York in February, 2000.

The Biology Undergraduate Students Advisory Committee Award for Excellence in Teaching, 2001 was awarded to Biology Professor Ellen Rothenberg.

Howard and Gwen Laurie Smits Professor of Cell Biology Alexander Varshavsky has received the following honors and awards during the past year: the 2001 Wolf Prize in Medicine (Wolf Foundation, Israel); the 2001 Horwitz Prize (Columbia University); the 2001 Merck Award (American Society for Biochemistry and Molecular Biology); and the 2001 Massry Prize (Massry Foundation). He shared these awards with Avram Hershko (Technion, Israel). Varshavsky also received the 2001 Max Planck Research Prize (Germany), the 2001 Pasarow Award in Cancer Research (Pasarow Foundation), and was elected to the American Philosophical Society and the European Molecular Biology Organization. In 2001, he gave Harvey Society Lecture at the Rockefeller University, Nelson Lecture at the Yale University, and Edsall Lecture at the Harvard University.

Left to right: Alex Benzer, Carol Miller Benzer, Seymour Benzer, Emperor Akihito, Doug Feldman, Empress Machiko, Renny Feldman
Pictures taken at Lake Arrowhead Retreat. Photos taken by Paul Patterson.
Pictures taken at Topping Off Ceremony for Broad Center for the Biological Sciences.
Photos taken by Bob Paz.
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Summary: Motor enzymes — dyneins, kinesins, and myosins — convert energy from ATP dephosphorylation into most of the movements performed by eukaryotic cells. We think that myosin and kinesin are reasonably well understood, although new experimental results from time to time surprise us. On the other hand, we have very little knowledge or understanding of the functioning of the axonemal dyneins that power the movements of flagella and cilia; these molecular complexes are a major challenge for the future. My current work uses computer simulation methods to explore ideas about motor enzyme function in situations ranging from experimental studies on individual motors to an intact flagellum containing tens of thousands of dyneins. Most of the simulation programs, as Macintosh applications, are available at www.cco.caltech.edu/~brokawc/software.html

1. Protein-protein ratchets

Charles J. Brokaw

Interaction between a protein and a series of binding sites on a cytoskeletal substrate can create a resistance, or "protein friction," as the protein is moved along the substrate. If attachment and detachment rates are specified asymmetrically, this resistance can depend on the direction of movement, and the binding interaction acts as a ratchet. Stochastic computer simulations have been used to examine this type of protein–protein interaction. The performance of a protein–protein ratchet in the picoNewton and nanometer range is significantly limited by thermal fluctuations, which in experimental measurements with single molecules are evident as Brownian movement. Simulations with a two–component model combining a conventional motor enzyme model with a protein–protein ratchet confirm previous suggestions that the processive movement of a single motor enzyme molecule against a load, as seen in experiments with inner arm dynein molecules, might be made possible by an accessory protein interaction that prevents backward slippage. When this accessory protein interaction is defined so that it acts as a ratchet, backward slippage can be prevented with minimal interference with forward progression.

2. Generation of helical bending waves by local curvature control of active sliding

Charles J. Brokaw

Generation of planar bending waves by a model flagellum containing local curvature control of active sliding was demonstrated by computer simulations 30 years ago. Many cilia and flagella generate three-dimensional, often nearly helical, bending waves. In these waves, regions of active sliding propagate circumferentially around the axoneme as well as propagating along the length. It has been suggested that the circumferential propagation may be related to rotation of the central pair microtubules, which appears to occur during three-dimensional bending. However, it might be the case that helical bending waves are the easiest mode of bending if the dyneins on each outer doublet are independently regulated by the curvature of that doublet. In this case, no circumferentially propagated signal, other than the mechanical interactions between outer doublets, would be required. The computer programs for simulating flagellar movement are now being expanded to deal with three-dimensional movement. Preliminary results suggest that local curvature control is sufficient to generate regular helical bending waves, but the prediction that a slight anisotropy of elastic bending resistance would induce planar bending has not been confirmed.

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

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

Over the past few years, we have focused our effort on two cell cycle transitions: the entry into S phase and the exit from mitosis. Surprisingly, both of these transitions are triggered by the ubiquitin-mediated destruction of key cell cycle regulatory proteins. Ubiquitin is a small protein that can be ligated post-translationally to substrate proteins. Upon attachment of ubiquitin, the substrate protein is rapidly destroyed by a multisubunit protease complex known as the proteasome. The realization that ubiquitination plays such a critical role in cell cycle control has led my lab to focus increasingly on understanding how selective proteolysis via the ubiquitin pathway is employed to regulate a variety of processes inside cells, including cell proliferation, signal transduction, and transcription.

Below, I summarize in more detail the four major areas of investigation in the lab, and provide thumbnail descriptions of all current projects.

Mechanism and regulation of protein degradation by the SCF pathway. Our prior investigations into the biochemical mechanism by which budding yeast cells transit from the G1 phase of the cell cycle to the S phase led to the discovery of the SCF ubiquitin ligases. SCF ubiquitin ligases are comprised of four subunits: Skp1, the Cullin family member Cul1 (or Cdc53), an E-box protein, and the RING-H2 domain protein Hrt1 (also known as Rbx1 or Roc1). The F-box protein serves as a receptor that docks substrates to the catalytic core of SCF, which is comprised of the cullin and RING-H2 subunits. We have proposed that the catalytic core of SCF recruits the E2 (ubiquitin-conjugating) enzyme Cdc34, and switches on the ability of Cdc34 to transfer ubiquitin to substrate that is docked to the F-box subunit. Skp1 serves as a tether that links together the substrate-binding and catalytic modules. Interestingly, there are ~20 F-box proteins encoded in the yeast genome, and many more in metazoan genomes. At least some of these F-box proteins can assemble with the cullin/RING-H2 catalytic core to form SCF complexes, suggesting that eukaryotic cells harbor many distinct SCF ubiquitin ligases, each with a unique substrate specificity.

The prototype for the SCF family - SCFCdc4 (where Cdc4 refers to the identity of the F-box subunit) - enables the transition from G1 phase to S phase in budding yeast by promoting the ubiquitination and degradation of the S-phase cyclin-dependent kinase inhibitor Sic1 (see Figure 1). Five ongoing projects in the lab build upon our discovery of SCFCdc4. Rati Verma is using a purified system comprised of SCFCdc4, 26S proteasome, and Sic1/S-phase CDK to investigate the mechanism by which
ubiquitinated Sic1 is recognized by the proteosome and selectively degraded to yield active S-phase CDK. Matt Petroski is investigating the biochemical mechanism by which SCF\textsuperscript{Cdc4} activates the ubiquitin-conjugating enzyme Cdc34 to transfer ubiquitin to Sic1. Yong Chi is investigating how SCF\textsuperscript{Cdc4} regulates transcription via ubiquitin-dependent degradation of transcriptional regulatory proteins. Kathleen Sakamoto’s project addresses the hypothesis that tethering a protein to the F-box subunit of SCF is sufficient to activate its ubiquitination and eventual destruction. A small molecule-based approach to achieve this goal might lead to the development of a novel class of therapeutics. Finally, Nazli Ghaboosi is attempting to isolate conditional lethal temperature-sensitive mutants of the ubiquitin-activating enzyme Uba1. These mutants will enable us to address how ubiquitin metabolism modulates the recruitment of SCF\textsuperscript{Cdc4} and a host of other proteins to the 26S proteosome (see abstract by R. Verma).

**G1 phase**

**S-phase CDK off**

**S-phase CDK switched on**

**DNA synthesis proceeds**

Figure 1: A molecular model for the G1/S transition in budding yeast.

**Mechanism of action, substrates, and regulation of the COP9 signalosome.** During the course of our studies on SCF ubiquitin ligases in animal cells, Svetlana Lyapina (now departed) discovered that SCF co-purifies with the COP9 signalosome (see abstract by S. Lyapina). The COP9 signalosome (CSN) is an eight-protein assemblage that is evolutionarily related to the lid subcomplex of the 26S proteosome. CSN was originally discovered by Xing-Wang Deng and colleagues as a regulator of photomorphogenetic development in plants, and has since been implicated in a wide range of cellular and developmental processes. However, essentially nothing was known about the mechanism of action, biochemical targets, and regulation of this fascinating protein complex. In the past year, we have shown that CSN promotes cleavage of the ubiquitin-like protein Nedd8 from the Cul1 subunit of SCF. This finding suggests that the diverse biological activities of CSN may all relate to its ability to promote the removal of Nedd8 from Nedd8-conjugated proteins. This discovery has opened up new opportunities to investigate how CSN works, and what its role is in cell physiology. Sylvia Schwarz is investigating the substrate specificity of both CSN and the related lid subcomplex of the 26S proteosome. Meanwhile, Greg Cope seeks to define the catalytic subunit that harbors CSN-dependent Nedd8 deconjugating activity. Once the catalytic site is defined, he will attempt to construct dominant-negative mutants to use as a tool to identify CSN substrates.
Figure 2: A model for how CSN controls photomorphogenesis, and how this may relate to the Nedd8-deconjugating activity of CSN. In the dark, CSN activates COP1, which in turn mediates turnover of the transcription factor HY5. In sunlight, the stimulatory action of CSN on COP1 is abrogated, rendering COP1 inactive and thereby allowing the accumulation of HY5. HY5, in turn, activates transcription of light-induced photomorphogenic genes. We postulate that one of the components of this circuit (e.g., COP1, HY5, or affiliated polypeptides) is regulated by reversible modification with Nedd8. In the example drawn, Nedd8 modification of COP1 would promote its export from the nucleus, which is exactly what is seen in CSN-deficient cells.

Proteomics. Budding yeast - with its formidable arsenal of genetic, molecular genetic, biochemical, and cell biological techniques - is an ideal system in which to develop and test new approaches in proteomics. Currently, there are two proteomic-type projects in the lab. Johannes Graumann proposes to use a mutant construction technique devised by Alex Varshavsky ('ts degron') combined with mass spectrometry approaches developed in this lab, to investigate the heretofore unknown functions of a set of conserved, essential genes. Jae Hong Seolís project is to devise new technologies for protein purification, and to apply these approaches, in conjunction with a novel mass spectrometry technique, to investigate the yeast protein "interactome".

Functions of the RENT complex in cell cycle control and nucleolar biogenesis. Several years ago, Wenying Shou and Jae Hong Seol discovered the RENT complex, and characterization of this complex led to the model shown in Figure 3. RENT is comprised of the nucleolar anchor protein Net1, the cell cycle regulatory protein phosphatase Cdc14 and the chromatin silencing protein Sir2. Cdc14 is required for the exit from mitosis, which it promotes by dephosphorylating (and thereby activating) proteins that mediate the inactivation of cyclin/CDK activity at the end of mitosis. Throughout the cell cycle, Cdc14 is confined to the nucleolus through its interaction with Net1. At the end of mitosis, the successful completion of anaphase activates a signaling pathway referred to as the mitotic exit network (MEN), which disengages Cdc14 from Net1. The emancipated Cdc14 goes on to inactivate cyclin/CDK and thereby trigger the exit from mitosis. This hypothesis for how the exit from mitosis is controlled in budding yeast was dubbed 'RENT control' by Shou et al. Intriguingly, in addition to its role in cell cycle control, Net1 also mediates silencing of RNA polymerase II transcription in the nucleolus, activation of transcription by RNA polymerase I, and retention of a broad set of resident proteins within the nucleolus (see abstract by W. Shou). The molecular mechanisms underlying the cell cycle regulated disassembly of RENT and the role of RENT in organizing nucleolar chromatin remain largely unknown. Over the past year, Wenying Shou has focused her efforts on characterizing the diverse roles of RENT in nucleolar biology. Ramzi Azzam seeks to identify and characterize molecules involved in the regulated disassembly of RENT at the end of mitosis. Angie Mah has discovered a protein kinase signaling
module in the MEN that appears to be conserved in multiple signaling pathways throughout eukaryota, and plans to investigate the generality of this module. Dane Mohl seeks to understand how the MEN component protein kinase Cdc5 and protein kinase A contribute to the regulation of exit from mitosis. Finally, Rusty Lipford intends to use Net1 as a springboard to understand how a unique chromatin domain is assembled to form the nucleolus.

**Figure 3:** The mitotic exit network (MEN) triggers release of Cdc14 from Net1 during late anaphase. During interphase (left side), Cdc14 is sequestered in the nucleolus by virtue of its interaction with Net1. Upon activation of the MEN, Cdc14 is released from Net1, and diffuses throughout the nucleus and cytoplasm (right side). Free Cdc14 triggers the telophase G1 transition in part by activating APC-dependent ubiquitination and degradation of mitotic cyclin.

3. **Regulation of the mitotic exit network (MEN) via casein kinase II (CKII)**  
   
   Ramzi Azzam  

   Exit from mitosis is an essential step in the progression of cells through the cell cycle. A network of seven genetically interacting proteins collectively known as the mitotic exit network (MEN) is required for the exit from mitosis in budding yeast. A model for how the exit from mitosis is controlled involves the retention of the protein phosphatase Cdc14 in an inactive form within the nucleolus while complexed to the nucleolar protein (Net1). Upon activation of the MEN, Cdc14 is released from Net1, diffuses throughout the cell, and activates cyclin proteolysis and mitotic exit by dephosphorylating Hct1/Cdh1. In MEN mutants, Cdc14 remains in the nucleolus and these mutants fail to exit mitosis.

   To further understand the regulation of this network, epitope tagging/immunoaffinity chromatography/mass spectrometry was used to look for factors that interact with the Net1/Cdc14 complex in vivo. Casein kinase II (CKII) was identified as an interacting factor and subsequent co-immunoprecipitation experiments provided further evidence for the validity of this interaction. Upon further analysis of CKII temperature sensitive (ts) mutants, it was found that these mutants fail to exit mitosis and display a similar phenotype to MEN mutants in which Cdc14 remains trapped in the nucleolus. Furthermore, CKII mutants genetically interact with components of the MEN as determined by synthetic phenotypic analysis of double mutants. CKII mutants appear to arrest at a late stage in the cell cycle after sister chromatid separation yet prior to late anaphase as judged by immunofluorescence studies. Unexpectedly, the late mitotic arrest phenotype of CKII mutants appears to require the RAD9-dependent DNA damage checkpoint. These observations along with further mutant characterizations potentially point to a role for CKII in preventing or repairing DNA damage during mitosis, such that loss-of-function alleles of CKII display a RAD-dependent, checkpoint arrest in late mitosis.

   To further our understanding of how Cdc14 is released from Net1 in late mitosis, future efforts will be dedicated to making Net1 cis-acting mutants in which in vivo phosphorylation sites on Net1 have been mutated to alamines in the hopes of generating dominant negative alleles that may point to factors responsible for RENT complex disassembly.

4. **Negative regulation of transcriptional activators**  
   
   Yong Chi  

   We have investigated the role of proteolysis in transcriptional regulation by studying the turnover of Gcn4, an activator of the amino acid biosynthetic genes. Our studies have identified Srb10 cyclin-dependent kinase (CDK) as a protein kinase that phosphorylates Gcn4 and promotes its degradation by the SCF ubiquitin ligase pathway. We also find that Srb10 contributes to the negative regulation of Msn2, a transcription factor
involved in stress response, possibly by promoting phosphorylation-dependent nuclear export. Since Srb10 is a component of the RNA polymerase II holoenzyme, our findings raise the interesting idea that transcriptional activators are marked for negative regulation by phosphorylation as a consequence of their ability to activate transcription. Such a self-limiting regulatory arrangement would prevent transcriptional regulatory proteins from hyperactivating transcription. This hypothesis predicts that negative regulation of transcription factors may be restricted to the vicinity of the promoter DNA. To test this, we have constructed a DNA-binding domain (DBD) point mutant of Gcn4 and find that this mutant protein is significantly stabilized compared to wild-type Gcn4. We also find that the nuclear export of a DBD point mutant of Pho4, an activator involved in phosphate metabolism, is also compromised when cells are grown in phosphate-rich medium, under which condition wild-type Pho4 is kept in the cytoplasm by phosphorylation-dependent nuclear export. These results suggest that phosphorylation and consequent negative regulation of transcriptional activators may be coupled to DNA binding. We are currently investigating the general molecular mechanism underlying the distinct modes of negative regulation of transcription factors.

5. Nedd8 protein modification and the COP9 signalosome
   Gregory Cope

The COP9 signalosome (CSN) is a multi-subunit complex conserved from humans to the fission yeast S. pombe. This complex has been attributed to play a role in multiple processes, including photomorphogenesis in plants and cell cycle control in S. pombe. Given that all eight subunits of the CSN are homologous to components of the lid subcomplex of the 26S proteasome, a role in proteolysis has been proposed for the CSN. However the mechanism(s) underlying the true function of the CSN have yet to be elucidated.

We have recently identified the CSN to be associated with multiple cullin proteins (termed Cul1-5) in mammalian and yeast cells (Lyapina et al. (2000) Science 292:1382-1385). Cullins are members of a class of E3 ubiquitin ligases which target specific substrates to ubiquitin dependent proteolysis. There are multiple ways in which cullins are regulated, one of which is through the covalent modification with the ubiquitin-like protein Nedd8. This modification increases Cul1 ubiquitin ligase activity toward substrates in vitro and is essential in the fission yeast S. pombe. Interestingly, we have found that CSN can promote the cleavage of the ubiquitin-like molecule Nedd8 from S. pombe Cul1 in vitro and in vivo. In an effort to elucidate the mechanisms behind these actions, we are currently attempting to identify the catalytic site that carries out this activity. Once the enzymatic component is identified, we hope to test whether this activity underlies the multiple functions previously attributed to CSN.

6. Role of ubiquitin-activating enzyme in ubiquitin-dependent degradation
   Nazli Ghaboosi

Ubiquitin-dependent proteolysis by the multi-subunit 26S proteasome is the major non-lysosomal pathway for protein degradation in eukaryotic cells. The pathway begins with the activation of ubiquitin by the E1 ubiquitin-activating enzyme. The ubiquitin moiety is transferred to one of several E2 ubiquitin-conjugating enzymes and is subsequently attached to the substrate, sometimes with the aid of an E3 ubiquitin ligase. The multi-ubiquitinated substrate is then targeted for degradation by the 26S proteasome through a poorly understood mechanism. It was originally thought that all substrates for the 26S proteasome must be ubiquitinated for proper targeting. However, there is convincing evidence that several proteins, notably ornithine decarboxylase and p21Cip1, are targeted to the proteasome without ubiquitin modification. These ubiquitin-independent proteasomal substrates call into question the necessity of ubiquitination in proteasomal targeting as well as the mechanism of substrate-proteasome interactions.

We would like to characterize the role of ubiquitination in substrate targeting to the proteasome by identification of ubiquitin-independent substrates and ubiquitin-independent proteasome interacting proteins. While the list of known E2s, E3s, and substrates is steadily growing, there is only one E1 enzyme in all somatic eukaryotic cell types. At the apex of this intricate network, E1 offers a unique perspective from which to address the many unanswered questions regarding proteasomal targeting. Temperature-sensitive mutants of the yeast E1 gene, UBA1, will be created using PCR mutagenesis. These mutants will allow conditional disruption of the entire downstream ubiquitination pathway. The effect of UBA1 mutations on ubiquitin-protein conjugation and protein degradation will be assayed. E1-independent proteasome substrates will be identified using quantitative proteomics to screen for short-lived proteins which are not stabilized in uba1 mutant cells. Finally, the ubiquitin-independence of proteasome-protein interactions will be determined by comparing mass spectrometric profiles of affinity-purified 26S proteasome interacting proteins from wild-type and uba1 mutant cells.

7. Analysis of a set of unknown genes in the yeast Saccharomyces cerevisiae
   Johannes Graumann

The completion of the genome of Saccharomyces cerevisiae (Nature (1997) 387:1-105) revealed more than one-third of the identified genes to be of unknown function. In the wake of the yeast genome sequencing project, the Saccharomyces Genome Deletion Project (Winzeler et al. (1999) Science 285:901-906) systematically deleted every open reading frame (ORF) in the yeast genome and identified about 750 essential genes. Matching the genes of unknown function with those reported to be essential resulted in a list of about 180 open reading frames, which are essential and unknown.
Blasting this list against the complete GenBank database (Benson et al. (2000); Nucleic Acids Res. (2000) 28:15-8) revealed a set of 30 ORFs that are essential in yeast, highly conserved among eucaryota and which have no known functions. This set of candidates for novel biological functions is being explored by combining conditional knock-out methods developed in the Varshavsky lab (‘ts degron’) and mass spectrometry-based approaches to identify associated factors (Seol et al. (2001) Nat. Cell. Biol. 3:384-91).

8. **Net1 and the structure of the nucleolus**
   
   Rusty Lipford

   The eukaryotic nucleolus, conserved from yeast to man, is a remarkable nuclear organelle that contains the genes and machinery required to synthesize and assemble ribosomes. As ribosomes manufacture proteins, the nucleolus is uniquely positioned to allow the cell to respond appropriately to growth signals and to cell-cycle transitions. In addition, recent studies have established a direct link between the nucleolus and the molecular circuitry of tumor formation and cell-cycle progression. Proliferation factors and cell-cycle activators appear to be sequestered within the nucleolus until the appropriate signal stimulates their release. Tumor cells are often defective in this nucleolar sequestration step, underscoring the importance of the nucleolus in preventing tumor formation.

   Despite the importance of the nucleolus, the factors that assemble and maintain the unique structure of the nucleolus are poorly understood. In the yeast *Saccharomyces cerevisiae*, Wenying Shou and others in the Deshaies lab have recently characterized a nucleolar factor, Net1, that may be a key player in the structure and function of the nucleolus. From its location in the nucleolus, Net1 regulates mitosis, nucleolar chromatin structure, and nucleolar ultrastructure. The broad functionality of Net1 makes it an attractive candidate as an important structural determinant for the nucleolus. Therefore, we hope to characterize the interaction of Net1 with the nucleolus and whether this interaction is direct or requires a partner. In addition, we will attempt to reconstruct the Net1-dependent steps in the assembly of a nucleolus. These studies should lead to a greater understanding of the role of Net1 in the structure and function of the nucleolus and may shed light on the connections between the nucleolus, the cell cycle and cancer.

9. **Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome**
   
   Svetlana Lyapina

   SCF ubiquitin ligases control various processes by marking regulatory proteins for ubiquitin-dependent proteolysis. To illuminate how SCF complexes are regulated, we sought proteins that interact with the human SCF component CUL1. The COP9 signalosome (CSN), a suppressor of plant photomorphogenesis, associated with multiple cullins and promoted cleavage of the ubiquitin-like protein NEDD8 from *Schizosaccharomyces pombe* CUL1 *in vivo and in vitro*. Multiple NEDD8-modified proteins uniquely accumulated in CSN-deficient *S. pombe* cells. We propose that the broad spectrum of activities previously attributed to CSN subunits—including repression of photomorphogenesis, activation of JUN, and activation of p27 nuclear export—underscores the importance of dynamic cycles of NEDD8 attachment and removal in biological regulation.

10. **The mitotic exit network in *S. cerevisiae***
   
   Angie Mah

   Exit from mitosis in budding yeast is triggered by the loss of cyclin-dependent kinase (Cdk) activity caused by accumulation of Cdk inhibitors and degradation of mitotic cyclins. Cdc14 plays a critical role in triggering these events as it dephosphorylates (and activates) both Cdk inhibitors and a key regulator (Hct1/Cdh1) of cyclin proteolysis. Cdc14 activity is thought to be dependent on its release from a nucleolar storage depot, and genetic evidence has suggested that a regulatory group of proteins, the mitotic exit network, modulates Cdc14 activity by controlling its localization. The mitotic exit network consists of the group of genes TEM1, LTE1, CDC15, DBF2, DBF20, MOB1 and CDC5. Mutations in these genes yield similar phenotypes: arrest in late anaphase with separated chromosomes, an elongated spindle, and elevated Cdc28/Cln2 kinase activity. Tem1 is a ras-like GTPase, which is likely to be a target of Lte1, a guanine nucleotide exchange factor, whereas Cdc15, Dbf2, and Cdc5 are all protein kinases. The function of Mob1 is still unknown.

   The sequence of steps in the signaling pathway that ultimately leads to release of Cdc14 from the nucleolus and mitotic exit remains poorly understood. In order to characterize this pathway, we have determined the functional interactions between Cdc15, Dbf2, and Mob1 by reconstituting the activation of Dbf2 using recombinant proteins expressed in the baculovirus system. We have demonstrated that protein kinase Cdc15 directly phosphorylates and activates protein kinase Dbf2, but only when Dbf2 is bound to Mob1. Curiously, in the fission yeast *S. pombe*, an additional protein kinase complex known as Sid1/Cdc14 is thought to function between the steps carried out by the Cdc15 and Dbf2 homologs, and thus we are currently investigating whether other factors may be involved in the physiological activation of Dbf2. We are also in the process of determining whether the role of Mob1-like proteins in promoting activation of Dbf2-like protein kinases defines a novel, conserved signaling module.

11. **Regulation of mitotic exit by *Saccharomyces cerevisiae* polo-like kinase CDC5**
   
   Dane Mohl

   Faithful segregation of chromosomes requires tight control over exit from mitosis with respect to the completion of DNA partitioning. In budding yeast, mitotic exit involves the proteolytic removal of B-type cyclins
(Cbl) and concomitant accumulation of Sic1, a Cbl/Cdk inhibitor. The mitotic exit network (MEN) is believed to link mitotic exit and cell division to DNA segregation, by controlling the activity of the anaphase promoting complex, SIC1 expression, and Sic1 stability with respect to the movement of a single spindle pole body and one complement of the genome into the daughter cell compartment. Furthermore, the MEN pathway represents an important target of checkpoint control, including those that respond to DNA replication, damage, and mitotic spindle function.

** CDC5 is a member of the MEN pathway. It also belongs to the conserved family of polo-like kinases that have been implicated in the establishment of mitotic events and the initiation of cytokinesis in other organisms. In order to understand the regulation of mitotic exit in *Saccharomyces cerevisiae*, I will attempt to determine the target of Cdc5 activity by employing both genetic and biochemical strategies. Currently, my work is focused on the interaction of Cdc5 with other MEN components as well as the identification of novel proteins that interact with Cdc5 in either the spindle pole body associated or nucleolar localized regulatory complexes.

12. **Mechanism of substrate ubiquitination by ubiquitin ligases**

*Matthew Petroski*

The ubiquitin-proteasome pathway has emerged as a central mechanism in eukaryotes for regulating protein stability in response to diverse cellular stimuli. Through the concerted action of ubiquitin activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s), ubiquitin molecules are transferred onto specific lysine residues of a target protein. The poly-ubiquitinated protein is now "tagged" for destruction by the proteasome. The generality of this mechanism is striking—similar pathways containing homologous proteins have been identified from yeast to humans.

There appear to be two major classes of ubiquitin ligases: the HECT domain ligases and the RING-containing ligases. The RING class is by far the predominant one, and its members have been implicated in diverse aspects of cell and organismal function. Despite the importance of RING-based ligases, little is known about their mechanism of action. We are pursuing biochemical and genetic approaches to better understand how these ligases work. We have utilized SCF as a prototypical RING-type ubiquitin ligase. SCF is found in budding yeast and humans, and consists of Skp1, a Cullin protein, an F box protein that confers substrate specificity, and the RING-H2 protein Hrt1. Preliminary evidence suggests that SCF oligomerizes its corresponding E2. This observation has led us to hypothesize that SCF-mediated oligomerization of E2, possibly via the RING subunit, activates the E2 to transfer ubiquitin to substrate. We are testing this hypothesis by developing novel substrates that will allow us to measure the rate of ubiquitin chain assembly on a single lysine in the presence and absence of SCF and E2 enzyme.

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13. **Targeting proteins for proteolysis by ubiquitin ligases: Potential therapeutic applications**

*Kathleen M. Sakamoto, M.D.¹*

A central goal of the pharmaceutical and biotechnology industries is to identify small, stable, cell-permeable molecules that inhibit the activity of cellular proteins that contribute to cancer. Despite the importance of identifying new protein inhibitors for use as therapeutics, there is no simple, straightforward strategy for doing so that works for all target proteins. We propose a general method for developing inhibitors that exploits the unique characteristics of the ubiquitin-dependent proteolytic system of eukaryotic cells. The ultimate goal of the research program proposed here will be to identify a cell-permeable molecule that binds to the substrate-docking site of a ubiquitin ligase. By covalently linking this molecule to other compounds that bind specific cellular proteins, we intend to develop a novel class of drugs or Protacs (Proteolysis Targeting Chimerical) that can trigger the destruction of any protein in eukaryotic cells for which there exists a small, cell-permeable ligand. The goals of my project are to develop an experimental system to prove that this novel concept for drug design is experimentally feasible. At one end, Protac contains a peptide that binds with high affinity to the substrate-docking domain of the ubiquitin ligase β-TRCP. We then chemically linked this peptide to the fungal metabolite ovalicin, which binds specifically to the cellular enzyme methionine aminopeptidase-2 (MetAP-2). We have demonstrated as "proof of principle" that the resulting peptide-ovalicin Protac chimera is able to tether MetAP-2 to β-TRCP, and target MetAP-2 for ubiquitination and degradation. We are now testing whether this approach can be applied to other ubiquitin ligases and different targets, such as the androgen receptor. The ability to target androgen receptor for ectopic degradation might serve as a useful strategy for treating prostate cancer.

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14. **Cullin/RING-H2-based ubiquitin-protein ligases**

*Sylvia E. Schwarz*

The evolutionary conserved protein family of cullins comprises of at least seven different members in human and three in *S. cerevisiae*. One of the members, Cul1, and its budding yeast ortholog, Cdc53, both assemble into SCF complexes, a class of ubiquitin-protein ligases (E3s). SCF consists of Skp1, Cdc53/Cul1, an F-box protein that is responsible for substrate binding, and a fourth essential subunit, the RING-H2 protein Hrt1.
Substrates ubiquitinated by SCF include cell cycle regulatory proteins (e.g., the CDK inhibitor Sic 1) and transcriptional regulators (e.g., IkB).

All human cullins can interact with RING-H2 proteins. Furthermore, three different families of putative substrate receptors have been identified: F-box proteins for SCF, SOCS-box proteins for the Cul2-based E3, and the WD-40 repeat proteins Cdc20 and Hct1 for the Anaphase-Promoting Complex/Cyclosome. These results suggest that other cullins might also work as multisubunit ubiquitin-protein ligases. However, to date, little is known about the function and potential substrates of those cullins. To address these questions, we have established mouse and human cell lines that express Myc9-TEV-tagged versions of human Cul3-Cul5. Furthermore, yeast strains have been constructed to express two epitope-tagged versions of the chromosomal copy of the yeast cullin homologues, namely ScCulB and C. Cullin-containing complexes from these cells are currently being utilized for immunooaffinity purification on anti-myc resin and elution by cleavage with TEV protease. Eluted proteins are fractionated by SDS-PAGE, detected by silver staining, and identified by subsequent mass spectrometry (in collaboration with Dr. A. Shevchenko, EMBL Heidelberg). The COP9 signalosome/CSN, a multi-subunit complex first identified in plants, was found to be among the proteins interacting with human cullins (see abstract by S. Lyapina). We are in the process of trying to elucidate the significance of this interaction by using several biochemical methods.

15. RAVE, a Skp1-containing complex, regulates assembly of vacuolar (H⁺)-ATPase

Jae Hong Seol

SCF ubiquitin ligases are comprised of Skp1, Cdc53 (human Cul1 or cullin), Hrt1 (human Roc1 or Rbx1) and one member of a large family of substrate receptors (F-box proteins, or FBPs). To investigate how the subunits of SCF are integrated into protein networks in vivo, we employed sequential rounds of epitope-tagging, affinity purification, and mass spectrometry. Sixteen Skp1- and Cdc53-associated proteins were identified in budding yeast, including all components of SCF, nine FBPs, Yjr033 (Rav1), and Ydr202 (Rav2). Reiterative purification revealed that Rav1 and Rav2 form a novel protein complex with Skp1 named RAVE (Regulator of the (H⁺)-ATPase of the Vacuolar and Endosomal membranes), which associates with the V₅ domain of the vacuolar membrane (H⁺)-ATPase (V-ATPase). V-ATPases are conserved throughout eukaryotes, and have been implicated in tumor metastasis and multidrug resistance. Genetic and mechanistic dissection revealed that RAVE promotes the glucose-triggered assembly of V-ATPase holoenzyme. Systematic genome-wide two-hybrid screens yielded 17 Skp1- and Cdc53-interactors, only three of which overlap with those that we found. Thus, reiterative rounds of purification/mass spectrometry provide a distinct view of the web of interactions that link proteins into a comprehensive cellular network.

To fully exploit the potential power of mass spectrometry for deciphering networks of interacting proteins, we have developed an epitope tag system (HTM for His8-TEV2-Myc9) that enables a C terminally-tagged protein to be purified in consecutive affinity steps. The target protein is bound to anti-myc affinity resin, eluted with Tobacco Etch Virus protease (which cleaves at the TEV recognition sites in the tag), and recaptured on NTA resin. Using Skp1 as a test-bed, we have shown (in collaboration with John Yates at Scripps) that the MudPIT (multidimensional protein identification technology) approach developed in the Yates laboratory (Washburn et al. (2000) Nature Biotechnol., 19:242) can be used to rapidly identify multiple interacting partners of Skp1 in a single LC/MS run. We plan to tag dozens of proteins with the HTM epitope, and use MudPIT to identify the interacting partners. Iterative application of this approach on a large scale has the potential to reveal the web of protein interactions that underlie cell function and regulation.

16. Net1 stimulates RNA polymerase I transcription and regulates nucleolar structure independently of controlling mitotic exit

Wenyong Shou

The budding yeast RENT complex, consisting of at least three proteins (Net1, Cdc14, Sir2), is anchored to the nucleolus by Net1. RENT controls mitotic exit, nucleolar silencing, and nucleolar localization of Nop1. We discovered two new functions of Net1. First, Net1 directly binds Pol1 and stimulates rRNA synthesis both in vitro and in vivo. Second, Net1 modulates nucleolar structure by regulating rDNA morphology and proper localization of multiple nucleolar antigens, including Pol1. Importantly, we show that the nucleolar and previously-described cell cycle functions of the RENT complex can be uncoupled by a dominant mutant allele of CDC14. The independent functions of Net1 link a key event in the cell cycle to nucleolar processes that are fundamental to cell growth.

17. Determining the requirements for proteolysis by purified 26S proteasomes

Rati Verma

Sic1 is a budding yeast S-Cdk inhibitor that has to be degraded before cells can enter S phase (Verma et al., 1997). Degradation is mediated by the ubiquitin pathway. To elucidate the mechanism by which ubiquitinated Sic1 is degraded, we developed a one step affinity method to purify 26S proteasomes for functional studies (Verma et al., 2000). Sic1, complexed to S-Cdk, and ubiquitinated by immunopurified SCFCdc4 ligase, was presented to purified 26S proteasomes. As predicted by the ubiquitin-targeting hypothesis, ubiquitinated Sic1 was degraded, whereas unmodified Sic1 was spared. Concomitant with Ub-Sic1 degradation, S-Cdk was liberated in an active form (Verma et al., Mol. Cell. In Press).

The conjugation of ubiquitin to Sic1 is mediated by the ubiquitin conjugating enzyme Cdc34. Cdc34 was
extensively autoubiquitinated in this reaction, yielding high molecular weight conjugates similar to Sic1. However, consistent with prior observations that Cdc34 is stable in vivo, ubiquitinated Cdc34 was not degraded by purified 26S proteasomes, whether Sic1 was present in the reaction or not. To determine if sequences within Cdc34 were inhibitory to 26S proteasome, a chimera between Cdc34 and Sic1 was generated. The chimeric molecule was degraded as efficiently as wild-type Sic1. The nature of the ubiquitin chain linkages on Sic1 and Cdc34 was next examined using known ubiquitin chain terminators. It is believed that a K48-linked tetraubiquitin chain, present anywhere in a long multiquitin chain, constitutes the minimum targeting signal to the 26S proteasome. Whereas the multiquitin chains on Sic1 were composed of K48 linkages, those assembled upon Cdc34 appeared to be of mixed linkages, although a minimum K48-linked tetraubiquitin chain was indeed present. Additionally, Cdc34 modified by a homogenous K48 chain (using K48 ubiquitin) was nevertheless not degraded by the 26S proteasome. We are now exploring possible explanations for why ubiquitinated Cdc34 is not degraded.

We have also initiated studies aimed at understanding the function of the individual subunits of the 19S cap of the 26S proteasome in vivo. It has been reported that two of the 19S subunits – Rpn3 and Rpn9 – are not required for Sic1 turnover in vivo, even though disruption of the RPN9 gene results in gross disassembly of the complex. As a first step towards understanding the molecular basis for this observation, we have tagged several subunits of the 19S to determine if 26S proteasomes exist as discrete subparticles within the cell, and whether the degradation of Ub-Sic1 is compartmentalized.

References

Publications


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

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

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

1. What controls the timing of MPF activation so that it occurs at a defined interval following the completion of DNA replication?
2. How do various checkpoint or feedback controls influence the Cdc2/cyclin B complex?
3. What are the molecular differences between the simple biphasic cell cycle found in early embryonic cells and the more complex cell cycles that arise later in development?

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

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

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

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

19. Regulation of Xenopus Plx1, a protein kinase that phosphorylates and activates the Cdc25 protein phosphatase
Hae Yong Yoo, Akiko Kumagai

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

We set out to purify the unknown kinase responsible for activating Cdc25 from Xenopus extracts. This attempt was aided greatly by our finding that a major Cdc25-specific kinase distinct from Cdc2/cyclin B associates with the N-terminal domain of Cdc25. Using a Xenopus Cdc25 affinity column and several conventional chromatographic methods, we purified a Cdc25-specific kinase activity approximately 2500-fold. We isolated several micrograms of this protein (p67) and sequenced four of its tryptic peptides. PCR primers designed for two of these peptides were used to amplify a segment of the cDNA encoding p67. The PCR fragment was used to isolate a full-length 2.4 kb cDNA that contains all four tryptic peptide sequences. The amino acid sequence indicates that it is a typical Ser-Thr kinase with its catalytic domain in the N-terminal half of the polypeptide. It is clearly a member of the Polo family of protein kinases. Accordingly, we have named it Plx1, for Polo-like kinase from Xenopus. Recombinant six-histidine-tagged Plx1 was then produced in Sf9 insect cells and purified on nickel agarose. This kinase phosphorylates and activates Cdc25 in vitro. We are now studying the regulation of this enzyme, which may be a key regulator of mitosis in all eukaryotic cells.

20. Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25
Akiko Kumagai

The entry into mitosis is triggered by maturation-promoting factor (MPF), which contains the protein kinase Cdc2, a B-type mitotic cyclin, and a Suc1/Cks protein. In higher eukaryotes, the activity of Cdc2 is suppressed during interphase by inhibitory phosphorylations on Thr-14 and Tyr-15. The activation of MPF at the G2/M boundary occurs when the dual-specificity phosphatase Cdc25C dephosphorylates these sites. The action of Cdc25C is strictly regulated during the cell cycle. Cdc25C is activated at mitosis by a kinase cascade containing Cdc2-cyclin B itself and Plx1. Furthermore, during interphase, Cdc25C is suppressed by the binding of inhibitory 14-3-3 proteins. 14-3-3 proteins bind to a critical phosphoserine (Ser-216 and Ser-287 in human and Xenopus Cdc25C, respectively) after phosphorylation by Chk1 and other kinases. The association between 14-3-3 and Cdc25 is ultimately reversed at mitosis, but is maintained in the presence of unreplicated or damaged DNA.

Binding of 14-3-3 proteins to a critical phosphoserine residue near the nuclear localization sequence of Xenopus Cdc25 suppresses its ability to induce entry into mitosis. We have examined the intracellular localization of hybrids between the green fluorescent protein (GFP) and either wild-type Cdc25 or a mutant (S287A) that cannot bind 14-3-3 proteins. Upon coexpression with Myc-14-3-3, GFP-Cdc25-WT was predominantly cytoplasmic in transfected tissue culture cells, whereas GFP-Cdc25-S287A was exclusively nuclear. Treatment with leptomycin B, an inhibitor of nuclear export, elicited a prompt redistribution of GFP-Cdc25-WT to the nucleus. Mutagenesis experiments demonstrated that Cdc25 contains multiple nuclear export sequences. These studies indicate that the binding of 14-3-3 proteins and nuclear export regulate the shuttling of Cdc25 between the nuclear and cytoplasmic compartments.
21. **The Xenopus Chkl protein kinase mediates a caffeine-sensitive pathway of checkpoint control in cell-free extracts**

Akiko Kumagai, Seong-Yun Jeong

The entry into mitosis is controlled by regulatory proteins that ensure the proper segregation of replicated chromosomes to daughter cells. The integrity of chromosomal DNA is under constant surveillance during the cell cycle. In eukaryotic cells, when chromosomes become damaged or cannot be replicated completely, mitosis is prevented by checkpoint mechanisms until two intact copies of the genome can be produced. A key target of these checkpoint pathways is the Cdc2-cyclin B complex, also known as maturation or M-phase promoting factor (MPF). The Cdc2-cyclin B complex, once activated at the G2/M transition, phosphorylates a myriad of proteins that carry out the various processes of mitosis such as nuclear disassembly and chromosome segregation.

In the presence of damaged or unreplicated DNA, Cdc2-cyclin B is kept inactive due to inhibitory phosphorylation of the Tyr-15 and Thr-14 residues of Cdc2. These phosphorylations are carried out collectively by the kinases Wee1 and Myt1. At the onset of mitosis, the phosphatase Cdc25C removes these inhibitory phosphate groups and thereby activates Cdc2-cyclin B. The activity of Cdc25C is strictly regulated, being low during interphase and high at mitosis. In recent studies in humans and Xenopus, it has been shown that Cdc25 is negatively regulated during interphase by the binding of 14-3-3 proteins. The inactive form of Cdc25 found prior to mitosis is phosphorylated on Ser-216 and Ser-287 in humans and Xenopus, respectively. This phosphorylation, which occurs in a consensus 14-3-3 binding site, mediates the association between Cdc25 and 14-3-3 proteins. This phosphorylation-dependent interaction appears to suppress the activation and/or action of Cdc25. Mutants of Cdc25 that cannot be phosphorylated at this residue and thus cannot bind 14-3-3 proteins override the unreplicated/damaged DNA checkpoint(s), suggesting that Cdc25 is a target of checkpoint regulation.

We have analyzed the role of the protein kinase Chkl in checkpoint control by using cell-free extracts from Xenopus eggs. Recombinant Xenopus Chkl (Xchk1) phosphorylates the mitotic inducer Cdc25 in vitro on multiple sites including Ser-287. The Xchk1-catalyzed phosphorylation of Cdc25 on Ser-287 is sufficient to confer the binding of 14-3-3 proteins. Egg extracts from which Xchk1 has been removed by immunodepletion are strongly but not totally compromised in their ability to undergo a cell cycle delay in response to the presence of unreplicated DNA. Cdc25 in Xchk1-depleted extracts remains bound to 14-3-3 due to the action of a distinct Ser-287-specific kinase in addition to Xchk1. Xchk1 is highly phosphorylated in the presence of unreplicated or damaged DNA, and this phosphorylation is abolished by caffeine, an agent which attenuates checkpoint control. The checkpoint response to unreplicated DNA in this system involves both caffeine-sensitive and caffeine-insensitive steps. Our results indicate that caffeine disrupts the checkpoint pathway containing Xchk1.

22. **Positive regulation of Wee1 by Chkl and 14-3-3 proteins**

Joon Lee

Wee1 inactivates the Cdc2-cyclin B complex during interphase by phosphorylating Cdc2 on Tyr-15. The activity of Wee1 is highly regulated during the cell cycle. In frog egg extracts, it has been established previously that Xenopus Wee1 (Xwee1) is present in a hypophosphorylated, active form during interphase and undergoes down-regulation by extensive phosphorylation at M-phase. We report that Xwee1 is also regulated by association with 14-3-3 proteins. Binding of 14-3-3 to Xwee1 occurs during interphase, but not M-phase, and requires phosphorylation of Xwee1 on Ser-549. A mutant of Xwee1 (S549A) that cannot bind 14-3-3 is substantially less active than wild-type Xwee1 in its ability to phosphorylate Cdc2. This mutation also affects the intranuclear distribution of Xwee1. In cell-free kinase assays, Xchk1 phosphorylates Xwee1 on Ser-549. The results of experiments in which Xwee1, Xchk1, or both were immunodepleted from Xenopus egg extracts suggested that these two enzymes are involved in a common pathway in the DNA replication checkpoint response. Replacement of endogenous Xwee1 with recombinant Xwee1-S549A in egg extracts attenuated the cell cycle delay induced by addition of excess recombinant Xchk1. Taken together, these results suggest that Xchk1 and 14-3-3 proteins act together as positive regulators of Xwee1.

23. **Respective roles of Xchk1 and Xcds1 in checkpoint pathways**

Zijian Guo

In eukaryotic cells, biochemical pathways have evolved to ensure that initiation of a late cell cycle event is dependent upon an early event. Particularly, entry into mitosis normally depends on successful completion of DNA replication and of DNA repair. Perturbation of DNA synthesis or the presence of irreparable DNA damage arrests the cell cycle at a point termed the mitotic entry checkpoint. Mutants that disrupt the checkpoint control have been isolated from various organisms, indicating that the checkpoint control is due to an active mechanism involving kinase cascade signaling.

We have isolated one of the checkpoint kinases, Xenopus Cds1 (Xcds1), by using database analysis, polymerase chain reaction (PCR) amplification, and library screening. Xcds1 is phosphorylated and activated by the presence of some simple DNA molecules with double-stranded ends in cell-free Xenopus egg extracts. Xcds1 is not affected by aphidicolin, an agent that induces DNA replication blocks. In contrast, another checkpoint kinase Xenopus Chkl (Xchk1) responds to DNA replication blocks, but not the presence of double-stranded DNA ends. Both Xcds1 and Xchk1 phosphorylate Cdc25 within a 14-3-3 binding site, which is required for a normal checkpoint
immunodepletion of Xcds1 (and/or Xchk1) from egg extracts does not attenuate the cell cycle delay induced by double-stranded DNA ends, suggesting that there are redundant mechanisms in this system that prevent mitosis in the presence of this type of DNA damage. Our findings indicate that in *Xenopus*, and perhaps other vertebrates, there are two pathways that respond to different kinds of signals from the DNA. Xcds1 and possibly another kinase(s) are activated by double-stranded DNA ends. In contrast, Xchk1 is unaffected by double-stranded DNA ends, but responds efficiently to replication blocks and UV damage, which also may act in at least in part by perturbing replication.

24. **Xe-p9, a *Xenopus* Suc1/Cks protein, promotes the phosphorylation of G2/M regulators**

Debabrata Patra, Sophie Wang, Akiko Kumagai

The entry into mitosis is controlled by Cdc2/cyclin B, also known as the M-phase promoting factor (MPF). In *Xenopus* egg extracts, the inhibitory phosphorylations of Cdc2 on Tyr-15 and Thr-14 are controlled by the phosphatase Cdc25 and the kinases Myt1 and Wee1. At mitosis, Cdc25 is activated and Myt1 and Wee1 are inactivated through phosphorylation by multiple kinases, including Cdc2 itself. Notably, the immunodepletion of p9 from *Xenopus* interphase egg extracts also prevents the entry into M-phase; moreover, the capacity to enter mitosis is fully restored by the addition of His6-p9. This suggests that p9 is essential for entry into mitosis. In the absence of p9, Cdc2/cyclin B complex accumulates in the inactive Tyr-15 phosphorylated form, which suggests that lack of p9 results in some defect in a reaction which is crucial for entry into M-phase. We find that p9 strongly stimulates the regulatory phosphorylations of Cdc25, Myt1, and Wee1 that are carried out by the Cdc2/cyclin B complex. These findings suggest that p9 promotes the entry into mitosis by facilitating phosphorylation of the key upstream regulators of Cdc2. Overexpression of the prolyl isomerase Pin1, which binds to the hyperphosphorylated forms of Cdc25, Myt1, and Wee1 found at M-phase, is known to block the initiation of mitosis in egg extracts. We find that Pin1 specifically antagonizes the stimulatory effect of p9 on phosphorylation of Cdc25 by Cdc2/cyclin B. This observation could explain why overexpression of Pin1 inhibits mitotic initiation. Thus, the Pin1 and p9 proteins play antagonistic roles and may thus balance the decision to enter mitosis.

25. **Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts**

Akiko Kumagai, William G. Dunphy

We have identified Claspin, a novel protein that binds to *Xenopus* Chk1 (Xchk1). Binding of Claspin to Xchk1 is highly elevated in the presence of DNA templates that trigger a checkpoint arrest of the cell cycle in *Xenopus* egg extracts. Xchk1 becomes phosphorylated during a checkpoint response, and we demonstrate directly that this phosphorylation results in the activation of Xchk1. Immunodepletion of Claspin from egg extracts abolishes both the phosphorylation and activation of Xchk1. Furthermore, Claspin-depleted extracts are unable to arrest the cell cycle in response to DNA replication blocks. Taken together, these findings indicate that Claspin is an essential upstream regulator of Xchk1.

26. **Activation of Chk1 by mutagenesis of its TRF motif**

Sophie Wang

*Xenopus* Chk1 (Xchk1) is required for the checkpoint-associated delay of the cell cycle in frog egg extracts containing unreplicated or UV-damaged DNA. Phosphorylation of Xchk1 at multiple sites in the SQ/TQ domain (residues 314-366) in response to unreplicated or UV-damaged DNA results in elevation of its kinase activity. We have found that mutagenesis of Thr-377 in the conserved Thr-Arg-Phe (TRF) motif of Xchk1 also leads to a substantial increase in kinase activity. Thr-377 does not appear to be a site of phosphorylation in Xchk1. These findings suggest that Thr-377 may play a role in suppressing the activity of Xchk1.

27. **Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in Xenopus egg extracts**

Zijian Guo

The checkpoint kinase Xchk1 becomes phosphorylated in *Xenopus* egg extracts in response to DNA replication blocks or UV-damaged DNA. Xchk1 is also required for the cell cycle delay that is induced by unreplicated or UV-damaged DNA. In this report, we have removed the *Xenopus* homolog of ATR (Xatr) from egg extracts by immunodepletion. In Xatr-depleted extracts, the checkpoint-associated phosphorylation of Xchk is abolished, and the cell cycle delay induced by replication blocks is strongly compromised. Xatr from egg extracts phosphorylated recombinant Xchk1 in *vitro*, but not a mutant form of Xchk1 (Xchk1-4AQ) containing nonphosphorylatable residues in its four conserved SQ/TQ motifs. Recombinant human ATR, but not a kinase-inactive mutant, phosphorylated the same sites in Xchk1. Furthermore, the Xchk1-4AQ mutant was found to be defective in mediating a checkpoint response in egg extracts. These findings suggest that Xchk1 is a functionally important target of Xatr during a checkpoint response to unreplicated or UV-damaged DNA.

**Publications**


Developmental Biology and Genetics

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The neural crest is comprised of multipotent stem-cell-like precursor cells that migrate extensively and give rise to an amazingly diverse set of derivatives. As important precursors of the peripheral nervous system, neural crest cells have been of interest to the neuroscience community for decades. In addition to their specific neuronal and glial cell populations, the neural crest and the paired opV placodes. Pax3 is the earliest known marker of opV placode ectoderm in the chick. Here, we show that Pax3 expression in opV ectoderm correlates with commitment to a cutaneous sensory neuron fate. We previously showed that Pax3 is induced in opV placode ectoderm by a diffusible signal from the midbrain, and that a Pax3-inducing signal is also present in the trunk neural tube. When grafted into the trunk region of chick hosts, quail Pax3-positive opV ectoderm cells, whether induced by the midbrain or by the trunk neural tube, associate with host neural crest migratory streams and form neurons in dorsal root and sympathetic ganglia. They also form neurons in several ectopic sites, including along the ventral root, where they sometimes form ganglia. In all these locations, quail neurons survive until at least E8, when they are large in diameter and express the neurotrophin receptor TrkB. This marker is expressed both by large-diameter cutaneous sensory neurons and by large-diameter mechanoreceptive dorsal root ganglion neurons. When present in the dorsal root ganglion, opV ectoderm-derived TrkB-positive neurons are found together with host TrkB-positive neurons in the ventrolateral region of the ganglion. Further, they project to the lateral region of the dorsal horn at E8, suggesting that they are cutaneous sensory neurons. These results confirm and extend our previous results to show that Pax3-positive opV ectoderm cells are committed to forming cutaneous sensory neurons.

28. **Pax3-expressing ophthalmic trigeminal placode cells are committed to a cutaneous sensory neuron fate**

*Clare Baker, Marianne Bronner-Fraser*

The cutaneous sensory neurons of the ophthalmic trigeminal (opV) ganglion are derived from two embryonic cell populations, the neural crest and the paired opV placodes. Pax3 is the earliest known marker of opV placode ectoderm in the chick. Here, we show that Pax3 expression in opV ectoderm correlates with commitment to a cutaneous sensory neuron fate. We previously showed that Pax3 is induced in opV placode ectoderm by a diffusible signal from the midbrain, and that a Pax3-inducing signal is also present in the trunk neural tube. When grafted into the trunk region of chick hosts, quail Pax3-positive opV ectoderm cells, whether induced by the midbrain or by the trunk neural tube, associate with host neural crest migratory streams and form neurons in dorsal root and sympathetic ganglia. They also form neurons in several ectopic sites, including along the ventral root, where they sometimes form ganglia. In all these locations, quail neurons survive until at least E8, when they are large in diameter and express the neurotrophin receptor TrkB. This marker is expressed both by large-diameter cutaneous placode-derived trigeminal neurons and by large-diameter mechanoreceptive dorsal root ganglion neurons. When present in the dorsal root ganglion, opV ectoderm-derived TrkB-positive neurons are found together with host TrkB-positive neurons in the ventrolateral region of the ganglion. Further, they project to the lateral region of the dorsal horn at E8, suggesting that they are cutaneous sensory neurons. These results confirm and extend our previous results to show that Pax3-positive opV ectoderm cells are committed to forming cutaneous sensory neurons.
29. **MOX, a gene with homology to dopamine β-hydroxylase, is expressed in the neural crest throughout early development**  
*Anne Knecht, Marianne Bronner-Fraser*

In a screen for genes involved in neural crest development, we identified chick MOX, a putative monoxygenase with low homology to dopamine β-hydroxylase (DBH). Here we describe novel expression patterns for MOX in the developing embryo and particularly the neural crest. MOX is an early marker for prospective neural crest, with earliest expression at the neural plate border where neural crest is induced. Furthermore, MOX expression persists in migrating neural crest and in many, though not all, crest derivatives. MOX is also expressed in the myotome, from the earliest stages of its formation, and in distinct regions of the neural tube, including even-numbered rhombomeres of the hindbrain. In order to investigate the signals that regulate the segmented pattern in the hindbrain, we microsurgically rotated the rostrocaudal positions of rhombomeres 3/4. Despite their ectopic position, both rhombomeres continued to express MOX at the level appropriate to their original location, indicating that MOX is regulated autonomously within rhombomeres. We conclude that MOX is a divergent member of a growing family of DBH-related genes; thus MOX represents a completely new type of neural crest marker, expressed throughout the development of the neural crest, with possible functions in cell-cell signaling.

30. **Timing and competence of neural crest formation**  
*Martin Basch, Mark Selleck, Marianne Bronner-Fraser*

Neural crest cells can be induced by an interaction between neural plate and ectoderm. To clarify the timing and nature of these inductive interactions, we have examined the time of competence of the neural plate to become neural crest as well as the time of neural fold specification. The neural plate is competent to respond to inductive interactions with the non-neural ectoderm for a limited period, rapidly losing its responsive ability after stage 10. In contrast, non-neural ectoderm from numerous stages retains the ability to induce neural crest cells from competent neural plate. When neural folds are explanted to test their ability to produce neural crest without further tissue interactions, we find that folds derived from all rostrocaudal levels of the open neural plate are already specified to express the neural crest marker, Slug. However, additional signals may be required for maintenance of Slug expression, since the transcript was later down-regulated in vitro in the absence of tissue interactions. Taken together, these results suggest that there are multiple stages of neural crest induction. The earliest induction must have occurred by the end of gastrulation, since the newly formed neural fold population is already specified to form neural crest. However, isolated neural folds eventually down-regulate Slug, suggesting a second phase that maintains neural crest formation. Thus, induction of the neural crest may involve multiple and sustained tissue interactions.

31. **Excess lunatic fringe causes cranial neural crest over-proliferation**  
*Christine Nelleman, Maria Elena deBellard, Meyer Barenbaum, Ed Laufer, Marianne Bronner-Fraser*

Lunatic fringe is a vertebrate homologue of Drosophila fringe, which plays an important role in modulating Notch signaling. This study examines the distribution of chick lunatic fringe at sites of neural crest formation and explores its possible function by ectopic expression. Shortly after neural tube closure, lunatic fringe is expressed in most of the neural tube, with the exception of the dorsal midline containing presumptive neural crest. Thus, there is a fringe/non-fringe border at the site of neural crest production. Expression of excess lunatic fringe in the cranial neural tube and neural crest by retrovirally-mediated gene transfer resulted in a significant increase (~60%) in the percentage of cranial neural crest cells one day after infection. This effect was mediated by an increase in cell division as assayed by BrdU incorporation. Infected embryos had an up-regulation of Delta-1 in the dorsal neural tube and redistribution of Notch-1 to the lumen of the neural tube, confirming that excess fringe modulates Notch signaling. These findings point to a novel role for lunatic fringe in regulating cell division and/or production of neural crest cells by the neural tube.

32. **The secreted glycoprotein Noelin promotes neurogenesis in Xenopus**  
*Tanya Moreno, Marianne Bronner-Fraser*

In this study, we have cloned and characterized the Xenopus homologues of Noelin-1 and -2. We find that these genes are expressed in the early differentiating neurons in the central and peripheral nervous system. Over-expression of neurogenic genes causes ectopic expression of Noelin. Furthermore, co-expression of Noelin and Noggin causes early neurogenesis in animal cap assays. Thus, Noelin may function as a positive modulator of neurogenesis in the Xenopus embryo.

33. **Sonic hedgehog rescues neural crest cell death induced by fetal alcohol syndrome**  
*Sara Ahlgren, Vijaya Thakur, Marianne Bronner-Fraser*

Maternal exposure of the embryo to alcohol results in a constellation of craniofacial and brain malformations termed fetal alcohol syndrome (FAS). The observation that blocking Sonic hedgehog (Shh) signaling results in similar abnormalities prompted us to examine whether there was a link between fetal alcohol syndrome and loss of Shh. We demonstrate that administration of alcohol results in a loss of Shh, as well as a loss of transcripts involved with Shh signaling pathways, including the Patched receptor and the Gli genes. In contrast, other signaling molecules like FGF8 and Wnt1,
appear unchanged. Importantly, addition of exogenous Shh rescues ethanol-induced cranial neural crest cell death. These data suggest that craniofacial anomalies resulting from maternal alcohol exposure are caused at least partially from loss of Shh and subsequent neural crest cell death.

34. **Isolation and distribution pattern of chick Id1, Id3 and Id4 suggest diverse roles in early nervous system development.**

*Yun Kee, Marianne Bronner-Fraser*

Members of the Id (inhibitor of differentiation or inhibitor of DNA-binding) helix-loop-helix (HLH) family proteins play important roles in cell cycle entry, apoptosis, proliferation, and cellular differentiation. The first member of this family, Id1, was originally identified as a protein that inhibits DNA-binding of basic helix-loop-helix (bHLH) protein. There are four Id genes known in vertebrates, and this raises the interesting questions of whether and how their gene expression patterns and functions are unique or overlapping in early embryonic development. We isolated the chick orthologue of the Id1, Id3 and Id4 and analyzed their expression patterns during early chick embryo development by whole-mount *in situ* hybridization. The Id1 expression pattern is dynamic and confined to discrete locations including the neural plate border, prospective olfactory placode, hindbrain, mesenchyme of distal branchial arches and adjacent to placodes, and the distal mesoderm of the limb buds. Id3 is dynamically expressed in the olfactory, lens, and otic placodes. It is also observed in the epiphysis, nephric primordium, stomodeum, dermomyotome, distal branchial arches, dorsolateral hindbrain, foregut endoderm, dorsal spinal cord, and somites. Its distribution pattern is discrete, with transcripts transiently expressed in subsets of migrating neural crest cells and in the dorsal myocardium, as well as in the segmental plate mesoderm and tail bud at early stages. Later, expression is also observed in the telencephalic vesicles and corneal epithelium. Our results suggest that vertebrate Ids have largely complementary patterns during early nervous system development with some areas of overlap, showing that these important transcriptional regulators have dynamic patterns of expression consistent with a role in multiple events in early embryonic development.

35. **Isolation of a new chick Spalt gene**

*Meyer Barendbaurn, Marianne Bronner-Fraser*

*Spalt* is a zinc finger transcription factor, which was first isolated from *Drosophila* as a region specific homeotic gene. It was also found to be involved in trachea formation and wing vein patterning later in development, where it is under the control of hedgehog, wg and dpp. Vertebrate homologues of spalt have been isolated in chicken, frog, chicken, mouse and human. In medaka, spalt was seen to be under the control of hedgehog. In chicken, *csal1* was shown to be under the control of FGFs and Wnts in the limb. In humans, mutations in the gene *SALL1* cause Townes-Brocks syndrome, which has a combination of anal, renal, limb and ear defects. In humans there are at least three spalt family members. In chicken there are two members already described.

We have isolated a third member of the chicken spalt family, *csalx*. Although it is closest to the *Xenopus Xenopushxsal3*, it may not be its ortholog since the identity between the two genes is lower than expected. *Csalx* is expressed in a variety of tissues, sometimes in a very dynamic pattern. Early in development it is expressed in the ventral and medial neural tube, and later it is expressed only dorsally. The *csalx* RNA can also be detected in the pharyngeal endoderm early, and then in the mesoderm of the branchial arches. Other regions of prominent *csalx* expression include the somatic lateral plate and limb buds, the nephric primordia, the caudal somites and the tail bud.

Thus csalx may be involved in a number of different developmental processes. In the future we will study its possible functions by over-expressing the protein in the embryo as well as assess the its regulation by a number of growth factors.

36. **Conservation of Pax gene expression in ectodermal placodes of the lamprey**

*David McCauley, Marianne Bronner-Fraser*

Ectodermal placodes contribute to the cranial ganglia and sense organs of the head and, together with neural crest cells, represent defining features of the vertebrate embryo. The identity of different placodes appears to be specified in part by the expression of different Pax genes, with Pax3 class genes being expressed in the trigeminal placode of mice, chick, frogs and fish, and Pax2/5/8 class genes expressed in the otic placode. Here, we present the cloning and expression pattern of lamprey Pax 7 and Pax2, which mark the trigeminal and otic placodes, respectively, as well as other structures characteristic of vertebrate Pax genes. These results suggest conservation of Pax genes and placodal structures in basal and higher vertebrates.

37. **AP-2 expression in amphioxus and lampreys**

*Daniel Meulemans, David McCauley, Marianne Bronner-Fraser*

To explore the genetic changes driving the evolution of a critical vertebrate innovation, the neural crest, we have analyzed the structure and regulation of amphioxus and lamprey homologs of vertebrate neural crest markers. Amphioxus, a cephalochordate, shares many morphological features with vertebrates, but diverged from the vertebrate lineage before the origins of neural crest. Lamprey, a basal craniate, diverged from vertebrates shortly after neural crest arose. We have isolated amphioxus and lamprey homologs of the neural crest marker AP-2 and have shown that the origin of neural crest correlates with an extensive expansion of AP-2 expression in the neural tube and branchial arches. Furthermore, comparison of lamprey AP-2 expression with AP-2 expression in tetrapods demonstrates a refinement of AP-2 expression in true vertebrates. Whereas lamprey AP-2 is expressed throughout the neural tube, vertebrate AP-2
genes are excluded from ventral and lateral levels, and the odd-numbered rhombomeres. This suggests that the evolution of jawed vertebrates from an agnathan ancestor involved the elaboration of neural tube and neural crest patterning mechanisms.

**Publications**


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Summary: The major focus of research in our laboratory is on gene networks that control development and their evolution. Our areas of research include the transcriptional mechanisms by which specification of embryonic blastomeres occurs early in development; structure/function relationships in developmental cis-regulatory systems; sea urchin genomics; and regulatory evolution in the bilaterians. Most of our work is carried out on sea urchin embryos, which provide key experimental advantages. Among these are an easy gene transfer technology; virtually unlimited availability of embryonic material, necessary for isolation of rare molecules such as transcription factors; an optically clear, easily manipulated embryo that is remarkably able to withstand micromanipulation; blastomere recombination and disaggregation procedures; a very well understood and relatively simple embryonic process; and in-house egg-to-embryo culture of the species we work with, *Strongylocentrotus purpuratus* (in a special culture system we have developed located at Caltech's Kerckhoff Marine Laboratory). At this point there is also an unusually rich collection of arrayed cDNA and genomic libraries for sea urchins, a fair amount of EST and genomic data and a very extensive repertoire of effective molecular technologies.

We pursue an integrated, "vertical" mode of experimental analysis, in that our experiments are directed at all levels of biological organization, extending the transcription factor-DNA interactions that control spatial and temporal expression of specific genes in the embryo to the systems level analysis of large regulatory networks. Current projects in the laboratory are as follows:  
1. Analysis of the regulatory gene network underlying endomesodermal specification in *S. purpuratus* embryos. At present over 40 genes have been linked into this network. The architecture of the network is emerging from an interdisciplinary approach in which computational analysis is applied to data emerging from gene expression knockouts, genomic sequence, and gene discovery projects, combined with experimental embryology. We are isolating both regulatory and downstream genes required for skeletonogenesis, for territorial specification, utilizing high density arrayed cDNA libraries. A predictive model which indicates expected inputs and outputs of key cis-regulatory elements has been built and is being tested as the relevant cis-regulatory systems are found. Many individual projects reported below are contributing to understanding of this network.  
2. Analysis of cis-regulatory systems of territorially expressed genes: We are using in vitro mutagenesis and reconstruction of regulatory systems with synthetic DNA fragments in order to unravel the cis-regulatory "information processing system" by which genes determine where they are in the embryo, and whether to be expressed. Computational models are essential for interpretation and as guides to experimental analysis of these systems. Among genes expressed in the definitive territories and cell types of the embryo that are being studied in this way are: the *endo16* gene; the *brachyury* gene, *Hbox12*, a gene encoding a homeodomain regulator of the very early skeletonogenic lineage; *krox 1*, a gene encoding a Zn finger transcription factor; *foxA*, a gene encoding a sea urchin HMF3β winged helix-type factor; *gata-e*, an endodermal regulatory gene; *delta*, a Notch ligand; and *otx*, a transcriptional regulator which acts in many domains of the embryo, but which also plays a key role in endomesoderm specification.  
3. Isolation, cloning, and functional analysis of transcription factors: We have developed technologies for purification of transcription factors from embryo nuclear extract, followed by microsequencing, and hence cloning. Transcription factors that interact with target sites of known function can thus be characterized, and their embryonic provenance determined. Current research targets include several different transcription factors and a repressor that serves as a terminator of a signal pathway, all identified in studies on the *endo16* gene; a repressor of the aboral ectoderm specific *Spec2a* gene; and a transcriptional regulator of the *cyIIa* aboral ectoderm-specific actin gene.  
4. Sea urchin homeobox gene cluster: We have isolated the sea urchin *HOM-C* genes and shown that they reside in a single genomic cluster.
Their genomic organization and pattern of expression during development are of particular comparative interest, since echinoderms (together with hemichordates) are the surviving sister group of all other major deuterostome members of the Animal Kingdom, i.e., chordates. An objective is to determine the spatial pattern of expression of *hox* genes in the larva, in order to elucidate the relation between gene order in the cluster and the radially symmetric echinoderm body plan. This has been accomplished for the posterior *hox* genes in the last year.

**v. Regulatory evolution:** We are engaged in comparative assessments of *cis*-regulatory evolution in echinoderm species, comparing some key regulatory linkages in the endomesoderm gene network of a starfish to those discovered in the sea urchin. Starfish and sea urchins have evolved separately almost since the Cambrian. We are also embarking on a comparative study of the endomesoderm regulatory network in other non-chordate and chordate deuterostomes. **vi. The sea urchin immune system:** Sea urchins have a very effective immune system. Levels of expression of a coelomocyte GATA factor, an NfXB factor, and a Runt factor respond sharply to immune challenge, and a number of effectors genes expressed in mammalian myeloid cells are also expressed in coelomocytes. Specific cDNAs representing genes expressed strongly in response to bacteria are being isolated from high-density arrays, using amplified probes. A particularly interesting set of coelomocyte-specific genes, the SR2R genes, has been discovered which are expressed in individual-specific patterns. In addition, methods of transferring sea urchin coelomocytes were worked out. **vii. Sea urchin genome project:** We are engaged with a consortium of other sea urchin laboratories in an effort to generate genomic research tools for *Strongylocentrotus purpuratus* particularly relevant for studies of development. A suite of arrayed libraries covering embryonic stages, larval stages and adult tissue types has been constructed and is being made available to the sea urchin research community. We have developed techniques for screening the libraries using linearly amplified probes derived from manually injected embryos or isolated portions of embryos. Several million base pairs of BAC genomic sequence has been obtained from our collaborators at JGI and the Institute of Systems Biology in Seattle and annotated. We maintain a genomic data web site in which the annotated BAC sequence; some EST data; the results from a BAC-end sequencing project that includes 76,000 genomic sequence tags; and some further information is included: (http://sea-urchin.caltech.edu-genome/).

**Transcriptional Factor Research Resource Center**

The mission of the Transcriptional Factor Research Resource Center of the Beckman Institute is to develop, extend and invent new technologies for working with transcription factors, particularly on a very small scale. The innovative research that is carried out in the Center can be divided into two components: (i) affinity purification and microsequencing factors; (ii) computational models of the transcriptional regulatory processes and the development of the sea urchin genome database. The Research Center also shares responsibility for the Genomic Technology Facility. The Facility and the Center are an integrated unit in which advanced technologies are applied to the molecular biology of gene regulation. The main research material used in the Center is sea urchin embryos; these provide enormous technical advantages for transcription factor research, as reviewed elsewhere on this Site. Requests for collaborative research focused on transcription regulation are welcome: please contact Chiou-Hwa Yuh.

**Purification, isolation, cloning of genes encoding transcription factors**

Since its inception, a core objective of the Transcription Factor Research Center has been to improve methodology for direct isolation of transcription factors from DNA-protein complexes by affinity chromatography; to fractionate and partially purify the relevant proteins; and to determine enough of their structure by microsequencing to clone the mRNAs encoding them. This has never been easy to do, but it remains the only general approach by which a gene regulatory protein can be directly isolated. In the last year our capability to bring this technology to fruition has dramatically improved. In the last few months we have successfully obtained high quality protein sequence for the first time from an unprecedented five different previously unidentified transcription factors. These are an HNF6-class factor (with Dr. Ochan Otim); a CREB-class repressor; a previously unknown transcriptional repressor isolated and characterized in collaboration with Drs. Xiaotao Li and William Klein of the University of Texas Health Sciences Center; a gut-specific activator of the *endo16* gene; and an activator of an aboral ectoderm gene that had remained uncharacterized for about a decade (with C. Titus Brown, a graduate student).

**Cis-regulatory analysis**

Experiments during the past year on the *cis*-regulatory system of the *endo16* gene led to the creation of a regulatory logic model, which provides a predictive DNA-level description of regulatory output for all perturbations of the *cis*-regulatory system and for all spatial and temporal conditions to which the gene can be exposed during development. Focus has now shifted to three other projects: (i) development of an equivalent *cis*-regulatory model for another sea urchin gene (*cyllia*: with C. Titus Brown); (ii) DNA-protein interaction mapping on a *cis*-regulatory element of the *brachury* gene (with Ms. Jane Wyllie and Dr. R. Andrew Cameron); (iii) discovery, isolation, functional characterization, and analysis of the multiple regulatory modules of the *otx* gene. This gene plays a key role in the endomesoderm specification gene network.
Computational aspects of endomesodermal gene network projects

With the addition of a Beowulf parallel computer stack to the Center, this is now the location of the major computational activities required for a large-scale gene network analysis in which we are involved, as a demonstration project. A computational scientist (Peter Clarke) is now in residence in the Center. A series of new computational research tools has emerged in the last year, developed under supervision of Hamid Bolouri: (i) the BIOARRAY image processing package for quantitative and locational analysis of macroarray screens; (ii) a custom-built genomic DNA sequence annotation program called SUGAR (Sea Urchin Genome Annotation Resource); (iii) a new software package for trouble shooting and symmetry analysis of sets of shotgun genomic sequence reads (with Roy Britten and John Williams); (iv) statistical detection package for identification of cis-regulatory elements (this is still in the middle stages of development). In addition, a powerful new program, NETBUILDER, is being adapted for creation of cis-regulatory logic models and networks. We have also available a newly-created program, FAMILYRELATIONS, built independently by C. Titus Brown for use in comparing large stretches of genomic sequence from related species. The overall result is that the Center is developing powerful and novel capabilities for regulatory network genomics. These computational resources are all available, or soon will be, to anyone who wishes access to them (except for BIOARRAY, which is proprietary, but which can be made freely available on a collaborative basis).

Deployment and function of a transcription factor

The HNF6 factor mentioned above poses a typical suite of problems: what does this regulator do; where is it active; what controls its activity? The effort to answer these questions has generated a multidisciplinary transcription factor demonstration project being carried out by Dr. Ochan Otim. The technologies he has applied range from DNA-protein physical chemistry and mutational site mapping and double-label confocal immunocytochemistry (in collaboration with the PI's Imaging Center) to gene transfer and knock-out technology. In an ongoing project these approaches have revealed that the factor is encoded by a maternal mRNA and later by zygotic messages transcribed in skeletogenic cells. Entry into nuclei is selective and developmentally controlled. The factor recognizes a specific target site in the cis-regulatory element of a skeletogenic target gene, and interference with its function prevents skeletogenesis. Its activity rises and then disappears as development progresses. Understanding HNF6 function presents a typical complex of problems, similar to those encountered with many animal gene regulators.

Technological repertoire of the Center

The Center is open to any outside scientists for whom its technological expertise is relevant, on a collaborative basis, to be arranged ad hoc. The current technologies and capabilities of the Center can be summarized as follows. These are capabilities that relate specifically to gene regulation and transcription factor isolation and analysis (though of course they might be useful for other problems as well).

- Measurement of quantitative parameters of transcription factor/DNA interaction, by gel shift and capillary electrophoresis
- Affinity chromatography, HPLC, microscale protein purification, and protein microsequencing
- Miscellaneous technologies for cytological and biochemical characterization of transcription factor behavior and function
- Design and execution of experimental cis-regulatory analysis
- Computational analysis of DNA sequence similarity problems, array data reductions, and all aspects of gene regulatory network modeling

38. The endomesoderm regulatory gene network

Jonathan P. Rast, Paola Oliveri, R. Andrew Cameron, Andrew Ransick, Chiou-Hwa Yuh, Cristina Calestani, César Arenas-Mena, Ochan Otim, Takuya Minokawa, C. Titus Brown, Pei Yun Lee, Carolina Livi, Roger Revilla, Ping Dong, Jane Wyllie, Miryong Yun, Chi H. Yun, Peter Clarke*, Leroy E. Hood1, Lee Rowen1, Hamid Bolouri*

We have undertaken to solve the regulatory gene network that underlies endomesoderm specification in the sea urchin embryo. In this embryo three territories defined by blastula stage give rise to all the embryonic mesodermal and endodermal cell types and the structures they generate. These are the skeletogenic cells which arise at the vegetal pole as a special lineage descended from four founder cells that are segregated at 5th cleavage; the definitive endoderm; and the secondary mesodermal territory. Most of the endoderm and all of the secondary mesoderm descend from a 6th cleavage ring of eight vegetal cells (veg_2). These initially produce the endomesodermal "vegetal plate." Its specification as such depends on signaling from the underlying skeletogenic precursors occurring during 4th-6th cleavage, and also on the autonomously occurring (i.e., not dependent on intercellular signaling) nuclearization of maternal β-catenin, which takes place after 7th cleavage. At this time the skeletogenic cells emit a second signal expressing the ligand Delta, and the surrounding veg_2 cells in the central region of the vegetal plate which receive this signal become the secondary mesodermal territory. The skeletogenic cells ingress into the blastocoel before gastrulation, while the secondary mesoderm delaminates into the blastocoel in the course of gastrulation. Toward the close of the gastrular invagination that generates the embryonic gut from the vegetal plate endoderm some additional cells descend from the ring of eight cells originally overlying the veg_2 ring also invaginate,
contributing mainly to hindgut endoderm. In addition there are eight cells present at the gastrula stage which contribute only to the post-embryonic mesoderm of the adult rudiment. These cells are descendants of the sister cells of the skeletogenic founders (small micromeres), but since they contribute little to the embryonic structures per se, they are not further considered in the following (though they do ultimately give rise to mesoderm). Our knowledge of the process of embryonic endomesoderm specification derives from previous work in many different sea urchin laboratories, including our own, of which the contributions from the group of David R. McClay at Duke University have been exceedingly important (reviewed by Davidson, 2001).

The first problem to be met in solving the gene network was to find the relevant genes, both those encoding transcription factors and downstream genes encoding other cellular proteins. About two dozen endodermal or mesodermal genes were already known from other work, and to discover additional relevant genes we applied a new and very powerful differential subtraction and hybridization method designed for use with macroarrayed cDNA libraries. This technology was developed earlier in this laboratory (Rast et al., 2000). Differential screens have been carried out utilizing three different regulatory perturbations. The first resulted in the isolation of cDNAs representing the products of genes expressed in embryos producing too much endomesoderm (LiCl-treated embryos), but not in embryos producing no endomesoderm at all (embryos in which β-catenin nuclearization is prevented by overexpression of cadherin). The second resulted in isolation of genes expressed in LiCl-treated embryos, but not in embryos unable to produce secondary mesoderm (embryos in which the effect of the Delta signal is cancelled by overexpression of a truncated form of the Notch receptor). The third resulted in isolation of genes expressed ectopically in embryonic cells carrying a brachyury gene expression construct, but not in embryos prior to the time the brachyury gene is activated. The brachyury gene is normally expressed in hindgut endoderm (and also the mouth). In another version of this screen we generated a probe from embryos in which Brachyury protein synthesis was blocked by a morpholino antisense oligonucleotide against brachyury mRNA. These screens are described in separate reports below. We then obtained BAC recombinants bearing the selected endomesodermal genes, including those endomesodermal genes known previously. These BACs are being sequenced at DOE’s Joint Genome Institute. Simultaneously, we have isolated BACs from other sea urchin species bearing the same genes, and these are being sequenced at the Institute for Systems Biology through a collaboration with Lee Hood. As described below, a novel computational approach to sequence comparison for the purpose of identifying putative cis-regulatory elements by their interspecific conservation has been developed by C. Titus Brown of this laboratory. Additional computational tools developed under the direction of our collaborator Prof. Hamid Bolouri of the University of Hertfordshire for the endomesoderm network project include a package ("Bioarray") for automated analysis of macroarray screens that is tailor made for differential screening protocols such as we used for gene discovery in this project; a sea urchin genome annotation program ("SUGAR"); and a system for building network models ("Netbuilder").

The general location of genes in the logic structure of the regulatory network is initially indicated by their spatial (as observed by whole mount in situ hybridization) and temporal patterns of expression. To determine the regulatory relationships among the genes we are carrying out an extensive series of experiments in which expression of given genes is knocked out by use of morpholino antisense oligonucleotides, or the regulatory factor encoded by the gene is converted into an obligate repressor of target genes by fusing its DNA binding domain to the Engrailed repressor domain. The morpholino reagent or the mRNA encoding the Engrailed fusion is introduced into embryos, and by use of a real-time quantitative PCR instrument the effect on transcript levels of every other possibly relevant gene in the network is determined. In addition we have tested the effects of cadherin mRNA and mRNA encoding the truncated N receptor on expression of all other network genes (at present there are about 40 genes in play and a total of over 90 genes that will ultimately be incorporated in the network). The resulting network of regulatory interactions now includes the key genes that start off the process of endomesoderm specification, and that account for the specification of the three endomesodermal territories indicated above. The current version of the network, which is continuously updated according to new information, can be viewed at our website, (http://www.its.caltech.edu/~mirsky/).

The network architecture emerges from the specific inputs into each cis-regulatory system included. These are predictions of DNA target site sequences based on functional analysis. They are testable predictions, that can be, and in some cases already have been verified by cis-regulatory analysis. Our objective is to obtain a true representation of the regulatory network architecture that is hardwired into the genomic DNA sequence, and which causally determines the way this aspect of the developmental process works.

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References


Screen for genes involved in early endomesoderm specification

Andrew Ransick, Jonathan P. Rast, Takuya Minokawa, Cristina Calestani

In *S. purpuratus* embryos, initial specification of the endoderm and mesoderm territories begins during the middle cleavage stages and proceeds through the hatched blastula stage (a period spanning 5 hr to 20 hr post fertilization). In an effort to identify new genes involved in this specification process we took the approach of screening 12 hr and 15 hr arrayed cDNA libraries with complex probes resulting from a subtractive hybridization procedure. The subtractive hybridization was designed to enrich for endomesoderm specific (or enriched) genes by using as a selectate population the cDNAs derived from LiCl-treated embryos (which expands the endomesodermal territory). As a driver for the subtraction, cDNAs were prepared from embryos that had depleted endomesoderm resulting from injection with message for the cytoplasmic fragment of cadherin (which knocks out the β-catenin signaling pathway required for endomesoderm specification). One round of subtraction yielded approximately 15-fold enrichment in the selectate pool of cDNAs, which was then minimally amplified and used to make radioactive complex probes. By screening the macroarrays sequentially with probes prepared from the presubtraction and postsubtraction cDNA pools, we were able to identify 1434 cDNA clones on the 12 hr library filters that were enriched postsubtraction. Analysis of this group yielded 264 identifiable nonredundant gene products, which fell into a broad range of protein types. Of particular interest to us were transcription factors and signaling pathway proteins, and we obtained ~70 candidates in these categories. These clones were subjected to further analyses with quantitative PCR and *in situ* hybridization methodologies to confirm an endomesodermal specific pattern of expression. While many genes already known to be relevant to this developmental process were cloned and confirmed (as we expected), we have also identified three new transcription factor proteins from this pool of candidates. These include the *S. purpuratus* homologues of the genes even skipped, glial cells missing and foxC. Currently functional analyses of the role of these factors in embryogenesis are underway, as well as a *cis*-regulatory analysis of these genes. The analysis of the original pool of candidates continues, as does the analysis of the screen of the 15 hr arrayed library.

Identification of secondary mesenchyme cell specific genes in sea urchin

Cristina Calestani

Sea urchin secondary mesenchyme cells (SMC) are the progenitors of pigment cells, blastocoelar cells, muscle cells and coelomic sac cells of the embryo. SMC are specified at the blastula stage from a vegetal embryonic territory, the vegetal plate. The central disc of cells of the vegetal plate is specified as SMC, while the ring of cells around it is specified as the endodermal progenitor cells. Notch signaling has been proven to be necessary for SMC specification. It has also been shown that LiCl treatment of embryos, which expands both presumptive endoderm and mesoderm territories, shifts the boundary of the Notch receptor localization toward the animal half. A more detailed understanding of the molecular and cellular mechanisms underlying mesoderm versus endoderm specification is a major goal.

In this work, a differential screening of a hatched-blastula macroarray cDNA library was performed using a comparison of mRNA populations from LiCl-treated embryos and dominant negative Notch (dnN) receptor-expressing embryos. The differential screening involved a subtractive hybridization between dnN RNA (driver) and LiCl-treated embryo single-strand cDNA (selectate), in order to lower the limit of detection on hybridized macroarray filters to genes expressed at about five copies per cell.

About 500 putative positive clones were identified and sequenced at the 5′ and 3′ ends. About 50% of the clones had a significant homology (E-value <1x10^−4) with genes in the NCBI DataBank. These genes encode enzymes, transcription factors, signal transduction proteins, extracellular matrix, membrane and cytoskeleton associated proteins. Temporal and spatial analyses of gene expression are being carried out using QPCR and WMISH. The most abundant SMC specific transcript found is homologous to the polyketide synthase gene cluster (*PKS*), which encodes a multifunctional enzyme of the biosynthetic pathway of antibiotics and antifungal molecules in fungi and bacteria. In the hatched sea urchin blastula, *PKS* is expressed in the precursors of pigment cells and its expression is maintained throughout the gastrula and pluteus stages. Analysis on the other SMC candidate genes is in progress.

Transcriptional targets of Brachyury in the gastrulating sea urchin embryo

Jonathan P. Rast, R. Andrew Cameron, Ping Dong

Brachyury is a transcription factor that is best known for its role in vertebrate notochord development. However, its ancestral role in development is likely to be in gastrulation and endoderm formation since this function is observed in phylogenetically diverse bilaterians including vertebrates. We are identifying genes downstream of Brachyury that are expressed during early gastrulation. Screens with two complex probes were made on high-density arrays of embryonic cDNA libraries. These probes are derived by subtractive hybridization using: (1) message sequence from cells that misexpress Brachyury before it is usually present minus sequence from embryos in which Brachyury function has been "knocked-out" by injection of an antisense morpholino. These screens target overlapping but distinct sets of downstream genes. After partial sequence characterization, promising genes are independently analyzed by quantitative PCR and by *in situ*
hybridization. Three classes of genes that show significant down-regulation upon brachyury antisense morpholino injection emerge from this study: genes expressed in the primary mesenchyme cells (PMCs) which produce the larval skeleton, genes expressed in a subset of the secondary mesenchyme cells (SMC), and genes that are expressed in portions of the endoderm coincident with brachyury expression. The latter class are candidates for direct transcriptional targets of Brachyury and we are in the process of searching for Brachyury target sites in the sequence of BAC clones containing these genes. The PMC and SMC genes may be indirect targets of Brachyury-induced signaling. Some of the endodermal genes are cytoskeletal modulators that may play a role in cell movement, consistent with the block in gastrulation induced by antisense morpholino injection and Brachyury function in other species. Characterization of the genes downstream of Brachyury will lead to a more precise understanding of the relationship between this transcription factor and the biological phenomena with which it is associated. Because of the phylogenetic breadth of brachyury function in gastrulation its upstream inputs and downstream targets may serve as a comparative model for understanding the evolution of gene networks.

42. **SpHbox12 an early repressor involved in PMC specification**

*Paola Oliveri, Deanna Carrick*

The large micromere give rise to the Primary Mesenchyme Cells (PMC) which later form the larva skeleton. These are the first type of cells specified during the sea urchin development and the only cells autonomously specified. Blastomere recombination experiments have shown that the micromeres also play a key role in patterning the endomesoderm through short range cell signaling. Our interest is focused on understanding the molecular mechanisms that underlie the very early endomesoderm specification process. To this purpose we studied the transcription factor SpHbox12, a paired-like homeobox gene, which starts to be expressed at the 16-cell stage, then to the large and the small micromere at peak expression and later is turned off in the small micromeres. In order to understand the role of SpHbox12 in the PMC specification and the function in the PMC regulatory network we ectopically expressed, by mRNA injection, the wild-type SpHbox12 and a dominant-negative version En-SpHbox12. In both cases the phenotype consists of a great expansion of the PMC territory, creating a blastula with the blastocoel filled with mesenchyme cells (normal embryos have only 16 ingressing cells). The consistency of the phenotypes of these two constructs suggests that SpHbox12 functions as a repressor. A further analysis of the phenotype has been obtained with QPCR and WMISH using known marker genes on mRNA injected embryos. The results showed that SpHbox12 is immediately downstream of β-catenin and otx. The ectopic expression of SpHbox12 or the dominant negative form causes the ectopic up-regulation of micromere specific genes like SpDelta (a good candidate for the late micromere signal) and the transcription factor TBr and later on PMC markers such as SM50, SM30.

43. **Finding downstream target genes of SpKrox1 using a dominant repressor construct**

*Carolina B. Livi*

As part of a larger effort in the Davidson lab, we are investigating the gene network downstream of SpKrox1, an endomesodermal transcription factor expressed in the early development of the purple sea urchin. As a strategy to search for genes downstream of this transcription factor, we have made a fusion construct containing the DNA binding domain of this transcription factor fused to the N-terminal repression domain of engrailed (eng-krox1). This construct acts as a dominant repressive transcription factor shutting off the transcription of genes that have binding sites for SpKrox1. We find that injecting eng-krox1 mRNA into sea urchin zygotes prevents embryos from gastrulating and differentiating endoderm. Additionally, patterning of the ectoderm is affected as oral ectoderm does not differentiate and embryos appear radialized. Checking for the expression of genes by real time quantitative RT-PCR revealed several endomesodermal genes that were affected. Among these are brachyury, wnt8, foxB, even skipped, and krox1 (which turns out to autoregulate its expression). This information facilitates cis-regulatory analysis as some of the connections predicted by this method might be the result of binding sites for Krox1 present in regulatory regions of the genes affected.

44. **A mesodermal GATA transcription factor expressed in early sea urchin embryogenesis**

*Paola Oliveri, Kisha Young, Jonathan P. Rast*

In sea urchin embryos mesoderm gives rise to at least five cell types. The primary mesenchyme cells (PMCs) are autonomously specified and ingress into the blastocoel of the blastula to form the larval skeleton. The secondary mesenchyme cells (SMCs) are established as a ring of cells around the PMCs in the center of the vegetal plate. These cells egress from the tip of the archenteron at various times during gastrulation. Four categories of cells originate from the SMC territory: pigment cells, blastocoelar cells, gut muscle cells and cells of the coelomic pouches (that will later participate in adult development). Cell labeling strategies and gene expression patterns indicate that some SMCs are fated to particular categories by the late blastula stage. We have identified a Gata transcription factor (SpGataC) that is expressed in a subset of these cells at a time when the specification of some SMC types is taking place and later is expressed in
the adult coelomocyte cells. This gene is a homolog of the vertebrate Gata-1,2,3 factors which are well known for their role in various aspects of hematopoiesis. In the sea urchin embryo the time course of expression was determined by both probe excess titration and quantitative PCR. Expression is first detected in the early blastula (20 hr) and peaks at early gastrulation with roughly 1000 transcripts per embryo. Later it drops to lower levels but persists throughout gastrulation. In situ hybridization localizes this expression to a patch of 6-8 SMCs in late blastula embryos. Antisense morpholino injection designed to shut down SpGataC translation disrupts aspects of both skeletogenesis and SMC specification. Effects on skeletogenesis are likely to be caused by interference with cell signaling, because the PMCs are established before SpGataC is expressed and SpGataC is never detected in these cells. In order to begin to study the cis-regulation of the SpGataC gene we have sequenced a 138 kb BAC clone containing the gene. We have also isolated a BAC clone from a second sea urchin species (*Lytechinus variegatus*) which will be sequenced and used in comparative approaches to isolate relevant regulatory sequence. An understanding of how this gene is regulated and its transcriptional targets will lead to a more complete picture of how the sea urchin mesoderm is specified.

45. **SpDRi:** A transcription factor required for the late specification of the oral ectoderm and PMCs  
*Gabriele Amore, Robert Yavrouian, Andrew Ransick*

A cDNA clone for SpDRi, a putative transcription factor falling into the ARID transcription factor family, has been isolated in the purple sea. SpDri is a single gene. It first appears to be transcribed around 15 hr and its transcripts accumulate quickly in the embryos through development. Its transcription is initially observed in the Primary Mesenchyme Cells (PMCs) precursors right before and after their ingression in the blastocoel. Starting at the mesenchyme blastula stage, SpDri expression is spatially restricted to the oral ectoderm. Interference with SpDRi translation, by means of anti-SpDRi morpholino-oligos microinjection, results in the lack of oral ectoderm differentiation and in a severe impairment of gastrulation and spiculogenesis. Analysis of the expression of several marker genes assayed by *in situ* hybridization and by quantitative PCR in microinjected embryos demonstrates a critical role for SpDRi in the commitment of the oral ectoderm. Chimeric embryos that only contain the morpholino molecules in the micromeres are able to gastrulate and form a normally polarized ectoderm in synchronous with the controls. However, the inability of these embryos to form spicules shows an autonomous role for SpDRi in the process of spiculogenesis. These results also point to the existence of a signaling mechanism between the oral ectoderm and the vegetal plate, downstream of the specification of the vegetal plate that is required by the latter territory to initiate gastrulation.

46. **Cis-regulatory analysis of the sea urchin Delta gene**  
*Roger Revilla*

During the specification of the endomesoderm of the sea urchin embryo, the 6th cleavage ring of eight vegetal cells (veg2) will give rise to endodermal cells and to mesodermal cells (secondary mesenchyme cells). As shown elsewhere the segregation between these two fates depends on a mechanism of local cell signaling that takes place between 7th and 9th cleavage. The signal is emitted by the skeletogenic cells at the center of the vegetal pole expressing the ligand Delta, and the surrounding veg2 cells which receive the signal become secondary mesenchyme cells. The expression of genes that encode for signaling molecules has to respond to a great amount of inputs, and it is expected that the regulation of these genes must be very complex. We are interested in unraveling the cis-regulatory information processing system that determines the expression of delta during the specification of secondary mesenchyme cells.

To locate genomic regions that may be responsible for the regulation of delta gene, we have isolated and sequenced genomic BACs from both *S. purpuratus* and *L. variegatus*. Using the comparative sequence analysis program FamilyRelations, we have found several highly similar regions. A reporter construct containing one of these regions has been made and preliminary results indicate that this region is capable of driving expression of the reporter gene in the skeletogenic cells. *In vitro* mutagenesis and reconstruction of the regulatory system with synthetic DNA fragments will be used in order to characterize the hard-wired transcriptional control region driving delta expression during the specification of secondary mesenchyme cells.

47. **SpHNF6, a transcription factor involved in spiculogenesis**  
*Ochan Otim*

In the past three years, our focus has been on the C-element of the *SM50* regulatory sequence. This element is sufficient for the correct spatial expression of the *SM50* gene in the purple sea urchin. By sequence homology with a different species of sea urchin, a 52 kD *S. purpuratus* transcription factor that binds the element was cloned. The factor was found to belong to the novel ONECUT transcription factor family of hepatocyte nuclear factor 6 (HNF6). Immunocytochemistry suggests that this factor (SpHNF6) is ubiquitous in the cytoplasm in the early stages of development, but enriched in the primary mesenchyme cells at the gastrula stage. The observation was confirmed by measuring relative amounts of SpHNF6 transcripts in micromeres and whole embryonic extracts by quantitative PCR (QPCR).

Blocking the expression of SpHNF6 target genes by a *Drosophila* engrailed repressor domain linked to the DNA binding domains of SpHNF6 (cut and homebox) confirms statistically the locator role of the C-element in spiculogenesis. We are currently quantifying this repression by QPCR.
Mobility shift analysis of a 2 hr nuclear extract using the C-element of SM50 suggests that there are probably more than one protein that binds the element specifically, and that only a 10 bp fragment of the element is responsible for the binding. Mutation at any position in this 10 bp abolishes all complex formation. Antibodies raised against the cut-domain of the SpHN6 supershifts this complex only one of the complexes. We are currently studying the in vivo activity of these mutants using SM50-GFP DNA constructs.

We have also affinity purified the endogenous S. purpuratus C-factor from a homogenous 24 hr embryonic nuclear extract. Three distinct bands with in vitro binding activity were fractionated and are currently being sequenced.

48. Sequencing an early ectodermal transcription factor
   C. Titus Brown, Chiou-Hwa Yuh

   Transcription of the clylla gene begins at about 20 hr in the presumptive aboral ectoderm, and continues throughout larval embryogenesis. The 2.3 kb cis-regulatory region required for recapitulation of the clylla expression pattern contains binding sites for two distinct positive-acting transcription factors, SpRunt and a hitherto-uncharacterized transcription factor known only as "P1."

   The P1 transcription factor drives early expression in the entire ectoderm, and binds with high specificity to a DNA motif in the proximal module of the clylla cis-regulatory region. We have used affinity chromatography to selectively enrich the P1 factor in 24-hr sea urchin nuclear extract and are in the process of sequencing it.

   Determination of the sequence will allow us to determine homologous genes in other, related organisms, as well as enabling further investigation of the role of P1 in early development. Since P1 is zygotically transcribed, drives activation only in the ectoderm, and is active fairly early (at the hatching blastula stage), investigation of the regulation of P1 transcription may provide clues to the specification of ectoderm in the sea urchin.

49. The role of SpGata-e in the endomesodermal network
   Pei Yun Lee

   SpGata-e, the S. purpuratus homolog to vertebrate Gata genes 4/5/6, is a zinc-finger transcription factor. Its expression is first detected at 18 hr, persists throughout gastrulation and decreases at the end of embryogenesis. When the translation of SpGata-e protein is prevented by injection of an antisense morpholino oligonucleotide, the resulting embryos have a reduced vegetal plate and fail to gastrulate. These results suggest that SpGata-e plays a role in the specification of endoderm identity.

   To test the role of SpGata-e in the endomesodermal network, I examined the levels of expression of other endomesodermal genes when SpGata-e function was knocked out. Embryos are injected with either a morpholino oligonucleotide specific to SpGata-e or a control morpholino oligonucleotide. The levels of gene expression were then assessed using quantitative PCR performed on cDNA isolated from the injected embryos. The results show that knocking out SpGata-e function affects most of the endodermal genes such as SpBrachyury and SpFoxA. In addition, a subset of mesodermal genes are also affected. Further experiments will be necessary to determine if the effects of SpGata-e on those genes are direct or indirect. Overall, the results show that SpGata-e is likely to be an important regulator during endomesoderm specification.

   A Gata-e probe was used to screen sea urchin BAC libraries and the identified clones were sequenced. Comparison of the sequences between S. purpuratus and L. variegatus using the FamilyRelations program has identified regions with very high similarities between the sequences from the two species. Because non-coding regions that have important cis-regulatory functions are likely to be conserved during evolution, the non-coding regions displaying high similarities may contain cis-regulatory elements.

50. Cis-regulatory study on the sea urchin Otx locus using comparative sequence analysis
   Chiou-Hwa Yuh, Leah Vega, Eric Davidson

   The otx (orthodentical-related) gene in S. purpuratus plays an important role in early embryogenesis. SpOtx exists in two forms (α and β) that are generated by alternative RNA splicing from a single otx gene. Previous studies have located two separate transcription start sites that drive expression in different tissues, indicating distinct regulatory influences. To locate genomic regions that may be responsible for these regulatory inputs, we have isolated and sequenced genomic BACs from both S. purpuratus and L. variegatus. Using the comparative sequence analysis program FamilyRelations, we have found seven highly similar regions, between 0.8 kb and 3 kb in length, near the otx gene in both organisms. We have made reporter constructs containing these regions and are in the process of determining the in vivo expression patterns.

51. Transcriptional control of the sea urchin Brachyury gene
   R. Andrew Cameron, Niño Le, Jane Wyllie, Paola Oliveri

   Brachyury is a transcription factor involved in mesoderm specification in vertebrates. The sea urchin homolog of this gene is expressed in the oral ectoderm and the blastopore region of the archenteron. In order to determine the role of cis-regulatory sequence in the transcription of this gene, we adopted a new approach wherein random fragments of the genomic BAC clone were screened as GFP reporter genes. From this approach we have identified a fragment that confers the expected spatial pattern of expression. In collaboration with the Joint Genome Institute of the Department of Energy we have undertaken a large sequencing project to study the
genetic regulatory networks of endomesoderm specification, of which Brachyury is a part. We have acquired from the BAC containing the brachyury gene one large contig in which the gene is found. This 22 kb stretch of DNA contains no other coding regions recognizable from Genbank. Within this genomic sequence the cDNA identifies seven different exons that lie over a 14 kb interval. The entire cDNA is only 3.7 kb in length and several of the introns are over 3 kb. Our functional fragment has been mapped to a 2 kb fragment that contains most of the intron between the 6th and 7th exons. A BAC containing a similar region from the genome of Lytechinus variegatus has been sequenced at the Megabase Sequencing Center of the Institute for Systems Biology. Using the FamilyRelations software developed by Titus Brown, we identify conserved tracts of sequence in this intronic region. Gel mobility assays are currently underway do define protein binding sites in this segment. In other studies of the endomesoderm specification pathway, it has been shown that foxA inhibits the expression of brachyury. We have identified a canonical FoxA binding site among the conserved tracts of sequence. These experiments highlight the utility of comparative sequence analysis to the determination of cis-regulatory function.

52. Determination of the complete wiring diagram for the cyIIIa regulatory region
C. Titus Brown, Chiou-Hwa Yuh
The cyIIIa cis-regulatory region drives expression of the cyIIIa actin gene in the aboral ectoderm with great precision. We are systematically modifying this control region in combination with a CAT reporter gene and assaying the results to determine the complete "wiring diagram" for the regulation of this gene.

In addition to continuing the characterization of the hard-wired transcriptional control region driving cyIIIa expression, we are in the process of measuring the concentration of the input transcription factors at several developmental stages. When complete, we will be able to create a kinetic model of the regulation of cyIIIa; this will result in the most detailed model of the regulation of a eukaryotic gene to date.

53. Cis-regulatory analysis of an endomesodermal transcription factor: SpKrox1
Carolina B. Livi, Sangeeta Bardhan
One way of knowing which factors are responsible for direct regulation of genes is to analyze their regulatory region and find functional binding sites. We have begun to characterize the cis-regulatory region of SpKrox1. This gene is expressed very early in sea urchin development in the precursors of the endomesoderm, and later its expression is restricted to the endoderm. Its regulatory sequence has inputs from the β-catenin pathway, such that LiCl expands its domain of expression while blocking this pathway greatly reduces its expression. Bacterial artificial chromosome (BAC) genomic libraries are available for several species of sea urchins as well as a starfish. We have sequenced clones containing Krox1 from Strongylocentrotus purpuratus as well as from Lytechinus variegatus. These two sea urchin species diverged approximately 50 million years ago. This has allowed enough time (empirically shown to be adequate from analysis of other genes) for nucleotide substitutions to occur so that conserved regions are likely to reflect functional regions. We are using the FamilyRelations program to compare genomic sequences from these two species in an attempt to deduce where binding sites are clustered. We have also searched for known consensus binding sites and have found sites for many factors. For example, there are two Otx sites separated by eight base pairs present immediately 5’ of the first LvKrox1 exon in the L. variegatus BAC. We also find several Otx sites in the same region in the S. purpuratus BAC. These are of interest since experiments done in the lab indicate that a dominant repressive form of Otx represses the expression of krox1. We intend to make reporter constructs containing fragments from the BACs containing krox1 to find regions capable of driving expression of the marker gene specifically recapitulating Krox1 expression.

54. hox gene cluster studies in sea urchins
César Arenas-Mena, R. Andrew Cameron, Peter Clarke, Takuya Minokawa
The hox gene cluster is an archetype of a gene battery involved in axial patterning processes which is conserved throughout the bilaterian animals. The structure and function of the Hox cluster informs our understanding of developmental mechanism of adult body plan formation and the evolution of developmental programs. Of the 10 genes of the single sea urchin hox cluster we have shown that only two are expressed during embryogenesis and it appears that they are not involved in axial patterning per se. However, during post-embryonic stages, the five posterior genes of the cluster are expressed in a colinear fashion in the posterior coelomic compartment of the bilateral larva. Considering the time and place of expression, these gene products must play a role in the development of the adult body plan. Viewed in this light, the expression patterns of the more anterior genes and their relationship to the pentameral rudiment of the adult is of interest with regard to the evolution of echinoderms from a bilateral ancestor. To this end we are isolating and characterizing cDNA clones for the anterior hox genes.

We have begun to use a comparative approach to reveal the genomic structure of individual genes in the cluster in order to better define exon-intron boundaries, 5’ and 3’ untranslated mRNA regions and noncoding control regions. In a large collaboration with the Joint Genome Institute, DOE and the Institute for Systems Biology, we have obtained sequence for a number of individual BACs from Strongylocentrotus purpuratus and Lytechinus variegatus which carry hox genes. These two species diverged 50 million years ago and neutral mutations have sufficiently obscured unconserved sequence elements so that conserved features are readily apparent. Using bioinformatics tools including the FamilyRelations
package written by Titus Brown in the laboratory, we are annotating the genomic sequence of the hox cluster.

The more posterior of the hox genes (SpHox11/13b) has an embryonic domain of expression in the endoderm, later restricted to the hindgut. Morpholino knockout experiments and cadherin misexpression indicate that the transcription factor Krox, Otx and the pathway of β-catenin TCF positively regulate SpHox11/13b. We are using a random subclone strategy to insert genomic fragments from a BAC containing SpHox11/13b, in front of a basal promoter and a GFP coding region. The regulatory elements shown to be responsible for spatially restricted expression will be further scrutinized by site directed mutagenesis. This analysis will be extremely facilitated by the Strongylocentrotus versus Lytechinus comparison mentioned earlier. We will thus identify the sites responsible for the Krox, Otx and TCF regulatory inputs.

In order to identify SpHox11/13b function, morpholino knockdown experiments will be used to identify target endomesodermal genes by QPCR. Among the targets there may be other transcription factors, signaling molecules and differentiation genes. To identify more endoderm differentiation genes than the ones currently available, we are conducting differential screens with probes generated from embryos dissociated in endomesodermal and ectodermal fractions.

The study of SpHox11/13b in the hindgut-anus has an additional evolutionary interest. In all animals studied so far the more posterior hox gene is expressed in the hindgut-anus region. This may represent an ancestral function in the posterior digestive tract. It is important to know how this domain is established in a bilaterian larva, which appears to be the ancestral level of organization for bilaterians.

55. S. purpuratus repeated sequences estimated from genomic sequences

Roy Britten

As a part of the Sea Urchin Genome Project, repeated sequences have been studied in the sequence samples as they become available. At present almost complete and accurate sequences are available for 25 BACs, adding up to 3.55 million base pairs (Mbp). Previously there were first-pass sequences of BAC ends adding to 46.4 Mbp. Based on the 25 BAC sequences the highest frequency family of repeat contains the sequence GAGAGAGA . . . in various lengths and accuracies at about 100,000 copies in the genome. The two next highest frequency families contain GAGA . . . related simple sequences. Then there are many families of more complex sequences. The number of families rises rapidly and smoothly to lower frequencies. The fraction of the genome contained in them rises much more slowly to lower frequencies. The 3.55 Mbp sample is only 1/223 of the genome, so we are unable to estimate families with less than 1000 copies in the genome from this data. The following tabular estimate uses the BAC-end data for the lower frequency region. The BAC-end sequences are not useful at high frequencies because the GAGA sequences were filtered out and certain families of repeats with conserved EcoRI sites were highly exaggerated in the cloning process. However, intercomparison gives a fairly reliable result. The estimates below are from the 25 BAC sequences except when marked *, which are from the earlier BAC-end sequences.

<table>
<thead>
<tr>
<th>Frequency Range</th>
<th>Number of Families</th>
<th>Percent of Genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>65 k to 131 k</td>
<td>1</td>
<td>2%</td>
</tr>
<tr>
<td>32 k to 65 k</td>
<td>1</td>
<td>1%</td>
</tr>
<tr>
<td>16 k to 32 k</td>
<td>7</td>
<td>2%</td>
</tr>
<tr>
<td>8 k to 16 k</td>
<td>37</td>
<td>4%</td>
</tr>
<tr>
<td>4 k to 8 k</td>
<td>48</td>
<td>4%</td>
</tr>
<tr>
<td>2 k to 4 k</td>
<td>125, 130*</td>
<td>5.7%</td>
</tr>
<tr>
<td>1 k to 2 k</td>
<td>213, 317*</td>
<td>5.8%</td>
</tr>
<tr>
<td>512 to 1 k</td>
<td>577*</td>
<td>8%</td>
</tr>
<tr>
<td>256 to 512</td>
<td>994*</td>
<td>8%</td>
</tr>
<tr>
<td>128 to 256</td>
<td>1663*</td>
<td>8%</td>
</tr>
<tr>
<td>64 to 128</td>
<td>4786*</td>
<td>14%</td>
</tr>
</tbody>
</table>

56. Immune functions of sea urchin coelomocytes

Zeev Pancer

Coelomocytes, a heterogeneous population of sea urchin putative immune cells, were found to express a complex set of transcripts featuring scavenger receptor cysteine-rich (SRCR) repeats. SRCR domains define a metazoan superfamily of proteins, many of which are implicated in development and regulation of the immune system of vertebrates. Coelomocytes transcribe multiple SRCR genes from among a multigene family encoding an estimated number of 1,200 SRCR domains in specific patterns particular to each individual. Transcription levels for given SRCR genes may range from pronounced to undetectable, yet all tested animals harbor the genomic loci encoding these genes. Analysis of several SRCR genes revealed multiple loci corresponding to each type. In the case of one SRCR type, a cluster of at least three genes was detected within a 1330kb bacterial artificial chromosome insert, and conserved as well as unique regions were identified in sequences of three genomic clones derived from a single animal. Array hybridizations with repeated samples of coelomocyte messages revealed substantial alterations in levels of expression of many SRCR genes, with fluctuations of up to 10-fold in 1 week and up to 30-fold over a period of 3 months. This report is the first demonstration of genomic and transcriptional complexity in molecules expressed by invertebrate coelomocytes. In addition, preliminary data show that the S. purpuratus genome includes a large family of Toll receptor-like genes. Coelomocytes provide a very accessible system for study of innate immune mechanisms in a lower deuterostome. We recently learned how to transform them so that they will express any exogenous plasmid in vivo, when reintroduced into an adult sea urchin. This finding opens the way to functional tests on
both immune effector and regulatory genes.

57. High density arrayed library technology in support of molecular studies on sea urchin development

  Julie Hahn, Ted Biondi, Arnulfo Lorico, Ping Dong, Jina Yuh, Jonathan P. Rast, R. Andrew Cameron

High density arrayed libraries and the automated technology associated with them greatly facilitate the identification of individual genes, differential screening strategies, the study of genomes and many other aspects of molecular developmental biology. Our automated facility, centered around a colony arraying and spotting robot, has been used in support of several library preparation and sequencing efforts that together constitute a Sea Urchin Genome Resource. The collection of materials in the Resource now includes 11 cDNA libraries and two genomic libraries.

In terms of total production for this year we picked 1,280,000 individual bacterial clones during 39 weeks of operation. Of these, 1,005,000 clones were for the Sea Urchin Resource and 275,000 clones were prepared by visitors to the Resource facility. From the libraries we gridded 416 filters and visitors gridded 212 for a grand total of 628. The clones and filters came from five different libraries besides our own. In all cases the visitors who made use of the Sea Urchin Genome Resource prepared library materials at a remote location and came to our facility to process the library using the Q-Bot. These visiting investigators included representatives of Caltech laboratories in Biology and Chemistry as well as individuals from other academic institutions.

The sea urchin community has made numerous requests for library filters which have resulted in continuing requests for clones since each filter set can be screened many times. Since libraries requested in previous years may result in clone requests this year we document 17 laboratories requesting clones and filters in the past two years. Outside of our own laboratory, 360 clones from the arrayed libraries were prepared as glycerol stocks and distributed to the users listed above. In addition, five users have requested libraries but not requested clones. This level of response indicates that the Sea Urchin Genome Resource continues to be useful to the greater biological community.

58. Modification and improvement on protein purification techniques and amino acid sequencing of several transcription factors from sea urchin

  Chiou-Hwa Yuh, Jina Yun, Ochan Otim, C. Titus Brown

In the Transcription Factor Center, our mission is to invent, develop and extend technologies for working with transcription factors, with emphasis on techniques that facilitate analysis of small samples. We recently bought a highly sensitive protein sequence (Procise cLC) that can sequence approximately 250 femtomoles of sample. This new tool, along with modifications to our sequencing protocols, has improved our ability to obtain amino acid sequence from low-abundance transcription factors.

To elucidate the mechanisms of transcriptional regulation in sea urchins, we purified several transcription factors and obtained a partial amino acid sequence for each. Our initial material came from a large-scale nuclear prep of 20-hr embryos. After affinity column purification, proteins were separated on SDS-PAGE and zinc-stained bands were cut from the gel. In-gel digestion of the protein by LysC was followed by HPLC fragment separation and sequencing of the fragments on the Procise cLC. We then constructed degenerate oligos from the amino acid sequence and used these to screen an arrayed library for cDNA clones.

We are currently purifying several transcription factors, including a late stomach spatial control factory (UI); a repressor of ectodermal expression (UC); a repressor of oral ectoderm expression (OER); an endoderm repressor (ENR); and a positive spatial factor for the cyIIIa gene (P1). All of these transcription factors play an important role in early territorial specification in the sea urchin.

59. Sequence annotation and computational methods to identify the network connections involved in embryonic endomesoderm specification in S. purpuratus

  Peter Clarke*, Mark Robinson*, Alistair Rust+, Hamid Bolouri*

In view of the large amount of genomic sequence information that we are accumulating, a convenient viewer for annotation was needed. We have customized an annotation viewer for use with sea urchin BAC sequences. The viewer displays results from a number of different sequence analyses. Protein coding regions are identified using BLAST homology searches against:

a) public protein databases  
b) S. purpuratus EST data  
c) S. purpuratus cDNA sequences  
d) orthologous genomic sequence from closely related species; together with three different computational gene finding programs.

Repetitive sequence regions are identified using BLAST searches against Roy Britten's S. purpuratus repetitive sequence database, and against BAC end linker DNA sequence generated for the Sea Urchin Genome project.

In a parallel effort, we are developing software to identify non-coding sequences predicted to function in transcriptional regulation. Using this software we aim to identify clusters of sequence motifs that may represent transcriptional control regions. This approach, together with comparative sequence analysis (provided by FamilyRelations), gives us two complementary computational approaches for identifying transcriptional regulation.
control connections at the sequence level. Sequence features identified by these two methods can be further investigated in the laboratory.

*University of Hertfordshire
†European Bioinformatics Institute, Cambridge, U.K.

60. A computational platform for comparative sequence analysis
C. Titus Brown, Tristan De Buysscher

Comparative sequence analysis of orthologous genomic regions is perhaps the most reliable way to detect functionally significant regions of genomic DNA. Use of comparative sequence analysis to discover regulatory regions has been slow to develop, because of the lack of easy-to-use computational tools.

We are developing a suite of tools to provide batched analysis of DNA (seqcomp) written in Java. Our design focus has been, firstly, to provide an easy to interface; secondly, to use a simple and understandable algorithm for generating significant matches; and thirdly, to enable correlation of these results with the results of other widely used tools (e.g., BLAST).

All tools are freely available under the GNU Public License. For more information, including screenshots, installation instructions, analysis instructions, and the programs themselves, visit http://family.caltech.edu/.

61. Sea Urchin Genome Project web site and database
C. Titus Brown, R. Andrew Cameron

The Sea Urchin Genome Project has generated a significant amount of data, including over 80,000 BAC end sequences and several thousand EST sequences. We are developing, maintaining, and extending a Web-accessible database of this information.

The database currently consists of two sets of sequences from large-scale sequencing projects: a collection of approximately 80,000 BAC-end sequences, or STCs; and approximately 2000 EST sequences from our PMC arrayed library. In addition, we are facilitating the entry of sequences from individual probes done in the Sea Urchin community, along with data linking the sequence to our collection of arrayed libraries. This has resulted in a continually expanding collection of annotations for our arrayed libraries.

The Sea Urchin Genome Project Web site is publicly accessible at the URL http://sea-urchin.caltech.edu/genome/.

62. A strategy for finding cis-regulatory elements
R. Andrew Cameron, Paola Oliveri, Jane Wyllie

We have developed an efficient method to scan the genome for sequences that support expression of a reporter gene in embryos of the sea urchin. A vector containing a green fluorescent protein (GFP) coding region, a known basal promoter and a small cloning site was used to construct a library of genomic fragments. These reporter gene constructs were injected into sea urchin zygotes and observed for expression. Detectable expression of GFP resulted from 14 of the 108 individual constructs tested. Some of these constructs produced tissue specific expression and others were ubiquitous. The temporal expression patterns were also diverse. We are currently sequencing two 3 kb fragments that confer spatially restricted expression: one in the endoderm and one in the pigment-cells, a mesodermal type of cells. The sequences will be analyzed by comparison to known elements in our database from the endomesoderm specification pathway and with the regulatory region of endo16, the most well understood endomesodermal gene.

Extrapolations from the insert sizes of the injected clones, the genome size and the incidence of positive constructs indicate that about 27,000 cis-regulatory regions are employed during embryonic development. This estimate is considerably higher than the 8500 transcripts per developmental stage estimated by hybridization kinetics. However, the value is similar to the estimate of 27, 350 genes in the sea urchin genome as estimated from a comparison between 5.8% of the genomic sequence and published sequences. The full complement of embryonic expression patterns and the various cis-regulatory sequences that produce them could be determined from a library of genomic fragments as little as 10-20 times the size of the one used in this study.

63. Biophysical nature of polymers–biomolecular interactions under electric field
Ochan Otim

We are interested in understanding the mechanism by which bimolecular solutes migrate and, therefore, separate in the polymer matrices under electric field. The consequences of insight into such mechanism should lead directly to a clear understanding of the relationship between polymer properties (such as polydispersity, rigidity, molecular size and hydrophilicity) and solute behavior in a given electric field strength. This, we hope, should provide informed choices from the possible and yet unexplored electrophoretic systems. In addition, true values of electrophoretically determined parameters such as binding constants can be compared directly in a given polymer matrix irrespective of how they were obtained.

The foregoing statements are especially true for electrophoresis performed in capillary formats (CE). In traditional CE, data analysis usually involves elimination of electroosmotic force (EOF) contribution to observed mobility of a solute either by numerical reduction or physically minimizing EOF by coating a capillary dynamically or covalently. We seek to eliminate the necessity for either of the two methods of obtaining mobilities by using raw mobility data.

Reference
64. **Evolution of endomesodermal specification in echinoderms**

Veronica F. Hinman

We are interested to understand how regulatory networks of transcription factors underlying processes in early animal development may evolve. Toward this aim we are undertaking a comparative analysis of the large-scale project ongoing in the laboratory which is aimed at producing a regulatory network map for mesendoderm specification during development of the purple urchin *Strongylocentrotus purpuratus*. A representative of another major class of echinoderms, the starfish *Asterina miniata* is an ideal candidate for such a comparative regulatory analysis.

For the initial stages of this project we have isolated and analyzed *A. miniata* clones for homologues of transcription factors known to be involved in endomesodermal specification in *S. purpuratus*. An arrayed *A. miniata* BAC genomic library with an average insert of 80 Kb has been prepared along with a late gastrulation cDNA library. We have isolated BAC and/or cDNA clones for *A. miniata* homologues of **foxA**, **foxB**, **foxC**, **evx**, **otx**, **gata-e** (**gata4/5/6**), **krox**, **sox17**, **brachyury** and **T-brain**. Spatial expression of the mRNA product of the genes examined thus far shows that, as predicted, they are localized to progenitors of the endomesoderm, the proximal ectoderm and in some cases, the ectoderm of the oral plate. These expression patterns are essentially homologous to those reported for sea urchin species.

The further aims of the project are to disrupt endogenous gene expression using morpholino antisense oligonucleotides to determine downstream impacts on other endomesodermal transcription factors. These effects may then be related to the structure of their **cis**-regulatory modules. A comparison of this network of **trans**- and **cis**-regulation between *A. miniata* and *S. purpuratus* will reveal the nature of the regulatory evolution that has occurred during the 400-500 million years since their divergence.

To determine regulatory interactions of the candidate transcription factors it is essential to be able to readily form transgenic *A. miniata* embryos. An efficient method for microinjection of recombinant nucleic acids has been established whereby many hundreds of oocytes may be injected in a day. A construct containing a 4.5 kb segment of DNA immediately 5' to the translation start of the *A. miniata* FoxA gene associated with a GFP reporter has been injected into oocytes of *A. miniata*. Preliminary results are encouraging as this construct expresses GFP in the invaginating archenteron and proximal ectoderm in a domain overlapping with the mRNA in situ localization pattern for this gene.

65. **Putative Precambrian embryos and adult forms from the Doushantuo formation of China**

Paola Oliveri

Almost all major bilaterian clades are represented in Lower Cambrian deposits suggesting their existence during at least the late Neoproterozoic. Even the most conservative calculation of the molecular divergence indicates that the latest bilaterian common ancestor could have existed no less than 600 million years ago. So far unequivocal paleontological evidence of bilaterian forms has extended only to the final period of the Neoproterozoic, a few million years prior to the Cambrian "explosion." To better understand bilaterian evolution we analyzed the amazingly well preserved microfossils of the phosphatized deposit of the Doushantuo formation from South West of China (570±20 Ma). Two types of evidences are produced to support the morphological analysis of a metazoan affinity. First: the thin sections of rock are observed under polarized light to determine if the boundaries of the crystal domains are the same as the morphological boundaries of the analyzed structures. Second: measurement of the sizes of the internal and external structure of several examples of each morphotype are compared for consistency. The end products of genetic programs show a remarkable consistency in dimensions, not observed in structure of abiotic origin. We have reported the identification of highly convincing remains of not only adult and embryonic sponges, but cnidarian developmental stages and most importantly a variety of small (≤200 µm) structures that greatly resemble gastrula stage embryos of modern bilaterian forms, including both deuterostomes and protostomes. Lately we have focused our attention on the great variety of forms resembling cnidarians present in the deposit. We identified not only many different developmental forms and larval stages belonging to different cnidarian classes but also little adult Anthozoans and Hydrozoans showing features specific to these groups of metazoans. Such diversification within the cnidarians, the sister group of Bilateria, is consistent with the existence of the previously described bilaterian forms and the hypothesis that the latest common ancestor of deuterostomes and protostomes must lie deeper in time.

**Publications**


Erwin, D.H. and Davidson, E.H. The last common bilaterian ancestor. In preparation


Summary: The recent explosion of molecular data available in a wide variety of experimental systems has offered tremendous insight into the machinery underlying biological processes. The challenge now is to carry these important insights forward to deduce their function in intact cells and organisms. Much of our research focuses on the patterning of the vertebrate embryonic nervous system, where key events are largely invisible because of the large number of seemingly identical cells that participate. Thus, an understanding of the cellular events requires some means to reliably and indelibly mark cells and to follow these marked cells without perturbing development or killing the labeled cells.

Recent progress in the laboratory has ranged from the refinement of the techniques used for the imaging of cells and organisms, as well as the application of the techniques to issues of cell lineage, cell migration and cell patterning. For this purpose, we employ video, laser scanning confocal, laser scanning two-photon, or magnetic resonance microscopy. To label cells so they can be uniquely followed over time, we have used a variety of microinjection, transgenesis and viral vector techniques. Parallel developments of improved MRI contrast agents, fluorescent dyes, and genetically-encoded markers such as GFP have moved research in the laboratory closer to our dream of non-invasively eavesdropping on cell and molecular events critical for embryonic development.

66. Interdisciplinary study of the molecular mechanisms underlying somite formation in chick

Paul Kulesa, Scott Fraser

One of the most striking patterns of segmentation in the vertebrate embryo is the formation of somites. During the assembly of the vertebrate axis, mesodermal tissue along either side of the neural plate and neural tube, becomes subdivided into individual segments. This pattern of segmentation repeats with a fixed periodicity (every 90 minutes in mouse and chick), beginning near the head and moving in the posterior direction, in the same direction as the elongating axis. As the somite matures, it will form skeletal muscle, dermis, axial skeleton and ribs.

Over the last decade, molecular studies have implicated a large number of genes in somitogenesis, but it is still not clear what mechanisms control somite size and the periodicity of the segmentation. The dynamic nature of gene expression, where genes turn on and off as the segments form, coupled with the lack of specific somite shaping information has made it difficult to correlate distinct genes with morphological changes.

To fill this void, we are using a two-fold approach. First, we have developed a new culture and imaging technique to visualize cell and tissue shaping and individual cell movements in the chick during somite
formation. We find that an individual somite is sculpted in a series of six events which follow a precise spatial and temporal order. The separation of the forming somite from the segmental plate involves a complex series of cell movements in which groups of cells cross between the forming somite and the segmental plate. The exchange of cells is crucial to the formation of the anterior and posterior borders of the somite. Simultaneous with the separation of the somite from the unsegmented mesoderm, local cell rearrangements and cell shape changes within the forming somite create its structure, consisting of an epithelial-like circumferential ring surrounding a mesenchymal core of cells. Together, these results suggest that the mesoderm is sculpted into somites in a choreographed shaping process which coordinates a series of local and non-local cell movements and tissue shaping events.

To coordinate gene expression with the morphological shaping events, we have developed a technique to more accurately pinpoint (to within a few minutes of the 90-minute interval) where and when a gene is expressed within the unsegmented mesoderm. First, we time-lapse image somite formation until a specific point, then fix the animal and perform the *in situ*. The *in situ* data shows the position of gene expression at the exact time within the 90-minute interval of the last forming somite and allows us to link gene expression and cell movements in the same animal. We are currently analyzing gene expression data for four key genes.

67. **Modeling a gene network: The interaction of retinoic acid, HoxA1, HoxB1, and HoxB2 in rhombomeres 3-5**

   *Jason Kastner, Jerry Solomon*

Shortly after the closure of the neural tube, the chick hindbrain develops a series of axial bulges called rhombomeres. The initial descriptions of the rhombomeres in the early part of the twentieth century resulted in some arguments about the importance of these structures, but these arguments were finally quelled in the past decade when experiments showed that the segmentation of the hindbrain is a crucial process in hindbrain development.

In vertebrates, the *Hox* gene family is a set of transcription factors that play a pivotal role in regulating patterning and axial morphogenesis. Molecular expression studies have shown that members of the *Hox* family display rhombomere-restricted patterns of expression. Recent studies have show the importance of Retinoic Acid (RA) in this process as it is able to directly regulate *Hox* family members. But by virtue of the fact that these data have been gathered using molecular techniques, the data produced generally describes very localized events. The sheer amount of data that is now available allows us to take the next important step: integrating the results and constructing a higher level model for the interaction and regulation of members of the *Hox* family.

To accomplish this, we have adopted a stochastic simulation approach (Gillespie, 1976) to modeling the biochemical reactions between RA, HoxA1, HoxB1 and HoxB2 in rhombomeres 3-5. This method is able to capture more of the actual biology (including the inevitable fluctuations and the low number of molecules) than the traditional continuous-valued kinetic equations and allows for a direct connection between the model parameters and the biologically meaningful parameters such as binding affinities and transcription rates. This model also allows us to track in detail the behavior of each component of a particular biochemical pathway and to produce computerized movies of the time evolution of the system that is a result of the dynamic interplay of these various components. The simulation has been able to reproduce several key observations seen in the laboratory. In addition, the simulation has suggested several new results from experiments (including perturbing endogenous RA levels and introducing a dominant negative RA receptor) performed *in silico*. For validation and enhancement of the model, these experiments are currently being performed *in vivo*.

**References**


68. **Quantitative GFP imaging in living embryos: Applications for sea urchin genomic studies**

   *Ivan J. Dmochowski, Jane E. Dmochowski*, Eric H. Davidson, Scott E. Fraser

Confocal laser scanning microscopy (CLSM) allows the study of cellular processes with high spatial (~0.1 µm) and temporal (millisecond) resolution, but has traditionally provided limited quantitative information. Ratiometric analysis of fluorescence collected at multiple wavelengths can be employed, for example, to study *in vivo* enzyme activity towards fluorogenic substrates (Zlokarnik et al., 1998). However, this method reports only relative fluorescence intensities, whereas the elucidation of many biological processes requires absolute quantification of cellular analytes (e.g., cytokines, proteins, or nucleic acids). Current efforts to measure gene activity, and, specifically, comparisons of promoter regions on genes transcribed during sea urchin embryogenesis (Yuh, *et al*., 1998), often rely on *in situ* hybridizations or biochemical analyses of homogenates of hundreds of embryos. Gene expression studies involving green fluorescent protein (GFP) are potentially much faster, but methods have not yet been developed to quantify this versatile reporter in living embryos. My goal is to establish CLSM as a quantitative tool for measuring GFP fluorescence intensity in living cells.

Currently, I am working in collaboration with the Davidson lab to quantify the number of GFP molecules expressed per cell in sea urchin embryos injected with a well characterized expression construct: the hatching enzyme (HE) *cis*-regulatory system driving the GFP reporter gene. Scatter from living tissue dictates that laser excitation and fluorescence collection efficiencies vary with the position of the fluorophore in the embryo. By co-injecting HE-GFP constructs with an internal fluorescent
standard that migrates uniformly throughout the embryos, it is possible to correct the GFP fluorescence intensity for the depth at each confocal slice. We have written software using IDL, in conjunction with the image processing package ENVI, to select fluorescent voxels, depth correct the confocal images, and integrate intensity values. To normalize the total depth-corrected fluorescence intensity with the number of GFP molecules, the imaged embryos are lysed and the GFP concentration quantified using a fluorometer calibrated with GFP protein standards. In this manner, protein expression levels will be determined as a function of time and cellular localization.

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References

69. A detailed study of the G-protein signaling pathway using exact stochastic reaction-diffusion simulations
Jerry E. Solomon

It is now widely recognized that traditional mass-action-derived kinetic equations are not suitable for modeling the biochemical reaction networks that drive intracellular behavior. This approach assumes that the concentrations involved are large enough to be treated as continuous variables, that reaction rates are relatively fast, and that fluctuations can be ignored. These assumptions are almost never valid when treating intracellular reaction systems. Over the past several years, we have developed software tools which allow us to compute "exact" stochastic simulations of coupled biochemical reaction-diffusion systems. Such simulations are "exact" in the sense that they reproduce exactly the results one would obtain from solving the appropriate stochastic master equations describing the system.

One of the systems that we have been studying recently, using stochastic simulation, is the ubiquitous G-protein signaling system. The importance of this system arises from the fact that it is involved in a large number of developmental processes that require intercellular signaling coupled with intracellular responses. There are of course a number of distinct G-protein pathways that have been identified as crucial in a variety of developmental processes. The specific G-protein pathway that we have chosen to study is one that begins with the activation of a G-protein complex by a particular agonist/receptor binding event, and ends with the release of large amounts of free Ca2+ from internal calcium stores maintained in the endoplasmic reticulum. Depending on context, this Ca2+ release event triggers various cellular responses to external signals.

An interesting aspect of this pathway, from a biochemistry standpoint, is the fact that most of its components undergo two-dimensional diffusion on the inner surface of the cytoplasmic membrane. Thus, most of the reactions that constitute this pathway are diffusion-limited, with effective reaction rate coefficients that depend on the diffusion constants of the reacting partners. In applying our simulation approach to this problem, we first define the pathway explicitly in terms of the individual reaction events that make up the complete G-protein signaling pathway. Our stochastic simulation code then "solves" this reaction system in order to provide a complete kinetic description of the behavior of each of the molecular components of the pathway. As noted above, our stochastic simulation incorporates explicitly the fluctuations present in the system so we are able to see directly the effects (if any) of "noise" on the system.

The reaction rate coefficients and Michaelis-Menten thresholds were taken from the rather extensive literature on the G-protein system. Where such quantities were not available, we used estimates derived from previous experience on similar reactions. The results that we obtained from our simulations were in good agreement with observations reported in the literature; in particular, the timing of major events, such as time to Ca2+ release from initial agonist/receptor binding, was found to be in good agreement with experimental observations. Since, aside from timing data, there are not many quantitative experimental measurements on the kinetic response curves of the signaling components, we can only report that the overall behavior of the simulation is in good qualitative agreement with what is reported in the literature. Given these results, we believe that we now have a very good stochastic model of the G-protein pathway that can be used to study a variety of intercellular processes that utilize this pathway to accomplish various specific functions.

70. Finding in vivo biochemical reaction coefficients from measured reaction response curves
Jerry E. Solomon

It is generally recognized that direct, in vivo measurements of biochemical reaction rate coefficients, e.g., ligand-receptor binding coefficients, is a very difficult task. Thus, most of these measurements are conducted on in vitro systems, with the consequence that one is generally left with serious questions as to the applicability of the results to the in vivo behavior of the system in question. We have recently developed a robust method for computing estimates of the reaction parameters of a coupled biochemical reaction-diffusion system from measured response curves for the system. These response curves are simply in vivo measurements of species concentrations as a function of time, and are generally easier to carry out than are direct measurements of the reaction parameters.

The method that we have developed is based on the use of our exact stochastic reaction-diffusion simulation algorithm inside a multiobjective genetic algorithm (MOGA) which acts as a multi-function optimizer. The solution vectors produced by the GA are
the so-called "chromosomes," and are simply the real-valued reaction rate parameters for the system under study. These solution vectors become the input for our stochastic reaction-diffusion algorithm which computes the response curves associated with these parameters. Within the GA we then compute the mean-squared differences between the calculated response curves and the measured response curves in order to compute the fitness values associated with each of the solution vectors (chromosomes). The MOGA computation begins with a fixed-size population of randomly generated chromosomes, to which we apply (in a probabilistic manner) the standard GA operators: reproduction, cross-over, and mutation, in order to generate the next "generation" of chromosomes. The chromosome population size and the maximum number of generations for the computation are input parameters for the code. We have applied this procedure to two test cases that are simple enough to check the behavior of the algorithm in detail, but, at the same time, complex enough to demonstrate the general applicability of the method. The two cases we have chosen are a one-stage Michaelis-Menten reaction, and a two-stage Michaelis-Menten reaction, i.e.,

Enzy + Sub $\rightarrow$ Cmplx1 $\rightarrow$ Enzy + Prod,
Enzy + Sub $\leftarrow$ Cmplx1 $\leftarrow$ Cmplx2 $\rightarrow$ Enzy + Prod.

For these two cases we find that the MOGA procedure produces reaction rate coefficient values that are within one percent or so of the correct values. In order to obtain these results it was necessary to use population sizes of 300 or more, and at least 100 generations. This represents a significant amount of CPU time, and we found it necessary to use the fastest serial workstation available in order to produce these results in a reasonable amount of time. We are currently working on a parallel implementation of the MOGA code using the discrete event simulation language, Parsec, produced by the Parallel Computing Laboratory at UCLA. We should be able to achieve significant improvements in the compute time for this code using parallelization techniques offered by the Parsec language.

An interesting feature of the MOGA procedure is the fact that, in certain cases, one can actually use fewer than the total number of possible response curves for the system. That is to say, if $N$ is the total number of possible response curves, then we can, by judicious selection, use only $M < N$ measured response curves to completely constrain the system and compute all of the system reaction parameters. This is obviously a very valuable feature, since it reduces the difficulties involved with making a large number of in vivo response curve measurements. The next step in this project is to test the MOGA procedure against "real" laboratory in vivo measurements of the response curves for a specific biological reaction network.

71. Guidance of avian neural crest cells by ephrins-B ligands
Andrew J. Ewald, Scott E. Fraser

The neural crest is a transient cell population in the developing vertebrate embryo, which migrates out from the dorsal neural tube to form a broad range of derivatives in the adult animal. Trunk neural crest cells migrate over long distances in a topologically complex manner. We are interested in understanding the molecular basis of neural crest migration guidance. Based on previous work done in the Fraser, Bronner-Fraser, and Anderson laboratories, we have decided to focus our efforts on the Eph family of receptor tyrosine kinases and their cognate ligands, the ephrins.

Trunk neural crest cells express Eph B receptors and their migratory pathways avoid regions of ephrin-B signaling. We are taking two related approaches to understanding the cell biological basis of ephrin-B signaling on chick neural crest cells. The first is a novel cell migration choice assay, in which primary neural crest cells are presented with a choice between regions of a glass coverslip containing ephrin-B protein plus fibronectin and regions containing fibronectin alone. We photolithographically pattern the proteins on glass, explant primary neural crest onto the surface, then image the cells as they respond to the pattern using time lapse confocal microscopy. With this assay and related experiments we have been characterizing the normal cytoskeletal and membrane dynamics associated with encountering ephrin-B ligands. We are currently using a pharmaceutical inhibitor-based approach to determining the signal transduction requirements for ephrin-B signaling.

In parallel with these in vitro experiments we are mapping the normal trunk neural crest cell migration routes in three-dimensions, in the 10-20th somites of the chick embryo. Using vital dyes and GFP fusion proteins we can selectively label the neural crest and reveal their location with confocal and two-photon microscopy. In older embryos, we are applying a novel form of sectioning microscopy, that we have developed in collaboration with Resolution Corporation. We fluorescently label the neural crest and surrounding tissues, then automatically section through the tissue at one micron increments, capturing a fluorescent image of the block face after each section. By serially repeating this process we are able to digitally reconstruct large volumes of the chick embryo, currently up to four cubic millimeters, at subcellular resolution. Since the embryo is growing dramatically during the period of neural crest migration, this increased size allows us to follow neural crest cells in three dimensions over longer times, allowing us to score for defects in normal development, rather than early migration behavior. We expect that these whole embryo studies will inform and refine our in vitro understanding of crest migration, guiding new rounds of in vitro experiments, which ultimately lead us back to the embryo again for validation.
72. The role of the sacral neural crest in development of the chick enteric nervous system

Helen J. McBride, Scott E. Fraser

The enteric nervous system (ENS) is primarily derived from neural crest cells. The neural crest is a mobile population of cells derived from the dorsal neural tube. Both the vagal and sacral regions of the neural tube contribute neural crest cells to the ENS, but the vagal level is the most well described.

Congenital megacolon (Hirschprung's Disease/HD) affects one in 5,000 live births and is characterized by a varying length of intestine, usually the distal colon and rectum, that is lacking in neurons because neural crest cells failed to properly colonize the hindgut during development. Several genes have been identified in humans and in mouse models of the disorder which are critical for proper neural crest colonization of the hindgut, but their function is not well understood. Our goal is to better understand how normal development proceeds in the hindgut and how the genes involved in the disease work to ensure that all the gut is colonized by neural crest cells.

We have chosen to study gut and ENS development in the chicken embryo. Not only is the gut of these animals similar to humans during development, but the embryos are easy to manipulate and culture outside of the egg.

The sacral neural crest contributes cells to the ENS which form both neurons and glia. The sacral neural crest is of particular interest because those cells are normally restricted to the hindgut. They also colonize that region prior to vagal crest cells.

Why don't sacral crest cells within the hindgut compensate for the lack of vagal cells during HD? It may be that an interaction between the two is required for sacral crest cells to develop into neurons. By surgically ablating the vagal neural crest and comparing the development of the ENS in the presence and absence of that cell population, we hope to better understand what happens during HD. By using normal development of the ENS as a standard, we can also perturb expression of some of the genes involved in HD to examine how they affect colonization of the hindgut by both vagal and sacral crest cells.

73. 3D imaging and modeling of the primary neuronal scaffold of the zebrafish forebrain

Magdalena Bak, Mary E. Dickinson, Scott E. Fraser

We are interested in studying the early phases of the forebrain development in zebrafish embryos. We would like to understand how the primary neuronal scaffold is formed, first by studying changes in cell behavior and morphology that occur as cells differentiate, and second by gaining insight into molecular signals that guide this process.

Building upon previous descriptions of the primary neuronal scaffold in zebrafish, we have used fluorescence immunocytochemistry and confocal scanning microscopy to create 3D renderings of neuronal cells and axon tracts in the developing zebrafish forebrain. These models offer a 3D view of the scaffold at several key time points which allows for the analysis of how cells become arranged in the scaffold over time. With these models as a starting point, we have now begun to analyze the differentiation of individual cells using time-lapse microscopy in order to study the precise path these cells take as they become part of the scaffold. Using transient Green Fluorescent Protein (GFP) expression introduced by injecting a plasmid expression construct into the one cell stage embryo, we have followed neuronal differentiation from 20-36 hpf.

Our preliminary results show that in young embryos (20-22 hpf), we see cells that oscillate between the outer boundary of the neuroepithelium and the midline, divide and differentiate into neurons with a clearly distinguishable axon. In older embryos (24-36 hpf), we continue to see neurons differentiate, as more cells are recruited to the clusters from the midline. In addition, we have observed very pronounced and dynamic filopodial activity at the cell bodies of neurons within established clusters, suggesting a mechanism for cell-cell communication between cells within a cluster or the immediate environment. More detailed characterization of the neuronal differentiation process is currently underway.

The zebrafish primary neuron scaffold in the forebrain represents a simple model system for understanding the influence of environmental cues on the development of complex neuronal circuitry. Our results provide valuable insights into neurogenesis that may be relevant to other, less tenable, vertebrate systems.
References

74. Time-lapse imaging of cell behaviors driving convergence and extension in the zebrafish gastrulae

*Ying Gong, Scott Fraser*

Gastrulation is an important period in vertebrate embryogenesis, which transforms a morphologically undifferentiated spherical egg into an embryo with distinct germ layers and body axes. Convergence and extension, which refers to the narrowing and lengthening of a tissue, is important for the establishment of the antero-posterior (AP) axis during gastrulation. Due to the complexity of these movements, it has been a great challenge to define the cellular and molecular mechanisms that generate and regulate these movements. Using a combination of vital dye labeling and *in vivo* imaging techniques, we are tracking cell motile behaviors that underlie convergence and extension in the zebrafish gastrulae. The zebrafish embryo lends itself to such a study because its optical clarity allows the movements of individual cells to be followed *in vivo* and because it is amenable to embryological manipulations and genetic analysis.

We are using a number of labeling techniques to allow visualization of individual cells in the embryo, including intracellular injection of fluorescent dextrans or GFP mRNA and soaking with membrane associated or membrane permeable vital dyes. Preliminary time-lapse confocal imaging studies have revealed a number of cell behaviors in the dorsal tissue during convergence and extension movements. These behaviors include cell division; cell shape change from an AP elongated morphology to a medio-lateral elongated one; and cell intercalation. We are continuing these experiments to establish a reliable and complete picture of the cellular machinery driving convergence and extension. In addition, we have obtained the zebrafish *silberblick/wnt11* mutant that has convergence and extension defects. Direct comparison of cell behaviors in the mutant embryos with those in the wild-type embryos will provide important information on the specific role of *Wnt11* in regulating convergence and extension.

75. A role for calcium in the control of gastrulation motions in frogs

*Andrew Ewald, John Wallingford, Richard Harland, Scott E. Fraser*

During *Xenopus* gastrulation, cell intercalation drives convergent extension of dorsal tissues. Convergent extension in turn leads to the elongation of the embryo's anterior-posterior axis. This process requires the coordination of motility throughout a large epithelial sheet of cells.

To investigate the potential contribution of calcium signaling to the control of morphogenetic cell movements, we visualized calcium dynamics during convergent extension using a calcium-sensitive fluorescent dye, Calcium Green. We found that dramatic intercellular waves of calcium mobilization occurred in cells undergoing convergent extension in explants of gastrulating *Xenopus* embryos. These waves arose stochastically with respect to timing and position within the dorsal tissues. Waves propagated quickly and were often accompanied by a wave of contraction within the tissue. Calcium waves were not observed in explants of the ventral marginal zone or prospective epidermis. Pharmacological depletion of intracellular calcium stores abolished the calcium dynamics and also inhibited convergent extension without affecting cell fate.

In order to better understand the cellular basis of the gastrulation defect induced by interference with calcium signaling, we have begun a detailed three-dimensional mapping of the *Xenopus* embryo during gastrulation. Using a novel form of sectioning microscopy, developed in collaboration with Resolution Corporation, we fluorescently label frog embryos, embed them in a hard polymer, then robotically section through them, collecting an image of the block face after each cut. With this technique we have reconstructed frog embryos at each of the recognized stages of gastrulation and are using that knowledge of the normal embryo to interpret the cellular basis of a number of gastrulation perturbations.

Publication
Figure 1: Four frames from a time-lapse movie of calcium activity in the explanted dorsal mesoderm of a frog embryo. Calcium concentration is revealed by the indicator Calcium Green dextran; lighter colors indicate higher calcium concentration.

76. **MR microscopy with intermolecular multiple quantum coherences**

*P.T. Narashimhan, S. Sendhil Velan, Russell E. Jacobs*

An important parameter in biological magnetic resonance imaging (MRI) is contrast, since it enables one to distinguish different tissues. Some of the well known mechanisms that contribute to contrast in conventional MRI are spin density, T1, T2, and diffusion of water molecules. Using appropriate pulse sequences and choices of sequence parameters one can highlight areas of high/low spin density or different diffusion characteristics. Recently, a new type of contrast has been introduced in MRI based on intermolecular multiple quantum coherences or i-MQC’s (Warren et al., *Science*, 1998). These coherences arise from distant dipolar field of magnetic dipoles, namely, protons located on different water molecules that are separated by distances larger than the diffusion length on the NMR time scale (a few milliseconds). The pulse sequences used for i-MQC imaging excite intermolecular zero quantum coherences (i-ZQC’s) or double quantum coherences (i-DQC’s) and subsequently convert them into single quantum coherences (SQC’s), which are phase/frequency encoded. We have obtained 2D and 3D i-MQC images of the mouse brain at microscopic resolution. One of the salient features of this type of imaging is that the distance scale of the interacting spins can be adjusted by varying the strength and/or duration of the coherence gradient in these pulse sequences. The contrast that arises in this manner allows us to probe distances ranging from a few microns to a few millimeters in the object that is imaged.

In order to clearly identify the signals arising from i-MQC’s a few tests are carried out. In the presence of a gradient oriented at magic angle (54° 44’) with respect to the main magnetic field (Z-axis) the i-MQC signals vanish. The signal intensity with the coherence gradient along the X-axis is half of that with the gradient along the Z-axis and the sign of the signal is reversed. In the case of i-DQC imaging two coherence selection gradients are used along the Z direction in the ratio of 1:2. Any deviation from this ratio reduces the intensity of the i-DQC signal drastically. This serves as an additional test for i-DQC’s. Suitable phase cycling schemes are incorporated in the pulse sequences to eliminate contamination from unwanted coherences. i-MQC signal intensity depends on the square of the magnetization. As a result dark areas appear darker and bright areas appear brighter. The i-ZQC signal arises from spins on water molecules at locations characterized by a difference of frequencies. Thus, the i-ZQC signal can be made sensitive to susceptibility differences at the two locations separated by the distance scale mentioned above. The i-DQC signal, on the other hand arises from spins on water molecules at locations characterized by the sum of frequencies. i-MQC imaging can therefore provide unique contrast as compared to conventional MR imaging. Figure 1a shows i-ZQC slice image of a mouse brain obtained from a 3D data set. Figure 1b shows a 2D i-DQC slice image of a mouse head.

The i-MQC images were obtained with a Bruker 500 MHz (11.7 T) AMX spectrometer equipped with an actively shielded microimaging probehead. Solenoidal coil of 10 mm was employed for i-ZQC imaging and 20 mm birdcage coil was employed for i-DQC imaging. The 1-ZQC slice (390 μm) image was obtained with a FOV of 1.25 cm x 1.25 cm, Dim = 128 x 128, TR 2s and Gc = 5.6G. The 2D i-DQC image (slice thickness of 2 mm) was obtained with a FOV of 1.5 cm x 1.5 cm, TR= 2s and Dim = 256 x 256. The coherence gradients were in the ratio of 3.7G : 7.4G.
77. **In vivo neuronal tract tracing utilizing Mn²⁺ enhanced MRI (MEMRI)**
Robia G. Pautler, Raymond Mongeau, Russell E. Jacobs

It has been previously been shown that Mn²⁺ is taken up through Ca²⁺ channels and can be transported along axons. Because Mn²⁺ ion is also paramagnetic, these combined properties of Mn²⁺ allow for the use of the ion as an effective MRI detectable, neuronal tract tracer. A previous study has demonstrated Mn²⁺ tract tracing along the olfactory and visual pathways in mice. Whereas the previous study traced from sensory receptors leading into the brain, the purpose of the present study was to delineate tract tracings from focal points located within the brain.

Nanoliter injections of 5 mM MnCl₂ into either the striatum or the amygdala demonstrated contrast enhancement along the correct neuronal circuitry. Furthermore, enhancement of homotopic connections was observed in both cases. In the event of the amygdala tracings, a total of six synapses were traversed as evidenced by positive contrast enhancement due to Mn²⁺ accumulation. Ten days post-injection, the Mn²⁺ appeared to wash out as evidenced by lack of enhancement within the brain. Incorporation of this refined methodology should allow for repeated mapping of neuronal circuitry in experimental animal paradigms.

In Figure 1a, fine tracts were delineated leading from specific structures. It was possible to view a small bundle of fibers leading from the striatum to the pre-limbic cortex. Fig 1b, the projections (fornix) leading from the hippocampal fimbria are visible. The fornix enhancements lead to the lateral septal nucleus which then terminate in the medial habenula. Fig 1c, 48 hours post-injection, MEMRI tract tracings were visible from the amygdala. Fig 1d T₁–weighted 3D MRI image of the same mouse scanned 10 days later. At this time point, the Mn²⁺ appears to have washed out.

78. **3D time lapse analysis of Spemann organizer dynamics using µMRI**
Cyrus Papan, S. Sendhil Velan, Scott E. Fraser, Russell E. Jacobs

A main function of Spemann organizer in *Xenopus laevis* is neural induction. In recent years, much insight has been gained into the molecular correlation of this process. Many aspects of this induction event have a clear prediction about the spatial relations and morphogenetic behavior of the tissues involved.

However, models about tissue interactions are controversial. Because of the total opacity of the *Xenopus* embryo, it is not possible to track morphogenetic movements by optical methods. Thus, none of these relationships have been observed in vivo.

We seek to define the dynamics of Spemann organizer and its spatial relationship with its surrounding tissue during late blastula and gastrula stages. To overcome the problem of opacity, we have utilized NMR imaging to conduct a 3D time-lapse analysis of the morphogenetic movements of the live developing *Xenopus* embryo.

Intrinsic image contrast and resolution allow us to identify the blastocoel, archenteron, and the blastopore, and to distinguish between animal and vegetal tissue.

Single cells can be labeled by microinjection of contrast agent. Dynamics of clone movement can then be followed in 3D-time lapse series within the context of the whole embryo.

By labeling the C1-blastomere alone, which gives rise to most of the organizer tissue, or together with the C4 blastomere, our imaging experiments allow us to identify the relationship between the organizer and ectodermal and endodermal tissue during blastula and gastrula stages. In summary our results show:

1. The animal cap rotates dorsally during late blastula, resulting in a higher cell mass at the dorsal marginal zone.
2. Brachet’s cleft forms anteriorly by apposition between endoderm and ectoderm and posteriorly by shearing between ectoderm and deep mesoderm.
3. Dorsal mesoderm does not involute underneath ectoderm but ectoderm moves over dorsal mesoderm by epiboly.
4. Axial mesoderm does not move animal-wards before the end of gastrulation.
5. Axial mesoderm is already internally localized and is in vertical contact with the overlying posterior neuroectoderm throughout blastula.
6. Anterior neuroectoderm is located in the animal cap and thus is not in contact with deep cells. Only after vegetal rotation, it comes in contact with anterior endoderm.
79. **Tracing transgene expression in living zebrafish embryos**

*Reinhard W. Köster, Scott E. Fraser*

Imaging developmental processes and cellular behavior during stages of organogenesis requires a long-lasting labeling method ideally encoded genetically. We adopted the Gal4-UAS-system commonly used in *Drosophila* to develop a transient transgenic labeling method that can be easily performed by plasmid injection into single cell-stage zebrafish embryos. Activator constructs containing the transcriptional activator Gal4-VP16 under control of an enhancer drive the expression of effectors that encode a transgene under control of Gal4-binding sites. Both units can be placed on a single expression vector. These vectors give rise to a significantly higher number of transgene expressing cells as well as to higher expression levels compared to conventional expression plasmids in injected zebrafish embryos. Using different tissue-specific enhancers driving the expression of Gal4-VP16, transgene expression could be restricted to the notochord, somitic muscles or the nervous system. Strong expression lasts over several weeks, which allows cells to be imaged over several days. Furthermore, multiple Gal4-dependent effectors can be expressed simultaneously from a single plasmid allowing one to monitor a cell's response to the expression of any transgene by simultaneous expression of the fluorescent reporter GFP. The combinatorial nature of the established expression system facilitates changing the tissue-specificity, the transgene expressed, or the cell compartment-specific GFP reporter.

Reference

80. **In vivo imaging of neuronal migration in the developing zebrafish cerebellum**

*Reinhard W. Köster, Scott E. Fraser*

Due to its relatively simple organization, which is mainly obtained by neuronal cell migration, the cerebellum since long has been of special interest to analyze migration of neuronal precursor cells. Recently, several cell fate studies have questioned the long established migratory pathways in the cerebellum making a direct analysis of cell migration in the zebrafish embryo an interesting goal for imaging.

Neuronal precursor cells in the cerebellar neuroepithelium, the rhombic lip, were labeled using Gal4-VP16-amplified GFP-expression vectors (see above) and subsequently time-lapse imaged in vivo by culturing zebrafish embryos on a confocal microscope over 1.5 days. Rhombic lip descendants, which were believed to exclusively migrate dorsally to become cerebellar granule cells, were found to migrate anteriorly towards the midhindbrain boundary (MHB) to subsequently turn into a ventral migratory pathway along the MHB. Finally, they settle in the ipsilateral ventral brainstem underneath the cerebellum to contribute to precerebellar nuclei.

High magnification analysis of individual cells revealed that migration, which occurs in characteristic chains, is accomplished by alternating phases of exploration and translocation of the nucleus into an established growth cone-like lamelllopod. Some neuronal precursors having left the rhombic lip migrate backwards along their chain of migrating neurons to undergo another round of cell division. At the MHB the migratory behavior changes dramatically, with cells becoming postmitotic, changing cell polarity, losing contact to the migratory chain and the extension of an axon. The latter is very intriguing as it suggests that axonal pathfinding and neuronal migration can happen simultaneously suggesting that both processes are intimately linked. Currently, molecular cues underlying cerebellar neuronal migration are being analyzed using in vivo imaging.

Reference

81. **Analysis of fluid dynamics in the developing zebrafish heart**

*Jay R. Hove, Reinhard W. Köster, Arian S. Forouhar, Gabriel Acevedo-Bolton, Mehrdad M. Zarandi, Scott E. Fraser, Morteza Gharib*

Fluid forces are known to influence cell and tissue morphology during embryogenesis but little is known about the intracardiac hemodynamics of developing vertebrate embryos. In collaboration with the Bioengineering group at Caltech we have imaged the intracardiac blood flow in the valve-less and valved zebrafish embryonic heart. Blood flow was found to be laminar by imaging the flow pattern of fluorescent-labeled serum. Using both indirect velocity analysis by ejection fraction calculation and direct erythrocyte displacement by Digital Particle Image Velocimetry (DPIV), we find that even in the early valve-less heart high-speed, high-shear laminar fluid dynamics are already present which are well above the threshold of cellular sensitivity. These findings suggest that hemodynamic forces have been underscored in influencing cardiogenesis.

Reference

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The neurally-controlled Animat: Multi-electrode culture dishes, high-speed optical recording, and two-photon microscopy for studying learning in vitro

Thomas DeMarse, Axel Blau, Daniel Wagenaar, Jerome Pine, Steve Potter

Neurons from embryonic rat cortex spontaneously connect and form intricate patterns of electrical activity when grown in culture. We and others studying neuronal cultures call stimulation-induced changes in this activity "synaptic plasticity." Learning is usually defined in behavioral terms, as a change in behavior as a result of experience. Thus, it is presently not appropriate to study learning in cultured neural networks, since they lack a body with which to behave.

We have developed a system, illustrated in Figure 1, that provides a living network of neurons cultured on a multi-electrode array (MEA), a body with which to behave and an environment with which to interact. These noninvasive electrodes allow us to record from, and stimulate, cultured neurons for days or even weeks. Neural activity from cultured cortex is interfaced to custom software that creates a simulated body with the ability to receive sensory feedback and initiate movements within a virtual environment of our choosing. Our real-time software processes over ~3 MB per second, separating neural activity from noise, filtering artifacts from feedback. A custom artificial neural network (ANN) then examines the spatial activity on the 8x8 grid of electrodes (upper left panel of Fig. 2) over time, categorizing and clustering recurrent patterns which are used to control the Animat's movements (right two panels). The output of the ANN then initiates movement of the Animat's body within a simulated environment (Fig. 2, bottom left). Sensory information is then provided in the form of real-time feedback via custom-built electronics that rapidly induces patterns of neural activity within the living neural network. In essence, creating a closed-loop dynamical system, an artificial animal that is based on a biological brain, whose mechanisms we can investigate in detail.

For example, one of the fundamental questions is information is encoded within these living neural networks. In our case, how is information we provide about the simulated environment via feedback encoded. By studying how patterns of neural activity change over time as a result of experience, we can learn about how that information is transformed, encoded, and then produces changes in the resulting behavior (e.g., movements of the simulated body) over time. In fact, one of the advances of our system is that we can conduct repeated and long-term experiments with our cultures, allowing us to follow these changes as they occur over hours, weeks, and even months using special custom culture chambers.

In conjunction with this global analysis, we are also investigating what the cellular basis for these changes might be. By integrating our extra-cellular recording methods with a combination of other technologies, we can view activity on a wide variety of both spatial and temporal scales. For example, on the millisecond time-scale we image neural activity using a high-speed CCD camera, and voltage sensitive dyes. This allows us to investigate the global network dynamics, but over a much wider spatial area than current MEA technology allows.

One mechanism for that change, for example, are the changes in the morphology of the synapses which we study using two-photon time-lapse imaging which can measure the minute morphological changes across minutes, hours, or even days. By studying the changes we observe in each of these measures, using the detailed analysis each can provide, we will learn more about learning within this artificial animal, and perhaps the neural basis of learning in general with applications to computing, control systems, robotics, etc. For more information, please see our web page at: http://www.caltech.edu/~pinelab/PotterGroup.html

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Fig. 1: Real-time feedback loop of the Neurally Controlled Animat System. Neural activity from a grid of 60 extra-cellular electrodes are analyzed for patterns using custom software resulting in a movement within a virtual world, and sensory feedback in the form of electrical stimuli delivered through custom electronics.

Fig. 2: An example of a session in which the Animat is moving around a 3-dimensional room (lower left) based on the current neural activity (upper left) and the performance of an artificial neural network that looks and responds to patterns of activity (right two panels).
83. **Teflon-sealed culture dishes for long-term neural cultures**

*Steve M. Potter, Thomas B. DeMarse*

We have developed a new method for culturing cells that maintains their health and sterility for many months. We have employed this technique to grow dissociated cortical cultures from rat embryos on multi-electrode arrays. After more than a year in culture, the neurons still exhibit robust spontaneous electrical activity (Fig. 1). Using conventional techniques, primary neuron cultures seldom survive more than two months. Increases in the osmotic strength of media due to evaporation are a large and under-appreciated contributor to the gradual decline in the health of these cultures. Because of this and the ever-present likelihood of contamination by airborne pathogens, repeated or extended experiments on any given culture have until now been difficult, if not impossible. We surmounted survival problems by using culture dish lids that form a gas-tight seal, and incorporate a transparent Teflon membrane that is selectively permeable to oxygen (O₂) and carbon dioxide (CO₂), and relatively impermeable to water vapor (Fig. 2). This prevents contamination and greatly reduces evaporation, allowing the use of a non-humidified incubator. The combination of sealed culture dishes with extracellular multi-electrode recording and stimulation enables study of development, adaptation, and very long-term plasticity, across months, in cultured neuronal networks, and is a key element in our Neurally Controlled Animat system. Membrane-sealed dishes will also be useful for the culture of many other cell types susceptible to evaporation and contamination.

Fig. 1. **Top:** Phase-contrast image of the world's oldest living dissociated rat cortical culture, taken at 15 months *in vitro*. Neuron somata are mostly obscured by abundant glia and fascicles. Scale: 200 μm between electrodes. **Bottom:** Spontaneous activity of this network, recorded after one year in culture using MultiChannel Systems MEA60. Each dot is an action potential recorded by one of the multi-electrode array channels.

Fig. 2. **Multielectrode array with FEP-membrane-sealed lids.** Lids are machined PTFE rings, with grooves for O-rings that are used to hold on the 12.7 μm FEP membrane and to form an airtight seal with the MEA. The materials (PTFE, O-rings, and FEP membrane) are autoclavable and re-usable.
A toolset for realtime analysis of network dynamics in dense cultures of cortical neurons

Daniel A. Wagenaar, Thomas B. DeMarse, Steve M. Potter

As part of our lab's effort to study learning in vitro by connecting living neural networks to a simulated body in a simulated environment (the Animat project), we have developed a set of tools for online and offline analysis of data collected from MEAs (multi-electrode arrays).

We grow cultures of thousands of neurons dissociated from cortex of E18 rat embryo on dishes with a rectangular array of 60 microelectrodes spaced at 200 μm. Although such cultures are enormously simplified model systems when compared to the intact brain, they still exhibit rich dynamics at a variety of timescales.

Recording from 60 electrodes at 25 kHz produces a data stream of 3 MB/s, so it is clear that significant online data reduction is a prerequisite for experiments of any duration. We developed a fully extendable modular toolset for online MEA data analysis, featuring spike detectors tailored to data streams with large stimulation artifacts, online data inspection, and hooks for connecting online feedback stimulation. These tools are implemented as a set of C++ programs with a common core library which we run under Linux on a four CPU SGI machine.

We used this system to make 20 minute recordings of spontaneous spiking activity in two dishes on 30 consecutive days starting on the second day after plating cells. Most of this activity occurred in dishwide bursts of varying magnitude and duration. There was large day-to-day variation in bursting behavior. On some days, the culture bursted quite regularly about 30 times per minute. On other days, the burst intervals were longer, or much less regular. Finally, on some days bursts mainly occurred in 'superbursts' - periods lasting up to 30 seconds with burst rates as high as 60 bpm.

The spatiotemporal fine structure of bursts as well as inter-burst activity are currently under investigation.

References

Figure 1. Overview of the data analysis system
85. Towards high-speed imaging of neural network activity in vitro
Axel Blau, Steve M. Potter

In addition to electrical recording with multi-electrode arrays (MEAs), we pursue a high-speed optical recording technique to trace neural activity spread within cultured neural networks. It is intended to overcome limitations in electrical recording, namely, the lack of spatial resolution. Optical recording will allow us to separate concerted network activity, and attribute neural activity to single neurons or even neuronal compartments. Furthermore, it will be possible to record from neurons that have been stimulated electrically, which is limited by artifacts in an electrical recording approach. Recording optical activity by means of fast-response potential-sensitive fluorescent membrane dyes (e.g., di-8-ANEPPS) will therefore complement electrical recording to compensate for the shortcomings of the latter method. However, due to photo-bleaching of the currently available dyes, and due to their gradual decomposition through cell metabolism, optical recording is generally limited to taking temporary snapshots of activity between subsequent staining procedures.

Our optical recording system is based on a custom-made high-speed digital camera system with a 64x64 pixel CCD chip, which is mounted on a regular, vibration-isolated upright microscope. The sub-frame readout times of arbitrarily selectable pixels on the chip are below one millisecond, allowing imaging at over 1000 subframes per second. It will be used with a highly stable tungsten-halogen illuminator.

Spectroscopic studies in vitro have elucidated the response mechanism of di-8-ANEPPS and its magnitude in cultured neural networks from rats and mice. The neurons were cultured on 14x14 mm² cover slips for 2 to 20 days. They have been stained with the potential-sensitive dye di-8-ANEPPS, and then analyzed with a UV/vis fluorimeter while exposing them to different concentrations of potassium chloride in artificial cerebrospinal medium. This approach allowed us to depolarize the membrane potential to a defined value, and thereby mimic neural activity. Care has been taken to exclude any secondary factors that have turned out to affect the shape of the spectra (e.g., shift of the coverslip in the cuvette; changes in pH, osmolarity, and temperature). 3D and 2D spectra have provided information on the general spectral characteristics of the dye. Time-series spectra revealed the actual dye response mechanism and its expected magnitude for the particular type of tissue. From our studies, we could confirm an electrochromic blue-shift as the main response mechanism, which can be as high as 10% per 100 mV change in membrane potential. The results will allow us to optimize the signal to noise ratio in the camera setup by selecting the proper filter set, which best matches the response characteristics of the dye with the chosen illumination source.

86. Two-photon time-lapse of liposome-transfected GFP neurons
Natalia Lukina, Yan Wu, Steve M. Potter

In order to make connections between neuron morphology and network activity, we are developing ways to image living cultured networks that are interfaced to a computer via a multi-electrode array substrate. In order to observe changes in morphology and connectivity over weeks in culture, we need a fluorescent label that persists and does not interfere with cellular processes. Professor Kenneth Longmuir, UC Irvine, created a novel liposome-based transfection system that we used to transfect cultured neurons with DNA coding for the green fluorescent protein. The liposomes incorporate a fusogenic peptide, a component to prevent serum inactivation, and a cationic nuclear localization signal. They were used to transfect cultures of dissociated rat embryonic cortical cells. We observed bright labeling persisting for over two weeks, in 1-5% of the cells per dish. The liposomes appear to have a preference for transfecting glia over neurons, perhaps due to their increased surface area. Cultures with labeled neurons older than three weeks in vitro showed numerous dendritic spines. We imaged these using a custom computer-controlled two-photon microscope stage that allows us to
visit multiple sites in the dish in succession, to within one micron accuracy, for several hours. We observed that the spines in these cultures, even without exogenous electrical stimulation, exhibit morphological dynamics even at the 2-3 minute time scale.

**Publication**  

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Summary: We are interested in how cell fate choice is regulated and carried out. A large focus of our work is directed towards understanding the genetic and molecular mechanisms that regulate and bring about cell death. Specifically, we are using *Drosophila melanogaster* as a model system to identify genes that function to regulate cell death, and to identify important roles that cell death plays in normal development. Important cellular regulatory pathways are evolutionarily conserved; thus molecules identified as important regulators of cell death in *Drosophila* are likely to have homologs in vertebrates and the pathways that link these molecules are likely to be regulated similarly. A second set of goals is to take the molecules and pathways uncovered in *Drosophila* and apply this information to the study of cell death in vertebrates, with the ultimate goal of determining the role that aberrations in this process play in human pathologies. In this context we see *Drosophila* as a powerful tool for uncovering conserved components and modes of death regulation. Toward this end we have developed several genetic screening approaches in the fly, and in the yeast *Saccharomyces cerevisiae* (described below), to identify genes important in the regulation of cell death.

We have also become interested in identifying and characterizing site-specific proteases, and in identifying regulators of their activity. We have developed an approach to monitoring the activity of site-specific proteases in the yeast *Saccharomyces cerevisiae*. We are using these yeast as backgrounds in which to carry out screens for these proteases and their regulators.

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

Although the signals and stimuli that trigger cell death are diverse, once initiated, apoptosis is thought to proceed via one or several common pathways. The identification in worms, flies and mammals of homologous proteins that function similarly to regulate cell death indicates that, as with other important signal transduction pathways, components and modes of death regulation are likely to be conserved throughout evolution (reviewed in Vernooy et al., 2000). *Drosophila* is an ideal system in which to do screens for genes important in death signaling because it is a complex organism with multiple life stages in which death plays important roles, it has powerful genetics, and we are able to manipulate death signaling in individual tissues using tissue-specific promoters.

References  

The dominant modifier approach  
Since normally occurring cell death is essential for *Drosophila* viability, genetic approaches to cell death must take account of this fact as well as the potentially important role of maternal effect gene products. We have circumvented these problems by creating a sensitized system that exploits a tissue dispensable for viability and fertility, the eye. In this system a cell death signaling pathway is made hyperactive (giving rise to flies with small eyes) or partially nonfunctional (giving rise to flies with large, rough eyes). In this sensitized background a decrease in gene dosage of 50% in genes functioning downstream or in parallel to the point at which the signaling pathway has been manipulated might be expected to result in an eye phenotype change, if these components are now rate limiting.
To carry out this approach we use a P element expression vector, pGMR (Glass Multimer Reporter), which drives eye-specific expression of reporter genes (Hay, et al., 1994). To identify genes important in cell death regulation, proteins from Drosophila or other systems that are known to be able to activate or inhibit cell death in some context are expressed in the eye under GMR control, and their capacity to perturb the normal patterns of cell death determined. To the extent that the ability of these proteins to manipulate cell death is conserved, such expression should result in the creation of flies with visible phenotypes (increased cell death=small eye; decreased cell death=large, rough eye). These flies can then be used as screening backgrounds to identify interacting genes.

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

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

References

The Drosophila IAP family of cell death inhibitors
Overexpression of either DIAP1 or DIAP2 blocks death in response to multiple stimuli, suggesting that they act on components common to multiple death signals. Though these proteins are homologous, they are different enough from each other in sequence (30% identity), genetic interactions and expression pattern that they probably function in distinct cellular contexts and have preferred substrates of action. DIAPs have two major functional domains: an N-terminal region containing two or three BIR repeats, sufficient for death-preventing activity, and a C-terminal RING finger, which may be
functioning to negatively regulate or antagonize this death-preventing activity.

As described below, Drosophila IAPs can interact with a number of proteins. Our evidence shows that DIAP1 is required for cell survival, and that a major function of the protein is to inhibit caspase activity. Death activators such as RPR, HID and GRIM act, at least in part, by blocking DIAP1 function, unleashing latent caspase activity. We are interested in understanding how proteins like RPR, HID and GRIM block DIAP1 function. We would also like to determine if peptides or other small molecules can mimic the function of these proteins. Because IAPs are the only known family of caspase inhibitors and, based on our observations, their function is required for cell survival, we think that modulating their ability to interact with caspases is likely to be an important mechanism by which cells can be made more or less sensitive to caspase-dependent cell death.

**Gene activation screens**

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

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

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

**References**


**Yeast-based screens for caspases and their regulators**

Site-specific proteolysis plays a number of critical roles in regulating cellular processes. For each of these cleavage events we would like to know what the protease is that is doing the cleavage, what the targets of the protease are, how the activity of the protease is regulated, and where and when the protease is active? A useful tool for addressing these questions would be a reporter for protease activity whose presence or absence can be visualized and quantitated. We have devised such a protease reporter that uses living cells. The basis for our approach is the creation of chimeric proteins that consist of two protein domains separated by a protease target site. One of the two domains, domain A, when released from association with domain B, is able to act as a signal transducer. Domain B functions to inhibit the signal transducing function of domain A when they are linked. Thus, in the absence of a protease that cleaves between domains A and B, no signal is transduced, while in the presence of the appropriate protease, the target site is cleaved, releasing domain A to transduce a signal which is subsequently detected.

There are a number of possible ways of constructing such a system and we have pursued several of these. One approach that works well involves creating a fusion protein that consists of a transcription factor (domain A) tethered to the intracellular domain of a type 1 plasma membrane protein (domain B). In this fusion, the C-terminal of the transmembrane protein is separated from the transcription factor by a linker domain that contains protease target sites. In the absence of a protease that can cleave the target site, the transcription factor remains at the membrane where it is nonfunctional. When the target site is cleaved by an introduced or endogenous protease, the transcription factor is released from the membrane and is free to go to the nucleus and activate transcription of a reporter.

A central step in carrying out apoptosis is the activation of members of a family of cysteine-dependent, aspartate-specific proteases, known as caspases. In many cell types, caspases sufficient to carry out cell death are expressed ubiquitously, indicating that their activation and activity must be tightly controlled in normal cells. Because caspases are central to cell death regulation, they
are important potential therapeutic targets. In order to take advantage of this potential, it is important to identify and characterize the organismal complement of caspases, and to understand how their activity is regulated by other cellular factors. With these goals in mind, we have developed yeast that act as reporters for caspase activity. Yeast provides an ideal background in which to screen for caspases and their regulators because they can be transformed with high efficiency, allowing for the screening of large numbers of proteins for activity in any particular assay; 2) Strains with a number of nutritional markers are available, allowing for the introduction and maintenance of a number of different plasmids at defined copy number, 3) Powerful selections can be carried out, and 4) It is likely that the intracellular environment of yeast constitutes a null, or close to null, state with respect to the activity of higher eukaryotic cell death regulators, including caspases.

Our caspase reporter yeast express a fusion protein substrate for caspase cleavage in which the type 1 transmembrane protein CD4 has linked to its cytoplasmic tail the transcription factor LexA-B42 (LB). Separating these two domains is a short linker (DG6) consisting of six different caspase cleavage sites that bracket the specificities of known caspases and the serine protease granzyme B, which cleaves caspases at sites of similar sequence (Thornberry et al., 1997). The emerging N-terminus following cleavage at any of the caspase target sites is a glycine residue, which acts as a stabilizing residue in the N-end rule degradation pathway in yeast (Varshavsky, 1996). When this molecule, referred to as CLBDG6 is expressed in a yeast strain that carries a plasmid in which expression of the lacZ gene is under the control of a LexA-B42-dependent promoter (designated as the LexA/B-gal reporter strain), expression of lacZ requires caspase cleavage at one or more of the introduced target sites. This cleavage releases LexA-B42 from membrane association, allowing it to activate lacZ transcription. Using this strain and other related strains, in conjunction with Drosophila and human cDNA yeast expression libraries we have generated, we are screening for caspases and caspase inhibitors (Hawkins et al., 1999).

The transcription based reporter strategy to monitor caspase activity in yeast also lends itself to screens designed to identify other site-specific proteases. One class of proteases that has generated a large amount of interest are those that cleave membrane proteins in the transmembrane domain. As described below we are pursuing screens designed to identify molecules that modulate an activity known as gamma secretase, which is critical for the cleavage of the Amyloid Precursor Protein (APP).

We also found that overexpression of an active caspase kills yeast. This fact provides the basis for a very powerful set of screens for proteins that function as caspase inhibitors as molecules that block caspase-dependent cell death. As described below, we have used this screen as a tool to show that the Drosophila cell death inhibitor DIAP1 functions as a caspase inhibitor (Hawkins et al., 1999). Cells that live because they express a caspase inhibitor as well as an active caspase provide a background in which to identify molecules that can disrupt caspase inhibitor-caspase interactions by virtue of their ability to kill these yeast, but not naive yeast. As described below, we have used this approach, as well as in vitro assays using purified proteins, to show that the Drosophila cell death activators REAPER, HID and GRIM block DIAP1ís ability inhibit caspase activity, suggesting a mechanism by which they induce cell death (Wang et al., 1999).

![Figure 2](image.png)  
**Figure 2.** A yeast strain that acts as a reporter for caspase activity. (A) Six caspase cleavage sites are placed between an N-terminal fragment of CD4 and a C-terminal LexA-B42 transcription factor. (B) Caspase cleavage results in release of the LexA-B42 protein, and activation of lacZ transcription.
References

87. Yeast-and fly-based screens for proteases that can cleave in a transmembrane environment
Ming Guo, Jolene Fernandez, Bruce A. Hay

Alzheimer's disease is genetically heterogeneous, but it is invariably associated with the accumulation in the brains of affected individuals of senile plaques consisting largely of amyloid beta peptide (A-beta), which is derived by proteolytic processing from the amyloid precursor protein (APP). A large body of evidence suggests that A-beta deposition is a cause rather than a consequence of Alzheimer's disease. Thus, blocking A-beta deposition is an important therapeutic goal. APP is initially translated as a type I transmembrane protein, but it can be processed by different pathways (see Figure 2). In the major pathway, alpha secretase releases the APP N-terminal ectodomain into the lumenal and extracellular space. Alpha secretase cleaves in the middle of the sequence that could give rise to the A-beta peptide, thus precluding its formation. In an alternative pathway, A-beta peptides are formed through the action of beta and gamma secretases. Gamma secretase activity (which may consist of distinct proteases) cleaves in the transmembrane region of APP to generate, in conjunction with beta secretase, two major A-beta species of 40 or 42-43 residues in length, differing in the length of their C-termini. The longer forms of A-beta aggregate and are thought to seed the formation of amyloid plaques. The molecular nature of the gamma secretase(s) is unknown.

Figure 3

Generation of a yeast strain that acts as a reporter for gamma secretase activity. APP is made as a type I transmembrane protein. In (A) APP (open and shaded boxes) is shown with LexA-B42 (diagonal lined domains) fused to its C-terminus. Cleavage by alpha secretase (A) results in release of an N-terminal ectodomain. Alpha secretase cleaves within the A-beta peptide sequence (shaded box). Alternatively, cleavage by beta- (B) and gamma secretase (G) results in formation of the A-beta peptide 40 and 42-43 forms and release of a C-terminal fragment consisting of the APP cytoplasmic domain. (B) In the presence of gamma secretase the APP cytoplasmic domain-LexA-B42 fusion is released from the membrane and translocates to the nucleus where it activates transcription of lacZ.
We have developed two screens to search for proteins that either are gamma secretase, or that regulate its activity. The first screen, diagrammed in Figure 2, is a simple variant of the caspase reporter system in yeast, in which lacZ expression is the readout. We generated a form of APP in which a cleavable signal sequence lies just N-terminal to the APP beta secretase cleavage site. APP C-terminal sequences are then followed by the transcription factor LexA-B42. We are using yeast expressing this construct, as well as one of the presenilins, as a background in which to screen for proteins that show potential gamma secretase activity: cleavage-dependent reporter activation.

We have also set up a related screen in Drosophila, in which a similar APP fusion protein (in which the transcription factor is GAL4) is expressed in the eye in flies that carry a UAS-rpr construct. In this system release of GAL4 from the membrane as a result of gamma secretase activity creates a cell death signal, and thus flies with small eyes. This readout is very convenient for us because we can compare modifiers identified in GMR-rpr screens with those identified in GMR-APP-GAL4 screens. Those modifiers that are specific for GMR-APP-GAL4 are potentially interesting in terms of identifying genes that regulate APP cleavage. A hint that this screening approach will give us some interesting molecules is that mutations that decrease the dose of the Drosophila presenilin also strongly suppress the GMR-APP-GAL4 phenotype, but not that of GMR-rpr.

Doing a screen for modifiers of gamma secretase activity in a higher eukaryote is also important for the following reason. While gamma secretase is of course critical for cleavage of APP it is also likely to be important for the cleavage of other transmembrane signaling proteins such as Notch. Thus, drugs targeted directly at gamma secretase may have pleiotropic effects. A genetic approach that focuses more generally on identifying modifiers of this activity may project towards new ways of modifying its activity or specificity in ways that more specifically affect APP processing.

88. Gene activation screens for cell death regulators
Stephanie Vernooy, Soon Ji Yoo, Peizhang Xu, Cain H. Yam

The GMREP vector contains an eye-specific promoter near one P-element end, as well as sequences sufficient for plasmid rescue of genomic DNA flanking the site of P-element insertion. When this P element inserts near the 5' end of a gene it causes the gene to be misexpressed at high levels in the developing eye (Hay et al., 1997). We have mobilized this P element throughout the genome and are characterizing the insertions that act as cell death regulators. We first score the lines for dominant phenotypes that may be due to increased cell death (a small eye) or decreased cell death (a large, rough eye). We then cross these insertions to lines of flies that express the cell death activators REAPER, HID or GRIM specifically in the eye (GMR-rpr, GMR-hid, or GMR-grim flies), and that thus have small eyes. The progeny of these crosses are then scored for the ability of the GMREP insertion to alter the GMR-rpr, GMR-hid or GMR-grim-dependent small eye phenotype. These modifiers identify new cell death regulators. Genomic DNA that is likely to contain a portion of the gene being overexpressed can be quickly isolated using plasmid rescue. The Drosophila genome is now finished. Thus a small sequence tag from the end of the P element serves to tell us exactly where in the genome our insertion is. Because GMREP-dependent phenotypes are primarily due to insertions near the transcription start site, and because GMREP carries the dominant eye color marker white, imprecise P-element excision using a genomic source of transposase or X-rays can be carried out to rapidly generate deletions that create loss-of-function phenotypes for the overexpressed gene.

We generated and screened 7,000 transposon insertions for their ability to suppress rpr- hid-, or grim-dependent cell death and identified a modest number of new loci that specifically suppress death due to overexpression of one or the other, or both, of these genes in the eye. We are in the process of characterizing some of these lines.

Reference

89. Structure-function analysis of the DIAPs
Soon Ji Yoo, Jun Huh, Mark Zarnegar, H. Arno J. Muller

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

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

DIAP1, as with many IAPs, also shows E3 ubiquitin-protein ligase activity. The function of this activity in vivo is unclear. One possibility is that this
activity simply constitutes a mechanism for conferring a short half life to the IAP, thus serving a proapoptotic function. Alternatively, ubiquitination of IAP-bound proapoptotic proteins may provide a prosurvival mechanism by which IAPs can catalytically remove these molecules. IAPs may also engage in substrate choice, preferentially degrading themselves when not bound to proapoptotic molecules, but degrading binding partners when the opportunity arises. In this way IAPs could serve to create, through a post-transcriptional mechanism, a relative balance between pro-and antiapoptotic proteins, at different levels of proapoptotic proteins.

90. Intracellular dynamics of the IAPs and caspases
Jun Huh, H-Arno J. Muller, Hong Yu, Lijuan Wang
We have antibodies that are specific for DIAP1, DIAP2, as well as a number of the caspases. We are using these reagents to determine the expression patterns of these proteins and their interrelationships throughout the fly life cycle. This analysis will tell us where these proteins are likely to be functioning to regulate cell death, and thus where loss-of-function phenotypes may be apparent.

91. Loss-of-function phenotypes of the DIAPs
Bruce Hay, Jun Huh, H.-A. J. Muller
We are characterizing the loss-of-function phenotypes of DIAP1 and DIAP2. These phenotypes will identify DIAP gene functions that cannot be substituted for by the activity of other genes or developmental compensatory mechanisms. Homozygous DIAP1 mutant embryos arrest early during embryonic development, and essentially all cells appear to die. Clones of homozygous mutant cells are also not seen in other tissues. These observations all suggest that DIAP1 is required for cell survival, perhaps in all cells. We are characterizing these phenotypes in further detail, focusing particularly on the early embryonic phenotype. We have also generated double mutants that remove DIAP1 as well as genes contained within the 75C region (rpr, hid and grim). These mutants have a phenotype similar to that of the DIAP1 mutant alone, consistent with models in which rpr, hid and grim act through DIAP1 to induce cell death (Fig. 3). Biochemical evidence supporting this model is presented below. We have also identified several P elements near the DIAP2 gene and are in the process of using these to generate DIAP2 mutants.

92. Identification of DIAP1-interacting proteins
Soon Ji Yoo, Hong Yu, Lijuan Wang
DIAP1 is required for cell survival in Drosophila. This suggests that its activity is likely to be regulated through interactions with other proteins. Genetic screens for cell death regulators provide one approach to identifying proteins that may interact with DIAP1. However, a more direct approach to identifying proteins that regulate DIAP1 function involves identifying proteins that physically bind DIAP1 in living Drosophila. We are replacing the endogenous DIAP1 with a multiply epitope-tagged version of DIAP1. Immunoprecipitation of DIAP1 from specific life stages or genetic backgrounds, followed by mass spec of eluted proteins, should identify such proteins.

93. DRONC: An apical caspase required for RPR-, HID-, and GRIM-dependent cell death
Christine J. Hawkins, Soon Ji Yoo
One of our goals is to identify the caspase cascades used to promote rpr, hid and grim-dependent death. We (Hawkins et al., 2000 and Meier et al., 2000) have found that the Drosophila caspase DRONC is critical for transducing these death signals because flies in which DRONC function is compromised show a dramatic suppression in RPR-, HID-, and GRIM-dependent death. DRONC also interacts with DIAP1 and HID and GRIM in Drosophila, in yeast and in vitro assays. The DRONC active site is very different from known caspases, suggesting that DRONC might have unique properties. We showed, using protein sequencing of DRONC substrates and in vitro mutagenesis that DRONC, unlike all other known caspases, processes itself following a glutamate. However, DRONC cleaves other caspases following an aspartate. DRONC activity is also not inhibited by the baculovirus p35 protein, generally thought of as a pancaspase inhibitor. These observations, in conjunction with the results of a series of experiments in which we examined the ability of the four published Drosophila caspases to process each other, suggest a model in which DRONC and only DRONC is able to process itself at a glutamate residue within the catalytic domain, generating an active caspase. This presumably serves to prevent DRONC activation by other caspases. Other caspases, however, are able to process the DRONC prodomain, providing a second level of regulation of DRONC activity. Once activated, DRONC rapidly cleaves downstream caspases, presumably triggering cell death.

Our observations with DRONC are important for several reasons. There are many situations in which caspase inhibitors are used to determine if particular cell deaths are apoptotic and caspase-independent. If the death is not suppressed by caspase inhibitors it is usually argued that the death is caspase-independent. Our results suggest, however, that cell deaths that are clearly caspase-dependent (those mediated by rpr, hid and grim) involve a caspase-dependent step which has remained effectively invisible using inhibitors such as p35. It may well turn out to be the case that other pathological cell deaths that are thought to be caspase-independent (and thus not amenable to caspase-based therapeutics), are in fact regulated by upstream proteases with nontraditional cleavage specificities.

References
which RPR, HID and GRIM act through DIAP1 to suppress DIAP1's ability to inhibit DCP-1 activity. They found that all three proteins, while nontoxic on their own, killed yeast coexpressing DCP-1 or drICE, and DIAP1, suggesting that they were blocking DIAP1's ability to inhibit caspase activity (Wang et al., 1999). They found that the DIAP1 loss-of-function phenotype consists of an embryo-wide set of cellular changes reminiscent of apoptotic cell death, and that these were associated with the activation of DIAP1-inhibitable caspase activity. Furthermore, double mutants that remove zygotic rpr, hid, and DIAP1 function showed phenotypes similar to those of the DIAP1 loss-of-function mutant alone (Wang et al., 1999).

Together, the above observations suggest that a principal function of DIAP1 is to promote cell survival by blocking caspase activity, and that at least one mechanism by which REAPER, HID and GRIM promote apoptosis is by disrupting IAP-caspase interactions (Fig. 4). These early studies left several important questions unanswered: 1) how does RPR, HID or GRIM binding to DIAP1 suppress DIAP1’s ability to inhibit caspase activity? 2) Do RPR, HID and GRIM regulate DIAP1 function through other mechanisms? Domain analysis of RPR and GRIM suggests that these proteins have apoptotic domains distinct from their N-terminal DIAP1 binding motifs. One question we are interested in is whether these other domains regulate DIAP1 through other mechanisms. 3) Finally, it is interesting to ask if there exist other proteins that function similarly to RPR, HID and GRIM. The yeast survival-based assay we used to show that these proteins disrupt IAP-caspase interactions provides a straightforward approach to screening for such molecules. Importantly, because such a screen is a function-based screen, and does not rely on identifying candidates based on sequence homology, we may identify proteins that disrupt IAP-caspase interactions even if they have only minimal homology to RPR, HID or GRIM.


94. How does Drosophila activate caspase-dependent cell death?
Soon Ji Yoo, H.-A.J. Muller, Jun Huh

An important set of questions is how different cell death signals, which in many cases are initiated through distinct signal transduction pathways, converge to activate a set of common downstream effector pathways. In Drosophila many different death signals lead to the transcriptional activation of one or more of three genes, reaper (rpr), hid and grim. The function of these genes is required for most normally occurring and induced cell death in Drosophila. Thus, their transcriptional activation acts as a point of death signaling convergence. One of our primary goals has been to identify mechanisms by which these different proteins activate cell death. Three important facts are known about these proteins that suggested testable mechanisms of action. These are 1) that death induced by their expression requires caspase activity, 2) that their death-promoting activity is suppressed in a dose-dependent manner by DIAP1, and 3) that REAPER (RPR), HID and GRIM bind to DIAP1 in insect cells (reviewed in Miller, 1999). These results have suggested several models of how DIAP1, caspases and RPR, HID and GRIM might interact to regulate cell death. In one model, death activating proteins such as REAPER, HID or GRIM activate caspases through an IAP-independent pathway. This model postulates that Drosophila IAPs act at two different points to suppress apoptosis: by acting as a sink for death activators such as REAPER, HID or GRIM, preventing them from interacting with their normal targets, and by inhibiting the caspase activity initiated by their action. In a second model, DIAP1 is proposed to function primarily as a caspase inhibitor, and REAPER, HID and/or GRIM initiate caspase-dependent cell death by preventing IAPs from productively interacting with caspases, thereby promoting their activity, and ultimately cell death.

Susan Wang, a former graduate student, and Christine Hawkins, a former postdoc, carried out experiments in Drosophila, yeast and in vitro to test the idea that RPR, HID and GRIM promote apoptosis by blocking DIAP1’s ability to inhibit caspase activity (Wang et al., 1999). They found that all three proteins, while nontoxic on their own, killed yeast coexpressing DCP-1 or drICE, and DIAP1, suggesting that they were blocking DIAP1’s ability to function as a caspase inhibitor. They pursued the basis for this activity further with HID and found, both in yeast and in vitro, that proteins containing the N-terminal 37 residues of HID, which are sufficient to induce apoptosis in insect cells (Vucic et al., 1998), suppressed DIAP1’s ability to inhibit DCP-1 activity.

These results are consistent with a model in which RPR, HID and GRIM act through DIAP1 to promote death-inducing caspase activity. This model predicts that DIAP1 should be essential for cell survival, and that a loss of DIAP1 function should result in an increase in DIAP1-inhibitable caspase activity. To test this idea they carried out a second set of experiments in which we characterized the phenotype of a DIAP1 loss-of-function mutation, as well as the phenotype of a double mutant that removed DIAP1, as well as rpr, hid and grim. They found that the DIAP1 loss-of-function phenotype consists of an embryo-wide set of cellular changes reminiscent of apoptotic cell death, and that these were associated with the activation of DIAP1-inhibitable caspase activity. Furthermore, double mutants that remove zygotic rpr, hid, and DIAP1 function showed phenotypes similar to those of the DIAP1 loss-of-function mutant alone (Wang et al., 1999).

Together, the above observations suggest that a principal function of DIAP1 is to promote cell survival by blocking caspase activity, and that at least one mechanism by which REAPER, HID and GRIM promote apoptosis is by disrupting IAP-caspase interactions (Fig. 4). These early studies left several important questions unanswered: 1) how does RPR, HID or GRIM binding to DIAP1 suppress DIAP1’s ability to inhibit caspase activity? 2) Do RPR, HID and GRIM regulate DIAP1 function through other mechanisms? Domain analysis of RPR and GRIM suggests that these proteins have apoptotic domains distinct from their N-terminal DIAP1 binding motifs. One question we are interested in is whether these other domains regulate DIAP1 through other mechanisms. 3) Finally, it is interesting to ask if there exist other proteins that function similarly to RPR, HID and GRIM. The yeast survival-based assay we used to show that these proteins disrupt IAP-caspase interactions provides a straightforward approach to screening for such molecules. Importantly, because such a screen is a function-based screen, and does not rely on identifying candidates based on sequence homology, we may identify proteins that disrupt IAP-caspase interactions even if they have only minimal homology to RPR, HID or GRIM.
Figure 4. Model for how DIAP1 regulates apoptosis. DIAP1 inhibits caspase activity (---) and is essential for cell survival. DIAP1’s ability to inhibit caspase activity is suppressed by RPR, HID and GRIM (-----), thus promoting caspase-dependent cell death (bold arrow). RPR and GRIM have other activities (Evans et al., 1997; Thress et al., 1998; Avdonin et al., 1999), suggesting that these proteins may also promote apoptosis through other pathways (dashed arrows). Free DIAP1 may inhibit apoptosis by binding these proteins, sequestering them from their targets (-----). Thus DIAP1 may act both upstream and downstream of RPR, HID and GRIM to prevent cell death. Genes within the H99 interval are not required for some cell deaths (White et al., 1994; Foley and Cooley, 1998), indicating that other death pathways exist (dashed lines). It is not known if these pathways act through DIAP1.

References

95. Autophagic cell death
Jeffrey Copeland
While much cell death is apoptotic, a number of cell deaths share features with a process known as autophagy, which has been described in some molecular detail in yeast. In yeast, starvation leads to a cellular response in which double membrane-bound vesicles are formed that take up and hydrolyze organelles as well as bulk cytoplasm. This process of autodigestion provides the cell with nutrients, allowing survival under starvation conditions. It has been clear for some time that there are a number of situations in which cell death in animals shows morphological features similar to those of autophagy rather than apoptosis. However, the molecular mechanisms that mediate these deaths has remained unexplored. Drosophila homologs are available for many of the yeast proteins involved in autophagy. The goal of my project is to explore the molecular mechanisms underlying autophagic cell death in Drosophila.

Death-Related Publications


Professor Emeritus: Edward B. Lewis
Visiting Professor: Andrew Dowsett
Postdoctoral Fellow: John Burr, Joanne Topol
Research Staff: Jae Hur, Corey Olson, Mitzi Shpak
Laboratory Staff: Lydia Bowman, Blanca Granados, A. Hernandez, A. Marquina

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National Institutes of Health, USPHS

Summary: Our experiments involve genes that control the body plan of all higher animals, including vertebrates and invertebrates. Mutations in these genes tend to convert one structure or organ into a homologous one, a phenomenon known as homeosis. Such genes have come to be called homeotic or, more precisely, homeobox (HOX) genes, since they invariably contain a HOX, or a 180 bp DNA sequence that codes for the DNA-binding region of the transcription factor, or protein product, of a HOX gene (see Guidebook to the Homeobox genes, 1994). The cluster of HOX genes that controls the body pattern is known as the HOX complex (HOX-C). In contrast to most organisms known, the HOX-C in Drosophila melanogaster has split into two: the bithorax (BX-C) and the Antennapedia (ANT-C) complexes. The ANT-C has 5 major HOX genes which program the anterior half of the body and the BX-C has 3 HOX genes that control the posterior half, with the dividing line being in the thoracic region. The HOX genes of each complex are arranged colinearly: the order of the genes in the chromosome parallels their order of expression along the longitudinal axis of the organism.

We have identified by statistical analysis (see Lewis et al., 1995 for the methodology) numerous significantly over represented DNA motifs in the ANT-C and BX-C. In the first section, we discuss one example, the hexanucleotide, AGATAC. Secondly, we report on molecular experiments designed to identify: (a) transcription factors that bind to over represented hexanucleotide motifs in the BX-C; and (b) downstream genes of the three HOX genes of the BX-C. Finally, we report on an analysis of a miniature ring chromosome that expresses an unusual type of position-effect variegation (PEV) of the BX-C.

Reference

96. Cis-regulation of the homeotic complex (HOX) of Drosophila

Edward B. Lewis, David Mathog, Jae Hur

We are making use of the nearly complete DNA sequence now available for the non-heterochromatic regions of the genome of Drosophila melanogaster. A few of the highly over represented clusters of a given hexanucleotide that we had already identified in the BX-C and/or ANT-C are also highly over represented in the non-coding region of the whole genome. Especially interesting is the sequence, AGATAAC, that has perfect concatemers (with no spaces between them) of three or, in one case in the Abdominal-B region of the BX-C, four concatemers of that hexanucleotide. In the whole genome sequence, there is a perfect concatemer of 58 contiguous AGATAC's in the X chromosome; and in addition several concatemers with 10 or more AGATAC in other regions of the genome. The possible function of such concatemers is unknown. An over abundance of AGATAC's in the BX-C was first noted by Lipshitz et al., 1987.

The Ultrabithorax (Ubx) and Antennapedia (Antp) genes control development of the wing-bearing and halter-bearing thoracic segments. They have the largest introns of the HOX genes; namely, 78,051 and 102,958 bp, respectively. In a search for DNA sequences that are unique to these introns, one stands out as promising: namely, a 34 bp sequence in the Antp intron that consists, almost exclusively, of a concatamer of the tetranucleotides, TACA, and its complement, TGTA, as follows: TACATATGTCATCATATGATGATGATG. The reverse of this sequence with five transitions occurs in only one other region: the Ubx intron. For the Antp and Ubx introns, the consensus sequence is: TACATATTRYAYATRYATGATGATGATG. A search for it in the entire genome finds only these two cases. Presumably this sequence has some special functional, and/or possibly evolutionary, significance.

Reference

97. Functional analysis of the BX-C

John Burr, Corey Olson, Joanne Topol

Previous work by the Lewis group has identified over-represented hexanucleotide clusters or "hexamer pairs" (e.g., two AGATAC's separated by 30, or fewer, base pairs) in cis-regulatory sequences of the BX-C (Lewis et al., 1995). It is likely that such over represented hexamer pairs play a role in controlling the expression of BX-C genes. In order to test the possibility that these hexamer pairs are recognition sequences for transcription factors, we have performed band shift assays. For this purpose, we have synthesized oligonucleotides that carry two copies of the hexamer pair as a direct repeat separated by three base pairs; and have isolated soluble nuclear proteins from 0-24 hour Drosophila embryos. Among the 88 over-represented hexamer pairs tested, eight are specifically bound by unknown nuclear factors. DNA-binding proteins present in crude extracts of the nematode, C. elegans also specifically recognize a subset of these eight hexamer pair sequences.

We have begun a microchip assay to identify possible downstream targets of the three BX-C transcription factors; namely, those coded for by the
Ultrabithorax (Ubx), abdominal A (abd-A), and Abdominal B (Abd-B) genes. For this purpose we have isolated mRNA from early Drosophila embryos in which those factors have been independently over expressed. As an example, over expression for Ubx was achieved by mating females transgenic for a P element that ubiquitously expresses the gal4 transcription factor in their eggs and early embryos, to males transgenic for a P element that has the Ubx cDNA under the control of gal4. From this mating and the corresponding abd-A and Abd-B matings, 1-5 hour embryos and 4-8 hour embryos have been collected and processed for their mRNAs. These mRNAs will be used to probe chips that carry a microarray of a Drosophila cDNA library. As a control, mRNAs from wild-type embryos will be used jointly as a probe. Any cDNAs on the chip that are expressed differentially in wild-type embryos vs. embryos having over expressed BX-C genes are expected to be downstream genes, either directly or indirectly, of the BX-C transcription factors.

98. Position-effect variegation involving the BX-C
Andrew Dowsett, Edward B. Lewis

We have continued our studies of a small, x-ray induced, ring-X chromosome, Ring(1)a2, that contains little more than the BX-C and part of the euchromatic regions of the X chromosome. This ring is highly unusual in that it exhibits heterochromatin or "position-effect" variegation (PEV) for the Ubx gene of the BX-C. The flies showing PEV have small to large patches of third thoracic tissue transformed towards tissue of the second thoracic segment. This is most striking in the halteres, which exhibit patches of wing tissue. Although most genes that are brought next to heterochromatic regions become unstable in their expression, the genes of the BX-C have, until this example, not exhibited this phenomenon in many rearrangements that have brought the complex adjacent to heterochromatic regions of the X and other chromosomes. We find that Ring(1)a2 obeys two important criteria for PEV: (1) the variegation is almost completely suppressed by the addition of an extra Y chromosome and (2) the variegation is polarized, in that there is a gradient of inactivation of the cis-regulatory regions of the Ubx gene. This gradient is colinear in that it proceeds with diminishing intensity from anterobithorax (abx) to bithorax (bx) to postbithorax (pbx), paralleling the order of these regions in the chromosome. We have failed to detect PEV for the remaining abd-A and Abd-B genes, indicating that the variegation is not the result of somatic loss of the ring. Experiments to determine the molecular breakpoints that unite the BX-C with X heterochromatin in Ring(1)a2 have thus far been unsuccessful.

Publications
Professor: Elliot Meyerowitz  
Postdoctoral Scholars: Marcio Ferreira, Venugopala Gonehal, Elizabeth Haswell, Marcus Heisler, Toshiro Ito, Jeffrey Long, Kazuaki Ohashi, Michael Vishnevetsky, Frank Wellmer, Eva Ziegelhoffer  
Senior Research Fellow: Carolyn Ohno  
Senior Research Associate: Leonard Medrano  
Visiting Associate: Jose Luis Riechmann  
Graduate Student: Catherine Baker  

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Summary: Our lab is working to understand plant development, and the role played by each of the genes of the flowering plant *Arabidopsis thaliana* in that process. We concentrate on development of flowers, and on the formation and maintenance of shoot apical meristems, which are the collections of stem cells at the tips of growing shoots.

*Arabidopsis* flowers have four different types of organs (sepals, petals, stamens and carpels) disposed in concentric whorls. Each of the organ types normally appears in a fixed number, as well – four sepals, four petals, six stamens and two carpels. We have for many years been studying a set of master regulatory genes that is expressed in concentric rings, and that specifies the identity of the organs that develop in each region of the flower. One open question is the control of the spatial domain of expression of the master regulators, the so-called ABC genes. The B function genes are normally expressed in the second and third whorls, where they specify that petals and stamens will develop. Last year we reported the discovery of *MOB*, a gene that acts in the specification of the position of B function gene activity. An additional gene that acts in the same process, *OOK*, is reported this year. While *MOB* codes for a transcription factor, *OOK* encodes a histone deacetylase, implicating chromatin changes in B gene regulation. Another question raised by the ABC genes, which for the most part code for transcription factors of the MADS box family, is what downstream genes they activate. Several methods for finding such downstream genes are in use, including computer analysis of the *Arabidopsis* genome for binding sites, and use of extensive microarrays of flower- and meristem-expressed genes to assess changes in RNA levels in mutant and developing tissues. A third question is how the numbers of floral organs are controlled. Several of our research projects reported this year reveal new genes involved in this process, including enhancers of the mutant phenotype of the *CLV1* receptor kinase such as *SKA*, *HAN*, and *15UAS*, genes downstream of the floral organ number locus *PAN*, and new results in understanding the function of the *SUP* gene. Additional and new types of mutageneses have revealed additional novel genes such as *G245* and *SHD*, and their involvement in various aspects of flower development, and new descriptive studies are revealing some of the processes of stamen development.

In the area of meristem formation and maintenance we report new results on the *TPL* gene, mutant embryos of which can make two root meristems instead of the normal one root and one shoot meristem; and new studies aimed at understanding protein movement in meristems, and the functions of the plant hormone auxin in meristem patterning.

99. DNA microarray analysis of *Arabidopsis* flower development

Frank Wellmer, Jose Luis Riechmann, Marcio Alves-Ferreira

We are interested in the differences in gene expression between different floral organs and different floral stages as well as in the gene expression networks that control and execute the process of *Arabidopsis* flower development. In order to study flower development on a genomic level we have constructed a cDNA microarray that is composed of flory-expressed transcripts.

The *Arabidopsis* cDNA collections that are publicly available have been mainly prepared with mixed RNA populations from the different tissues of the plant. Therefore, these collections are not ideal for constructing a microarray to characterize flower-specific processes, since genes that are specifically expressed in meristems and flowers are under-represented. In order to increase the representation of genes that are involved in the processes we are interested in, we have generated subtracted flower-specific cDNA libraries. We have processed about 7,700 clones from those libraries for printing our microarray. In addition, we have specifically cloned or obtained cDNA fragments of approximately 250 genes that are known, or suspected, to play a role in flower development. Furthermore, the microarray also comprises about 100 elements representing constitutive and control genes, as well as sequences that can be spiked in the experimental samples to monitor the performance of the technique. The resulting microarray with 8,096 elements was recently augmented by the addition of 2,700 elements from a non-redundant *Arabidopsis*-EST collection which was derived from flowers, courtesy of Kazusa DNA Research Institute.

We are currently using the cDNA microarray to analyze the spatial and temporal differences in gene expression during flower development. Furthermore, we are trying to identify the target genes of several of the many transcription factors that have been shown to be involved in the regulation of flower development.
100. Cloning of OOKPICK, a gene involved in floral organ identity
Carolyn Ohno

To identify additional negative regulators of B function floral organ identity, a screen of T-DNA mutagenized mob mutant plants was conducted. The ookpick (ook) mutant was isolated as an enhancer of the mob mutant phenotype. In mob ook double mutant flowers, first whorl petaloid sepal and fourth whorl stamenoid carpel homeotic transformations are pronounced. In ook single mutants some petaloid sepal and stamenoid carpels are also produced and the inflorescence meristem eventually terminates in stamens. Ectopic expression of AP3, PI and AG is detected in sections of ook mutant inflorescences which is consistent with a role for OOK as a negative regulator of B and C function organ identity gene expression. Thus OOK is partially redundant with MOB in keeping homeotic genes off in specific whorls of the flower. The phenotype of ook mutants is pleiotropic which suggests that the gene affects multiple developmental processes. The ook single mutant is late flowering in long days, the leaves are narrow, the pedicels are elongated, phyllotaxy of emerging flowers is abnormal and sepal and petal number of early arising flowers is increased slightly.

The OOK gene was tagged with a T-DNA mutagen. The recessive ook mutant phenotype cosegregated with Basta resistance and Southern analysis of T3 individuals revealed a tandem inverted repeat insertion of the T-DNA at a single locus. Plasmid rescue of flanking genomic DNA on both sides of the T-DNA insertion of the T-DNA at a single locus. Plasmid rescue of T3 individuals revealed a tandem inverted repeat cosegregated with Basta resistance and Southern analysis of T3 individuals revealed a tandem inverted repeat insertion of the T-DNA at a single locus. Plasmid rescue of Basta resistance and Southern analysis of T3 individuals revealed a tandem inverted repeat insertion of the T-DNA at a single locus. Plasmid rescue of Basta resistance and Southern analysis of T3 individuals revealed a tandem inverted repeat insertion of the T-DNA at a single locus. Plasmid rescue of Basta resistance and Southern analysis of T3 individuals revealed a tandem inverted repeat insertion of the T-DNA at a single locus.

101. Interaction of OOK with CURLY LEAF
Carolyn Ohno, Elizabeth Haswell

Both OOK and CURLY LEAF (CLF) are negative regulators of floral homeotic gene expression. The phenotype of ook clf-2 double mutants reveal an enhanced phenotype where flowers display nearly complete transformation of petals to stamens in the second whorl. In clf-2 single mutants only partial transformation is observed in the second whorl. AG mRNA shows patchy misexpression in the second whorl of ook clf-2 flowers. This result is consistent with OOK acting as a repressor of AG in the second whorl. Interestingly the ook mutation suppresses the clf-2 early flowering phenotype. While clf-2 mutants grown in long day conditions produce 6.6 ± 0.5 rosette leaves at bolting, ook single mutants or ook clf-2 double mutants produce 8.3 ± 0.6 rosette leaves. This suggests that the clf-2 early flowering phenotype is dependent on OOK activity. ook and clf single-mutant and double-mutant combinations display partial homeotic phenotypes similar to mutations in Polycomb Group (PcG) genes in Drosophila. PcG proteins in Drosophila function to maintain repressed states of homeotic transcription via chromatin through a mechanism which is unclear. Coimmunoprecipitation experiments demonstrate an in vivo interaction between RPD3 and EnZ as part of a 600 kDa PcG complex (Reference: Tie et al., 2001 Dev. 128:275-286). Since OOK and CLF proteins show sequence similarity to RPD3 and EnZ we are first testing if OOK and CLF interact in vitro using GST pull-down and yeast two-hybrid assays. To determine if a PcG complex exists in plants antibodies/tagged proteins in plants are being generated to demonstrate in vivo protein interactions.

102. Searching for the AGAMOUS target genes based on in vitro binding sequences
Toshiro Ito

In order to reveal the downstream activities of the floral homeotic gene AGAMOUS, we have been searching for the target genes of the AG protein. AG encodes a MADS box transcription factor, which localizes in the nucleus. The AG protein makes a homodimer and binds to the consensus DNA sequences (CArG box) of 5'-TT(A/T/G)CC(A/T)6GG(A/T/C)AA-3' with a relatively high affinity (the equilibrium dissociation constant is 9.6 x 10^-10). We searched for this CArG box in the Arabidopsis genome sequence by using the TAIR PatMatching database search program and found 137 different AG CArG boxes in the Arabidopsis genome. Next, we retrieved the BAC clone sequences containing the AG CArG boxes from the GenBank database and searched for target candidate genes located nearby the AG CArG boxes by looking into the annotation databases of Stanford, TIGR and Kazusa. As a result, fifty-one candidate genes that have AG CArG boxes in the upstream regions, the introns or the 3' regions were selected. Next, we checked the expression of each candidate by RT-PCR using RNA isolated from wild-type roots, leaves, flower and ag-1 mutant flowers. Out of 51 candidates, all genes were expressed in the wild-type flower and 15 genes showed reduced or no expression in the ag-1 flower.

In order to check the inducibility of putative target genes by the AG, we produced an inducible system of AG by making a fusion between AG and the hormone binding domain of a glucocorticoid receptor. The transgenic plants with a 3SS promoter:AG-GR transgene in the ag-1 mutant background showed partially rescued phenotypes upon DEX induction. The flowers produced six stamens and two carpels, even though no homozygous seeds were obtained due to the reduced amount of pollen and underdeveloped ovules. We considered that the AG-GR fusion could almost fully replace the endogenous AG, and started RT-PCR experiments using this transgenic plant. Out of 15 candidate genes that showed reduced or no expression in the ag-1 mutant background, six genes were clearly induced in the transgenic flowers six hours
after DEX induction, showing that these six genes are early inducible genes of AG. Out of these six genes, five genes encode MADS box, homeobox, MYB-like and HLH type transcription factors. The remaining one gene encodes a TFL-like protein.

The detailed expression analyses and the functional analyses of these target genes are under way. I would like to acknowledge Natsuko Ito for the database search as a volunteer.

103. Searching for the AGAMOUS target genes based on the binding sequences containing one mismatch

Toshiro Ito

In order to reveal the downstream activities of the floral homeotic gene AGAMOUS, we have been searching for the direct target genes of the AG protein based on the binding consensus sequences (CArG box) of AG determined in in vitro binding experiments. It is possible, however, that sequences that have weaker affinity to the AG protein work as a cis-regulatory element in vivo. It's also possible that other unknown co-factor(s) that bind nearby to CArG box sequences enhance the binding of the AG proteins to partial consensus sequences in vivo. So, we started considering one mismatch from the AG CArG box in the database searches of the type described in the previous abstract. As a result, extra 870 sequences were found by using the TAIR PatMatching database search program. Next, we retrieved the BAC clone sequences containing the putative AG binding sites and searched for target candidate genes by looking into an annotation of each clone. As a result, fifty-nine candidate genes that are located close to the CArG box were selected. We made gene-specific primers for these 59 target candidates and did a RT-PCR experiment. Out of 59 candidates, all genes were expressed in wild-type flowers and six genes showed reduced or no expression in the ag-1 mutant background. Out of six candidate genes, four genes seem to encode MYB-like, AT hook-type or zinc finger-type transcription factors. Two genes encode protein kinases.

In order to check the inducibility of putative target genes by the AG protein, we performed a RT-PCR experiment using 35S promoter::AG-GR transgenic plants in the ag-1 mutant background. Out of six candidate genes, only one gene was induced in the transgenic flowers six hours after DEX induction. The expression of three genes were further down-regulated and the expression of the remaining three genes did not change. Detailed expression analysis and the functional analysis of these target genes are under way. I would like to acknowledge Natsuko Ito for the database search as a volunteer.

104. Sidekick of clavata is an enhancer of clv1 defects in floral and shoot meristems

Catherine C. Baker

The shoot apical meristem (SAM) is a population of cells at the growing tip of the plant; this group of cells is first organized in the embryo and is the source for all of the above-ground tissue of the plant. A small set of self-renewing stem cells resides in the central zone of the meristem, whereas cells nearer the periphery are thought to be more committed to organ formation. It seems crucial that the conflicting forces of stem cell proliferation and differentiation remain under subtle control, so that the SAM neither terminates prematurely nor becomes overgrown and disorganized.

CLAVATA1 (CLV1) is one of several genes known to regulate meristem size and structure. A clv1 loss-of-function mutation results in enlarged shoot and floral meristems, with a corresponding increase in stamen and carpel number. CLV1 was cloned and found to be a receptor-like serine/threonine kinase, suggesting that the protein might be part of a signaling pathway. In order to learn how CLV1 acts to restrict meristem size (for example, whether by blocking proliferation, promoting differentiation, or both), we have performed an activation-tagging screen in an intermediate clv1 background, with the goal of finding enhancers and suppressors of clv1.

We have identified a strong enhancer of clv1-1, which we named sidekick of clavata (ska). ska;clv1-1 plants have meristems which are typically 1 mm in diameter [wild-type meristems are ~100 microns], and the flowers have extra carpels and additional whorls of tissue inside the gynoecium. By itself, ska does not have any marked effect on shoot or floral meristems. Two CLV1-independent phenotypes which co-segregate with the set described above are distorted trichomes and reduced fertility. Preliminary data indicate that the severity of the ska meristem phenotype is sensitive to the level of CLV1 activity. In situ hybridization of ska and ska;clv1-1 tissues with a CLV1 probe show that CLV1 expression is not disrupted in either the single or the double mutant.

Unfortunately, ska is not a tagged allele, as has been determined by Southern blot. ska co-segregates with chromosome 2, but we have been unable to map it with any precision, since zero recombinants are recovered between ska and markers spanning 90% of chromosome 2. In order to identify new alleles of ska that are more conducive to being mapped, we have begun a non-complementation screen, using gamma-irradiated clv1–2 pollen crossed to clv1–1; ska flowers.

105. Characterization and molecular cloning of HANABA TARANU, a gene controlling meristem size

Kazuaki Ohashi, Leonard J Medrano

The shoot apical meristem (SAM) is a group of stem cells that forms the shoot and its attendant structures, such as leaves and flowers. Highly controlled patterns of cell division are observed in the SAM, and are crucial for forming the proper structure of plants. So far, numerous Arabidopsis mutants with defects in SAM and floral meristem (FM) development have been identified. One such mutant, hanaba taranu (han) is characterized by reduced floral organ number. Confocal laser scanning microscopy of han showed a reduction in the size of SAM and FMs, suggesting HAN regulates meristem development and/or meristem cell differentiation.
Two new *han* alleles, named *han-3* and *han-4* were identified as enhancers of fasciation in the *clv1-1* mutant. When crossed out of the *clv1* mutant background, *han-4* plants have normal stem width like *han-1* and *han-2*, however, *han-3* plants still show the fasciation phenotype. Analysis by scanning electron microscopy shows *han-3* plants have large flattened SAMs.

As stated above, *han* has a strong interaction with *clv1-1*. *han* also interacts strongly with other *clv* mutants. *han-3* *clv2-1* plants and *han-3* *clv3-2* plants show more severe phenotypes than *han-3* *clv1-1* plants. Double mutants with *clv2-1* or with *clv3-2* have extremely flattened stems, although the stem-fasciation phenotype of *clv2-1* is much weaker than that of *clv1-1*. These results, including meristem enlargement in *han-3*, suggest *HAN* may function as a modulator in the CLV signaling pathway responsible for regulating meristem size.

To characterize molecular mechanisms of *HAN* in SAM and FM development, the *HAN* gene has been cloned by two independent methods: *han-1* was cloned using a map-based approach, where as *han-3* and *han-4* were tagged alleles identified by TAIL-PCR. We find the *HAN* gene encodes a putative transcription factor whose Zn-finger motif shows high similarity with that of mammalian GATA transcription factors. In the *Arabidopsis* genome, there are 28 genes that encode GATA-like Zn-finger domain(s), however, little is known of the functions of such GATA-like transcription factors in plant development. Database searches reveal *Arabidopsis* has at least two *HAN*-like genes among plant GATAs in its genome. We are planning to screen knock-out mutant plants of those *HAN*-like genes from a large collection of T-DNA transformed *Arabidopsis* lines.

To examine the *HAN* mRNA expression pattern during plant development, *in situ* hybridization experiments are currently under way.

106. Characterization of meristem organization mutant *cvs1*
Leonard J. Medrano

The stereotypical development of the shoot apical meristem (SAM) has been well documented in *Arabidopsis*. All aerial portions of plants are determined from a defined group of cells within the SAM. The *SHOOT MERISTEMLESS* (*STM*) and *CLAVATA* (*CLV*) genes are responsible for balancing proliferation and differentiation of cells to maintain the SAM. The *clv1* and *clv3* mutants cause overproliferation of undifferentiated cells resulting in SAM and floral meristem enlargement. *STM* is essential for the initiation of the SAM in late embryogenesis. Loss of function *stm* alleles fail to produce any organs from the SAM after formation of the cotyledons. Here we describe the *cvs1* line in which the SAM organization is disrupted, causing defects in leaf phyllotaxy, lateral branching, and flower development. Meristems of *cvs1* are fasciated and internode elongation is reduced causing abnormal clusters of flowers. Flowers consist of four first whorl sepals, two to three petals and stamens in the second and third whorls, and two to three carpels in the fourth whorl.

The *cvs1* line was obtained through the INRA-Versailles *Arabidopsis* *Agrobacterium* meditated T-DNA lines in the Wassilewskija (Ws) wild-type background. Genetic analysis of *cvs1* has shown a single recessive mutation is responsible for the mutant phenotype. Segregation analysis showed the mutation does not segregate with the drug resistance marker of the T-DNA insertion.

Double mutants between the *cvs1* mutant gene and the meristem mutants *clv1-4*, *clv3-1* and *stm-12* were constructed. Double-mutant combinations with *clv3-1* produce severely fasciated stems. The *cvs1* mutation *stm-12* double-mutant combination produced a large rosette with multiple whorls of leaves arising from the apex in a disorganized, indeterminate manner. This data suggests a possible role for the gene in maintenance of shoot meristem organization.

To map the mutant gene in the *cvs1* line (Ws background), the *cvs1* mutant line was crossed to wild-type Landsberg erecta (Ler). DNA was prepared from plants displaying the *cvs1* mutant phenotype for molecular mapping of the gene. CAPS markers were used in a PCR-based mapping strategy to identify recombination events between Ws and Ler in the mutant region. Preliminary analysis of 120 mutant F2 plants has mapped the mutation in the *cvs1* line to the bottom of chromosome 5 between markers nga129 and MDA7. Additional genetic and molecular analysis is underway.

107. 15UAS: A gene affecting floral organ number, isolated in a modular misexpression screen
G. Venugopala Reddy

We have designed a new screen to isolate molecules involved in meristem development. The objective of this screen is to direct the expression of random molecules in the genome to a tissue of interest, in this case meristems and screen for resultant dominant phenotypes. Screen utilizes the heterologous two component, GAL4;UAS system to achieve targeted misexpression. We have utilized a GAL4 line expressed in meristems, generated by fusing GAL4:*VP16* coding region to the *CLAVATA1* (*CLV1*) promoter (*CLV1::GAL4*). The *GAL4:*VP16 construct was kindly provided by Dr. Jim Haseloff, Department of Plant Sciences, University of Cambridge UK). The activity of *GAL4:*VP16-glucuronidase (β-glucuronidase (*GUS*)) and *WUSCHEL* whose misexpression results in bigger meristems. We constructed a vector, which can deliver UAS-promoter carrying ten GAL4 binding sites to different regions in the genome (UAS-trap). The homozygous *CLV1::GAL4* plants were transformed with the UAS-trap vector to generate independent transformants. The primary transformants were selected and subjected to phenotypic analysis. We have thus far screened close to 1200 independent transformants and have identified two transformants, which show alteration in floral organ
number and one resulted in fasciated meristems and radialized leaves. We have also noticed four transformants, which resulted in single cotyledon and cup-shaped cotyledons. We could not rescue these mutants due to meristem arrest at early stages in development.

We have analyzed one of the lines, 15UAS (isolation number) in greater detail. This line was isolated as a mutation in which the central floral organs, the carpels were absent and the meristem terminated in a single stamen and thus resembled wuschel mutant flower. In severe cases all the floral organs failed to develop except for nectary-like structures located on pedicel. The segregation analysis in subsequent generations confirms it as being a dominant phenotype. We rescued the genomic sequence flanking the T-DNA and found that UAS-promoter is located about 190 base pairs upstream of an open reading frame, which is predicted to encode a DNA-binding protein. Subsequent RT-PCR analysis revealed the overexpression/misexpression of the target gene. We are currently carrying out recapitulation experiments to further test whether the phenotype is due to misexpression/overexpression of the target gene.

We are planning to bring in a few modifications to this screen. Firstly, to increase the number of GAL4 binding sites to achieve higher levels of misexpression. We also would like to use GAL4 lines active only in floral meristems such as AGAMOUS::GAL4 and LEAFY::GAL4, in order to avoid the possibility of meristem arrest in early seedling stages.

108. G245: Characterization of a gene expressed in organ boundaries

G. Venugopala Reddy, M.G. Heisler

The gene was identified in a screen for GAL4 enhancer trap lines. We have characterized the GFP expression pattern by confocal scanning microscopy. Expression is restricted to the presumptive organ forming regions in torpedo stage embryos and in sub-epidermal cells of developing cotyledons. In post-embryonic development, expression is seen in boundary regions separating floral primordia and the inflorescence meristem. Within floral meristem, an elevated levels of expression is observed at the boundary regions that separate different whorls. At a later stage expression is restricted to the sub-epidermal cells of developing floral organs.

We have rescued the flanking genomic region and found that the T-DNA insertion is located at about 0.8 kb upstream of an open reading frame predicted to encode a protein with a zinc binding RING FINGER motif. We have attempted to characterize the loss of function phenotypes by double-stranded RNA interference (RNAi) and screening for T-DNA insertion lines. We have analyzed 394 primary transformants and 51 of them show defects in cotyledon development. Cotyledons are closely spaced as reflected by the position of cotyledon petiole on the main axis. In severe cases single cotyledon develops on an enlarged petiole. A fraction of the plants, 25/394 showed floral phenotypes. We have observed sepal fusion wherein only two sepals appear to occupy the entire whorl. Sepal-petal and stamen-carpel fusions are also observed resulting in chimeric organs. Another striking phenotype is seen in petals, which appear narrow. The phenotypes indicate its possible function in organ separation. But the variability associated with RNAi phenotypes makes it difficult to analyze these phenotypes at molecular level. Therefore, we have identified three independent T-DNA insertions located within the coding region by screening T-DNA knock-out pools generated by Arabidopsis functional genomics consortium (AFGC), University of Wisconsin, Madison. We are currently analyzing these lines for loss-of-function phenotypes. Phenotypic analysis and the expression pattern by RNA in situ analysis might yield further insights into gene function.

109. A gain-of-function mutation at the TOPIESS locus causes shoot to root transformations in Arabidopsis

Jeff Long

Arabidopsis embryos develop with a distinct apical/basal polarity. Two groups of stem cells, the shoot apical meristem (SAM) and the root apical meristem (RAM), form at opposite poles (the apical and basal poles respectively). The topless-1 (tpl-1) is a single, temperature-sensitive mutation that transforms the shoot pole of the embryo into a second root pole and was originally identified by Dr. Kathryn Barton. Currently, this is the only known Arabidopsis mutation that can cause this type of homeotic transformation.

TPL was cloned using a map-based strategy and was shown to encode an 1131 amino acid protein containing 11 predicted WD40 repeats. These repeats are thought to mediate protein-protein interactions. The first 300 amino acids of TPL show no homology to known motifs, but are conserved within a TPL gene family. There are at least eight other TPL-like genes in Arabidopsis, with four of these genes sharing at least 74% amino acid similarity with TPL.

In situ hybridizations with a TPL gene-specific probe show that it is expressed throughout the early embryo. Later in embryogenesis, expression remains throughout the embryo but becomes stronger in the vasculature. Post-embryonically, TPL expression is found in the vasculature, the SAM, floral meristems and in floral organ primordia.

In an EMS mutagenesis, we isolated three strong suppressors of tpl-1. All three of these suppressors are second site mutations in the TPL gene and are predicted to disrupt gene function. This shows that tpl-1 is a gain-of-function allele. When these intragenic suppressors are made homozygous, they appear to be wild-type indicating that TPL may be redundant. Other TPL family members are good candidates for these redundant genes.

In order to determine the wild-type role of TPL and to uncover any redundancy with other TPL family members, we are taking a three-pronged approach. First, we have isolated T-DNA insertions in seven of the eight TPL-like genes and have created RNAi lines for all of the family members. We are combining different loss-
of-function alleles of these genes and analyzing the resulting lines for phenotypes. Second, we are mutagenizing both the *tpl-1* gain-of-function allele and loss-of-function alleles to look for genetic interactors. Finally, we are expressing both wild-type and mutant forms of *TPL* under the control of different promoters in order to determine if *TPL* has distinct roles in different tissue types. These experiments should lead to a better understanding of *TPL* gene function.

110. Characterization of the *shreddie* mutant affecting floral organ morphology  
Carolyn Ohno, G. Venugopala Reddy

The *Arabidopsis* flower consists of four concentric whorls of organs that develop precisely with distinct organ identity, position, number and size. Two EMS-induced mutant alleles of the *SHREDDIE* (*SHD*) locus have been isolated that appear to affect cell division and growth of floral organs. All floral organs are narrow in the *shd* mutants. The *shd* phenotype most closely resembled that of the *aintegumenta* mutant. Flowers of *ant-9 shd* double mutants display an enhanced phenotype with extremely narrow organs indicating that both loci function in controlling floral organ growth. In contrast *clv3-3 shd* double mutants exhibit an additive phenotype, indicating that these genes act in independent pathways to control cell proliferation in the flowers. Flowers of *ap2-1 shd* double mutants show an enhanced phenotype with loss of lateral sepals and carpelloid medial sepals, suggesting that these genes may repress C function organ identity in the outer whorl. Scanning electron microscope (SEM) analysis of developing flowers will reveal whether cell division and/or expansion are altered in the *shd* mutant flowers. Further genetic and molecular analysis is in progress to understand the function of the *SHD* gene. The *SHD* locus has been mapped to the lower half of chromosome I using CAPS and SSLP PCR markers.

111. Identification of a small gene family, *PAR*, regulated by *PERIANTHIA*  
Chiou-Fen Chuang

We previously showed that *PAN* encodes a bZIP class of transcription factor, suggesting that *PAN* controls floral organ number and carpel development via transcriptional regulation of downstream genes. The identification of genes regulated by *PAN* is a step towards understanding the molecular mechanisms by which *PAN* controls floral organ number and carpel development. One way to search for targets of *PAN* is to compare mRNA populations between cells with differential *PAN* activity. In this study, transgenic *pan-2* mutant plants containing a gene fusion of *PAN* and a rat glucocorticoid receptor steroid-binding domain (*PAN-GR*) were constructed to regulate activity of the *PAN* protein.

We identified a small gene family of four members, designated *PARs* (*PAN-Regulated Genes*) (previous name: *TOTs*), by using differential display to examine messenger RNAs in *pan-2; 35S::PAN-GR* plants. These four closely related *PAR* genes are located on chromosome 5. *PAR2* represents the gene isolated by differential display. *PAR1*, *PAR2*, and *PAR3* are closely linked within a segment of 8 kb, while *PAR4* is about 20 cM away from *PAR1*. All the *PAR* genes except *PAR4* have intronless coding regions ranging from 630 to 657 bp. *PAR4* has a small intron within its coding region of 759 bp. Deduced amino acid sequences encoded by *PAR* genes show similarity to an *Arabidopsis* putative abscisic acid (ABA)-responsive protein (GenBank accession number CAB77770) whose function is still unknown. RT-PCR analysis shows that expression of these genes is up-regulated by *PAN*. In *vitro* DNA-binding assays demonstrated that PAN can directly interact with the *PAR* promoters, suggesting that these genes might be direct targets of PAN. By using dsRNA interference (RNAi) with the *PAR* genes, we also demonstrate that these genes act in the *PAN* pathway to pattern *Arabidopsis* flowers.

112. Analysis of the *SUPERMAN* functions in floral meristem development  
Toshiro Ito

The *superman* (*sup*) mutant has extra whorls of stamens and underdeveloped carpels. The *SUP* gene encodes a C2H2-type zinc-finger protein, which is likely to be a transcription factor. The *SUP* expression is initiated in the adaxial part of the stamen primordia at stage 3 like a cylinder to enclose the whorl 4. In order to investigate the *SUP* function in floral meristem development, an ectopic expression analysis was done. As a 35S promoter::*SUP* construct had a toxic effect in early plant development, we generated an inducible system for *SUP* activity by making a translational fusion between *SUP* and the hormone binding domain of a glucocorticoid receptor (GR). At first, we demonstrated that the *SUP-GR* fusion could replace endogenous *SUP*. Transgenic plants with *SUP-GR* under the *SUP* promoter in the *sup-1* mutant background was rescued in a DEX-dependent manner. Next, by inserting tandem 35S enhancers in the upstream region of the *SUP* promoter, the *SUP-GR* fusion was ectopically expressed in the wild-type flower. The transgenic plants got smaller and the flowers showed a weak *clavata* mutant-like phenotype. The carpel number was increased to four with a very low deviation, in contrast, the number of the sepals, petals and stamens was not changed. Additionally, the ectopic *SUP* showed an activity to change the inflorescence meristem into carpelloid organs. Most surprisingly, by a strong continuous DEX treatment, the flowers were transformed into leaves. These results suggest that the ectopic *SUP* activity can promote the differentiation of inflorescence meristem and floral meristem, and terminate them independently of flower-specific factors. The mutant phenotypes of *sup, sup ag, sup ap3* also support the hypothesis that *SUP* promotes the differentiation of the meristem in whorl 4. The four carpel phenotypes induced by the ectopic *SUP* also can be explained by this *SUP* function as follows. Ectopic *SUP* forces the differentiation of the whorl 4 meristem precociously. As *SUP* has no organ identity function, the primordia start to produce another flower as a default...
condition. By stage 3, AG starts to express and four organ primordia develop to four carpels.

In addition to the previously published model that shows SUP works as a boundary to prevent the proliferation of the whorl 3, we propose that SUP has an additional activity that promotes the differentiation of the meristem in the center. Some more experiments to test this hypothesis are under way.

113. Analysis of the non-cell-autonomous function of the Superman gene
Toshiro Ito

The Superman (SUP) gene has a function to prevent the proliferation from the outer whorls and at the same time, to promote the differentiation of the meristem in the center of a developing flower. The SUP is also involved in the growth of the outer integument in ovule development. The SUP gene starts to express at the adaxial part of the stamen primordia at late stage 3. No SUP expression is detected in the carpel primordia at the mRNA level. At stage 9, SUP starts to express in the developing ovary, first on the inner surface of the carpels, and later in the funiculus of ovules, but it is not expressed in the integuments. In order to explain the non-cell autonomous function of SUP, there are two possible explanations. (1) The SUP protein moves into the nearby cells. (2) The downstream factors of SUP move into the nearby cells. To reveal the molecular mechanism of the non-cell autonomous function of SUP in the floral meristem development, we started to test if SUP protein moves into nearby cells.

As the first experiment, a fusion protein between the SUP protein and the dipheria toxin (DT-A) protein were driven by the SUP promoter in order to check the effects of killing SUP-expressing cells. Out of 31 T1 transformants, three plants showed wild-type phenotypes, 19 plants showed a mild phenotype with fused stamens. In the mild lines, the stamens were fused to the carpels and some of the stamens were fused each other. The remaining nine plants showed a severe phenotype with no stamens. In the most extreme case, no carpels were produced. These results suggest a possibility that SUP protein itself moves into the nearby cells. In order to check more directly if SUP protein moves into nearby cells, we expressed a fusion protein between the SUP and a green fluorescent protein (GFP) under the control of the SUP promoter. The transgenic plant with pSUP::SUP-GFP rescued the SUP mutant phenotype. We are now preparing to observe the GFP signal by using confocal microscopy, to compare the expression pattern of fusion protein with that of the mRNA.

114. Searching for the Superman target genes by microarray experiments
Toshiro Ito, Jose-Luis Riechmann, Frank Wellmer, Marcio Alves Ferreira

The Superman (SUP) gene, which encodes a C2H2-type zinc-finger protein, has a determinacy function in the center of the floral meristem. SUP expression is initiated in the adaxial part of the stamen primordia at stage 3, the expression domain forming like a cylinder to enclose whorl 4. In order to reveal the molecular nature of the downstream activities that SUP induces in floral meristem development, we are using flower cDNA microarrays. We produced an inducible system of SUP activity by making a fusion between SUP and the hormone binding domain of a glucocorticoid receptor. The ectopic SUP activity promotes the differentiation of the meristem and terminates meristem activity. In order to get larger amounts of material, and enhanced effects of the transgene, we introduced the 35S::SUP-GR construct into a severe clavata mutant, clv3-2. The 35S::SUP-GR clv3-2 mutant flowers showed extra whorls of carpelloid organs in the center upon DEX induction. A time course experiment after induction of SUP activity has been performed using this material. Inflorescences from 35S::SUP-GR clv3-2 mutant plants were collected at 0, 4, 8, 12 and 24 hours after mock- or DEX-treatment, and the RNA was isolated from each time sample.

The RNA sample were used to prepare Cy3- and Cy5-labeled probes for cDNA microarray hybridization, allowing us to compare the RNA expression profiles of mock and DEX-treated plants at the each time point. Once candidates target genes are identified, we will determine if they are induced in the presence of cycloheximide, an inhibitor of protein synthesis, in order to identify genes that are directly regulated by the SUP protein. We are also planning to use transgenic plants with SUP promoter::SUP-GR in the sup-1 mutant background to see if the target candidates are controlled in the developmental context of endogenous SUP in the floral meristem.

115. Identification of argonaute1 enhancers in Arabidopsis
Eva C. Ziegelhofer, Jennifer C. Fletcher*

A number of genes involved in the silencing of transgene DNA have been identified in fungal, animal, and plant systems. Transgene silencing phenomena in these experimental systems have been labeled as quelling, RNA interference (RNAi), and post-transcriptional gene silencing (PTGS), respectively. Homologous genes involved in silencing from these different classifications of organisms have been identified, suggesting that these processes may share mechanistic similarities. One such class of homologous genes is comprised of QDE-2 from Neurospora crassa, RDE-1 from Caenorhabditis elegans, and ARGONAUTE1 (AGO1) from Arabidopsis thaliana. Mutations in these genes result in loss of transgene silencing, but ago1 mutants have additional phenotypes implicating a role for AGO1 during development. AGO1 is a member of a family of Arabidopsis genes comprised of nine members. Genetic analysis of single and double ago1 mutants suggest developmental roles for AGO1 in embryonic and postembryonic growth, polarity, and patterning. All single ago1 mutants reported in the literature have similar pleiotropic phenotypes, resulting in sterility (Bohmert et al., 1998; Lynn et al., 1999; Fagard et al., 2000). We have identified a weak ago1 mutant that is
fertile and, despite exhibiting numerous phenotypes, results in a dramatically different appearance than plants of the strong alleles. Using this weak allele, both chemical (EMS) and insertional (T-DNA) mutagenesis schemes have been undertaken to genetically screen for enhancers and suppressors. Several T-DNA enhancers which resemble the strong ago1 alleles to varying degrees are currently being characterized. The T-DNA insertion scheme allows for rapid molecular identification of tagged mutants using TAIL-PCR. Thus, other genes important for AGO1-related processes, including both silencing and normal development, should be readily identifiable by our genetic screen. Hopefully the genes we identify will include both AGO family members and unrelated genes, resulting in a better understanding of the mechanisms involved in silencing and plant development.

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References

116. Intercellular trafficking of nuclear factors in Arabidopsis thaliana
Elizabeth Haswell

Several recent lines of evidence demonstrate that nuclear factors are capable of moving in a directional and controlled fashion from cell to cell in plant meristems (1-4). The intercellular trafficking of nuclear proteins is an unsuspected and completely novel mechanism for cell-cell communication in plants, and may impart positional information and developmental cues. However, our understanding of the function and potential regulation of this process awaits the answers to more basic questions. At present, we do not know what proportion of plant proteins possess the ability to move from cell to cell, what processes these putative proteins may act in, nor have we identified the cellular and molecular nature of the movement pathway or pathways. Our research is directed towards the clarification of these issues.

We are executing a genetic screen to identify endogenous plant proteins that move, using a cDNA-GFP fusion library expressed under the control of a tissue-specific promoter. Transformed plants will be visually screened for movement of GFP signal to nearby cells. By identifying a pool of proteins capable of cell-to-cell trafficking, we hope to: 1) determine if intercellular movement is a common or a specialized function of plant nuclear proteins; 2) identify a domain or signal sequence capable of conferring intercellular movement; 3) gain insight into the number and variety of developmental pathways that may involve intercellular trafficking; and 4) provide tools for the subsequent identification of the cellular pathway or pathways for movement.

These studies will provide the first genetic and cellular description of the process by which transcription factors move from cell to cell within the plant meristem. A clear picture of the function and mechanism of this phenomenon is critical to our understanding of shoot apical meristem function and development.

References

117. Study of cell and tissue differentiation during Arabidopsis stamen development
Alves-Ferreira, M., Wellmer, F., Riechmann, J.L

Male reproductive processes in flowering plants take place in stamens. This sporophytic organ system contains diploid cells that undergo meiosis and produce haploid male spores or microspores. The stamens represent an outstanding system to study the differentiation of plant cell types. However, much remains to be learned about the mechanisms that are responsible for anther cell specification and those required for activation of cell-specific gene sets within specialized anther cells and tissues.

To understand the processes during Arabidopsis stamen development, we have chosen two different experiments: cDNA microarray analysis and the study of shaggy/glycogen synthase kinase-3 (SGG/GSK-3) plant kinase homologues.

DNA microarrays representing approximately 8000 Arabidopsis cDNA clones are being screened with probes corresponding to mRNAs from an ap3 loss-of-function mutant. Since our goal is to identify genes involved in early steps of anther differentiation, we divided Arabidopsis flower development in two stages according to bud size and pollen development. Preliminary results suggest that this strategy can be extremely valuable to generate a gene expression profile during anther development.

In several organisms SGG/GSK-3 homologues are involved in signal transduction pathways that establish cell fate and/or pattern formation. The high similarity of the homologous plant proteins to SGG/GSK-3 and preliminary data suggest their possible implication in these processes during plant development. In order to get a picture of the function of this family of genes during anther development, their expression patterns were studied by RT-PCR and in situ hybridization. This analysis showed that four members of the shaggy family are expressed in flowers and in situ hybridization analysis using gene specific probes demonstrated that two of them are expressed specifically in the flower meristem (SGG-Theta and Dzeta). In addition, the expression of SGG kinase Theta was also observed in stamen primordia. At the present time, knockout lines have been screened to access additional information concerning the role of SGG.
kinases in stamen development.

118. **The role of auxin in positioning plant organ primordia**

*Marcus Heisler*

The positioning of organ primordia is a fundamental patterning process that occurs during plant and animal development. In higher plants, organ primordia arise in a continuous predictable arrangement, or phyllotaxis, on the shoot apical meristem (Lyndon, 1990). Auxin, predominantly in the form of indole-3-acetic acid (IAA), is an intercellular signaling molecule that is actively transported in plant tissues in a polar manner (Davies, 1995).

Interestingly, when auxin transport is inhibited, either chemically or in *Arabidopsis* pin1 mutants, formation of flower primordia is suppressed (Okada et al., 1991). In contrast, flower or leaf primordial growth is induced near the site of exogenous auxin application (Snow and Snow, 1937; Reinhardt et al., 2000). These results suggest that endogenous auxin promotes primordial growth and that its distribution directly determines the site of primordium initiation.

To investigate the role of endogenous auxin in primordium positioning, a number of experimental strategies are planned. Firstly, we will try and determine the distribution of auxin within the meristem immuno-histochemically and by using an auxin-responsive reporter construct. The transport pathways that give rise to this distribution will also be examined by determining the subcellular location and expression patterns of auxin transport proteins such as PIN1 (Galweiler et al., 1998). Next, the causal link between auxin and primordium positioning will be tested by first co-localizing auxin and the activity of primordium marker genes using distinct fluorescent proteins and multi-photon microscopy. The endogenous auxin distribution will then be altered by manipulating the expression of auxin efflux carriers. A continuing correspondence between high auxin concentrations and early primordium marker gene expression patterns would confirm the hypothesis that endogenous auxin directly determines where primordia arise. Lastly, to identify genes that function in the primordium growth pathway triggered by auxin, RNA from apices treated with auxin will be compared to that of untreated apices using a flower specific cDNA micro-array.

**References**


**Publications**


The T-cell developmental program uses regulatory molecules recursively at successive developmental choice points to control different decisions. Thus another general interest of the group is how the mechanisms that normally prevent systemic autoimmunity: the autoimmune-diabetic NOD mouse strain has developing T cells that fail to respect this checkpoint. The genetic loci responsible for this failure are now being mapped to test their relationship to known diabetes susceptibility loci. These diverse approaches give us insights into the mechanisms that divide T-cell development into discrete phases.

A major question we are addressing is how specific gene isoforms can control the TCR αβ lineage at all; and later, T-cell receptor-dependent events, which determine whether or not particular immature T-lineage cells express antigen receptors that will be useful to the organism. Most current work in the Rothenberg group focuses on the early phase. The cells that undergo the events leading to T-lineage commitment are pluripotent hematopoietic precursors that can initially generate natural killer lymphocytes and dendritic cells as well as T cells, and possibly B cells as well. These alternative developmental potentials are lost as T-cell genes are increasingly turned on and non-T genes, which are initially expressed in the precursors, become silenced. A major question we are addressing is how specific gene expression changes result in T-lineage commitment.

The T-cell developmental program uses regulatory molecules recursively at successive developmental choice points to control different decisions. Thus another general interest of the group is how the mechanisms that normally prevent systemic autoimmunity: the autoimmune-diabetic NOD mouse strain has developing T cells that fail to respect this checkpoint. The genetic loci responsible for this failure are now being mapped to test their relationship to known diabetes susceptibility loci. These diverse approaches give us insights into the mechanisms that divide T-cell development into discrete phases.

The developmental program that gives rise to T lymphocytes is evolutionarily interesting because it is a
specific vertebrate innovation; most animals in the world do not have it. Lymphocytes are only found in vertebrates, and their molecular hallmark, the specialized receptor genes, are only found in jawed vertebrates. Clues to the origin of lymphocytes may be found in the lower vertebrates and in the nonvertebrate deuterostomes, which are the closest sister groups to vertebrates. Lymphocyte production must have become possible either through the appropriation of new roles by pre-existing transcription factors or through the generation of "new" transcription factors, for example by gene duplication and divergence and/or by exon shuffling. To explore where this developmental program came from, therefore, we are studying the evolutionary history of some major transcription factors that control early lymphocyte development. In the past two years, the laboratory has cloned some of the important regulators of lymphoid gene expression from cartilaginous fish and lampreys, and characterized them to determine what structural features and expression sites of these transcription factors are conserved between basal vertebrates and mammals. The results show differences in gene number, structure, and sites of utilization with a strikingly distinct break between the genes in cartilaginous fish and those in lampreys.

119. The transcription factor Runx1 specifically silences CD4 expression in immature CD4+CD8+ thymocytes

Janice Telfer

The transcription factor Runx1 (CBFα2, AML1, PEBP2αB), a member of the Runt family, is highly expressed in thymocytes. It is expressed in a complex array of isoforms that include or exclude different protein interaction domains and include more or less of the DNA binding domain itself. We examined the effect of over-expression of specific Runx1 isoforms in fetal thymic organ culture, in which thymic development is simulated in vitro. In vivo and in fetal thymic organ culture, CD4-CD8- thymocytes develop into CD4+CD8+ double-positive thymocytes, at which point potentially autoreactive thymocytes are deleted or negatively selected. Thymocytes expressing T-cell receptors having the proper level of affinity for class I or class II MHC are positively selected and allowed to develop further into CD8+ single-positive cytotoxic T cells or CD4+ single-positive helper T cells, respectively. Silencing of either CD4 or CD8 in double-positive thymocytes is directly related to the function of the resulting mature T cells and occurs through a mechanism that is not entirely understood.

Thymic lobes in which the donor thymocytes were transduced with full-length Runx1 had 10 times fewer mature CD4+ single-positive thymocytes and 50% fewer immature CD4+CD8+ double-positive thymocytes than controls. In order to examine the cell-autonomous effects of Runx1 expression on thymocytes, separate from the positive or negative selection that is seen in normal thymic development and in fetal thymic organ culture, retroviral vectors encoding Runx1 isoforms and mutants were also transduced into Bcl-2 transgenic CD4-CD8- thymocytes (DN1-DN4), which have a delay in death in in vitro suspension culture compared to normal thymocytes. In suspension cultures, such thymocytes survive and normally upregulate CD4, CD8, and TcRβ expression within 16 hours. Over-expression of Runx1 specifically silences CD4 expression in the CD4+CD8+ double-positive immature thymocyte compartment. The increase in yield of apparent CD8+ single-positive thymocytes resulting from Runx1 overexpression can occur without an increase in positive selection of CD8+ thymocytes or negative selection of CD4+ thymocytes, since there is no interaction between the T-cell receptor complex and either class I or class II MHC in Bcl-2 transgenic thymocyte cultures. The lack of signal through the TcR is confirmed by the lack of CD69 expression on the cultured Bcl-2+ thymocytes. CD69 expression is upregulated when cells are positively selected via signals induced by the interaction of the T-cell receptor complex with class I or class II MHC on thymic stromal cells. Heat-stable antigen (HSA, CD24) is normally downregulated as thymocytes mature to CD4+ or CD8+ single-positive thymocytes, just prior to emigration from the thymus as mature T cells. Apparent CD8+ single-positive thymocytes resulting from overexpression of full-length Runx1 do not downregulate HSA, indicating that they have not gone through the maturation process and are therefore, immature double-positive thymocytes with down-regulated CD4.

CD4 is known to be regulated by different enhancer and silencer elements in mature and immature T cells. Runx1 overexpression does not shut off CD4 expression in mature T cells isolated from the spleen, suggesting that Runx1 acts specifically on the CD4 silencer in conjunction with the immature cell ("double-positive") enhancer. This effect is localized to the C-terminal part of the molecule downstream of the DNA-binding Runt domain, as naturally occurring Runx1 isoforms truncated in the Runt domain and at the end of the Runt domain do not silence CD4 expression. However, the silencing effect is independent of the binding of the co-repressor Groucho, which normally binds to the last five C-terminal amino acids of Runx1.

120. The transcription factor Runx1 promotes the development of αβ T cells in a Groucho dependent manner

Janice Telfer

An early split in T-cell lineage commitment in the thymus occurs between αβ T cells and γδ T cells. αβ T cells develop into CD4+ helper T cells and CD8+ cytotoxic T cells. αβ T-cell receptors recognize an incredible diversity of polypeptide antigens in the context of class I or class II MHC; γδ T cells are a keystone of the mammalian immune system. In contrast, γδ T cells play a much more specialized role in mucosal immunity and immune surveillance in the skin. The regulation of the production of αβ T cells versus γδ T cells in the thymus is unclear.
We examined the role that the transcription factor Runx1 played in the development of αβ T cells versus γδ T cells in fetal thymic organ culture. To gain access to this early choice point, the cells assayed for responses to Runx1 overexpression were fetal liver cells enriched for hematopoietic stem cells and lymphoid precursors. Donor cells were infected with retroviral vectors encoding Runx1 isoforms and mutants. Overexpression of full-length Runx1 in the donor cells results in an increase in the ratio of αβ T cells to γδ T cells. This effect is dependent on the presence of the last five C-terminal amino acids of Runx1, which are necessary for interaction of Runx1 with corepressor Groucho. Overexpression of forms lacking just these five amino acids results in a decrease of αβ T-cell production relative to γδ-T cell production, similar to the effects of overexpression of severely truncated forms lacking all C-terminal domains including all known transactivation domains. Thus different isoforms of Runx1 can modulate the αβ:γδ ratio, with αβ production possibly depending on the ability of Runx1 to act as a repressor.

121. The role of GATA-3 in T-cell development

Gabriela Hernández-Hoyos, Michele K. Anderson

GATA-3 is a transcription factor that is expressed in immature and mature T cells. Studies performed in peripheral CD4 T cells showed that GATA-3 is sufficient and necessary to induce their differentiation into Th2 cells. Targeted deletion of this gene in mice leads to an early block in T-cell development, which demonstrates that GATA-3 is essential for all T-cell development. Unfortunately this early developmental block has precluded analysis of the role of GATA-3 at later stages of thymic differentiation. To study the role of GATA-3 during T-cell development, we analyzed the effects of GATA-3 overexpression in T-cell precursors at three stages of development: pre-thymic precursors (E14.5 fetal liver), double-negative thymocytes prior to β-selection (E14.5 thymocytes), and double-positive thymocytes prior to positive selection (E16.5 MHC-/- fetal thymocytes). We used retroviral transduction of fetal cells and fetal thymic organ culture for our studies. Our results indicate separate effects at each stage. GATA-3 overexpression blocks the generation of Thy-1+ CD25+ pro-T cells, inhibits expansion following β-selection, and promotes mature CD4 cell generation while inhibiting mature CD8 cell generation. This accords well with evidence that mature CD4 thymocytes express five times more GATA-3 than mature CD8 thymocytes. Inhibition of CD8 T-cell generation occurs in wild-type and in Rag2-/- thymocytes transgenic for a class-I restricted TCR (LCMV). Thus, GATA-3 appears to inhibit CD8 development and promote CD4 development, but is not sufficient to induce CD4 development of class-I restricted thymocytes. Using a GATA-3 construct with a mutation in a transactivation domain, we have determined that the inhibition after β-selection, but not the other effects, depends on the transactivation activity of GATA-3. We are now interested in studying the differential regulation of GATA-3 expression in CD4+ vs. CD8+ cells following positive selection and in the nature of the target genes that mediate the distinct GATA-3 effects.

122. Constitutive expression of PU.1 in fetal hematopoietic progenitors blocks T-cell development at the pro-T stage

Michele K. Anderson, Angela Weiss

PU.1 is a powerful transcription factor that is known to be essential for the development of macrophages, granulocytes, B cells, and fetal T cells. It is expressed at the earliest stages of thymocyte precursor development but is sharply downregulated just during the stages when T-cell lineage commitment occurs.

In order to investigate if this downregulation is functionally important for T-cell development to occur we used bicistronic retroviral vectors expressing PU.1/GFP or GFP alone to infect fetal liver-derived hematopoietic precursors. PU.1/GFP+ or GFP+ cells were sorted and allowed to differentiate in fetal thymic organ cultures (FTOC). We could show that PU.1 overexpression leads to an overall diminution in lymphoid precursor numbers in the FTOC system, indicating that high levels of PU.1 can inhibit development very early. Transduced T-cell precursors that express PU.1 at a modest level can progress through the early stages of T-cell development. This continues until they reach the pro-T-cell stage where they are blocked from further differentiation. This is at the same stage when normally developing T cells shut off PU.1 expression and undergo T-cell lineage commitment. If cells are forced to further express PU.1 in a microenvironment that promotes T-cell development, the cells are eliminated.

We also investigated if PU.1 expression interferes with B-cell or macrophage differentiation. If PU.1 transduced precursor cells are allowed to develop under conditions that also promote B-cell development, only GFP-transduced but not PU.1/GFP-transduced cells survive and develop into B cells, indicating that downregulation of PU.1 is also essential for B-cell development. Under culture conditions permissive for macrophage development, however, we not only see good cell survival but also differentiation along the myeloid/macrophage pathway of PU.1 overexpressing precursor cells. PU.1 overexpressing cells can even be rescued after five days of inhibited growth in fetal thymic organ culture if they are switched to a macrophage permissive environment that promotes T-cell development, the cells are eliminated.

We also investigated if PU.1 expression interferes with B-cell or macrophage differentiation. If PU.1 transduced precursor cells are allowed to develop under conditions that also promote B-cell development, only GFP-transduced but not PU.1/GFP-transduced cells survive and develop into B cells, indicating that downregulation of PU.1 is also essential for B-cell development. Under culture conditions permissive for macrophage development, however, we not only see good cell survival but also differentiation along the myeloid/macrophage pathway of PU.1 overexpressing precursor cells. PU.1 overexpressing cells can even be rescued after five days of inhibited growth in fetal thymic organ culture if they are switched to a macrophage permissive environment that promotes T-cell development, the cells are eliminated.

PU.1 consists of many different domains. In order to investigate which of these domains is essential for the effects on developing T cells we created a number of different mutants to test in our system. In one of these mutants the DNA binding ability is abolished by introducing a single amino acid change. When this mutated form of PU.1 is introduced into our FTOC system, overexpressing cells are not only able to survive in equal numbers to control cells but also to differentiate as well as untransduced cells. This indicates that the integrity of the
DNA binding domain is important for PU.1 to exert its effects.

123. Identification of PU.1 target genes in T-cell precursors
Michele K. Anderson

The transcription factor PU.1 is expressed in early thymic precursors (DN1, DN2) and downregulated the time of T-cell lineage commitment (DN3). Studies on PU.1−/− mice indicate that this factor is needed for efficient production of early T-cell precursors, although it is dispensable for later T-cell maturation and function. Retroviral expression of PU.1 in T-cell precursors, extending through the point at which it is normally downregulated, results in a block in T-cell development at the DN3 stage. Hematopoietic precursors expressing retroviral PU.1 are also at a growth disadvantage, leading to greatly decreased cell number. These effects are not produced by retroviral expression of a PU.1 mutant that cannot bind DNA, suggesting that PU.1 acts directly on target genes that mediate these effects. We are using the SCID.adh cell line, which roughly represents the DN3 stage of T-cell development, to investigate the nature of the genes regulated by PU.1 in early thymocytes. The SCID.adh cells, like normal DN3 cells, do not express PU.1. These cells can be infected with PU.1-encoding retroviruses, sorted, and cultured. Retroviral transduction of SCID.adh cells with PU.1 results in a growth disadvantage in the presence of non-transduced cells. In addition, a certain percentage of the sorted PU.1-expressing SCID.adh cells stably express the myeloid marker Mac-1 in addition to the T-lineage markers Thy-1 and CD25. Quantitative PCR analysis of specific transcript levels in SCID.adh cells expressing PU.1 indicate that certain genes essential for T-cell function, such as Rag-1, Rag-2, and preTα, are downregulated. These genes are downregulated to a greater degree in the subset of PU.1+ cells that are also expressing Mac-1. Furthermore, in fetal thymic precursors expressing PU.1, flow cytometry indicates that a percentage of the Thy-1+ cells upregulate macrophage colony stimulating factor receptor. Therefore, it appears that the ectopic expression of PU.1 in DN3 cells can lead not only to interference with essential early T-cell factors, but also to the activation of a competing myeloid gene expression program.

124. Differentially expressed transcription factors in early murine thymocyte development
Elizabeth-Sharon D. David

Early developing T cells in adults, as with other developing cell types, follow a sequence of molecular changes in time. Haematopoietic stem cells (HSCs) initially migrate from the bone marrow to the thymus where they continue to change and divide until they are committed to the T-cell lineage. It is this process of T-lineage commitment that we are investigating.

All developing cells are subject to changes via two mechanisms - cell-cell signaling, and autonomous changes that are fostered ultimately by the regulated expression of different transcription factors within the cell. While we and others have begun to work on transcription factors that are required for T-cell development, there is an important gap in current knowledge of the system. None of the known transcription factors appears to account for the initiation of development along the T lineage or for induction of T-lineage commitment. Therefore, we are trying to establish a more complete set of the regulators involved in early T-cell development. Our aim is to identify various transcription factors that are upregulated during thymocyte commitment and which, individually or in concert, are responsible for the early autonomous changes evident in T-cell development. The early stages of T-cell development that we study are collectively classified as the double-negative (DN) compartment. These cells are double negative with respect to the two cell-surface proteins CD4 and CD8 and can be divided into four sub-groups: DN1-DN4, where DN1 is composed of resting and dividing cells that are CD44+, CD25−. DN2 cells are actively dividing and are CD44+, 25+; DN3 is the stage during which β-selection occurs and the cells become CD44−, CD25+. Later, by DN4, they are dividing cells that are CD44−, CD25-.

Our strategy is to screen an arrayed cDNA library for genes encoding known transcription factor family motifs, compare expression patterns of these sequences during the different stages of development, and to focus our studies on those with a regulated pattern of expression. Certain cell lines have been chosen to represent the different stages of development that are being investigated. The FDCP, EML-C1 and BI3a lines are stem cell-like lines. The SCID.adh cell line represents the DN3a population that is arrested just prior to undergoing β-selection. Thymocytes from two immunodeficient mouse models are also being used to collect cells from appropriate stages of development. SCID mouse thymocytes are mostly at the DN2 stage, while thymocytes of Rag2−/− mice are enriched for the DN3 fraction (both pre-β-selection). Complex RNA probes from the cell lines and thymocytes of the mouse models have been used to screen DNA sub-arrays consisting of candidate transcription factor clones from a complete macro-arrayed SCID thymus cDNA library. In addition, new cDNA sub-arrays including a larger number of transcription factors have just been prepared. By using subtracted probes, we expect to enhance the sensitivity with which we can detect transcription factor genes that are developmentally regulated and thus candidates for roles in T-cell commitment.

Preliminary results suggest that some novel zinc-finger transcription factors are specifically upregulated during thymocyte commitment. Experiments to test whether these changes are significant are underway. Expression patterns will be confirmed by real time PCR on cDNA from cell-sorted populations of thymocytes. The actual functional relevance of these transcription factors will be tested by overexpression and dominant negative studies in the foetal thymic organ culture (FTOC) system.
125. Dose-dependent requirement for Fos family transcription factors in early T-cell development

Fei Chen

Fos family transcription factors, which are components of AP-1 heterodimers, are subject to developmental regulation during thymocyte differentiation. To determine the importance of Fos gene activity for T-cell development, we investigated the impact of homozygous or heterozygous disruption of the two Fos family genes, c-fos and fosB. Homozygous fosB knockout mice are viable and apparently normal, whereas homozygous c-fos knockout mice are deformed by osteoporosis with consequent extramedullary hematopoiesis and failure of tooth eruption. In spite of these apparent differences, both single-gene knockout genotypes support essentially normal T-cell development through the first 6 weeks of life. This could be due to functional redundancy of Fos family genes in developing T cells, or it could mean that Fos family transcription factors have no significant role. To distinguish these possibilities, we crossed the c-fos and fosB mutants to obtain mixed heterozygotes and mice in which three or four of the alleles at these two loci were mutant. Surprisingly, occasional c-fos<sup>-/-</sup> fosB<sup>-/-</sup> double homozygotes are viable from birth through weaning in spite of a severely runted appearance. Mice in which both c-fos alleles and at least one fosB allele are mutated show a reproducible and unique block in T-cell development.

When these mice are analyzed at 3-4 weeks of age, the number of immature CD4+ CD8+ (DP) thymocytes is sharply reduced, although the populations of mature CD4+ or CD8+ single-positive thymocytes remain substantial, indicating that at least one wave of complete thymocyte differentiation can be completed. The most distinctive phenotype is the distortion of subpopulations in the earlier immature CD4- CD8- (DN) subset. Cells with the CD25+ CD44- or CD25- CD44- phenotypes (DN3 or DN4) are greatly reduced, while the more primitive CD25+ CD44+ (DN2) cells are spared, and the most immature CD25- CD44+ (DN1) cells are dramatically enriched to 70-85% of all DN cells. This skewing is not seen in mutants of either c-fos or fosB alone. This phenotype is indicative of a powerful quantitative block in developmental progression around the DN1→DN2 transition and/or in the expansion of DN2 cells to generate DN3 progeny, critical stages in which T-lineage specification and commitment are taking place. Cells with four mutant alleles are more severely perturbed than cells with only three mutant alleles. The presence of CD4 and CD8 single-positive cells in normal ratios in these thymic populations nevertheless shows that none of the conditions required for positive selection per se is disrupted, i.e., the cells can rearrange and express T-cell receptor genes correctly and the microenvironment can shepherd them through to maturation. Therefore the main stage at which Fos family genes are required appears to be the early transition from thymic immigrant to early T-lineage precursor.

126. Thymocyte development in diabetes-prone NOD mice

Mary Yui, Nkechi Nzerem

Type I diabetes is a polygenic autoimmune disease in which insulin-producing pancreatic cells are destroyed in a process mediated by T lymphocytes. The non-obese diabetic (NOD) mouse is a model whose disease closely mimics human diabetes. A number of defects, which may contribute to autoimmunity, have been reported to occur in peripheral immune cells from these mice, including T, B, NK and dendritic cells, as well as macrophages. We are investigating whether any immunological defects can be detected early in the development of these cells. Mice unable to complete T-and B-cell receptor rearrangements, e.g., those with the scid/scid mutation or with an ablation of one of the Rag genes, are particularly well-suited for studying the earliest developmental stages of lymphocytes as well as non-lymphocyte populations. Normal mouse strains with these mutations accumulate specific precursor T-cell populations in the thymus because the cells are incapable of proceeding through the T cell "β-selection" checkpoint without a fully rearranged T cell receptor β chain. These mice are therefore an excellent source of thymic cells undergoing developmental choices to give rise to T, NK or dendritic cells.

The reported defective NK-cell activity in NOD mice prompted us to look for possible alterations in developing NK cells in the NOD-scid/scid mice. No inhibition of intrathymic NK-cell production was seen, demonstrating that the defect is due to function rather than production of these cells. Some alterations in cell-surface makers were noted, which may reflect their defective function. In addition, we have found that NOD- scid/scid and NOD-Rag<sup>1</sup>- mice do not encounter a block at the same T cell developmental stage as other immunologically normal mice which have the scid mutation or are Rag deficient. Immunodeficient NOD thymocytes proceed through the β-selection checkpoint to become CD4 and CD8 double-positive thymocytes even in the absence of a T-cell receptor β chain. This observation is especially intriguing because mature T-cell receptor-positive cells from NOD mice are known to be resistant to some forms of cell death and this defect has been postulated to be involved in the generation of autoimmunity. These results indicate that resistance to cell death may be present at the very earliest stages of T-cell development, possibly during lineage commitment or even earlier in hematopoiesis. We have found that this trait is recessive in an F1 cross with C3H/HeJ-scid/scid mice. F1 mice were then backcrossed to NOD-scid/scid mice and the offspring phenotyped. We are currently mapping the gene(s) involved in this trait using simple sequence repeat polymorphisms. We are also characterizing the cells that are proceeding through the β-selection checkpoint and plan...
to manipulate their development in vitro and in vivo to determine which pathways are defective.

127. A strategy for conditional rescue of developmentally arrested T-cell precursors

Chris Dionne

In the past number of years the Rothenberg group has been examining the early stages of T-cell commitment in the thymus. Cells enter the thymus in an uncommitted state, retaining the ability to give rise to B cells, NK cells, dendritic cells, or T cells. Developing T cells progressively shut off the ability to differentiate into other types until they are T committed and begin receptor rearrangement. To define the early stages of T-cell development, investigators have traditionally used lack of CD4 and CD8 expression to identify immature cells, populations that constitute 5% of a normal mouse thymus, and separated these cells into populations based on varying expression of the cell surface markers CD25 and CD44. Recently the Rothenberg group has examined the gene expression in these early populations and separated them into more precise subpopulations based on expression of various surface markers such as HSA, Sca-1, c-Kit, IL7Rα, and Thy-1. These populations had specific transcription factor and lineage-specific gene patterns that show the traditional early populations as being heterogeneous and suggest a more complex developmental relationship then previously thought. Testing the T-cell potential of these populations has not been possible, however. In the normal adult thymus these subpopulations can be less than 0.02% of the total cell number, and separating them without contamination becomes impossible. To remedy this, mice lacking the Recombination Activating Gene Rag2, necessary for DNA rearrangement and T-cell development, were used. In these mice, T-cell development is blocked just after commitment allowing access to the early subpopulations, but the potential to develop into T cells cannot be assayed. My project involves creating a retroviral construct that expresses Rag2 and could be introduced into Rag2-/- thymocytes and used to examine directly the T-cell developmental potential of these populations.

Currently I have inserted the Rag2 gene into the MSCV vector in tandem with the marker Green Fluorescent Protein (GFP). In an attempt to show functional DNA rearrangement, Rag2-/- stem-cell precursors with the potential to give rise to B cells (which are also blocked in Rag2-/- mice) were infected with the Rag2 retrovirus and cultured on a bone marrow stromal cell line that gives rise to B cells. The Rag2-infected cells showed a lower GFP levels (which corresponds to Rag2 levels) than controls and did not develop into B cells. There are a number of explanations for this. One is that there is a problem with the retroviral construct, another is that there are also some unresolved issues with the stromal cell culture, and finally the Rag2 gene may be detrimental to the cell. Rag2 is usually very tightly regulated and constant expression in cells could cause cell death. If Rag2 is detrimental one possibility is to use an inducible construct that can be shut off, while using cells that have an increased resistance to death is another possibility. Once the system is functioning the developmental potential of the early cell stages can be assayed and the vector can be modified to include another gene in tandem and be used to examine the role of various transcription factors in T development from these stages.

128. A new DNase hypersensitive regulatory region of the murine IL-2 locus that confers position-independent transgene expression

Mary Yui, Gabriela Hernández-Hoyos

IL-2 is an immunoregulatory cytokine which is first transcribed early in T-cell development and is then maintained in a transcriptionally silent state until induction by specific activating signals. The early activation of IL-2 expression in the most immature thymocyte precursors implies that the regulatory sequences of IL-2 are targets for the transcription factors specifically operating during this important state of development. Thus finding the enhancer elements responsible for this expression could provide access to novel factors acting prior to T-lineage commitment. However, although the well-characterized proximal promoter/enhancer of the IL-2 gene is sufficient for tissue-specific, inducible reporter gene expression in transfection studies, this region alone cannot drive consistent transgene expression in vivo, indicating that cis-acting chromatin elements, such as locus control regions, are missing. As described previously, thirteen potential sites of DNA/protein interactions were found in T cells using DNase hypersensitive assays. Inclusion of most of these upstream HS sites in an 8.4 kb IL2 promoter-GFP construct resulted in 10/11 of the mouse lines expressing the transgene in a tissue specific and inducible manner, whereas only 4/12 lines using the 2 kb proximal promoter sequence expressed the transgene at a similar level. Using quantitative real time fluorescent RT-PCR we showed that low copy number transgenic lines expressed GFP mRNA at levels similar to that of endogenous IL-2 in stimulated T cells. The few transgene-expressing 2 kb lines expressed GFP in the same cell types and with comparable inducibility as the lines utilizing the longer construct. Thus, the locus control elements allowing efficient integration site-independent transgene expression in T cells appear to map within the 6.4 kb of upstream sequence between -2 and -8.4 kb, while most of the elements required for tissue specificity and inducibility appear to be contained within the proximal 2 kb construct. An additional phenotype maps to the 6.4 kb sequence between -2 and -8.4 kb. Among 3/4 transgene-expressing 2 kb promoter lines, a higher percentage of thymic and splenic CD8 cells (relative to CD4 cells) express GFP while the 8.4 kb lines express equally well in both cell types. This is the first evidence that IL-2 regulation in these two T-cell subsets may be mediated by independent cis-elements.
129. **Differential IL-2 expression in T-cell subsets**

Mary Yui

Using the previously described IL2 promoter-GFP transgenic mouse lines, we found that GFP is inducibly expressed in all T-cell subsets known to express IL-2, including $\gamma\delta$-T, $\gamma\delta$-T, and $\alpha\beta$-NKT cells. Although we demonstrated that the transgene contains regulatory elements that allow expression in all of the correct subsets, we found that the transgene might be differentially expressed in these different subsets. We noted that $\gamma\delta$-T cells and NKT cells expressed particularly high levels of GFP even without exogenous stimulation. Upon stimulation of GFP-negative cells with antibodies to the CD3$\varepsilon$ molecule of the T-cell receptor and the costimulatory molecule, CD28, IL2 transcripts were found to still be relatively more abundant in $\alpha\beta$- than $\gamma\delta$-T cells in transgenic and normal mouse cells, while for GFP, the reverse is true. This result suggests that there are differences in the regulation of IL-2 between the different T-cell types. Possible explanations include differences in IL-2 mRNA turnover due to missing cell-type-specific regulatory elements in the transgene relative to the endogenous IL-2 gene, and/or a differential titration of cell type-specific silencing elements due to transgene copy number. We are currently investigating possible differences in rates of IL-2 mRNA turnover between $\gamma\delta$- and $\alpha\beta$-T cells. The IL-2 gene contains intronic and 3'-UTR elements, which stabilize the mRNA in response to costimulatory signals via CD28 in $\alpha\beta$-T cells. These sites are lacking in the GFP transgenes. Thus, it is possible that the differences in mRNA levels are a result of differences in the ability of $\gamma\delta$ vs. $\alpha\beta$-T cells to stabilize their RNA via CD28 costimulation, resulting in a more rapid turnover of IL-2 mRNA but not GFP in $\gamma\delta$-T cells.

130. **Evolution of the Runx gene family structure and utilization in vertebrate hematopoiesis**

Rashmi Pant, Janice Telfer, Michele Anderson

The lymphocyte-based adaptive immune system is a complex cellular structure, which seems to have evolved entirely since the vertebrate line diverged from all other deuterostome animals. Since vertebrate evolutionary relationships are mostly clear, the most basal vertebrates that possess lymphocyte-based immune systems can be compared with their nearest relatives that do not. We are therefore starting with cartilaginous fish, which do have lymphocytes, and proceeding to lamprey (jawless vertebrate, lymphocyte roles doubtful), amphioxus (non-vertebrate, unknown blood structure), ascidian (no lymphocytes), and sea urchin (no lymphocytes). One of four classes of regulatory molecules that control lymphocyte development in mammals is the Runx gene family. Runx was chosen because it has additional, nonlymphoid roles in mammals, and thus might predate the evolution of lymphocytes. Indeed, members of the same family play important roles in development of animals as phylogenetically divergent as *Drosophila*. We are examining the number of genes per family (since gene duplication gives genes an opportunity to acquire new functions), the structural relationships of the genes to their mammalian counterparts, and the types of cells or tissues in which they are expressed.

The sea urchin Runx gene family, which has been described by others, apparently consists of a single member, with little similarity to vertebrate Runx proteins outside of the DNA binding domain. The mammalian Runx family consists of three closely related members with different patterns of expression. Two of them, Runx1 and Runx2, also use alternative N-termini which apparently became associated with vertebrate Runx before the divergence of Runx1 and Runx2. The roles of the different family members and the tissue-specific use of the alternative N-termini are highly conserved between human and mouse, and structurally homologous N-termini are found in birds and amphibians as well. In mammals, the most prevalent form of Runx in the thymus is Runx1, and it uses the distinctive N-terminal domain that is characteristic of hematopoietic stem cells and T cells. In the skate, however, a cDNA library from the thymus has initially yielded Runx2, a family member that is used for bone development in mammals. Furthermore, the N-terminal domain used by this skate Runx2 isoform corresponds to the non-stem cell, non-T cell form in mammals. Runx1 also uses this N-terminal domain in skate. Because the form used in the skate is associated with shorter-lived, terminally-differentiating cells in mammals, and Runx may help coordinate differentiation vs. proliferation, we are attempting to confirm the altered Runx gene usage and explore the possibility that this is a clue to the evolution of long-term self-renewal in blood cells. So far, we have also obtained a partial sequence of the Runx3 molecule in skate thymus.

131. **Evolution of the PU.1/Spi transcription factor family in chordates**

Rashmi Pant, Michele K. Anderson

T lymphocytes and B lymphocytes are present in jawed vertebrates, including cartilaginous fishes, but not in jawless vertebrates or invertebrates. The origins of these lineages may be understood in terms of evolutionary changes in the structure and regulation of transcription factors that control lymphocyte development, such as PU.1 (Spi-1). We have identified and characterized three members of the PU.1/Spi family of transcription factors in a cartilaginous fish, *Raja eglanteria*. Two of these genes are orthologs of mammalian PU.1 and Spi-C, respectively, whereas the third gene, Spi-D, is a novel family member closely related to both PU.1 and Spi-B. In addition, a PU.1-like gene has been identified in a jawless vertebrate, *Petromyzon marinus* (sea lamprey). Both DNA-binding and transactivation domains are highly conserved between mammalian and skate PU.1, in marked contrast to lamprey Spi, in which similarity is evident only in the DNA-binding domain. Phylogenetic analysis of sequence data suggests that the appearance of Spi-C may pre-date the divergence of the jawed and jawless vertebrates, and that Spi-D arose before the divergence of the cartilaginous fish
from the lineage leading to the mammals. The tissue-
specific expression patterns of skate PU.1 and Spi-C
suggest that these genes share regulatory as well as
structural properties with their mammalian orthologs.

One model for the origin of small families of
genes in vertebrates is that two rounds of duplication of
parts of the genome occurred in the vertebrate stem group
at some point between the divergence of acraniates, such as
amphioxus, and the divergence of cartilaginous fish.
This raised the question of whether the skate has a Spi-B
ortholog as well as the three Spi family members we found
in hematopoietic tissue. Extensive screenings of skate
cDNA libraries did not yield Spi-B, and genomic DNA
blots yielded no cross-hybridizing bands other than the
ones accounted for by PU.1, Spi-D, and Spi-C. This
makes it less likely that Spi-B is expressed elsewhere and
not found yet. Instead, the data suggested that either SpiB
was lost in these organisms, or the newly discovered Spi-D
is a divergent form of SpiB.

Thus far the whole PU.1 subfamily of ETS
transcription factors appears to be confined to chordate
deuterostomes. No PU.1 subfamily genes can be found in
the genomic sequences of D. melanogaster or C. elegans.
Preliminary results from degenerate RT-PCR indicate that
the PU.1 family may be present in amphioxus and not in
sea urchins. If this is indeed true, then PU.1 appeared at
the base of the chordate lineage. This is unusual, as most
families of transcription factors are more ancient (found
both in vertebrate and invertebrates, including
protostomes), but have functions in those animals that are
different from, and older than, vertebrate-type blood cell
development.

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Summary: The major departure in our program this past year has been our association with the Alliance for Cell Signaling (http://www.cellularsignaling.org). The Alliance came about after a few years of discussion amongst researchers in the area of signal transduction. It became obvious from many kinds of experiments that signaling networks operate as complex systems. Information transduction in the cell involves many different components acting in concert and operating as a network. One way of approaching an understanding of the structure and function of these networks involves collecting data at different stages in the signaling process as a function of changes in the activation of various receptors. Thus, one can follow the transduction of information from ligand binding to the induction of specific protein-protein interactions, protein modification, and the activation of effectors or the deactivation of specific pathways that lead to changes in cellular function. Clearly this kind of comprehensive study requires enormous resources and diverse abilities. The notion was that such a project could be done by a consortium of laboratories that had a strong interest in collecting the data and eventually in generating the quantitative models that would allow the data to be reduced to knowledge and understanding. These models would generate predictions about system function that could not be easily deduced from understanding the individual interactions in portions of the pathways.

After due deliberation, a consortium with Dr. Alfred Gilman, University of Texas Southwestern Medical Center as Chief was organized. A grant proposal was written and funded by the NIH and a variety of private corporations. The alliance now involves laboratories in five universities including a cell preparation, protein and antibody laboratory in the University of Texas Southwestern Medical Center, a bioinformatics and central information database group at the supercomputer center in UCSD, La Jolla, the Molecular Biology Laboratory that is based at Caltech, a cell localization and cell morphology group at Stanford University, and an assay development and assay monitoring group at University of California, San Francisco. In addition to these groups there are large numbers of collaborators interested in studies of cell signaling drawn from the entire scientific community. Further details regarding the organization can be found on the website.

Our group at Caltech has focused in the last year on developing DNA microarray techniques for analyzing changes in transcript levels in the cell types that the Alliance is studying, i.e., the cardiac myocyte and the B cell. In addition to transcript analysis we have developed collaborations with a number of companies to do yeast two-hybrid analysis. We are developing the materials required for molecular biological experiments involving cell localization and protein translocation as a function of ligand treatment. Many of the planned experiments involve generating a variety of vectors and we have chosen to use the Gateway system (developed by Invitrogen) so that we can have flexibility in generating vectors that can express and deliver different fusion proteins as well as other proteins for expression in cells. We have also been developing anti-sense and RNAi techniques together with ISIS Pharmaceuticals for specific molecules to be used in the B-cell system in order to further explore the effects of blocking gene expression on signaling pathways.

In addition to our work with the Alliance for Cell Signaling, we continue to study G-protein signaling and bacterial chemotaxis. In the chemotaxis system we have focused our attention on the molecular mechanisms involved in generating signaling complexes. We have continued our studies on the histidine kinase, CheA, and on the interactions between CheA, a receptor, coupling proteins, and the signaling protein in bacteria. We are working primarily with the Thermotoga protein system where Alexandrine Bilwes and Brian Crane have developed crystallographic approaches and have solved the structures for many of the major components of this system.

We continue our analysis of the phototransduction system. We have focused primarily on the mechanisms involved in initiating cell death in photoreceptors in the presence of high levels of illumination. Working with mutants that block the phototransduction cascade at a variety of stages, we compare protocols of applying light to mutant mice and wild-type mice. Our data suggest that we can differentiate between at least two specific kinds of mechanisms. One that is the result of extended activation of the phototransduction cascade and requires the downstream components of the cascade including the transducin arm of the phototransduction cascade. Another different mechanism primarily involves the activation of rhodopsin and may not require the rest of the phototransduction
cascade but could involve the development and generation of reactive oxygen species. We have been using transcript analysis on affected retinas to identify the genes that might be involved in each of these processes.

Finally we continue our work with the G-protein signaling systems in two respects. One, in a collaboration with Dr. David Anderson’s laboratory, we are working on a set of receptors that he and his colleagues have discovered that are expressed in dorsal root ganglion cells. We are trying to determine the nature of the ligands that activate these receptors specifically and the function of these receptors (the MRG receptor family) in the dorsal root ganglion cells.

In addition we are continuing to pursue our work with mouse mutants and particularly with the cell lines derived from mice that are deficient in the Gα12, Gα13, and the Gαq family of signaling molecules.

132. Molecular biology laboratory of the Alliance for Cellular Signaling

Iain D.C. Fraser, Sangdun Choi, Eileen Fung, Geniee Lee, Sun-Young Lee, Qingli Mi, Sin-Yee Park, Rebecca Udwary, Mei Wang, Melvin I. Simon

The alliance for cellular signaling (http://www.cellularsignaling.org) is a research consortium established to study mechanisms of G-protein-mediated signal transduction in two murine cell types, the B lymphocyte and the cardiac myocyte. The consortium is organized around several core laboratories. The activities of the Caltech Molecular Biology lab break down into four main areas; transcript analysis, cDNA construction, nucleic acid based inhibition of gene expression and yeast two-hybrid screens.

Microarray analysis will be used to assess changes in gene expression levels in B cells and myocytes stimulated with a variety of ligands to both G-coupled and non-G-coupled receptors. Preliminary experiments have been carried out using a 16K mouse gene set and have assessed the transcript profiles of primary B cells and myocytes when maintained, unstimulated, in culture. The myocytes maintain a relatively stable profile over an incubation period of 8 hrs, whereas the B cells show moderate changes. These changes are distinct from those observed when B cells are stimulated with anti-IgM or IL-4, suggesting that individual ligands will elicit distinct and decipherable transcript profiles.

A further role of the lab is to construct and provide plasmids cDNAs for use in other alliance laboratories. We have established the use of the Gateway cloning system, which facilitates the transfer of cloned cDNAs into multiple expression systems in a single step using the site-specific recombination sites of lambda phage. We have constructed vectors that allow any Gateway cloned gene to be expressed with any of the following fusions at the C- or N-terminus (GFP, CFP, YFP, HA, Myc or Flag tag). Approximately 30 signaling genes and 20 subcellular markers have been cloned thus far. We have noted that almost 50% of the full-length genes cloned contain at least one base-pair change from their GenBank entry. Where possible we have checked the regions in question against mouse genomic sequence and this validates the sequence we have identified in our clones. However, it should be kept in mind that some of these changes could represent single nucleotide polymorphisms.

In collaboration with Isis Pharmaceuticals, we are using antisense to inhibit the expression of selected genes in B cells and myocytes. In preliminary studies in the B-cell lines, BCL1 and 2PK3, oligos targeted against isoforms of the stress-induced MAP kinase Jnk (Jnk1 and Jnk2) and the transcription factor STAT3, induced a >80% reduction in mRNA and protein levels 48 hr post-transfection.

In collaboration with Myriad Genetics, we will carry out 500 yeast two-hybrid screens per year. We are currently preparing specific activation domain libraries for B cells and myocytes and baits for the first 20 proteins selected for screening.

133. DNA microarray profiles to understand the relationships between signaling molecules

Sangdun Choi, Sun Young Lee, Mei Wang, Sin-Young Park, Rebecca Udwary, Melvin I. Simon

Gene expression profiles are useful for identifying unknown genes as well as contextualizing known genes into pathways. DNA microarrays are an extremely powerful technology for measuring mRNA expression levels and have been broadly used for this purpose. Our lab constructed 9K mouse cDNA chips in early 1999. As part of these continuing efforts, a mouse embryonic cDNA library from the National Institute of Aging was selected for the 2nd array set. The 16K genes were amplified by PCR and spotted onto the slides. Subsequently, several hundred chips were used for studying the functions of NF-κB, light-induced apoptosis, Galpha 13, memory, learning, etc., and proved to be excellent tools in the study of transcriptome. Gene grouping was addressed through hierarchical clustering and self organized map analyses. Gene filtering was carried out on hypothesis testing, followed by various types of carefully designed experiments.

Our (Alliance for Cellular Signaling; http://www.cellularsignaling.org) overall goal is to understand, as completely as possible, the relationships between sets of inputs and outputs in signaling cells, especially in B cells and cardiac myocytes. This also includes understanding how cells interpret signals in a context-dependent manner, identifying all the proteins that comprise the various signaling systems, assessing time-dependent information flow in both normal and pathological states, and creating a set of interacting biological models. As proof-of-principle, we have explored primary as many B-cell and myocyte preparations as possible. To find the best conditions for analyzing the transcriptional changes in stimulated cells with each of 50 different ligands, B cells and myocytes at several time points were compared to "0 time" control cells. Two ligands, IL-4 and anti-IgM, were selected first to determine
the responsiveness of the primary B cells to stimulation. The transcript profiles in primary B cells and myocytes were also compared to a number of cell lines. The transcript analysis carried out for the 'Alliance for Cellular Signaling' so far has greatly contributed to our understanding of the systems and of microarray design.

Currently, we are producing third-generation chips using RIKEN FANTOM 21,075 clones. In the mean time, we intend to develop new ways of making and using oligonucleotide chips, representing 13,443 known mouse genes. These significant transcription profiles will add enormous knowledge to the understanding of cellular signaling systems when further array chip data sets are analyzed. Integration of the observed mRNA profiles with the pathway-specific models and with other proteomics data will allow us to formulate new hypotheses to explain biologically complex systems such as apoptosis, aging, learning, and memory.

134 Functional analysis of the Thermotoga maritima CheA histidine kinase
Bryan D. Beel, Melvin I. Simon

The goal of this work is to study the biochemistry of CheA from the hyperthermophilic bacterium Thermotoga maritima. CheA is a histidine kinase which controls the flow of phosphate groups through the pathway controlling chemotaxis in the enteric bacteria Escherichia coli and Salmonella typhimurium. This signaling pathway is the best-studied of the two-component signal transduction systems in bacteria and the structures of many of its component proteins have been solved. CheA is in a complex at the bacterial cytoplasmic membrane with chemoreceptors and the coupling protein CheW; it controls chemotaxis by passing phosphate groups to intracellular response regulators. The crystal structure of a portion of the Thermotoga maritima CheA protein was solved in the Simon lab. Based on this structure, we have begun an investigation of the biochemistry of the Thermotoga CheA protein. The initial portion of this study involved isolating the genes encoding all the chemotaxis-like proteins from Thermotoga. These genes were cloned from Thermotoga genomic DNA and inserted into appropriate expression vectors for protein production in Escherichia coli. Next, purification protocols for these proteins were developed and the proteins successfully expressed. Currently, the Thermotoga chemotaxis proteins are available in pure form for use in in vitro biochemical assays. Our first experiments involve a search, in collaboration with the Shokat group at UCSF, for inhibitors of the CheA kinase; this protein has been shown to be essential for the establishment of host infection by pathogenic bacteria. Subsequent experiments will be directed toward establishing the requirements for effective signaling in the receptor/kinase complex. One important experiment will involve the use of FRET to test a hypothetical structure for the transmembrane signaling complex of receptor/CheA/CheW. Through the use of engineered, site-specific cysteine substitutions and energy transfer dyes, we will confirm or deny the relative orientations of the CheA protein and a transmembrane chemoreceptor. In addition, we intend to use the FRET probes to make a measurement of the dynamics of the protein complex during signal transmission.

135. Structure-function studies of the phosphotransfer domain of CheA, a histidine kinase involved in bacterial chemotaxis
Cindy M. Quezada, Alexandre M. Bilwes, Melvin I. Simon

Protein histidine kinases (PHKs) regulate a wide variety of cellular responses in bacteria, fungi, and plants by initiating "phosphorelays" in response to environmental stimuli. Bacterial chemotaxis, the directed movement towards attractants and away from repellents, uses a "two-component" signaling system with the autophosphorylating PHK CheA as the central element.

We are investigating the biochemical mechanism of CheA by studying the structures of its various domains and the effects of specific mutations on function. We have identified a single amino acid substitution (E67Q), located in the CheA phosphotransfer domain (P1), which completely inactivates the phosphorylation reaction. This residue is not only highly conserved in CheA, but also in a family of ATPases, where it is thought to play a key catalytic role. We propose that the CheA phosphotransfer domain provides the nucleophile for phosphate transfer (His45) and the activating glutamate (Glu67), completing the catalytic center of the CheA ATP binding domain. We are in the process of refining high resolution crystal structures of both wild-type and mutant phosphotransfer domains. In addition, we have collected x-ray data on the phosphorylated P1 domain. Coupled with kinetic data, these structures will provide further insight into the mechanism of the CheA histidine kinase.

136. Functional characterization of orphan G-protein coupled receptor, mrgAs
Sang-Kyou Han, Xinzong Dong, Mark J. Zylka, David J. Anderson, Melvin I. Simon

A large novel GPCR subfamily comprising almost 50 members was identified in David Anderson group. Based on sequence homology, this family is closed to Mas1, called Mas-related genes (mrgs). A subfamily of these genes, called mrgAs, is specifically expressed in subset of non-peptidergic, isolectin B4 cutaneous afferent sensory neurons, that has been implicated in neuropathic pain.

As a first step to characterize the function of receptors, we sought to determine whether these GPCRs could be activated by any known ligands. To this end, we cloned selected MrgA genes, including MrgA1 and MrgA4, into eukaryotic expression vector and confirmed that MrgA-GFP fusion proteins were expressed on membrane inHEK293 cells. We screened approximately 60 candidate peptides for their ability to activate mrgA1 and 4 in HEK293 cells modified to express Gtx15, which couples GPCRs to a signal transduction pathway leading to the release of intracellular calcium.
The most efficient response in mrgA1-expressing HEK cells were elicited by RF amide peptides, such as FMRFamide, FLRFamide, NPFF and NPAF. mrgA1 could be activated by FLRF amide at nanomolar concentration (EC50 ~20nM), and by NPFF at about an order of magnitude higher concentration (EC50~200nM), whereas NPAF was much less effective. In contrast, mrgA4 was well activated by NPAF (EC50~60 nM), and much more weakly activated by FLRF and NPFF. These data therefore confirmed that mrgA1 and mrgA4 display different selectivities toward different RF amide ligand. Furthermore, mrgA1 and A4 showed calcium activation and desensitization kinetics towards their preferred ligands that are characteristic of other GPCRs.

These data suggest that mrgA1 and A4 may be receptors for RFamide-family neuropeptides. The fact that these neuropeptides produce analgesic effects when injected intraspinally, further suggests that these receptors could be involved in the sensation or modulation of pain. Further studies to define signaling mechanism of these receptors are being investigated.

137.  Phosphorylation and regulation of Gα15 and Gα16 by protein kinase C
Jennifer L. Gu, Chunzhi Xia, Zhenmin Bao, Yihui Hu, Melvin I. Simon, Mingyao Liu
Heterotrimeric G proteins mediate cell growth and differentiation by coupling cell surface receptors to intracellular effector enzymes. The G-protein α subunit, Gα16, and its murine homologue Gα15, are expressed specifically in hematopoietic cells and their expression is highly regulated during differentiation of normal and leukemic cells. In this study, we examined the phosphorylation of Gα15/Gα16 and its role in receptor and effector coupling. We observed a PMA-stimulated intact cell phosphorylation of Gα15 in COS7 cells transfected with Gα15 and protein kinase C (PKC) alpha, and phosphorylation of endogenous Gα16 in HL60 cells. We also showed that recombinant Gα15 and Gα16, as well as peptides derived from the two G-proteins were phosphorylated in vitro using purified brain PKC. Furthermore, we identified the putative phosphorylation site and showed that mutation or deletion of this PKC phosphorylation site inhibited phospholipase C activation. The behavior of double mutants that include the constitutively active G-protein mutation (QL-mutant) and mutation in the putative phosphorylation site suggest that the phosphorylation site of Gα15/16 is essential for receptor-coupled activation of phospholipase C, but not for direct interaction of the G-protein with PLC-β.

138.  Interaction of Gα32 with Gα3 and Gαq signaling pathways
Stefan Muller, Jennifer L. Gu, Valeria Mancino, Stephan Offermanns, Melvin I. Simon
The G12 subfamily of heterotrimeric G-proteins consists of two members, Gα12 and Gα13. Gene targeting studies have revealed a role of Gα13 in blood vessel development as mice lacking the α-subunit of Gα13 die at embryonic day ten as a result of an angiogenic defect. In contrast, the physiological role of Gα12 is still unclear. To address this issue, we generated Gα12 -deficient mice. In contrast to the Gα13 -deficient mice, Gα12 -deficient mice are viable, fertile and do not show apparent abnormalities. However, Gα12 does not seem to be entirely dispensable since in the offspring generated from Gα12 +/- Gα13 +/- intercrosses, at least one intact Gα12 allele is required for the survival of animals with only one Gα13 allele. Furthermore, mice lacking both, Gα12 and Gαq, die in utero at about E12.5. These data indicate that the Gα12 mediated signaling pathway functionally interacts not only with the Gα13 - but also with Gαq-mediated signaling systems.

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139.  Modeling biological signal transduction pathways
Tau-Mu Yi‡, Hiroaki Kitano‡, John Doyle* Melvin I. Simon
Living organisms detect and respond to a variety of environmental cues (e.g., light, attractants, hormones, etc.) through receptor-mediated signal transduction pathways. The majority of pharmaceutical agents act by modulating the behavior of these biological networks. Many of the signaling systems possess remarkable performance characteristics including exquisite sensitivity, broad dynamic range, and robustness. Using techniques from control and dynamical systems theory, we have studied two well-characterized signaling pathways: (1) bacterial chemotaxis (two-component); and (2) mammalian visual phototransduction (G protein).

The chemotaxis signaling pathway in E. coli exhibits both sensitivity and dynamic range. E. coli are able to sense shallow gradients of attractant over five orders of attractant concentration in a robust fashion. We have constructed a model of this system that explains these properties. One key feature of the model is that robust perfect adaptation implemented through integral feedback control adjusts the response of the receptor to the background concentration of attractant. The gain is achieved through the highly cooperative interaction between the phosphorylated response regulator CheY-P and the flagellar motor. Finally, multiple levels of negative feedback enhance the robustness of the behavior and limit the sensitivity to noise. Taken together, this design is similar in many respects to feedback amplifiers.

The rod photoreceptor cell in the retina is able to detect single photons of light in a reproducible and quantitative fashion. This response is mediated by a G-protein signaling network. Combining biochemical, genetic, and physiological data, we have constructed a model of this system. Because only a single rhodopsin
molecule is activated, one would expect considerable stochastic noise in the response. Such variability in the output is not observed. It is our hypothesis that two separate calcium-mediated feedback loops are employed to suppress this noise, resulting in a reproducible single-photon response. We have proposed that the inner feedback loop acting through the calcium-binding protein guanylate cyclase activating protein (GCAP) resembles a lag controller. This feedback structure enhances the robustness of this transient response as well as providing a better fit to experimental data. Understanding the limits to the reproducibility of transient neural signals has implications for the neural code.

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140. Prolonged activated rhodopsin in Rhok\(^{-/-}\) and Arr\(^{-/-}\) mice mediates light damage through transducin-dependent pathway

Wenshan Hao, Ching-Kang Chen, Janis Lem*, Pamela Eversole-Cire, Melvin I. Simon

Excessive exposure to light can selectively induce apoptosis in photoreceptor cells and causes retinal degeneration in animals, and it has been suggested as an accelerating factor in some forms of human retinitis pigmentosa and age-related macular degeneration. Mice and rats have been widely used as experimental models to study light damage to the retina. Both chronic exposure at moderate intensity (1000-3000 lux) for days or weeks and acute exposure at high intensity (>5000 lux) for minutes or hours were used to induce retinal damage according to species and their genetic backgrounds. Despite difference in light exposure protocols, emerging evidence suggested that light damage was mediated by activated rhodopsin. Different hypothesis are proposed to explain how activated rhodopsin triggers damage. Although it appears that in Drosophila activated rhodopsin can mediate light damage through several independent pathways, there is lack of evidence that this is also the case in rodents. We have been using light sensitive Arr\(^{-/-}\) and Rhok\(^{-/-}\) (encoding arrestin and rhodopsin kinase, respectively) mice as the models to study the mechanisms of light damage. By crossing Gnat1\(^{-/-}\) mice (encoding transducin \(\alpha\)-subunit) to the two mutant mice, we found that in Gnat1\(^{-/-}\)/Arr\(^{-/-}\) and Gnat1\(^{-/-}\)/Rhok\(^{-/-}\) mice, moderate light-induced photoreceptor apoptosis and retinal degeneration was greatly reduced. Loss of c-Fos was shown to block high light-induced damage, but had little effect on the initiation of photoreceptor apoptosis in c-Fos\(^{-/-}\)/Rhok\(^{-/-}\) mice induced by moderate light. While tested under high light, Gnat1\(^{-/-}\) did not show the protection of the retina from damage as did by c-Fos\(^{-/-}\). Our findings clearly demonstrated that light damage to the retina can be mediated through multiple mechanisms depending on the genetic constitution and the level of light intensity.

141. Gene expression profiles of light-induced apoptosis in arrestin/rhodopsin kinase-deficient mouse retinas

Sanjuk Choi, Wenshan Hao, Ching-Kang Chen, Melvin I. Simon

In order to examine the molecular processes that lead to light-induced retinal degeneration, mutant mice deficient in arrestin and rhodopsin kinase were raised in the dark and then subjected to relatively low doses of white light. The kinetics of the subsequent induction of apoptosis, change in mRNA transcript level and photoreceptor cell death were monitored. Analysis of transcript profiles identified clusters of genes that responded differently to illumination, including a cluster of photoreceptor-specific genes that showed marked decreases in levels long before morphological damage could be readily ascertained. The behavior of other gene clusters demonstrate the coordinate induction of stress gene responses early in the course of irradiation. There was little if any change in transcript levels corresponding to genes associated with the initiation of apoptosis or anti-apoptotic effects. Transcript analysis provides insight into the patterns of gene expression that are associated with the different stages of retinal degeneration in this model system.

142. Decrease in Bax expression correlates with decline in ability of Bcl-2 to prevent photoreceptor cell death

Pamela Eversole-Cire, Jeannie Chen*, Judith Mosinger Ogilvie‡, Melvin I. Simon

Retinitis Pigmentosa (RP) refers to a group of inherited retinopathies that are clinically and genetically heterogeneous. The clinical presentation of the disorder begins with night blindness resulting from degeneration of rod photoreceptor cells. The loss of photoreceptors gradually leads to a generalized loss of retinal tissue that affects overall vision. The progressive nature of this disease suggests that a therapeutic intervention aimed at decreasing the rate of photoreceptor cell death may significantly increase the number of years of useful vision for an individual afflicted with RP. Recent evidence indicates that genetic mutations within several components of the visual transduction pathway may be involved in the pathogenesis of this group of diseases. Mutations within the genes encoding rhodopsin and the \(\beta\) subunit of rod cyclic guanosine monophosphate (cGMP)-phosphodiesterase (PDE), for example, have been linked to RP. Mouse models of RP are available to study the underlying pathogenic mechanism(s) leading to retinal degeneration such as the S334ter rhodopsin mutant mouse that overexpresses a truncated rhodopsin transgene specifically in photoreceptor cells and the rd mouse that contains a naturally occurring genetic alteration in the \(\beta\) subunit of rod cGMP-PDE. Retinal degeneration occurs at a rapid pace by an apoptotic mechanism in both of these mouse models.

Ectopic expression of Bcl-2 in photoreceptors in the S334ter rhodopsin transgenic mouse and the rd mouse
temporarily slows progression of the disease process (Chen et al. (1996) *PNAS* 93:7042-7047). The temporary effect produced by Bcl-2 may result from insufficient levels of proteins necessary for maintaining the anti-apoptotic activity of Bcl-2, such as Bax, which is thought to be a functional partner of Bcl-2. Therefore, endogenous levels of Bax expression were analyzed during early retinal development to determine if changing levels of the Bax protein would correlate with the decreasing ability of the Bcl-2 protein to prevent photoreceptor cell death. Bax expression was found to continuously decrease during the first month of age. The ability of Bcl-2 to prevent photoreceptor cell death in mice expressing either the S334ter mutant rhodopsin or that are homozygous for the rd mutation also decreases significantly during this developmental time period. Therefore, it is possible that the decreasing level of Bax expression is partially responsible for the decline in the anti-photoreceptor cell death activity of Bcl-2 in the S334ter and rd mouse models of RP. Experiments are currently underway to determine if Bax expression is necessary for Bcl-2 anti-cell death activity by determining if expression of the Bcl-2 transgene will still delay retinal degeneration in the photoreceptors of mice that are Bax-deficient and homozygous for the rd mutation.

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the uterine and vulval epithelia and allow them to connect
intercellular signaling that coordinate the patterning of
regulated at the level of ligand production as well as the responsiveness of the
receiving cells. LIN-3 is produced in a highly localized and regulator manner (see Abstract 143). A series of projects focus on the identification and analysis of negative regulators of LET-23 signaling (see Abstracts 144, 145, 146). The anchor cell then patterns the uterine epithelium (see Abstract 147). After the anchor cell induces the vulva, a complex program of further pattern formation, cell-type specification and morphogenesis follows. The primary (1°) vulval lineage generates an E-F-F-E pattern of cell types while the 2° vulval lineage generates an A-B-C-D pattern of cell types (see Abstracts 148, 149, 150). We now have our hands on a number of receptor proteins, transcription factors and regulated genes; we are trying to sort out this regulatory network to understand how organogenesis is genetically programmed. The anchor cell recognizes one of the seven vulval cell types and invades the vulval epithelium in a process akin to tumor metastasis (see Abstract 150). Regulation by the EGF-receptor, Wnt and HOM-C pathways impinge not only on vulval development but also P12 specification and male hook development. By comparing these examples with vulval development, we seek to understand the signaling specificity and signal integration (see Abstracts 152, 153).

Mating behavior, with its multiple steps, is arguably the most complex of C. elegans behaviors and because it is not essential for reproduction, given the presence of internally self-fertilizing hermaphrodites, is useful to elucidate how genes control behavior. We have found strong evidence for a soluble cue made by hermaphrodites and sensed by males (see Abstract 154). Males respond to the hermaphrodite using the sensory rays; this response requires C. elegans homologs of human polycystins 1 and 2 (see Abstract 155), mutated in autosomal dominant polycystic kidney disease. When the male contacts the hermaphrodite he alters his locomotory behavior dramatically (see Abstract 156). We have found that muscle function during spicule insertion involves a switch from rapid contraction/relaxation to prolonged muscle contraction (see Abstract 157). We are screening for genes that act in either the hermaphrodite or male to elicit sperm transfer (see Abstract 158).

Most C. elegans behaviors rely on the heterotrimeric G proteins Go and Gq, which we have found to act antagonistically (see Abstract 159). Sinusoidal movement provides an opportunity to study interactions among G-protein signaling pathways (see Abstract 160) as well as more general issues of how genes and cells program behavior. In collaboration with J. Bruck in EAS we have developed a system to observe and extract quantitative data on worm movement (Abstract 160). Ovulation involves complex regulation of a smooth muscle

Summary: Our laboratory uses a molecular genetic approach to study basic questions in developmental biology, neurobiology and evolutionary biology: What are the molecular mechanisms of intercellular signaling? How is the fate of a cell specified in response to several intercellular signals? How do genes and neurons control complex behavior? What types of changes in development occur during evolution? How can we use the full genome sequence of an organism? We primarily use the model organism Caenorhabditis elegans to study these questions. By understanding several aspects of this one organism in exquisite detail we hope to answer these fundamental biological questions. Our general approach is to use genetics to identify genes controlling the structure and behavior of cells and the whole organism, and to study those genes and their products, and how those genes interact to control development or behavior. In addition, we have a long-standing interest in the evolution of control mechanisms. Our research areas include: signaling by EGF-receptor and its negative regulation as a paradigm for signal transduction; vulval pattern formation and morphogenesis as a paradigm for organogenesis; sinusoidal movement including the role of G proteins Go and Gq; male mating behavior as a paradigm for the genetic control of development; modeling signal transduction; and bioinformatics. Our emphasis has been strongly focused on signal transduction but has more recently expanded to include transcriptional regulation.

Vulval development involves a remarkable series of intercellular signaling that coordinate the patterning of the uterine and vulval epithelia and allow them to connect

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by a variety of signal transduction pathways, including tyrosine kinases and G protein-coupled pathways; we have analyzed this year regulation of the second messenger inositol 1,4,5 triphosphate levels (see Abstract 161). We have also started to analyze a little understood signaling pathway involving spingosine phosphate (see Abstract 162). In the process of studying G-protein signaling we discovered genes necessary for heat shock response; this year we cloned the sag-3 locus and found it encodes C. elegans heat shock transcription factor.

To address some of the problems of the genomic era, we are working on three types of projects that use computer science approaches. One approach is the development of tools for "intermediate throughput biology," that is, automation useful for the molecular genetics researcher (see Abstract 160). A second approach is computer modeling. To understand the pathways and networks underlying development and behavior, it will be necessary to integrate quantitative modeling into our molecular genetic armamentarium. From conventional approaches, genomic approaches and the many "post-genomic" approaches, there is an increasing amount of information that each researcher must have accessible. A new project associated with the laboratory is WormBase, database of C. elegans biology (see Abstract 164). Through this project, we will provide a service to the C. elegans and broader biological communities, as well as work on problems in bioinformatics, such as analysis of cis-regulatory sequences, and methods of efficient extraction of information from the literature. As an initial approach to comparative genomics, we have established C. briggsae as a developmental genetic system; in terms of molecular sequence divergence, briggsae and elegans are roughly that of human-mouse (see Abstract 151).

143. Mechanisms that establish anchor cell-specific expression of lin-3, C. elegans epidermal growth factor (EGF) homolog

Byung Joon Hwang

The C. elegans lin-3 gene is required for multiple aspects of C. elegans development: vulva development, ovulation, cell fate specification of the P12 neuroblast of hermaphrodites and B-cell lineage in males, and for viability of males and hermaphrodites. LIN-3 has an extracellular domain with one EGF motif, a transmembrane domain, and a cytoplasmic domain that is longer than that of most other growth factors. It is expressed in anchor cells in the gonad during vulva induction. The induced LIN-3 likely binds to LET-23, its receptor in vulval precursor cells, and the binding activates the regulated proliferation of vulval precursor cells and pattern of the developing vulva. We took two different approaches to study mechanisms that establish its AC-specific expression. First, we identified the molecular nature of the vulva-specific e1417 mutation of lin-3. Second, the regulatory region of lin-3 for the anchor cell-specific expression was dissected by deletion and site-directed mutagenesis analysis. These approaches identified a 65-bp long cis-element before the 2nd lin-3 promoter that makes lin-3::gfp expressed only in AC in the gonad during vulva induction. We named this element ACEL (anchor cell element of lin-3). The ACEL contains three predicted binding sites for trans-acting factors. All of the sites are essential for expressing lin-3::gfp in anchor cell since mutation of any of the three sites abolished expression. In the L2 stage before AC becomes specified, lin-3::gfp expressed in the four pre-AC and pre-VU cells. By the L2 molt, when the AC/VU cell fates become specified, lin-3::gfp expression is repressed in the three VU cells but remains in the AC. Currently we are looking for proteins that bind to the ACEL and studying mechanisms that regulate lin-3::gfp expression during AC/VU cell fate specification.

144. Modulation of inductive signaling during C. elegans vulval development

Nadeem Moghal, Chieh Chang, L. René Garcia

In C. elegans hermaphrodites, the vulval precursor cells (VPCs) P3.p-P8.p can adopt vulval or hypodermal fates. In wild-type animals, the anchor cell secretes LIN-3, which activates its receptor, LET-23, and causes P5.p-P7.p to adopt vulval fates. Although P3.p, P4.p, and P8.p also express LET-23, it is thought that their distance from the anchor cell prevents levels of activated LET-23 from accumulating sufficient to overcome default hypodermal fates. We have used two approaches to study modulation of responses to this inductive signal. In one approach, we tested candidate loci for interaction with this pathway. We find that a loss-of-function mutation in the egl-15 negative regulator, clr-1, a receptor tyrosine phosphatase, increases responsiveness of all six VPCs to the inductive signal. In contrast, an activated allele of egl-30 Gqa facilitates responsiveness of P5.p-P7.p, but not P3.p, P4.p, and P8.p. In a second approach, we identified EMS-induced mutations that enhance the choice of vulval fates by P3.p, P4.p, and P8.p in the presence of a gain-of-function allele of let-23. Some of these mutations, such as sy622, strongly enhance the responsiveness of P3.p, P4.p, and P8.p, while only weakly affecting P5.p-P7.p. These results indicate that there are both pan-Pn.p, as well as Pn.p-specific mechanisms operating to modulate vulval induction. Pan-Pn.p mechanisms might function as buffers to lower the overall noise inappropriately contributing to vulval induction. In conjunction with pathways defined by egl-30(gf) and sy622 which increase responsiveness of P5.p-P7.p and decrease responsiveness of P3.p, P4.p, and P8.p, respectively, a robust response by only a subset of cells might be guaranteed during inductive signaling.

145. CLR-1 participates in EGFR signaling pathway during vulval development

Chieh Chang, Nadeem Moghal

We found that clr-1, a previously identified negative regulator for egl-15 mediated FGFR signaling, is involved in let-23 EGFR signaling. Specifically, a reduction-of-function mutation in clr-1 suppresses decreased activity in let-23 EGFR, the lin-10 PDZ protein, sem-3 Grb2, let-341 Sos, let-60 Ras, and lin-45 RAF
resulting in a rescue of vulval-less and lethal phenotypes. Further, a \textit{clr-1} mutation synergized with an activated \textit{let-60} Ras mutant and mutations in other loci defining negative regulators of \textit{let-23} mediated vulval signaling, including \textit{sli-1}, \textit{ark-1}, \textit{unc-101}, and \textit{gap-1}. We have shown that \textit{clr-1} is unlikely to regulate \textit{LET-23} signaling solely by an effect on FGF-receptor signaling since \textit{CLR-1} still inhibits \textit{LET-23} signaling in the absence of the FGF ligand \textit{EGL-17}. Since \textit{clr-1} encodes a single-pass transmembrane protein with two putative tyrosine phosphatase domains in its carboxy terminus, we hypothesize that \textit{CLR-1} acts to inhibit EGFR signaling by dephosphorylating activated \textit{LET-23}.

146. Suppression of mutation-induced vulval defects by activated \textit{EGL-30(Gq)}

\textbf{Nadeem Moghal, L. René Garcia}

During the course of other studies, we observed that hermaphrodites containing a gain-of-function allele of \textit{eql-30} occasionally (<0.5%) develop ectopic vulval tissue. To determine directly whether activated \textit{Gq} affects vulva development, we constructed double-mutant combinations of \textit{eql-30(gf)} with hypomorphic mutations in the EGF pathway. We find that \textit{eql-30(gf)} partially suppresses loss-of-function mutations in \textit{lin-3(n378), let-23(sy1), let-341(ku231), and let-60(n2021)}; however, activated \textit{Gq} neither suppresses all hypomorphs in \textit{lin-3} nor synergizes with \textit{gap-1(n1691)}, gain-of-function \textit{let-23(sa62)} or with \textit{lin-15(n765)} to generate ectopic vulval tissue. \textit{Gq}-mediated suppression requires wild-type EGL-19 voltage-gated calcium channels, but not synaptic transmission components such as \textit{UNC-64} (syntxin) or \textit{UNC-13}, suggesting that excitatory cells are involved in this phenomenon, but that the mechanism of suppression does not strictly require synaptic transmission. We hypothesize that in the mutant strains we have constructed, activated \textit{Gq} may amplify weak EGF signaling in \textit{P5.p}, \textit{P6.p}, and \textit{P7.p} through a parallel pathway to induce vulva development.

147. Regulation and function of \textit{lin-11} in vulval development

\textbf{Bhagwati P Gupta}

Vulval development in \textit{C. elegans} hermaphrodites allows us to address the questions of cell-fate specification. During the terminal differentiation of vulval cells, 22 cells differentiate to give rise to seven different cell types – \textit{vulA}, \textit{vulB}, \textit{vulC}, \textit{vulD}, \textit{vulE} and \textit{vulF}. Very little is known about the mechanisms that control this developmental process. \textit{lin-11} gene, a member of the \textit{LIM} Homeobox family, was previously shown to play a role in the specification of \textit{vulC} and \textit{vulD} cell types (1). The molecular basis of the defect is, however, not yet understood. We have begun to dissect the function of \textit{lin-11} in vulval differentiation and identify other components of the genetic pathway that involves \textit{lin-11}. As a first step towards characterizing the cellular defects in \textit{lin-11} mutants, we have used various cellular markers. Two experiments suggest that \textit{lin-11} is required for the specification of all vulval cells. First, in \textit{lin-11} animals \textit{1°} and \textit{2°} lineage vulval cells abnormally fuse together as revealed by the expression of \textit{jam-1::gfp}, a cell junction marker. Second, none of three vulval markers – \textit{egl-17::gfp (vulC, vulD)}, \textit{zmp-1::gfp (vulA, vulD, vulE)} and \textit{ceh-2::gfp (vulB1/vulB2, vulC)} express in \textit{lin-11} mutants.

Mutations in \textit{lin-11} cause fully penetrant egg-laying defect due to the requirements of \textit{lin-11} in uterine \textit{p} lineage cells (2). We have characterized the regulatory element of \textit{lin-11} and find distinct \textit{cis} elements for the vulva and uterine \textit{p} cells expression. Our \textit{in vivo} rescue experiments show that the normal egg-laying function requires \textit{lin-11} gene function in both tissues. A sequence analysis of the regulatory elements revealed putative binding sites for \textit{LAG-1} in the uterine element and \textit{TCF-1/LEF-1} in the vulval element. \textit{LAG-1} is homologous to \textit{Drosophila} \textit{Su(H)} and functions in the \textit{LIN-12}-mediated lateral signaling pathway. On the other hand, \textit{TCF-1/LEF-1} members such as \textit{POP-1}, have been shown to function in the \textit{Wnt} signaling pathway. \textit{LIN-17}, a frizzled receptor homolog in \textit{C. elegans}, is known to play a role in the vulval development; however it is not clear if it utilizes \textit{POP-1} function. Similarly, uterine \textit{p}-cell development is known to require \textit{LIN-12} signaling, but it remains to be shown if it involves \textit{LAG-1}. We are currently doing experiments to test these hypotheses. In addition to these experiments, we have also taken a genetic approach to identify the \textit{lin-11} regulators. An EMS screen was carried out to isolate mutants that enhance a weak \textit{lin-11::gfp (syIs53)} expression in vulval cells. A total of seven alleles have been identified and they belong to three different loci on chromosomes I and III. Genetic interaction studies and molecular cloning experiments are in progress.

**References**


148. Analysis of genes involved in late patterning of the vulva

\textbf{Takao Inoue}

Based on EM reconstructions, two and five terminally-differentiated cell types are generated by the primary and the secondary vulval lineages. We have assembled a panel of \textit{gfp, cfp, and yfp} markers that label subsets of cell types, and together enable us to distinguish between six of these cell fates. We are using these marker lines to screen for new mutations that affect the patterning of these cell fates, and to analyze previously isolated mutations that affect the secondary VPC lineage execution. The process is complex and likely involve multiple cell signaling events and transcription factors. Previous analysis based on lineage have suggested a role of a \textit{Wnt} signaling pathway in the \textit{P7.p} secondary lineage patterning. Mutations in \textit{lin-17} (Frizzled homolog) and \textit{lin-18} (homolog of Ryk = related to receptor-tyrosine kinases) cause a phenotype interpreted to be the anterior-
posterior reversal of the P7.p (secondary) lineage. Preliminary examinations of marker expression patterns in these mutants suggest a more complex defect. We are analyzing the defects in these mutants using additional gfp markers. We are also investigating how lin-18 Ryk is involved in the Wnt signaling pathway.

A number of transcription factors are also implicated in late patterning. egl-29, which affects expression of egl-17::gfp, was found to be allelic to the heterochronic gene lin-29, encoding a zinc-finger transcription factor. Re-interpretation of previous results and examination of additional gfp markers suggest that lin-29 mutations affect the timing of marker expression. Another gene under investigation is cog-1, encoding a GTX homeodomain protein (R. Palmer and Paul W. Sternberg). A missense allele, cog-1(sy275), causes ectopic expression of egl-17::gfp and ceh-2::gfp in the outer cells (vulE) of the primary lineage. A partial deletion allele cog-1(sy607) (generated by D. Sherwood) causes loss of ceh-2::gfp and cdh-3::gfp expression from cells of the secondary lineage. By lineage analysis, sy275 delays or eliminates the terminal division of the inner primary cells (vulF), whereas sy607 eliminates the terminal division of secondary cells P5.ppa and P7.pap (vulC). cog-1 encodes two alternative transcripts, and the differences in the phenotypes of two alleles possibly reflect different functions of the two transcripts.

149. Regulation of egl-17, cdh-3 and zmp-1
Martha Kirouac

After induction of primary and secondary fates by the anchor cell and lin-12, and undergoing two rounds of cell division, the final vulval cell types are specified A, B1, B2, C, D, E and F. A number of transcription factors have been identified that play a role in vulva development. However, we know little about how these transcription factors interact with each other, about their interactions with the inductive pathway, or about how they are regulated. The identification of cis-regulatory regions that confer cell specificity and respond to the ras pathway would be very helpful in determining such relationships. Three such target genes are egl-17, cdh-3 and zmp-1. These genes offer the opportunity to find response regions for multiple vulva cell types: vulE, vulF, vulC, vulD, and vulA, as well as the anchor cell. In addition, egl-17 is an early cell fate marker for the response to the inductive signal; the isolation of a cis-regulatory element that drives this early expression and identification of genes that regulate this expression would be informative. We are analyzing these regions using a PCR-based strategy to generate defined deletions to test the necessity and the sufficiency of fragments to confer regulation on the naive promoter pes-10.

egl-17::gfp comes on in P6.p, P6.p daughters and granddaughters; goes off in early L4; staying off in mid-L4; on in vulC and vulD cells in mid-L4. This GFP marker strain contains 3.8 kb of cis-regulatory region. Since this gene is the earliest known marker in response to the inductive signal, it makes a good candidate for a immediate response element to the ras pathway. We have found a 0.2 kb region that is necessary and sufficient for vulC and vulD cell specificity and a 1.5 kb region that confers early expression in P6.p daughters. zmp-1::gfp is expressed during late L4 in vulE and vulA cells and in the L3 animals it is also expressed in the anchor cell [J. Butler and J. Kramer, personal communication]. This GFP marker also contains 3.8 kb of upstream regulatory sequence. We have defined a 0.35 kb region that is necessary and sufficient vulE, vulA and anchor cell specificity. cdh-3::gfp is expressed during L3 in vulE and vulF cells and in L4 animals it is expressed in the vulC and vulD cells. cdh-3 is also expressed in the anchor cell starting in the late L2 stage. The GFP marker strain contains 6.0 kb of upstream regulatory sequence. We have found a 0.6 kb region that confers specificity to the anchor cell element and 0.5 kb region that drives expression in vulE and vul F cells.

150. Cellular mechanisms governing the specificity and timing of anchor cell invasion into the vulval epithelium
David R. Sherwood

We are examining the initial steps that regulate the connection of the uterus and vulva during C. elegans development. The initial contact between the developing uterus and vulva is established by the anchor cell (AC), which crosses the basement membranes separating both tissues and specifically attaches to the inner descendants of the 1° fated vulval precursor cell, P6.p, during the mid- to late-L3 stage. Following attachment, the AC extends cellular processes from its basolateral surface and invades between the inner descendants of the P6.p. cell, positioning itself at the apex of the developing vulva. Using mutants lacking vulval induction, we show that underlying vulval cells trigger AC attachment. We have further discovered that AC attachment is not stimulated by 2° fated vulval cells (the vulval cells that flank the centrally located 1° cells), but rather appears to be specific for the 1° vulval cells. Using a basement membrane marker, we show that isolated 1° cells do not break down the basement membrane in the absence of the AC, suggesting that 1° vulval cells do not stimulate attachment by removing the basement membrane. Instead we provide evidence indicating that the 1° vulval cells secrete a signal that triggers AC invasive behavior: when the AC is placed at a distance from the 1° vulval cells, it sends out cellular extensions toward the 1° vulval cells that are similar to those seen during normal invasion. We further show that the competence of the AC to respond to this signal is regulated: AC attachment is either absent or delayed in the heterochronic mutant lin-28, which causes precocious vulval development. To understand the molecular mechanisms that regulate AC attachment and invasion, we are examining many known mutants. Through these studies we have found that the evl-5 mutant, originally isolated in a screen for sterile mutants with everted vulvae (Seydoux et al. (1993) Dev. Biol. 157:423-436), has a
defect in AC attachment to the vulval epithelium. In evl-5 animals vulval induction and AC positioning over the P6.p cell is normal; however, AC attachment either does not occur or is severely delayed. Visualization of AC behavior in this mutant revealed the extension of cellular processes from the AC toward the vulva, indicating that the AC is still attracted to the developing vulva. However, in many cases the AC processes appeared to flatten or broaden out at the basement membrane of the developing gonad, suggesting that the AC may not be able to cross this basement membrane. We are currently molecularly cloning evl-5.

151. Developmental genetics of C. briggsae
Keith Brown, Shahla Gharib, Robert Johnsen*, David Baillie*, Shirley Phan*

We are studying the developmental genetics of Caenorhabditis briggsae in order to compare it with C. elegans. The two nematode species are morphologically similar but are approximately 25 - 40 million years apart, which is about one-half the evolutionary distance between mice and humans. Thus a detailed comparison will help think about the differences between human and mouse, especially concerning conservation of cis-regulatory sequences. The 100 million base pair C. elegans genome is effectively completely sequenced revealing approximately 19,000 genes. The sequencing of the C. briggsae genome, already 10% complete, is expected to be completed in 2001 by the Washington University Genome Sequencing Center and the Sanger Centre. The two nematodes show 30-50% divergence of unselected nucleotide sequence. We isolated over 300 mutations in C. briggsae, and have started to construct a genetic map. We are now focusing on mutations affecting vulval development. We previously found that there were subtle differences in vulval development between the two species: the division of the anchor-cell proximal VPC granddaughters (F cells) is induced by the anchor cell in species: the division of the anchor-cell proximal VPC.

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Development

Reference

153. Cell-fate specification during male hook development
Hui Yu

A completely sequenced genome and other features make Caenorhabditis elegans an excellent model system for functional studies of conserved signaling pathways, such as EGF-Ras, Wnt, and LIN-12/Notch pathways, using both the genetic manipulation and molecular approaches. The C. elegans male "hook" is one copulatory structure that aids in mating behavior. The hook sensillum, including structural cells, support cells and hook neurons, is derived from three multipotent hook precursor cells, P(9-11).p. The process of hook development involves initial cell-fate specification, further precise fate execution, and final morphogenesis and biological functions in behavior. Male P(9-11).p cells keep unfused at the L1 stage, and later from the mid-L3 stage each cell exhibits one of the three fates with an invariant pattern of 3°-2°-1° in intact wild-type animals. To examine whether HOM-C gene mab-5 has more roles in HPC patterning other than inhibiting the P(9-11).p cell fusion during L1 stage, we used a heat shock-inducible transgenic line encoding hs-mab-5 to manipulate the
MAB-5 activity. Overexpression of mab-5 during mid-L2 to early-L3 stage led to a high percentage of hook abnormalities in adult males, including no hook, misshapen hook, or anterior hook. hs-mab-5 can block formation of all three hooks in lin-12(gf) males. Activated LET-23/EGF-Receptor pathway induced ectopic hook formation in P9.p cell in which activity of LET-23 pathway is normally suppressed by negative regulator LIN-15. To elucidate the cell interactions among 1°, 2°, and 3° fate cells, cell ablations were performed within the P(9-11).p group. The 2° fate in an isolated P10.p was not altered by the disruption of 1°-fated P11.p in the early L3 stage after P10.p migrated to a posterior position. Within the 2° lineage, ablation of one of support cells, hook socket cell, or its parent cell P10.ppa, but not the hook neurons, had a negative effect on hook structure.

154. Hermaphrodite-derived, mate-finding cue implicated in C. elegans

Jasper M. Simon

When males of C. elegans come into association with their hermaphroditic counterparts they cease foraging behavior and begin to mate. This past year, we developed several assays to demonstrate that a soluble cue is correlated with this process. This cue is sexually dimorphic, given off only by the hermaphrodite and eliciting a response only in the male. From our studies we suggest a form of kinesis that works by attracting males to their mating partners from a distance and functions, once males arrive, in holding attracted males in close proximity. We have explored both the variability of this cue between geographically diverse C. elegans isolates as well as the extent of this cue in species in and outside the genus Caenorhabditis. Moreover, we continue studies of this cue in light of various factors, including reproductive mode (male/female vs. male/hermaphroditic), population structure (solitary vs. clumpers), mating experience, age, nutritional status, and heterospecific cue competition. Presently we are examining both candidate sensory mutants in the male and using classical genetics to work out the cue’s cellular source in the hermaphrodite.

155. Co-localization of polycystins in C. elegans

John DeModena, Barbara Perry, Maureen Barr*

Autosomal Dominant Polycystic Kidney Disease (ADPKD) strikes 1 in 1000 individuals, often resulting in end-stage renal failure. Mutations in either PKD1 or PKD2 account for 95% of all cases. Recently, polycystin-1 and polycystin-2 (encoded by PKD1 and PKD2, respectively) have been demonstrated to assemble to form a cation channel in vitro. We have clear evidence that the C. elegans homologs of PKD1 and PKD2, lov-1 and pkd-2, act in the same pathway in vivo. Mutations in either lov-1 or pkd-2 result in identical male sensory behavioral defects. Also, pkd-2; lov-1 double mutants are no more severe than either of the single mutants, indicating that lov-1 and pkd-2 act together. We have developed antibodies against LOV-1 and PKD-2 and find that they are expressed in the same male-specific sensory neurons and are concentrated in cilia and cell bodies. Cytoplasmic, non-nuclear staining in cell bodies is punctate, suggesting that one pool of PKD-2 is subcellularly localized in intracellular membranes while another is found in sensory cilia. In contrast to defects in the C. elegans Autosomal Recessive PKD gene osm-5, the cilia of lov-1, pkd-2, and lov-1; pkd-2 double mutants are normal as judged by electron microscopy, demonstrating that lov-1 and pkd-2 are not required for ultrastructural development of male-specific sensory cilia. Consistent with a role in sensory signal transduction, ciliary localization of PKD-2 is a prerequisite for function.

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156. Control of the backing and turning steps of male mating behavior of C. elegans.

Allyson J. Whittaker, Gary Schindelman, Shahla Gharib

Behavior in its simplest form consists of an organism sensing the environment and executing a motor output based on those sensations. The next level is coordination of distinct behaviors, that is, processing a number of signals from the environment and triggering the appropriate response. C. elegans male mating behavior provides an excellent model for studying how behavior is coordinated and also for furthering our understanding of sensory perception. Mating behavior in C. elegans consists of a series of sub-behaviors, response, backing, turning, vulva location and sperm transfer, with the initiation of one step correlating with the repression of the previous step. The hermaphrodite appears to play no active role in mating but does provide cues that trigger each step.

Understanding coordination of these behaviors will require furthering our knowledge of the molecular and neural pathways through which the different sub-steps are controlled. To this aim, we are currently doing a genetic screen to identify new mutations that effect male mating behavior. The design of the screen will also allow us to identify mutations in the hermaphrodite that effect male mating. One focus is on two of the earliest steps in mating behavior, backing and turning. Of the approximately 1300 genomes that have been screened so far, four mutations have been isolated that show consistent and significantly penetrant effects on response, backing and/or turning behavior. These mutations are being further characterized, mapped, and cloned. Additionally, a previously isolated mutation that effects response and turning behavior, cod-5, is being examined further. The role of mechanosensory and chemosensory pathways in response, backing and turning is being examined through the use of mutations that disrupt these pathways.

Previous research in our lab has established that the nine pairs of male sensory rays mediate response, backing and turning. In the absence of sensory rays, other ventral sensory structures such as the hook, post-cloacal sensillae and spicules are sufficient for these behaviors indicating redundancy of structures. The rays consist of three cells, two neurons, RnA and RnB and a structural
cell. Ablation studies will be done to determine to what degree, if any, the rays have distinct roles in the early steps of mating and if there is any functional difference between the ray neurons, RnA and RnB.

157. Characterization of the sperm transfer step of male mating behavior of C. elegans: Two sides to the story
Gary Schindelman, Allyson J. Whittaker, Shahla Gharib

C. elegans male mating behavior involves the proper execution of a series of sub-behaviors culminating in the transfer of sperm to the hermaphrodite (1). Prior to sperm transfer, the male must insert its copulatory spicules through the vulval opening into the uterus. Ablation of the SPV spicule neurons leads to premature sperm transfer at the vulva, thus the SPV neurons may regulate sperm transfer by inhibiting release (1). Once inserted into the uterus the male will not release sperm until he receives a cue from the hermaphrodite (2). This cue appears to originate in the hermaphrodite gonad, as its ablation (leaving the anchor cell intact) suppresses sperm transfer (2). This aspect of mating behavior therefore requires the perception and integration of a signal between cells of two separate organisms. We are interested in understanding this communication process. To that end we are currently doing a genetic screen designed to isolate both males and hermaphrodites defective for sperm transfer. Isolating and characterizing these mutants will help to elucidate the signaling process involved, and its regulation.

Reference

158. Regulation of multiple male C. elegans spicule muscle behaviors during mating
L. René Garcia

During mating, C. elegans males monitor the activities of hermaphrodites and react appropriately to impregnate their mates. To address how males interpret and integrate mating cues into appropriate responses, we are dissecting the spicule insertion step of mating behavior since spicule movements provide a readout for experimental manipulation. Observations of mating reveal that the spicule protractor muscles display at least three contractile behaviors. As the male tries to penetrate the vulval lips, the spicule muscles twitch at a frequency of 7 Hz. Partial spicule penetration through the vulva triggers the spicule muscles to switch from oscillating contractions to a 30 sec sustained contraction. Sustained muscle contraction can be increased for an additional 30 sec by the transfer of seminal fluid. By observing the effects of ablating male tail cells, we found that the PCA, PCB, PCC, HOA, and HOB sensory neurons initiate oscillating spicule muscle contractions, whereas SPC motor neurons trigger sustained muscle contraction. Also, modulation of sustained contraction requires the connection between the somatic gonad and the cloacal opening.

We have identified components that regulate spicule insertion by analyzing chemical agents and identifying novel genetic mutations that induce spicule muscle contraction. The acetylcholine (ACh) agonists levamisole, arecoline, nicotine, and oxotremorine induce the spicules to protract. This is consistent with our observations that the PCB, PCC, and SPV neurons express the ACh vesicular transporter UNC-17, suggesting that these cells use ACh to excite the spicule muscles. Multiple ACh receptors, defined by their agonist sensitivities, utilize different calcium channel genes to induce protraction. Levamisole receptors require *unc-68* (ryanodine receptor calcium channel): arecoline receptors require *egl-19* (voltage gated calcium channel subunit); nicotine and oxotremorine receptors require both *unc-68*, and *egl-19* to cause spicule protraction. Similar to agonist exposure, males mutant in *prc-1,-2,-3*, and –4, protract their spicules constitutively. These mutations require wild-type *egl-19* but not *unc-68* to induce the Prc phenotype. These *prc* genes might be involved in the regulation of extra-cellular calcium mobilization that is utilized by the arecoline-induced protraction pathway. During mating, *egl-19(3)* and *unc-68(null)* males show distinct defects during spicule insertion. *egl-19(3)* males produce normal oscillating spicule muscle contractions, but fail to trigger sustained contractions; whereas, *unc-68(null)* males display the opposite defect. Taken together, these observations suggest that multiple cholinergic pathways differentially regulate intra- and extra-cellular calcium to produce distinct muscle contraction behaviors during mating.

159. Characterization of Gαq-mediated signaling pathways in C. elegans
Carol A. Bastiani, Wen J. Chen, Shahla Gharib, Mel Simon

*egl-30* encodes the C. elegans homologue of the mammalian heterotrimeric G-protein alpha subunit, Gαq. Reduction-of-function and loss-of-function mutations in *egl-30* affect diverse behaviors in *C. elegans*. These include: viability, egg laying, pharyngeal pumping, movement, and spicule protraction. In a screen for suppressors of the reduction-of-function mutation, *egl-30(md186)*, two intragenic suppressor mutations and four extragenic mutations were recovered. These mutants phenotypically resemble worms that overexpress *egl-30*. The intragenic gain-of-function mutations have been used as a tool to examine interactions with mutations in genes that were expected to function in a pathway with *egl-30*, based on what is known about Gαq signaling in other organisms. Mutations that reduce signaling through the PLCβ/ IP3/ DAG pathway, the only well-characterized pathway downstream of the Gαq family, differentially suppress the activation of pharyngeal pumping and egg-laying behaviors exhibited by *egl-30* gain-of-function mutants. We conclude that there are multiple pathways downstream of EGL-30 in *C. elegans*, and that the
pathway(s) downstream of EGL-30 with respect to egg-laying behavior likely define uncharacterized pathway(s). We expect that the extragenic mutations that were isolated will aid in the characterization of additional components either downstream of EGL-30, or novel negative regulators of EGL-30.

We are also examining the interaction between the EGL-30 and Goα (GOA-1) signaling pathways in C. elegans. Mutations in the genes that encode the heterotrimeric G-protein alpha subunits, EGL-30 and GOA-1, produce opposite effects on a variety of the behaviors that they regulate: movement, egg laying, and pharyngeal pumping. Previous genetic analysis has suggested that activation of the GOA-1 signaling pathway leads to inhibition of EGL-30-mediated signaling through the RGS (regulator of G-protein signaling) protein, EAT-16 (1). Conversely, genetic epistasis suggests that another RGS protein, EGL-10, mediates inhibition of the GOA-1 signaling pathway in response to activation of EGL-30. We are further examining these interactions in mammalian cell lines. We found that cotransfection of EAT-16 with EGL-30 inhibits EGL-30-dependent activation of endogenous PLCβ in COS-7 cells and in Goα/Go11 knockout cell lines. Additionally, cotransfection of a constitutively active mutant, GOA-1(QL), enhances this inhibition. Immunoprecipitation assays reveal that both GOA-1 and EGL-30 can interact with EAT-16 and with EGL-10. These interactions are consistent with our genetic model that proposes that these RGS proteins can act as effectors for activated G proteins. We are further investigating how these interactions are regulated.

Reference

160. Worm locomotion described: A new method for extracting quantitative data on movement parameters
Jane Mendel, Saleem Mukhtar, Jehoshua Bruck, Paul Sternberg

We have developed an automated worm tracking system that enables us to record moving worms. Videos of recorded worms are analyzed by an automated worm recognition system that extracts quantitative data describing body posture as a function of time and then computes metrics for several parameters of locomotion. These metrics include frequency, which reflects muscle contractions per second, and amplitude, which reflects the degree of muscle contraction. The metrics are mathematically related but not identical to the wavelength and amplitude of the sinusoidal track left by worms on bacterial lawns. Comparison of metrics from anterior regions of the worm to those from posterior regions (the time delay) indicates the speed of propagation of the movement wave along the length of the worm. We are currently testing a variety of mutants affected in movement to both establish the limits of reliability of our analysis and to further our understanding of worm movement. For example, our analyzer has extended our understanding of movement differences in hyperactive mutants lacking goa-1. Previously, measurements using an ocular micrometer quantitated an increase in the amplitude and wavelength of tracks left by these mutants on bacterial lawns. Measurements using a key stroke recorder quantitated an increase in the number of body bends per second and an increase in the number of spontaneous reversals of direction in these hyperactive mutants. Our movement analyzer quantitated an expected increase in frequency and amplitude in these mutants, the increase in reversal frequency, and also an increase in the speed of propagation of the movement wave. The last difference is not easily detected without sophisticated quantitation. Mutations in the myosin heavy-chain gene cause differences in both amplitude and frequency. This observation is more consistent with a model in which the wave propagation involves feedback dependent on muscle contraction (as originally proposed by John White) rather than relying solely on a central pattern generator.

161. C. elegans inositol 5-phosphatase homologue IPP-5 and ovulation
Yen Kim Bui

During ovulation, the myoepithelial sheath contracts and pulls the dilating spermatheca, a bivalve muscular compartment full of sperm, over the proximal mature oocyte to expel it from the oviduct into this sac-like compartment where it is fertilized (1). Previous studies indicate the rhythmic motor pattern displayed in ovulation is dependent on LET-23/EGF receptor tyrosine kinase (RTK) signaling, which is thought to control the pattern by IP3-induced calcium release from internal stores. Worms that have reduction-of-function lin-3 or let-23 mutations are sterile because the spermatheca fails to dilate resulting in the Emo phenotype where oocytes remain trapped in the gonad arm and undergo multiple rounds of endomitotic DNA synthesis. In a genetic screen two different mutants were identified that can suppress the sterile phenotype: a gain-of-function (gf) in lfe-1(sy290), an inositol 1,4,5-triphosphate receptor (IP3 R) homologue which has been previously shown to act as a positive effector of LET-23, and a loss-of-function (lf) in lfe-2, an IP3K, which acts as a negative effector to mediate ovulation. lfe-2(lf) mutants do not have ovulation defects. This suggests that an alternate pathway to metabolize IP3 and down-regulate signaling must exist in C. elegans. The mammalian type I 5-phosphatase (5-Phs) plays a known role in IP3 metabolism by dephosphorylating the 5’ phosphate. The completely sequenced C. elegans genome contains only one ortholog (C09B8.1) of the Type I 5-phs, ipp-5. In an attempt to better understand how IP3 signaling downstream of the LET-23/RTK pathway affects ovulation and fertility, we have characterized the C. elegans ortholog of the Type I inositol polyphosphate 5-phosphatase, ipp-5. We carried out genetic analysis with a deletion mutant, sy605. The ipp-5 deletion mutant, sy605, shows a novel ovulation phenotype whereby the spermatheca extends
further to ovulate two oocytes during a single ovulation cycle; thus indicating that IPP-5 helps to regulate spermathecal contractile behavior during ovulation and is necessary to prevent hyper-extension of the spermatheca. This defect is opposite to the result of decreased EGFR function in which the spermatheca fails to dilate. Genetic analysis indicates ipp-5 acts downstream of let-23. ipp-5 suppresses the sterile defect of lin-3(rf) and let-23(rf). ipp-5 interacts with others in the let-23-mediated IP3 signaling pathway; it synergizes with let-23(gf), lfe-1/trr-1/dec-4, and lfe-2. Our data support the hypothesis that increased IP3 signaling causes increased extension of the spermatheca during ovulation and that either too much signaling or too little signaling prevents ovulation. Negative regulators that modulate signaling output are critical for precise behavioral events dependant on downstream RTK signaling. We propose that IP3 signaling levels modulate spermatheca contractions and dilations during ovulation, possibly through regulating calcium release. ipp-5, is expressed in the spermatheca, pharynx, and vulva. The expression is consistent with its function in ovulation in the adult spermatheca.

Reference
(1) McCarter et al., 1997.

162. Sphingosine phosphate lyase is required for fertility and behavior
Jane Mendel, Jianhui Zhou*, Julie D. Saba*
Eukaryotic sphingolipids are ubiquitous membrane constituents that function as second messengers. Sphingosine-1-phosphate, a breakdown product of membrane sphingolipids, has been shown to influence numerous cellular processes including cell proliferation, motility, invasiveness and programmed cell death. The actions of sphingosine-1-phosphate are mediated through two independent mechanisms: one mediated by G-proteins, and one dependent on intracellular sphingosine-1-phosphate concentrations. Sphingosine-1-phosphate levels are regulated by the actions of sphingosine kinase, which is responsible for its synthesis from sphingosine and by the two enzymes responsible for its catabolism, dihydrosphingosine phosphate phosphatase and sphingosine phosphate lyase. We are studying a C. elegans sphingosine phosphate lyase gene (spl-2). Inhibition of spl-2 expression in C. elegans through the use of RNA interference leads to highly-penetrant defects including poor feeding, slow growth, sluggish movement, slow egg-laying, and a delay in the production of mature oocytes in hermaphrodites. There are also less penetrant defects in embryonic development. We are continuing to analyze the function of sphingolipid metabolism in C. elegans by examining the expression pattern of spl-2, obtaining a null allele of this gene for further genetic studies by screening targeted deletion libraries, and cloning additional genes that regulate sphingosine-1-phosphate levels.
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163. A cyclin L homologue and a putative heat-shock transcription factor are involved in heat shock-mediated transcription in C. elegans
Yvonne M. Hajdu-Cronin, Wen J. Chen
In a screen for suppressors of activated GOA-1 (C. elegans Goα) under the control of a heat-shock promoter, we identified several genetic loci that affect heat-shock induction of GOA-1. sag-4 (linkage group IV) mutants are wild type in appearance, while sag-3 (I) and sag-5 (III) mutants are egg-laying defective. Western analysis indicated that mutations in all three genes suppress activated GOA-1 by decreasing heat-shock induced protein expression. Although endogenous GOA-1 expression is not affected, heat-shock induction of GOA-1 decreased in the suppressor strains. We cloned sag-4 and found that it encodes a cyclin homologue most similar to cyclin L, a novel type of cyclin with unknown function, but also similar to cyclin T, K or C, which was identified as a subunit of TFIIH, part of the RNA polymerase II complex that functions in basal transcription. Only transgenes with the hsp16-2 promoter can be affected by mutations in sag-4. These results suggest that SAG-4 is necessary for heat shock-mediated transcription in C. elegans. We propose that cyclin L is the type of cyclin acting in TFIIH during heat shock-induced mRNA transcription and that it functions similarly to cyclin T, K or C during basal transcription. We also found that mutations in sag-3 and sag-5, as well as sag-4, were able to suppress another transgene under the control of the hsp16-2 promoter. The suppression by sag-3 was especially striking. We have used single nucleotide polymorphisms to map sag-3 more precisely and found that it maps to a small region (Y53C10A) that includes a putative heat shock transcription factor. We have sequenced this candidate gene in the sag-3 mutant and have found that the sy441 mutation causes a truncation in the heat shock factor (W585stop). sag-5 has been mapped to a small interval on the left end of III, between unc-45 and daf-7. The molecular nature of sag-5 has not yet been determined.

164. WormBase
Wen Chen, Raymond Lee, Erich Schwarz, Juan Carlos Chan, Hans-Michael Müller, Eimear Kenny
WormBase (http://www.wormbase.org) is the successor to the C. elegans genomic database ACeDB. Its two fundamental goals are to make the C. elegans database more accessible through the Web, and to expand the database from gene structures to gene functions, networks, cellular expression patterns, and biological processes. A central element of WormBase is Ph.D.-level curation of the literature and of large-scale databases. Other goals of WormBase include: improving the accuracy of gene structures through literature curation and reanalysis of ESTs; general improvement of the Web interface through direct user feedback; an up-to-date user guide; a systematic checklist of literature curation topics (gene function and structure, transgene composition and gene expression
165. Finding new neuronal genes by microdissection and single-cell RT-PCR

Erich Schwarz, Miriam Goodman1, Mark Palfreyman2, Tali Melkman2, Martin Chalfie1, Piali Sengupta2

We intend to define the gene expression profiles of individual sensory neurons, and thus identify genes required for the specific sensory functions of each neuron type. To begin doing this, we have microdissected single GFP-labeled sensory neurons (AFD, ASER, AWA and AWC) using a modification of techniques previously used in single-cell neuronal recordings. We adapted the method of C. Dulac to carry out RT-PCR on single dissected cells. We validated RT-PCRs by secondary PCR with oligonucleotides directed against adjacent 3' exons of GFP, and of known cell-specific genes. In AFD, GFP and the AFD-specific gene gcy-8 were detected in 68% (32/47) and 19% (9/47) of dissected cells, respectively. In ASER, we had similar results for GFP (80%: 8/10) and ASER-specific gcy-5 (20%: 2/10). In AWC, GFP was detected in 80% (4/5) of dissected cells; in AWA, the AWA-specific odr-10 olfactory receptor gene was detected in 30% (1/3) of cells. Cross-contamination was assessed by amplification of GFP and cell-specific transcripts from RT-PCRs of adjacent unidentified cells, dissected as controls. 2/18 control cells were positive for GFP; 0/18 for gcy-5 or gcy-8. We also tested for the expression of potential thermosensory genes in AFD. AFD appears not to strongly express most members of the osm-9/trp superfamily, or homologs of most Drosophila genes used in visual transduction. Instead, RT-PCR detects expression of at least three membrane guanylate cyclases (gcy-8, gcy-23, and gcy-26), the most known for any C. elegans sensory neuron. We are currently expanding our search for new sensory genes by constructing AFD, AWA and AWC-specific cDNA libraries. Differential screening among these libraries should identify new genes specific to individual thermosensory and chemosensory neurons.

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pattern, cellular function) that allows rapid dissection of literature into specialized areas of curator expertise; annotation of protein-coding sequences gene ontology, conserved orthologous groups, and Interpro motifs; construction of a broadly usable structured vocabulary for detailed stages of embryonic and post-embryonic development; and incorporating several large-scale data sets (SNPs, mass RNAi phenotypes, and neural wiring diagrams) into WormBase. Presently, we have curated ~7% of the C. elegans literature; annotated 700 gene expression patterns; imported phenotypes of >2000 genes inactivated by RNAi; and computationally mapped gene ontology (GO) descriptions onto >6000 genes. Our current work includes continuing to curate the C. elegans literature, devising several computational tools to automate curation, and systematic extension of GO to all C. elegans genes.
Biochemical and genetic studies of the N-end rule pathway and other aspects of the Ub system in yeast and mammals continue to be a major theme of our work.

166. Altered activity, social behavior, and spatial memory in mice lacking the NTAN1 amidase and the asparagine branch of the N-end rule pathway

Yong Tae Kwon, Seth A. Balogh1, Ilia V. Davydov2, Anna S. Kashina, Jeong Kyo Yoon, Youming Xie, Arti Gaur 3, Lynn Hyde1,4, Victor H. Denenberg1,5, Alexander Varshavsky

The N-end rule is organized hierarchically. In S. cerevisiae, Asn and Gln are tertiary destabilizing N-terminal residues in that they function through their conversion, by enzymatic deamidation, into the secondary destabilizing N-terminal residues Asp and Glu, whose activity requires their conjugation, by Arg-tRNA-protein transferase (R-transferase), to Arg, one of the primary destabilizing N-terminal residues. The primary destabilizing residues are bound directly by N-recognin (E3), the recognition component of the N-end rule pathway. In metazoans such as mammals, but not in yeast, Cys is yet another secondary destabilizing residue. Previous work (Grigoryev et al., 1996) described the isolation and analysis of the mouse NTAN1 cDNA and gene that encode the Asn-specific N-terminal amidase (NiN-amidase). This enzyme is a component of the mammalian N-end rule pathway that confers short half-lives on N-end rule substrates bearing N-terminal Asn. Specifically, deamidation of N-terminal Asn by NiN-amidase leads to the arginylation of the resulting N-terminal Asp by the ATE1-encoded Arg-tRNA-protein transferase (R-transferase) (see Abstract 167). The resulting N-terminal Arg is bound by N-recognin, the UBR1-encoded E3 enzyme of the N-end rule pathway. The ~17 kb NTAN1 gene contains ten exons, ranging from 54 to 177 bp in length. The deduced amino acid sequence of mouse NiN-amidase bears no significant similarity to the sequence of NTA1, the NTAN1-encoded Nt-amidase of the yeast S. cerevisiae that can deamidate either N-terminal Asn or N-terminal Gln (Baker and Varshavsky, 1995). There are putative homologs of mammalian NTAN1 in the plant A. thaliana and the fly D. melanogaster. In mouse embryos, NTAN1 is strongly expressed in the branchial arches, and in the tail and limb buds, similarly to the expression pattern of the UBR1 gene. To address the function of NTAN1, we constructed NTAN1(-/-) mouse strains, using targeted mutagenesis in mouse embryonic stem (ES) cells (Kwon et al., 2000). The NTAN1(-/-) mice were fertile and outwardly normal. The relative levels of ATE1 and UBR1 transcripts remained unaltered in NTAN1(-/-) mice. NTAN1(-/-) embryonic fibroblasts (EFs) lacked the enzymatic activity of NiN-amidase but retained the enzymatic activity of a Gln-specific Ni-amidase (NiQ-amidase). In addition, an Asn-bearing N-end rule substrate was metabolically stable in NTAN1(-/-) EF cells, whereas Gln- and Arg-bearing substrates remained short-lived. The still uncloned...
NtO-amidase must therefore be encoded by a gene distinct from \textit{NTAN1} (instead of being derived, for example, from a differentially spliced variant of \textit{NTAN1} mRNA). The \textit{NTAN1}(-/-) mice differed from their congenic wild-type counterparts in spontaneous activity, spatial memory, and a socially conditioned exploratory phenotype that has not been previously described with other mouse strains (Kwon et al., 2000).

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References


167. The mouse \textit{ATE1} gene, encoding Arg-tRNA-protein transferases of the N-end rule pathway, is required for cardiogenesis and angiogenic remodeling

Yong Tae Kwon, Anna Kashina, Rong-Gui Hu, Jee Young An, Jai Wha Seo, Ilia Davydov1, Alexander Varshavsky

In vertebrates, the set of secondary destabilizing residues (see Abstract 166) contains not only Asp and Glu but also Cys, the latter being a stabilizing residue in the yeast N-end rule. The two known species of mammalian Arg-tRNA R-transferases (R-transferases), ATE1-1 and ATE1-2, are produced through alternative splicing of \textit{ATE1} pre-mRNA (Kwon et al., 1999). The ratio of \textit{ATE1}-1 to \textit{ATE1}-2 mRNA varies greatly among the mouse tissues: it is ~0.1 in the skeletal muscle, ~0.25 in the spleen, ~3.3 in the liver and brain, and ~10 in the testis, suggesting that the two R-transferases are functionally distinct. However, the substrate specificities of ATE1-1 and ATE1-2 are similar to that of the \textit{ATE1}-encoded R-transferase of \textit{S. cerevisiae}, in that they can arginylate N-terminal Asp and Glu, but cannot arginylate N-terminal Cys (Kwon et al., 1999). More recently, it was found (I. Davydov, unpublished data) that mouse \textit{ATE1} may contain an additional exon(s) upstream of the previously inferred start codon. This exon(s) is being mapped. Recent deletion analysis of the mouse \textit{ATE1} gene has shown that \textit{ATE1}(-/-) cells are incapable of arginyllating all three of the secondary destabilizing N-terminal residues, Asp, Glu and Cys, raising the possibility that N-terminal Cys may undergo an enzymatic modification that converts it into a substrate of ATE1 (Kwon et al., 2002). \textit{ATE1}(-/-) embryos die in \textit{utero} around day E15, primarily from hemorrhages. \textit{ATE1}(-/-) embryos exhibit defective cardiogenesis that involves hypoplasia of the ventricular compact zone, a ventricular and atrial septal defect, and insufficient septation between outflow tracts. The absence of \textit{ATE1} also caused defective angiogenic remodeling in the yolk sac and embryo proper. Thus, \textit{ATE1} is required for cardiovascular development, a new set of functions of the N-end rule pathway. Further genetic and biochemical investigations of the \textit{ATE1}-encoded mouse R-transferases and their physiological substrates are under way.

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References


168. Construction and analysis of mouse strains lacking the ubiquitin ligase \textit{UBR1} (E3α) of the N-end rule pathway

Yong Tae Kwon, Zanzxian Xia, Ilia Davydov1, Alexander Varshavsky

In mammals, \textit{UBR1}/E3α is a major recognition component of the N-end rule pathway. We have cloned and characterized the mouse \textit{UBR1} gene and cDNA (Kwon et al., 1998). Mouse \textit{UBR1} was expressed in \textit{ubr1Δ \textit{S. cerevisiae}}, and was found to be capable of rescuing the activity of the N-end rule pathway in yeast, provided that mouse mHR6A or mHR6B (E2-14K) Ub-conjugating (E2) enzyme was co-expressed as well. (The endogenous yeast RAD6 E2 did not support the complementing activity of mouse \textit{UBR1} in \textit{ubr1Δ \textit{S. cerevisiae}}.) To address the function of \textit{UBR1}, we
constructed UBR1(-/-) mouse strains, using targeted mutagenesis in ES cells (Kwon et al., 2001). The resulting deletion/disruption allele of UBR1 lacked the segment encoding both type 1 and type 2 substrate-binding sites of UBR1. UBR1(-/-) mice lacked the UBR1 protein, were slightly smaller that congenic +/+ mice, but did not exhibit significant impairments in motor coordination. The expression of UBR2 and UBR3 mRNAs was slightly increased in the skeletal muscle of ad libitum-fed UBR1(-/-) mice, in comparison to congenic +/+ mice. In fasting mice, the expression of UBR1, UBR2, and UBR3 mRNAs was significantly increased at least in the skeletal muscle. The magnitude of this increase, for UBR2 and UBR3 mRNAs, was larger in the skeletal muscle of fasting UBR1(-/-) mice, suggesting regulatory interactions among UBR1, UBR2 and UBR3. Although UBR1(-/-) embryonic fibroblast (EF) cells lacked UBR1, the activity of the N-end rule pathway was found to be essentially unchanged in these cells, in comparison to +/+ EFs. Thus, there must exist at least one other functional counterpart of UBR1 that is expressed at least in EF cells. (Recent work has shown it to be UBR2; see Abstract 4.) On the other hand, experiments with muscle extracts from UBR1(-/-) mice indicated the absence of the N-end rule pathway from these extracts, in contrast to otherwise identical extracts from congenic +/+ mice. The absence of UBR1 resulted in growth retardation throughout embryonic development; the 10-20% difference in mass between newborn UBR1(-/-) and +/+ pups was retained in adult mice as well. In UBR1(-/-) mice, the expression of fatty acid synthase mRNA was reduced, consistent with their (disproportionately) lower fat content. In addition, the expression of fatty acid synthase was not shut down upon fasting in UBR1(-/-) mice, in contrast to +/+ mice. We also analyzed plasma chemistry in +/+ and UBR1(-/-) mice, and found significant hypoglycemia in the latter, under both normal and starvation conditions (Kwon et al., 2001). Our data strongly suggest that UBR1(-/-) mice are mosaics in regard to the activity of the N-end rule pathway, owing to differential expression of E3(s) that can substitute for the ubiquitin ligase UBR1 (E3a).

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169. Male infertility and female lethality in mice lacking the ubiquitin ligase UBR2 of the N-end rule pathway
Yong Tae Kwon, Zanxian Xia, Ilia Davydov1, Jee Young An, Jai Wha Seo, Alexander Varshavsky
The absence of gross defects in UBR1(-/-) mice suggested that the E3 function of the mouse N-end rule pathway is mediated by at least two functionally overlapping genes, one of which is UBR1. The previously identified mouse gene, termed UBR2, encodes a 200 kD protein highly similar to UBR1 (Kwon et al., 1998). UBR2(-/-) mouse strains were recently constructed and characterized, revealing a sex-specific phenotype: male infertility and female lethality. The relative frequency of UBR2(-/-) males produced from heterozygous (+/- × +/+) matings was similar to that of +/+ males. Adult UBR2(-/-) males were outwardly normal, as judged by body weight and general behavior, but exhibited severe defects in spermatogenesis and were completely sterile. Until 4 weeks after birth, the size of their testes was close to that of congenic +/+ mice. However, during the 5th and 6th weeks, the mass of UBR2(-/-) testes decreased by ~2-fold. The degeneration of testes in UBR2(-/-) mice was caused by massive apoptosis of spermatocytes and their progeny, round spermatids. The number of apoptotic germ-line cells in the mutant testes dramatically increased at week 5 after birth. The first detectable abnormality of germ cell differentiation in the UBR2(-/-) testes was a much lower number of early meiotic spermatocytes at week 2. At week three, when meiotic divisions of spermatocytes produce round spermatids, ~2,800 round spermatids per 100 seminiferous tubules were observed in the +/+ testes, but virtually no round spermatids could be detected in the mutant testes. This and related data suggested that defective meiosis in UBR2(-/-) male mice was the primary cause of their sterility. Despite the meiotic defect, a small number of cells that appeared to be round spermatids could be detected in the UBR2(-/-) testes after week 4. However, most of these cells did not continue to differentiate, and died through apoptosis, together with meiotic spermatocytes, after week 6. As a result, the testes of two months old UBR2(-/-) males were severely shrunk and vacuolized; they nearly completely lacked spermatids and spermatozoa. In contrast, the spermatogonia, Sertoli cells, and Leydig cells remained apparently intact in the UBR2(-/-) testes. Thus, the absence of UBR2 (but not of its close homolog UBR1; see Abstract 168) leads to a severe defect in spermatogenesis which involves primarily, if not exclusively, a meiotic defect. Further analysis of this phenotype showed that UBR2(-/-) spermatocytes are arrested largely at the pachytenic stage of meiosis, and fail to form the synaptonemal complex that holds together the homologous chromosomes and is essential for meiotic recombination. Recent GST-pulldown binding assays with purified N-end rule substrates and extracts from S. cerevisiae overexpressing mouse UBR2 have shown that UBR2 can recognize N-degrons in vitro (see Abstract
182). Thus, mouse UBR2 functions in vivo as an E3 component of the N-end rule pathway that functionally overlaps with the previously known E3 of this pathway, UBR1 (E3α).

Yet another feature of the UBR2 (-/-) phenotype was extremely low frequency of the UBR2(-/-) female progeny from heterozygous (+/- × +/-) matings, in contrast to the normal (Mendelian) frequency of UBR2 (-/-) males. Strikingly, the rare UBR2(-/-) females that did get born were fertile and apparently normal otherwise. This phenotype is being analyzed. It is likely to arise from an impaired X-chromosome inactivation in early UBR2(-/-) embryos.

To investigate the functional interaction of UBR1 and its close homolog UBR we used [UBR1(+/+) UBR2(+/+)] mice to produce [UBR1(-/-) UBR2(+/+)] mice or cells. Disruption of both UBR1 and UBR2 was lethal: none of the [UBR1(-/-) UBR2(-/-)] embryos survived beyond day E12 (Y.T. Kwon and J. W. Seo, unpublished data). Whereas the double-mutant male embryos died at ~E9.5, most of their female counterparts died significantly earlier. Remarkably, UBR1(-/-)UBR2(-/-) embryonic fibroblasts did contain the N-end rule pathway, albeit of significantly lower activity, indicating the presence of at least one other (third) E3 component of this pathway.

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Reference

170. Construction and analysis of mouse strains lacking the ubiquitin ligase UBR3

Takafumi Tasaki, Yong Tae Kwon, Alexander Varshavsky

Kwon et al. (1998) identified two distinct mouse (and human) genes, termed UBR2 and UBR3, which encode proteins that are significantly similar to mouse UBR1 (E3α), the previously characterized E3 of the N-end rule pathway (see Abstracts 169). In contrast to the highly similar mouse UBR1 and UBR2 (47% identity and 68% similarity), the mouse UBR3 protein, while clearly a member of the UBR family, is less similar to UBR1 (25% identity and 51% similarity) and UBR2 (25% identity and 48% similarity) than they are to each other. In addition, mouse UBR3 lacks some of the residues in its N-terminal region that have been shown to be essential for the function of yeast UBR1, and are also present in mouse (and human) UBR1 and UBR2. We mapped and partially sequenced the mouse UBR3 gene, and more recently constructed UBR3(-/-) mouse strains (T. Tasaki and Y.T. Kwon, unpublished data). UBR3(-/-) mice are viable, and are being characterized.

Reference

171. Biochemical and genetic dissection of S. cerevisiae UBR1

Alisa Webster1, Michel Ghislain2, Fangyong Du, Alexander Varshavsky

In S. cerevisiae, the targeting machinery of the N-end rule pathway consists of UBR1 (called N-recoginin or E3) in a complex with UBC2, a specific E2 enzyme (Madura et al., 1993). The pathway’s other targeting components are the Arg-tRNA-protein transferase (R-transferase) ATE1, and the N-terminal amidohydrolase NTA1, which are required for the degradation of proteins bearing, respectively, the secondary (Asp, Glu) and tertiary (Asn, Gln) destabilizing N-terminal residues. Earlier dipeptide inhibition experiments identified two distinct substrate-binding sites of UBR1 (Gonda et al., 1989; Baker and Varshavsky, 1991). The type 1 site recognizes polypeptides bearing positively charged N-terminal residues (Arg, Lys and His). The type 2 site recognizes polypeptides bearing bulky hydrophobic N-terminal residues (Leu, Ile, Tyr, Phe and Trp). We employed genetic and biochemical methods to map these substrate-binding sites of UBR1.

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

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References


172. Peptides accelerate their uptake by activating the N-end rule pathway
Glenn Turner, Fangyong Du, Alexander Varshavsky

Previous work (Byrd et al., 1998) has shown that the UBR1-dependent N-end rule pathway regulates the import of di- and tripeptides in S. cerevisiae through the degradation of CUP9, a 35-kD transcriptional repressor of PTR2, which encodes peptide transporter (Alagramam et al., 1995). In the absence of UBR1, CUP9 accumulates to concentrations high enough to repress the expression of PTR2 to undetectable levels. Consequently, ubr1Δ cells are unable to import peptides. The UBR1-CUP9-PTR2 circuit is the first example of a physiological process controlled by the N-end rule pathway. In contrast to canonical N-end rule substrates, which are targeted by UBR1 through their destabilizing N-terminal residues, CUP9 is targeted by UBR1 through a distinct degron located near its C-terminus (see Abstract 173). UBR1 interacts with CUP9 through a separate substrate-binding site of UBR1, distinct from its type 1 and type 2 sites, which bind destabilizing N-terminal residues of N-end rule substrates. We discovered a physiologically critical property of the UBR1-CUP9-PTR2 regulation that involves the allosteric activation of UBR1 by imported dipeptides (Turner et al., 2000). In the resulting positive feedback circuit, imported dipeptides bind to the type 1 or type 2 sites of UBR1 and accelerate the UBR1-dependent degradation of CUP9, thereby derepressing the expression of PTR2 and increasing the cell’s capacity to import peptides. These findings identified the physiological rationale for the targeting of CUP9 by UBR1. They also established, for the first time, that the activity of an E3 can be directly linked to the presence of an environmental signal through an allosteric interaction with a small compound. As most cells have the capacity to import peptides, the results of this work suggest that peptide import may be regulated similarly by UBR1 homologs in mammalian cells (Du et al., 2001), we discovered that dipeptides cause dissociation of the C-terminal autoinhibitory domain of UBR1 from its N-terminal region that contains substrate-binding sites. Moreover, this dissociation, which allows high-affinity interaction between UBR1 and CUP9, is strongly increased only if both type 1 and type 2 binding sites of UBR1 are occupied by dipeptides (Du et al., 2001).

Autoinhibition is an essential feature of many regulatory proteins, including protein kinases and phosphatases, nitric oxide synthases, transcription factors, and regulators of actin polymerization. Autoinhibition has not been reported previously for Ub ligases. Mammalian cells express at least three distinct homologs of S. cerevisiae UBR1 that contain the conserved UBHC, RING-H2, and UBLC domains (Kwon et al., 1998), suggesting that these metazoan E3s are likely to be regulated through autoinhibition. Our findings also indicate that natural compounds (either small molecules or proteins) may regulate the activity of diverse Ub ligases through the abrogation or induction of autoinhibition.

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References

173. Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain
Fangyong Du, Federico Navarro-Garcia1 Alexander Varshavsky

Two binding sites of the S. cerevisiae Ub ligase UBR1, the E3 of the yeast N-end rule pathway, recognize basic (type 1) and bulky hydrophobic (type 2) N-terminal residues (see Abstracts 171 and 172). Previous work (Byrd et al., 1998; Turner et al., 2000; see Abstract 172) has shown that UBR1 mediates the Ub/proteasome-dependent degradation of CUP9, a transcriptional repressor of the PTR2 peptide transporter. These earlier studies also demonstrated that dipeptides with destabilizing N-terminal residues allosterically accelerate the in vivo degradation of CUP9, which is recognized by a third substrate-binding site of UBR1, located downstream from its type 1 and type 2 sites. In the present work (Du et al., 2001), we discovered that dipeptides cause dissociation of the C-terminal autoinhibitory domain of UBR1 from its N-terminal region that contains substrate-binding sites. Moreover, this dissociation, which allows high-affinity interaction between UBR1 and CUP9, is strongly increased only if both type 1 and type 2 binding sites of UBR1 are occupied by dipeptides (Du et al., 2001).

Autoinhibition is an essential feature of many regulatory proteins, including protein kinases and phosphatases, nitric oxide synthases, transcription factors, and regulators of actin polymerization. Autoinhibition has not been reported previously for Ub ligases. Mammalian cells express at least three distinct homologs of S. cerevisiae UBR1 that contain the conserved UBHC, RING-H2, and UBLC domains (Kwon et al., 1998), suggesting that these metazoan E3s are likely to be regulated through autoinhibition. Our findings also indicate that natural compounds (either small molecules or proteins) may regulate the activity of diverse Ub ligases through the abrogation or induction of autoinhibition.

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References


174. **Physical association of ubiquitin ligases and the 26S proteasome**

**Youming Xie, Alexander Varshavsky**

The Ub system recognizes degradation signals of the target proteins through the E3 components of E3-E2 Ub ligases. A targeted substrate bears a covalently linked multi-Ub chain and is degraded by the ATP-dependent 26S proteasome, which consists of the 20S core protease and two 19S particles. The latter mediate the binding and unfolding of a substrate protein before its transfer to the interior of the 20S core. It is unclear how a targeted substrate is delivered to the 26S proteasome, inasmuch as RPN10, the only known proteasomal subunit that binds multi-Ub chains, has been found to be not essential for degradation of many proteins in the yeast *Saccharomyces cerevisiae*. We discovered that UBR1 and UFD4, the E3 components of two distinct Ub ligases, directly interact with the 26S proteasome (Xie and Varshavsky, 2000). Specifically, UBR1 was shown to bind to the RPN2, RPT1 and RPT6 proteins of the 19S particle, and UFD4 was shown to bind to RPT6. These and related results suggested that a targeted substrate can be delivered to the proteasome either through a substrate-linked multi-Ub chain or through a substrate-bound E3 protein, the two routes being functionally complementary. This model, which is being verified, can account for the otherwise puzzling finding that a deletion of the currently known Ub-binding subunit from the 26S proteasome does not abolish Ub-dependent proteolysis. More recent work mapped the proteasome-interacting region of UFD4 to its first ~200 residues, and showed that a truncated UFD4 lacking this region is capable of mediating multiubiquitylation of its substrate protein, but is impaired in mediating its proteasome-dependent degradation (Y. Xie and A. Varshavsky, unpublished data).

**Reference**


175. **Characterization of the yeast ubiquitin ligase UBR2**

**Hai Rao, Youming Xie, Yong Tae Kwon, Alexander Varshavsky**

The 190 kD UBR2 is the only *S. cerevisiae* protein that is highly similar in sequence to the 225 kD UBR1, the E3 of the N-end rule pathway. We found, using two-hybrid assay, that UBR2 interacts with the Ub-conjugating enzyme RAD6, the E2 enzyme of the N-end rule pathway. In contrast to UBR1, UBR2 does not recognize N-end rule substrates. Moreover, overexpression of UBR2 partially inhibits the N-end rule pathway, presumably because of competition for the RAD6 E2 enzyme, a ligand in common between UBR1 and UBR2. Conversely, the N-end rule pathway is more active in a ubr2Δ strain. Phenotypes of mutants overexpressing or lacking UBR2 suggest a role for UBR2 in sporulation. Physiological substrates and degrons recognized by this UBR1-like E3 enzyme remain to be identified.

176. **Detecting and measuring cotranslational protein degradation *in vivo***

**Glenn Turner, Alexander Varshavsky**

Nascent polypeptides emerging from the ribosome and not yet folded may transiently present degradation signals similar to those recognized by the ubiquitin system in misfolded or otherwise damaged proteins. Thus, the folding of nascent proteins, including abnormal ones, may be in kinetic competition with pathways that target these proteins for degradation. It has been a long-standing question whether a significant fraction of nascent polypeptides is cotranslationally degraded *in vivo*. Determining whether nascent (being synthesized) polypeptides are actually degraded *in vivo* has been difficult because at any given time the nascent chains of a particular protein species are of different sizes, and therefore would not form a band upon electrophoresis in a conventional pulse-chase assay. The ubiquitin (Ub) sandwich technique (Turner and Varshavsky, 2000) makes it possible to detect cotranslational protein degradation by measuring the steady-state ratio of two reporter proteins whose relative abundance is established co-translationally. The polypeptide to be examined for cotranslational degradation, termed B, is sandwiched between two stable reporter domains A and C in a linear fusion protein. The three polypeptides are connected via Ub moieties to create a fusion protein of the form AUb-BUb-CUb. Ub-specific processing proteases (UBPs) cotranslationally cleave such linear Ub fusions after the last residue of Ub, yielding the independent polypeptides A Ub, B Ub and C Ub. UBP-mediated cleavage establishes a kinetic competition between two mutually exclusive events during the synthesis of the A Ub, B Ub and C Ub fusion: cotranslational UBP cleavage at the BUb-CUb junction to release the long-lived Cub module or, alternatively, cotranslational degradation of the entire BUb-CUb nascent chain by the 26S proteasome. In the latter case, the processivity of proteasome-mediated degradation results in the destruction of the Ub moiety between B and C before it can be recognized by UBP s. The resulting drop in levels of the Cub module relative to levels of AUb, referred to as the C/A ratio, reflects the cotranslational degradation of domain B. We used the Ub sandwich technique to demonstrate that more than 50% of nascent protein molecules bearing an N-terminal degradation signal can be degraded cotranslationally, never reaching their mature
size before their destruction by processive proteolysis (Turner and Varshavsky, 2000). If cotranslational degradation of natural proteins is found to be extensive \textit{in vivo}, it could be accounted for as an evolutionary trade-off between the necessity of identifying and destroying degron-bearing mature proteins and the mechanistic difficulty of distinguishing between posttranslationally and cotranslationally presented degrons. Conditional degradation signals that require post-translational phosphorylation for activation, such as those in $\alpha\beta\kappa\beta$-catenin, may have the additional feature of not contributing to cotranslational proteolysis. Co-translational protein degradation may also represent a previously unrecognized form of protein quality control, which destroys nascent chains that fail to fold correctly. These and other questions about physiological aspects of cotranslational protein degradation can now be addressed directly in living cells through the Ub sandwich technique.

Reference


177. Using microarray hybridization techniques to identify the functions of the N-end rule pathway

Hai Rao, Alexander Varshavsky

The aim of these experiments is to identify \textit{S. cerevisiae} mRNAs whose \textit{in vivo} concentrations differ significantly between wild-type cells and their congenic counterparts lacking a component of the N-end rule pathway. The power of this approach stems in part from efficiency of the recently developed methods for identifying differentially expressed genes, and also from the fact that an over- or under-expression, of a specific mRNA species from N-end rule-lacking cells should be easy to detect, whereas the mutant phenotype caused by this over- or under-expression may be far from obvious and difficult to detect. Our first target were \textit{ubr1}$\Delta$ cells, which lack the main recognition component of the N-end rule pathway. Using microarray hybridization techniques developed by Affymetrix, Inc., we have detected, in a collaboration with Affymetrix, several yeast genes whose expression differed from two- to at least ten-fold between congenic \textit{UBR1} and \textit{ubr1}$\Delta$ strains. To identify physiological substrates of the N-end rule pathway that underlie these differences in expression of specific genes between \textit{UBR1} and \textit{ubr1}$\Delta$ backgrounds, we plan to carry out suppressor screens analogous to those that yielded CUP9 as a UB1 substrate and the regulator of peptide import in \textit{S. cerevisiae} (Abstract 172).

178. On the mitochondrial function of N-terminal amidase NTA1 in \textit{S. cerevisiae}

Hong-Rui Wang, Alexander Varshavsky

The \textit{NTA1}-encoded N-terminal amidohydrolase (Nt-amidase) of \textit{S. cerevisiae} deamidates N-terminal Asn or Gln but not internal Asn and Gln in polypeptides. Another budding yeast, \textit{K. lactis}, contains a sequence homolog of \textit{S. cerevisiae} NTA1 (S. Grigoryev and A. Varshavsky, unpublished data), whereas mammals contain two distinct Nt-amidases specific, respectively, for N-terminal Asn and Gln (see Abstract 166). \textit{nta1}$\Delta$ \textit{S. cerevisiae} cannot degrade N-end rule substrates bearing N-terminal Asn, but appear phenotypically normal otherwise. Our experiments were prompted by the observation of two in-frame ATG start codons in the \textit{NTA1 ORF} (Baker and Varshavsky, 1995). These codons are separated by a region that resembles a mitochondrial translocation signal. Specifically, the 5' mapping showed that a significant fraction of \textit{NTA1} mRNAs has its 5' ends immediately upstream (or even downstream) of the inferred (+1) \textit{NTA1} start codon. Thus, there exist \textit{NTA1} mRNAs that either lack the (+1) start codon or contain it too close to the 5' end of the message for efficient initiation of translation at that position. An in-frame ATG is present 30 bp downstream of the (+1) \textit{NTA1} start codon. Initiation of translation at this (+31) ATG should yield a protein that lacks the first ten residues of the inferred NTA1. The (+31) ATG lies within a favorable context for translation initiation, with A's in positions -3 and +4 (AAGATGA), whereas the (+1) ATG is located in a less favorable context, with pyrimidines at -3 and +4 (TGAATGC). The amphipatic 29-residue region between Asp-4 and Asp-34 in the larger NTA1 strongly resembles mitochondrial translocation signals (Baker and Varshavsky, 1995). To address the possibility that NTA1 is present in both the cytosol and mitochondria, plasmids that expressed FLAG-tagged NTA1-GFP (green fluorescent protein) fusions from the natural NTA1 promoter were constructed, in which either the "upstream" (+1) or the "downstream" (+31) ATG codon of \textit{NTA1} was converted to another codon. The NTA1-GFP derived from the unaltered \textit{NTA1 ORF} was found to be located in both mitochondria and the cytosol, with the mitochondrial species being apparently predominant (H.R. Wang and A. Varshavsky, unpublished data). These findings should contribute to the eventual understanding of the function of Nt-amidase in mitochondria.

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Reference


179. Mouse RGS4 is arginylated and degraded by the N-end rule pathway \textit{in vitro}

Ilia Davydov, Jun Sheng, Alexander Varshavsky

We used an expression-cloning screen in reticulocyte lysate to search for mouse proteins that are degraded by the ubiquitin/proteasome-dependent N-end rule pathway. One substrate thus identified was RGS4, a member of the RGS family of GTPase-accelerating proteins (GAPs) that down-regulate specific G proteins. A determinant of the RGS4 degron was located at the N-terminus of RGS4, because converting cysteine-2 to
either glycine, alanine or valine completely stabilized RGS4. Radiochemical sequencing indicated that the N-terminal methionine of the lysate-produced RGS4 was replaced with arginine. Since N-terminal arginine is a destabilizing residue not encoded by RGS4 mRNA, we concluded that the degron of RGS4 is generated through the removal of N-terminal methionine and enzymatic arginylation of the resulting N-terminal cysteine. RGS16, another member of the RGS family, was also found to be an N-end rule substrate (Davydov and Varshavsky, 2000). RGS4 that was transiently expressed in mouse L cells was short-lived in these cells. However, the targeting of RGS4 for degradation in this in vivo setting involved primarily another degron, because N-terminal variants of RGS4 that were stable in reticulocyte lysate remained unstable in L cells. We also expressed mouse RGS4 and its N-terminus-altered variants in Xenopus oocytes. In contrast to the results with mouse L cells, the degradation of RGS4 in oocytes was mediated primarily, if not exclusively, by its cysteine-based N-degron (unpublished data).

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Reference

180. Dissection of c-MOS degron
Jun Sheng, Alexander Varshavsky

The MOS proto-oncogene product, c-MOS, is a Ser/Thr kinase that is normally expressed in germ line cells, and functions as a MAPKKK (mitogen-activated protein kinase kinase kinase). A major function of c-MOS (hereafter MOS) in females is the control of maturation of oocytes through activation of a specific MAP kinase cascade. This control is attained in part through regulation of the metabolic stability of MOS. Specifically, Xenopus MOS is short-lived during meiosis I but is stabilized during the metaphase of meiosis II, maintaining a mature unfertilized egg at metaphase II until fertilization, when MOS becomes short-lived again. Previous work (Nishizawa et al., 1992, 1993) suggested that the degradation of Xenopus MOS is mediated by a degron whose essential determinant is the N-terminal Pro of MOS (Pro-2 in the MOS ORF). The authors further suggested that phosphorylation of Ser-2 (Ser-3 in the MOS ORF) down-regulates the activity of this degron. If N-terminal Pro of MOS were indeed an E3-recognized determinant of the MOS degron, this degradation signal would be, by definition, a version of N-degron, a class of degradation signals recognized by the N-end rule pathway. (Pro becomes the first residue of the nascent, Met-Pro-bearing MOS after the cotranslational removal of N-terminal Met by Met-specific aminopeptidases.) At the same time, earlier studies by this laboratory, using engineered reporter proteins in both yeast and mammalian cells, suggested that N-terminal Pro is a stabilizing residue in the N-end rule. The aim of our MOS work was to advance the understanding of MOS degron, and thereby to address the above contradiction. Our results confirmed and extended the earlier finding that the presence of N-terminal Pro was essential for the MOS degradation in oocytes, in that the replacement of N-terminal Pro with Gly, a stabilizing residue in the N-end rule, rendered MOS long-lived in oocytes. However, we then found that the above replacement converted MOS into a long-lived protein not because a cognate E3 required Pro at the N-terminus of MOS, but because N-terminal Pro, in contrast to N-terminal Gly, impeded the degradation-inhibiting phosphorylation of MOS at Ser-2 (Ser-3 in the MOS ORF). More precisely, we showed that the steady-state level of degradation-inhibiting MOS phosphorylation at Ser-2 was significantly higher with MOS bearing N-terminal Gly instead of N-terminal Pro, thus accounting for stability of MOS bearing N-terminal Gly. We also showed that the (currently unknown) E3 that recognizes the N-terminus-proximal degron of MOS does not depend on the presence of N-terminal Pro for this recognition. Thus, N-terminal Pro of MOS is important for modulating, through phosphorylation, the activity of MOS degron, but N-terminal Pro is not, by itself, the degron’s recognition determinant. In other words, the degron of MOS is not an N-degron. Mapping analysis located the degron of MOS within its residues 2-24. We further showed that the stability of either Xenopus or mouse MOS in other tested cell types, such as Xenopus fibroblast XTC cells in culture, mouse 3T3 cells, and reticulocyte lysate, was independent of Pro at the N-terminus of MOS, possibly because the kinetics of MOS phosphorylation at Ser-2 is different in these cells, in comparison to oocytes.

References

181. Degradation of a cohesin subunit by the N-end rule pathway is essential for chromosome stability
Hai Rao, Frank Uhlmann1, Kim Nasmyth2, Alexander Varshavsky

The sister chromatids of a replicated chromosome are pulled apart by the centromere-attached microtubules that emanate from spindle poles at opposite sides of the cell. However, until the onset of anaphase the forces pulling sister chromatids apart are counteracted by cohesion that holds sisters together. A multisubunit complex termed cohesin is essential for sister chromatid cohesion in both fungi and metazoans. In the budding yeast S. cerevisiae, cohesin contains four subunits, termed SMC1, SMC3, SCC1 (MCD1, RAD21), and SCC3. Perturbations of chromosome segregation are among the
causes of human cancer and other diseases. Previous work by the laboratory of K. Nasmyth demonstrated that at the metaphase-anaphase transition in S. cerevisiae, the ESP1-encoded protease separase cleaves SCC1, a 63 kD subunit of cohesin. The resulting 33 kD C-terminal fragment of SCC1 bears N-terminal arginine, a destabilizing residue in the N-end rule. We have found that the SCC1 fragment is short-lived in vivo (τ<sub>1/2</sub>~2 min), being degraded by the N-end rule pathway. Overexpression of a long-lived derivative of the SCC1 fragment is lethal. These results led us to examine chromosome stability in ubr1Δ cells (which lack the N-end rule pathway), revealing a greatly increased frequency of chromosome loss in these cells (Rao et al., 2001). Further, we demonstrated that the bulk of increased chromosome loss in ubr1Δ cells is caused by metabolic stabilization of the ESP1-produced SCC1 fragment. This fragment is the first physiological substrate of the N-end rule pathway that is targeted through a substrate’s N-terminal residue. A number of yeast proteins bear putative cleavage sites for the ESP1 separase, suggesting other physiological N-end rule substrates and other functions of the N-end rule pathway (Rao et al., 2001).

We employed the glutathione transferase (GST)-pulldown assay to investigate the interactions between an N-degron-bearing test protein and either yeast UBR1, or mouse UBR1, or mouse UBR2. Both yeast and mouse UBR1 proteins are N-recognins, the E3 (Ub ligase) components of the N-end rule pathway in S. cerevisiae and mouse, respectively. One aim of this work was to ascertain whether mouse UBR2, a close homolog of mouse UBR1 (E3a), is also N-recognin, i.e., an E3 of the N-end rule pathway. The N-end rule substrate used was the 33 kD, ESP1-produced SCC1 fragment, a physiological substrate of the N-end rule pathway (see Abstract 181). X-SCC1 fragments, where X was a variable residues, were produced as X-GST-SCC1 fusions, using the IMPACT (Intein Mediated Purification with an Affinity Chitin-binding Tag) system (New England Biolabs). Three versions of X-SCC1-GST proteins were produced, Arg-SCC1-GST (Arg-SCC1), Leu-SCC1-GST (Leu-SCC1) and Met-SCC1-GST (Met-SCC1), which bore, respectively, Arg, Leu and Met at their N-termini. Each of these GST fusions was linked to glutathione-Sepharose, and GST-pulldown assays were carried out with extracts from S. cerevisiae that overexpressed either yeast or mouse UBR proteins. In the terminology below, the type 1 primary destabilizing N-terminal residues are the basic residues Arg, Lys and His, which interact with a distinct (type 1) substrate-binding site of N-recognin. The type 2 primary destabilizing N-terminal residues are the bulky hydrophobic residues Trp, Phe, Leu, Tyr, and Ile, which interact with the type 2 substrate-binding site of N-recognin.

As expected from the previous understanding of substrate-binding properties of S. cerevisiae UBR1, the E3 of the yeast N-end rule pathway, the yeast UBR1 bound to Arg-SCC1 and Leu-SCC1 but not to Met-SCC1, the latter bearing N-terminal Met, a stabilizing residue in the N-end rule. Remarkably, however, we found that the binding of UBR1 to Arg-SCC1 was inhibited not only by the type 1 dipeptide Arg-Ala (this was expected from the earlier understanding of UBR1), but also by the type 2 dipeptide Leu-Ala, an unexpected result. In contrast, the binding of UBR1 to Leu-SCC1 was inhibited by Leu-Ala but not by Arg-Ala. While the full-length, 1,950-residue S. cerevisiae UBR1 and its 1,140-residue N-terminal fragment UBR1(1-1140) exhibited indistinguishable X-SCC1 binding patterns in these dipeptide-competition assays, the 717-residue N-terminal fragment of UBR1, UBR1(1-717), yielded different results. The binding of UBR1(1-717) to Arg-SCC1 was inhibited by Arg-Ala, but was not inhibited by Leu-Ala, in contrast to the findings with full-length UBR1 and UBR1(1-1,140). In addition, UBR1(1-717) bound to Leu-SCC1 significantly weaker than the longer UBR1 fragment and full-length UBR1. Previous in vivo data with Arg-SCC1 versus Arg-Ala and Leu-Ala dipeptides (Rao et al., 2001) indicated that only Arg-Ala, not Leu-Ala, could inhibit degradation of Arg-SCC1. The apparent discrepancy between these two sets of results, produced with entirely different readouts and in settings of differing complexities, remains to be understood.

The same X-SCC1-GST proteins was used to examine the binding of N-end rule substrates to mouse UBR1 and UBR2 (mUBR1 and mUBR2). We already knew that mUBR1 is an N-recognin of the mammalian N-end rule pathway (Kwon, Y.T. et al., 2001). As expected, mUBR1 was found to bind to Arg-SCC1 and Leu-SCC1, but not to Met-SCC1, similarly to S. cerevisiae UBR1. The full-length mUBR1 and the mUBR(1-1,031) fragment exhibited the same binding patterns. Remarkably, mUBR2 also bound to X-SCC1 proteins in the N-end rule-dependent manner, thus identifying, for the first time mouse UBR2 as an N-recognin. mUBR2(1-1,041) exhibited the same binding patterns as the full-length mUBR2.

References

**Publications**


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Summary: In the Wold group we are interested in how mammalian cell fates are specified and executed during development and during regeneration. These biological problems include the intimately related problem of stem cell specification and utilization, in which we are trying to discover how subsets of progenitor cells with self-renewing properties are specified and how their activities are regulated. The approach we are taking to these problems is increasingly focused on genome-wide and proteome-wide studies. A key challenge is to understand the regulatory events that drive the progression from multipotential precursor cells to determined unipotential precursors (myoblasts). It has been postulated that some of these are important for specifying their precursor status of multipotential mesodermal precursors and in proliferating muscle tissue of vertebrates, and at the molecular level, pMesogenin1 is essential for putting in place the known regulatory machinery that governs vertebrate body segmentation, including genes of the notch/delta signaling pathway.

While "positive-acting" regulators like MyoD and pMesogenin1 receive much emphasis, negative-acting regulators also abound in muscle differentiation. At cellular and molecular levels, it is clear that negative regulators of skeletal myogenesis are probably just as important for regulating the outcome as are the positive regulators. The interaction between the two sets of regulators is of particular interest. Negative regulators of skeletal muscle are expressed in multipotential mesodermal precursors and in proliferating muscle precursors (myoblasts). It has been postulated that some of these are important for specifying their precursor status and/or maintaining precursor cells in an undifferentiated state. We are now working to develop a computational simulation to model the myogenic determination and differentiation regulatory network, and to use this simulator to probe and develop more sophisticated hypotheses for the function of Id, the MRFs, MEF2, etc. This will be used mainly as a hypothesis generating tool to explore possible roles of redundancy, positive and negative...
feedback loops and other features in contributing specific properties to the dynamics and switch-like character that is presently believed to characterize the transition from one cell state to another, in particular the binary quality of the decision and its dynamics.

A relatively new thrust for the lab is our interest in myogenic stem cells. Muscle satellite cells are muscle stem cells of the adult, and they are responsible for all muscle regeneration following injury or degenerative insult. We want to understand the lineal origin, cell cycle regulation, and regulation of myogenic status for this self-renewing progenitor cell population. D. Cornelison, a Ph.D. graduate of the lab, initiated this work by studying in satellite cells the expression and function of a panel of 80 different developmental regulatory genes, growth and trophic factor receptors, cdk/cyclin inhibitory proteins, and cdk/cyclin complexes in the context of mouse muscle regeneration for wild-type and MRF mutant animals. Marie Csete went on to study these stem cells and others from diverse tissues with respect to their responses to major environmental cues, focusing on oxygen availability which is often disrupted by injury. Recent collaborations with David Anderson's lab on campus and Ron McKay's at the NIH led to the conclusion that oxygen levels normally used in cell culture are both a physiologically high and deleterious. Lower, more physiological levels, Marie discovered, give dramatic improvement in yields of dopaminergic neurons in both CNS and PNS stem cell cultures. The oxygen levels were also found to influence the selection of cell fate in culture, leading to the provocative possibility that the oxygen microenvironment for a progenitor cell during development in vivo might affect cell fate selection. There are also intriguing effects of oxygen levels on muscle and fat developmental pathways, with a physiologically high levels of oxygen favoring the fat pathway and lower levels favoring an accelerated muscle differentiation pathway. The molecular basis for these effects of oxygen is not yet known and a present project involves reinvestigating them using microarray analysis.

An expanding area for the lab is further development and application of large-scale RNA expression analysis which is being done in collaboration with Drs. Steve Quake and Jim Brody from CIT Applied Physics, Dr. Eric Mjolsness at JPL and Chris Hart and Brian Williams in the Wold lab. The proximal goal is to apply large-scale microarray expression analysis to characterize the full gene expression status that describes cells at each point in the series of "decisions" taken on the way to becoming, for example, a skeletal muscle satellite cell or differentiated muscle cell. Conceptually similar studies interface with characterization of mutant mouse strains relative to their wild-type counterparts. A collaboration in this is with Dr. Timothy Triche and colleagues at Children's Hospital. It is aimed at using these methods to study the major solid tumors of youth: rhabdomyosarcoma, Ewing sarcoma and osteosarcoma. These correspond in cell types of origin with the paraxial mesoderm whose normal development we study.

183. Low oxygen culturing enhances CNS stem cell proliferation and affects developmental pathways
Marie Csete

Usual tissue culture is conducted at 37°C in a gas atmosphere of 5% CO₂ and 95% air. Thus, ambient temperature is adjusted to reflect mammalian core body temperature and CO₂ is adjusted to reflect approximate venous/arterial CO₂ concentrations. Room air contains 21% O₂, and a 95% air mixture contains 20% O₂. Under these standard culture conditions, the ambient oxygen levels are distinctly hyperoxic, not at all within physiologic ranges. Mean tissue intracellular oxygen concentration is 3% but tissue oxygen levels in parts of the brain are even lower, reflecting the high oxygen utilization in brain.

In collaboration with Lorenz Studer and Ron McKay at NIH, we compared rat CNS stem cell regeneration in vitro in traditional tissue culture (20% O₂) vs. cultures reflecting brain oxygen levels (generally 2-3% O₂). Rat CNS progenitors proliferated more in physiologic oxygen conditions than traditional cultures, yielding a four-fold increase in cell number over five days in culture. In addition to increased proliferation, stems cultured in physiologic oxygen were less likely to undergo apoptosis than cells cultured in 20% O₂. After differentiation of the stem cells by bFGF withdrawal, low oxygen culturing yielded a striking increase in the number of neurons which stained for tyrosine hydroxylase (about 82% of neurons in low oxygen vs. 22% of neurons in traditional cultures). HPLC quantitation of dopamine from culture supernatants confirmed that the increased TH staining was associated with increased functional enzyme. Semi-quantitative RT-PCR analysis of genes involved in the developmental pathway of dopaminergic neurons revealed a remarkable increase in message for FGF-8 under low oxygen conditions compared to traditional cultures. TH message itself was not dramatically different under the two conditions. Commitment to a dopaminergic pathway appears to be reinforced by day 4 of low O₂ culture. When such neuronal progenitors are returned to 20% O₂ culture conditions, the majority still assume a dopaminergic fate.

A variety of extracellular factors influence proliferation and differentiation of multipotent stem cells. Our results suggest that physiologic gases may also play an instructive role in CNS stem cell regeneration. Oxygen levels may be important in determining the response to signals from a variety of extracellular factors (much as precise temperature is critical to enzyme kinetics) or may act by specific regulation of intracellular pathways which promote survival, growth, or differentiation. We have not determined precise oxygen level optima for any of the functions assayed in these studies, but our results suggest that oxygen levels much lower than those traditionally used in culture should be used to adequately mimic in vivo cellular environments. In fact, traditional culture methods appear to be relatively toxic to CNS stem cells when compared with low oxygen cultures. Our results further suggest that oxygen levels may be used in the developing brain as part of the signals.
that regulate spatial and temporal cues for differentiation. In particular, low oxygen culturing may also prove a practical method for enriching for dopaminergic neurons that can be used in cellular transplants for the treatment of Parkinson’s disease.

184. **Lowered oxygen culture affects skeletal muscle satellite cell yield, development and phenotype**

*Marie Csete, Jean Walikonis*

Satellite cells are normally quiescent cells present in small numbers under skeletal muscle basal lamina. Satellites re-enter the cell cycle when skeletal muscle is traumatized, and produce myoblasts and ultimately new muscle fibers. They are thought to be the major source of new skeletal muscle after trauma or degeneration. An adult muscle fiber culture model of regeneration was established in the lab by D. Cornelison. That work demonstrated that satellites in culture activate in a relatively synchronous fashion, with single satellites emerging from the basal lamina at about one day in culture and the first satellite division common at 48 hr, with new muscle fiber apparent at 96 hr. A characteristic expression pattern of muscle transcription factors (MyoD, myf5, myogenin, and MRF4) accompanies these morphologic patterns.

Traditional tissue culture is usually conducted at oxygen levels that are aphysiologically high, even for skeletal muscle, a generally "high"-oxygen tissue. On average skeletal muscle tissue oxygen levels are approximately 6% O2. We compared early satellite regeneration under physiologic oxygen levels vs. usual 20% O2 culture conditions. Using sequential 12-hr BrdU pulses to label satellites adherent to the fibers, we found twice as many satellites proliferating at each time point (up to 60 hr) in physiologic oxygen vs. 20% O2. Immunohistochemical labeling of satellites using immunostaining for the presence of c-Met receptors (an operational molecular marker of quiescent and recently activated muscle stem cells) confirmed the presence of twice as many satellites on fibers in low oxygen conditions at 24 and 48 hrs. In parallel studies using human myoblast cultures, we confirmed that these cells undergo enhanced proliferation under 2% O2 conditions compared to traditional 20% O2 cultures.

Single-cell RT-PCR was also used to assess the satellite developmental process. We analyzed individual satellites for the expression of the four major muscle regulatory transcription factors of the MRF family: myf5, MyoD, myogenin and MRF4. Myogenin and MyoD expression is more prominent at 24 and 48 hr under low oxygen conditions than under 20% O2. At 24 hr, twice as many satellite in low oxygen have simultaneous expression of three MRFs. Both findings suggest that the satellites are further along in their developmental pathway under physiologic oxygen conditions.

Experience with these cultures suggests that the fiber-adherent satellites represent only a fraction of the satellite population. The population is free to migrate off the fiber, and cannot be captured for analysis in the established model. Our current efforts are directed at following the migrant satellite population to evaluate its regenerative capacity under varying oxygen conditions.

Reference


185. **Mouse gene microarrays**

*Brian A. Williams, Jean Walikonis*

cDNA microarray analysis has emerged as the most robust current technology for measuring the RNA "expression state" of a given cell type under a specific set of conditions. Microarray-based studies capitalize on the increasingly comprehensive genomic DNA sequence information becoming available from the whole genome sequencing of the major model organisms and man. Comparative measurements performed with microarrays allow us to learn how a cell or tissue expression state changes due to genetic mutation, developmental change, physiological change, or therapeutic treatment. The distinction between this approach and most prior techniques for measuring changes in gene expression phenotype is that microarrays make many different measurements (one measurement per gene chip), with each measurement monitoring the behavior of thousands of genes simultaneously. At present we are able to generate microarrays with 20,000 different spots on one glass microscope slide. The micro-scale allows one to work with relatively small samples of RNA for each determination (~1-2 µg of polyA RNA per sample). Even lower amounts of input sample can be used, if an intermediate biochemical amplification step is employed.

Mouse was selected for our studies because it is the central model organism used in our lab to study developmental pathways (see entries from Brian Williams and Libera Berghella below), and because the mouse has many pertinent transgenic and knockout strains. For the initial study, we generated a set of custom microarrays containing approximately 8700 different mouse cDNA clones, together with ~100 different custom designed 50mer oligonucleotides. Arrays were made using the robotic spotting method. The robotic spotter was built by Dr. Jim Brody of the Quake group in Applied Physics, according to the design of Pat Brown and colleagues at Stanford. The cDNA inserts in the collection were PCR amplified, and the products characterized by gel electrophoresis.

Using polyA RNA we synthesized fluorescently-labeled cDNA samples and hybridized them pairwise to the microarrays. We determined that we could readily detect RNAs encoding transcription factors that are known from other measurements to be present at ~20 transcripts per cell in cells of the line used here. We also compared and contrasted results using these conventional 200-2000 bp PCR products with the same genes represented by selected oligonucleotides of lengths 45-65 (see following entry) which showed that these printed oligos are, in our...
hands, superior to PCR products in absolute signal and in reproducibility.

186. **45-60mer oligonucleotide gene chips**

*Brian A. Williams, Susanna Horvath*

The two major forms of microarrays currently in use are at extremes for the lengths of target nucleic acid used to represent each gene: short 25mer oligonucleotides are used in Affymetrix commercial microarrays in which each oligo is synthesized *in situ* on the slide. In contrast, PCR products typically ranging between 500-1200 nt are synthesized by PCR and then arrayed mechanically to generate microarrays of the type originated by Pat Brown and his colleagues at Stanford. Both types of target DNA have significant shortcomings. 25mers are so short that there are significant problems achieving sequence specificity. There are additional technical ramifications for the lengths of labeled the RNA population that is being hybridized. Affymetrix deals with these problems, in part, by representing each gene as a tiling path of multiple sequences together with a comparison tiling path of negative controls containing one base mismatches. With time and experimental experience, they learn for each gene which tiles in the path most accurately represent the intended gene and show no interfering signals. A further practical problem is that the investigator cannot redesign an Affy chip to contain new genes he wants to add, or to create a reduced complexity gene chip suited to his special needs.

In contrast, PCR products and mechanical arraying in-house, offer more design flexibility, but they lack the uniformity and reliability of oligonucleotides. They also fail to take advantage of the design possibilities that are unique to oligonucleotides. Prominent among these is the opportunity to represent a gene on a microarray without ever having a cloned intermediate for to serve as template for PCR amplification. This means that candidate genes identified in genomic DNA, or by any other means, can be added to an oligonucleotide array by simply synthesizing the informatically identified gene. Also, while PCR is now a relatively robust laboratory procedure, material for every amplification calls for a new round of quality control every 500 - 1000 slides, and making material for microarrays containing ten's of thousands of different genes is a major and complex in-lab task. In contrast, oligo synthesis is a mature, fully automated out-of-lab procedure which can produce enough product for ten thousand microarrays or more from a single synthesis. Finally, oligonucleotides can be designed to be highly specific for different splice isoforms; PCR products typically are not specific for isoforms, because sequences in common among splice isoforms lead to positive signals for all species.

A remarkably good solution for the problems of both current procedures is use of oligonucleotides of lengths 45-70. We find that these can be mechanically spotted the same manner as PCR products. These sequence lengths, attached to an appropriate linker domain, afford significantly more sequence specificity than 25mers, eliminating the need for complex tiling paths and other special procedures that go with the shorter oligos. They also bring the opportunity to add elements to an array directly from deduced gene sequence without need of a cDNA template. These oligos are selected from a candidate gene sequence with attention to oligonucleotide base composition, to generate an array in which all elements react with their complements in labeled RNA or cDNA at a known and nearly identical stringency (temperature, salt and solvent). We have found that 50mer oligonucleotides are typically several fold more sensitive than 1 kb PCR DNA products, with the result of better signal noise and reproducibility fro the oligos. We have also shown that 50mer oligos are powerful tools for definitively measuring splice isoforms.

187. **Defining myogenic determination and differentiation on a whole-genome scale**

*Brian A. Williams, Tracey Teal, Shuling Wang*

The immediate biological goal in this project is to define comprehensively the gene expression states in a model vertebrate developmental pathway. Within this goal is the aim of dissecting out responses to multiple signaling pathways that act to enhance or suppress myogenic differentiation. This pathway has three defined stable cell states linked by dynamic transitions: 1) Proliferating multipotential mesodermal precursor cells that can elect the myogenic pathway or several other possibilities; 2) These multipotential precursors can be stimulated to convert to "determined" myoblasts which are proliferating unipotential muscle precursor cells; 3) Myoblasts can then be triggered to differentiate, at which point they express the genes and cellular properties of a mature muscle cell or myocyte.

The second goal is to use the data obtained to help evaluate our suite of different "clustering" algorithms to determine which ones are most useful in which ways for analyzing large-scale data of this kind (see entry from Chris Hart *et al.*, in this section). A third goal is to use the data to advance our current model of the regulatory circuitry that controls this developmental pathway. Clustered expression data is one key input in defining the circuit, which is then joined by comparative genomic DNA sequence data and protein:DNA binding studies (see entry of Tristan DeBuysscher).

Fluorescently-labeled cDNA populations representing skeletal muscle cells during several time courses of differentiation have been co-hybridized to the arrays in the presence of cDNA from each of several "reference RNAs." Biologically relevant reference RNA sets include undifferentiated proliferating myoblasts (mono-potential muscle precursor cells) and multipotential mesodermal precursor cells whose possible developmental fates include muscle cells. The comparisons of muscle differentiation courses with different reference RNA sets is designed to expose groups of genes whose expression in muscle changes relative to each of the different reference cell states. Through use of various clustering methods we have identified components of a contractile apparatus
regulon. Detailed kinetics reveal that this group is subdivided into kinetic classes, and the working hypothesis is that each of these groups depends differently on input signals (calcium dependent; FGF dependent; insulin dependent; etc.). These suggest, in turn, differential participation by specific transcription factors activated or repressed by each signal. The regulon was also shown to contain a number of expressed sequence tags representing previously unidentified genes in the mouse genome. To internally monitor the readout of these pathways dissected from each other, we created modified myogenic cell lines that allow us to measure the activities of individual transcription factors that we know are important in our system (MyoD family sites; MEF2 sites) to assist in attributing readout at natural complex genes. This information will be used in conjunction with computational tools to identify and categorize the cis-acting regulatory domains that drive genes within the regulon. Other groups of genes that belong to novel clusters have been identified and highlighted for further study, including some whose expression precedes any previously known regulator in the myogenic differentiation pathway, making them candidates for hitherto unknown functions from myogenin activation.

188. Comparative sequence analysis in vertebrate genomes

Tristan DeBuysscher, C. Titus Brown

The Seqcomp and FamilyRelations tools are described in detail in the entry from Titus Brown in the E. Davidson groups’ section of the Report. Briefly, they are designed to aid in locating cis-regulatory regions through comparative sequence analysis of orthologous non-coding sequence. Thus the basic seqcomp program performs a comparison between two sequences in a manner similar to the underlying comparison in dot-plot programs, but at a larger scale (presently, tens of kilobases). The Wold group emphasis has been on the underlying sequence comparison algorithm and on development of features especially important for large (vertebrate) genomes of primary concern to our group. This includes features designed to make and evaluate comparisons of more than two genomes and provision for masking known repeated DNA elements and certain kinds of “simple sequence” that generate a background of highly similar, but distracting features. The simple rationale for evaluating simultaneously, for example, three orthologs rather than two, is to sift out more sequences that have been conserved by chance between any two genomes. If the genomes are at auspicious distances from each other, the additional genome should highlight more clearly than the pairwise comparison those sequences that have been conserved because of pressure for function. Current work includes testing the triple view algorithm on multiple orthologous genes from various genomes over regions ranging from 25 kb to 200 kb. These comparisons begin to address basic questions we need to answer in order to maximize the efficiency of comparative genomic sequence searching to find regulatory elements. These include learning how much incremental resolving power each additional genome (beyond the first two) gives for identifying a regulatory module. Another question is which genomes are at optimal divergence distances from model organisms such as mouse and human. The answer to this may well be different for different neighborhoods within the genome. This simple comparison technique is proving to be a powerful tool for phylogenetic footprinting. Just how good it is, in terms of false positive and false negatives, is being evaluated experimentally (see entry below). Future goals in development include improving the speed of the comparison algorithm; automating selection of candidate regions; and coupling it with searches for binding site motif representation.

189. Identifying and testing candidate regulatory regions for genes of the myogenic regulatory circuit

Tristan DeBuysscher

Using the sequence comparisons and FamilyRelations tools, a dozen genes from the paraxial segmentation and myogenic regulatory circuits are being analyzed by mouse human comparisons. Mouse BAC sequences are from sequencing performed by collaborators and the DOE Joint Genome Institute. The BACs were originally obtained from the mouse BAC library made by Hiroke Shizuya and colleagues, with screening by Mai Wang of the Mel Simon genomics group at Caltech. The conserved noncoding candidate regulatory domains are amplified by PCR and cloned into reporter vectors designed for introduction into mice by transgenesis. Because the pathways in questions are active at mid-gestation, the transgenics can be productively assayed ~9 days after injection, giving an acceptably rapid assay compared with germline transgenesis.

190. Isolation of basic HLH genes expressed during early mouse development

Jeong Kyo Yoon

bHLH transcription factors play key roles in cell fate determination and cell differentiation during development, and both molecular identities and developmental functions often been substantially conserved in evolution. Two familiar examples are MyoD family proteins in skeletal myogenesis of vertebrates and the several neurogenic bHLH proteins in Drosophila and in the vertebrates. It is also now clear that other developmental lineages employ members of the bHLH system at key points to regulate specification decisions and execute differentiation functions. We are interested in the broader issue of how and why the bHLH factors are widely used for these developmental functions, and also in the specifics of how bHLH regulators function prior to specification of the muscle lineages. A cDNA library was constructed using mRNA from the body trunks of mouse embryos at embryonic day 9.5 (e9.5). The rostro-caudal gradient of maturity and patterning in paraxial mesoderm and in the neural tube within trunks at this time was expected to give good representation of the developmental
history of these lineages, including the spatiotemporal domains in which many progenitor cell fates are apparently being restricted and specified. Virtually all known lineage-specific bHLH proteins can form heterodimers with more widely expressed E12 and E47 bHLH proteins. We therefore used bHLH domain of E47 protein as an interaction bait. Two novel bHLH genes, originally designated T117 and T324, together with many known bHLH and HLH genes were isolated, and the expression pattern of the two novel genes during mouse development was analyzed. Based on their patterns of expression and behavior in functional studies, T117 and T324 were renamed Floorplate1(FP1) and pMesogenin1, respectively. Their functional characterization is outlined in the entries below.

191. pMesogenin1 regulates paraxial mesoderm specification and body segmentation in Xenopus

Jeong Kyo Yoon, Shuling Wang, Randall T. Moon*

Paraxial mesoderm in vertebrates gives rise to all trunk and limb skeletal muscles, the trunk skeleton, and portions of the trunk dermis and vasculature. A defining characteristic of all vertebrates is metameric segmentation of musculoskeletal and peripheral nervous systems. This body plan arises from primary segmentation of the paraxial mesoderm into tissue blocks called somites. The bHLH class gene we have named pMesogenin1, (isolated earlier as the E-protein heterodimerization partner from a two-hybrid screen in yeast), is specifically expressed in presomitic mesoderm in the as yet unsegmented block of paraxial mesoderm in mouse and in Xenopus. A striking feature of pMesogenin1 expression is that its expression terminates abruptly in the presomitic mesoderm shortly before formation of the next somite (somites are formed continuously in a rostral to caudal gradient). Its closest relatives, MESP1 and MESP2, are expressed just after pMesogenin1 is shut down and just before the next somite forms. Thus, the bHLH subfamily of pMesogenin and MESP1/2 collectively define discrete but highly dynamic prepatterned subdomains of the paraxial mesoderm. The pattern of expression for pMesogenin1 is consistent with a functional role in specifying one or more aspects of paraxial mesoderm phenotype, and in this project I set out to test this possibility using both gain-of-function and loss-of-function experimental designs. In collaboration with Randy Moon, I found that pMesogenin1 from either mouse or frog can efficiently drive non-mesodermal cells to assume a phenotype with molecular and cellular characteristics of early paraxial mesoderm. The assays were performed in Xenopus embryos or embryo explants that had been injected in early cleavage stages with RNA encoding pMesogenin1 or control RNAs of interest. Among genes induced by added pMesogenin1 is XWnt-8, a signaling molecule that induces a similar repertoire of marker genes and a similar cellular phenotype. Additional target genes induced by pMesogenin1 are ESR4/5, regulators known to play a significant role in segmentation of paraxial mesoderm. pMesogenin1 differs from other known mesoderm-inducing transcription factors because it does not also activate a dorsal (future axial) mesoderm phenotype, suggesting that pMesogenin1 is involved in specifying paraxial mesoderm. In the context of the intact frog embryo, ectopic pMesogenin1 also actively suppressed axial mesoderm markers and disrupted normal formation of notochord. In addition, we found evidence for cross-regulatory interactions between pMesogenin1 and T-box transcription factors, a family of genes normally expressed in a broader pattern and known to induce multiple types of mesoderm. Based on our results and results from prior studies of related bHLH genes, we propose that pMesogenin1 and its closest known relatives, MesP1/2 (in mouse) and Thylacine1/2 (in Xenopus), comprise a bHLH subfamily devoted to formation and segmentation of paraxial mesoderm.

To further define the function of pMesogenin1 in paraxial mesoderm formation, I created a germline deletion of mouse pMesogenin1 with the help of Shirley Pease and her colleagues in the Transgenic Mouse Facility. pMesogenin1 homozygous knockout embryos show a complete failure of trunk and tail somite formation and segmentation of the body trunk and tail. Although several genes, including members of the famous Notch/Delta intercellular signaling apparatus, have previously been shown to partially disrupt segmentation, none have a phenotype of complete blockade, as is seen in the pMesogenin1 animals. At the molecular level, the phenotype features dramatic loss of expression for components of the Notch/Delta pathway and the oscillating “somitic clock” genes that are thought to control segmentation and somitogenesis. The presumptive paraxial mesoderm also fails to execute patterning and specification steps, leading to a complete absence of all trunk paraxial mesoderm derivatives which include skeletal muscle, vertebrae and ribs. We infer that pMesogenin1 is an essential upstream regulator of trunk paraxial mesoderm development and segmentation in vertebrates.

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192. Isolation of target genes for pMesogenin1

Jeong Kyo Yoon, Jason Chua*

Functional analyses suggest that pMesogenin1 is a major regulator of paraxial mesoderm development and segmentation, based on mouse knockout and frog gain of function studies. We postulate that it functions at the molecular level, as do other bHLH proteins, as a sequence specific DNA binding protein. Based on precedents from other bHLH genes that have been studied in detail, it could act as a repressor, as an activator (the most commonly reported activity for these regulators), or as either one, depending on context in a given target gene. This raises the question of what genes are direct downstream targets of pMesogenin1 regulation and which genes are indirectly regulated by it. To address these questions, we generated
recombinant DNA constructs that encode chimeric proteins in which the ligand binding domain of either glucocorticoid receptor (GR) or estrogen receptor (ER) was fused to the carboxy terminus of pMesogenin1 protein. Normally, native GR or ER function as transcription factors in the presence of their respective ligands, but they are inactive in their absence. When no hormone is bound, they are complexed with heat shock protein (HSP). These complexes are transcriptionally inactive and are localized mainly outside the nucleus. However, upon exposure to their corresponding hormones, GR and ER are released from their HSP complexes, relocalize and function in the nucleus. Subsequently the hormone-activated native ER and GR activate their transcriptional target genes by binding to specific DNA sequences via their DNA binding domains. Similar to native GR or ER proteins, it has previously been shown that chimeric proteins containing the hormone binding domains (but not the ER or GR AND binding domain) reside in the cytoplasm, and can be translocated into the nucleus upon hormone treatment. Therefore, the functional activity of the chimeric protein as a transcription regulator can be controlled in a hormone-dependent manner.

To identify target genes, the chimeric protein will be expressed in both mouse cell culture and animal cap explant culture from Xenopus embryos. Then, RNA will be isolated from both naïve and cells/explants treated with hormone. An important option afforded by this hormone-dependent chimeric design, is separation of direct transcriptional targets from secondary and tertiary targets. Thus, if hormone is added in the presence of a protein synthesis inhibitor, direct targets can be up or down regulated. If hormone is added in the absence of a protein synthesis inhibitor, then primary, secondary and more indirect targets will all be affected. A microarray gene expression analysis will be applied to begin to identify genes that are either induced or repressed by the chimeric proteins in the context of appropriate cells from null animals.

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193. Expression and functional analysis of FP1 during mouse development
Jeong Kyo Yoon, Shuling Wang

Expression of T117 RNA is first detected in the rostral floor plate of the neural tube in e8.0 mouse embryos. Expression is extended more caudally as embryo develops. Expression of two previously known floor plate markers, HNF-3beta and shh, precedes that of T117 in the floor plate, suggesting that T117 may act downstream of HNF-3beta and shh. It is noteworthy, however, that unlike shh and HNF3beta RNAs, which are also expressed in other axial domains, T117 appears highly specific for the floorplate and for this reason has been named Floorplate1 (FP1). The floor plate is a very interesting ventral structure in neural tube that produces important signaling molecules such as SHH and netrins function as patterning signals for of CNS neurons and for some other axial domains as well. In order to investigate the functional role of FP1 in vivo, both "gain-of-function" and "loss-of-function" studies are in progress. First, the FP1 bHLH coding region was engineered for ectopic expression in the dorsal neural tube using either Wnt-1 or En-2 enhancer/promoters, and these have been introduced into fertilized eggs to generate transgenic embryos. The phenotypic consequences of ectopic expression, at least at the levels provided by these constructs, were unremarkable. For a loss-of-function study, the FP1 gene was disrupted in mouse ES cells and knockout mice were generated. The initial phenotypic analysis revealed that homozygous knockouts are also largely normal in development of the floorplate and of cells known to be affected by signals from the floorplate. However, a more detailed analysis will be needed to determine whether neuronal specification or pathfinding may have been altered in more subtle ways. A presumptive ortholog for FP1 has also been isolated from Drosophila, based on notable similarities in the bHLH domain protein coding sequences of the two genes. Preliminary in situ hybridization studies indicate that it is expressed in a specific subset of glial cells.

194. Role of a homeogene, msx-1, in adult muscle regeneration
Libera Berghella

Regeneration of adult skeletal muscle after an injury or disease is understood to be mediated primarily by muscle satellite cells. In an intact muscle this population of cells is composed of rare, mononucleate cells located beneath the basal lamina of the mature fiber. In healthy adult muscle the satellite cells are mitotically quiescent. Muscle injury triggers their metabolic and mitotic activation. They emerge from G0 mitotic arrest, proliferate and concomitantly undergo a series of developmental changes. This pathway ultimately ends, for the majority satellite cell progeny, in differentiation and fusion to form new myofibers or differentiation coupled with fusion into existing fibers. However, some cells apparently have a different fate. Instead of differentiating as muscle fibers, they return to replenish the satellite pool in order to support future regeneration.

Biochemical and molecular analysis of muscle regeneration has, in the past, been hampered by the rarity of satellite cells and the absence of reliable molecular markers for these cells, especially when they are in the quiescent state. Prior work in this lab led to a method of studying gene expression in individual satellite cells over the timecourse of an activation response (Cornelison and Wold, 1997; Cornelison et al., 2000). Among the genes studied, the homeobox gene msx-1 revealed a peculiar and interesting expression pattern, with transcripts being detectable only at extremely early timepoints (<30 minutes post-fiber isolation). This suggested that quiescent cells express msx-1 but quickly downregulate its expression once they are activated. It is also known that forced expression of msx-1 in cultured myoblasts inhibits differentiation and can suppress MyoD expression.
De Angelis et al., 1999). Based on these and other preliminary results, our working hypothesis is that msx-1 expression marks satellite cells that are in quiescence or are returning to it, and that it may be causal in suppressing expression of MRFs in quiescent satellite cells.

To test for a functional role of Msx-1 in adult muscle regeneration are trying to force the expression of Msx-1 in satellite cells in fiber explants and in muscle in vivo with a lentiviral vector system. The advantage of this system over other gene-delivery vehicles is the ability to transduce non-dividing cells. Taken together, the gene expression patterns and an over-expression assay will test the hypothesis of a significant role in regulating regeneration. One version of the vector is designed to permit visualization of the infected cells with the vital marker GFP. Mice in which the Msx-1 gene has been replaced by beta galactosidase will also studied. A final avenue of investigation is a model cell system provided by Dr. Shannon Odelberg of the University of Utah. It provides for reversible expression of mss-1. Its use in C2C12 cells has given evidence for reversal of the entire differentiation pathway.

Lentiviral vectors encoding lacZ with a nuclear localization signal and GFP, were produced to test the efficiency of the system in mice and rat muscle cells. Furthermore, a lentiviral vector encoding Mxs1 was generated. Titer of the vectors are estimated between 1 and 3X 107 I.U. From the preliminary results with markers genes in murine, rat and human cell lines and in murine and rat primary muscle cells, we observed a dramatic reduction of the efficiency of gene delivery in mouse cells compared to rat and human cells. In collaboration with Dr. Carlos Lois of the Baltimore lab, we have ameliorated this problem using a new generation of vector of his design.

One major goal in the analysis of large-scale gene expression data is to define sets of genes whose members have similar expression patterns. The current challenge is to detect similarities and differences among expression patterns when working with sample numbers ranging from 10's to 1000's of different individual tissues, cell types, tumors, or growth conditions. Inspection and deduction, which have traditionally served biologists well for comparing a few genes in two to ten RNA samples, fail when dealing with data sets of the new scale. The application of clustering algorithms provides a way to reveal hidden structure in large datasets. A variety of clustering algorithms have been developed over the past three decades to deal with problems of this general form, and a few of these have recently been applied to large-scale gene expression data. The various algorithms make substantially different assumptions and operate by different mechanisms. This raises questions about how they will differ in their ability to detect and report true underlying structure in these datasets. We expect that this will depend not only on intrinsic properties of the clustering algorithms, but will also depend on specific reference conditions.

References


195. Expression profiling various putative stem cell lines via cDNA microarrays
Libera Berghella, Sagar Damle, Brian Williams

In collaboration with the lab of Giulio Cossu at the University of Roma, we have initiated a large-scale gene expression characterization of cell lines isolated from the embryonic dorsal aorta of mice. The special interest in these cells comes from their capacity to differentiate into muscle, cardiocytes, bone or other derivatives when grown under various culture conditions. This mimics the in vivo potential of dorsal aorta multipotent cells implanted into a host animal via transfusion into the blood. Such transplanted cells can subsequently be found in mature muscle fibers, and in other tissues as well. Thus the isolation and initial characterization of this cell line supports the hypothesis that multipotent mesenchymal cells may be present embedded in vascular endothelium (De Angelis et al., 1999). Using cDNA microarrays, made in the lab, I have begun to analyze the transcription program of these cells in comparison with more conventional myogenic cell lines that are believed to be unipotential and myogenic (C2C12, MM14) or multipotential (10T1/2), or totipotent (embryonic stem cells). Results have revealed genes expressed in common only with 10T1/2, or with 10T1/2 and ES cells, but not myoblast lines. Other genes are novel to one or both of the aorta lines, whose developmental potentials are only partially overlapping. Among genes in common with stem-like cells is CD34, a putative marker of stem cells in several other contexts. These results are being verified by independent methods, and the impact of the compound trichostatin (a global pharmacological modifier of chromatin access) are next to be tested and correlated with phenotypic impact.

Reference


196. Comparison of clustering algorithms for use in large-scale gene expression analysis
C.E. Hart, Diane Trout, Sagar Damale, B. Bornstein*, J. Roden,* E. Mjolsness*

The advent of large-scale gene expression analysis provides us with unprecedented amounts of data that comprise gene expression profiles of thousands of genes assayed over many tissues, culture conditions, and genetic variants. In such experiments, the expression state of a cell is assayed by extracting RNA and then measuring the relative abundance each message. Currently, the two most prominent techniques for large-scale expression analysis involve 2-D DNA microarrays made by synthesizing complimentary DNA oligonucleotides on a glass slide via photolithography or by deposition printing of longer DNAs (usually cloned cDNA sequences prepared by PCR amplification) on a glass slide.

One major goal in the analysis of large-scale gene expression data is to define sets of genes whose members have similar expression patterns. The current challenge is to detect similarities and differences among expression patterns when working with sample numbers ranging from 10's to 1000's of different individual tissues, cell types, tumors, or growth conditions. Inspection and deduction, which have traditionally served biologists well for comparing a few genes in two to ten RNA samples, fail when dealing with data sets of the new scale. The application of clustering algorithms provides a way to reveal hidden structure in large datasets. A variety of clustering algorithms have been developed over the past three decades to deal with problems of this general form, and a few of these have recently been applied to large-scale gene expression data. The various algorithms make substantially different assumptions and operate by different mechanisms. This raises questions about how they will differ in their ability to detect and report true underlying structure in these datasets. We expect that this will depend not only on intrinsic properties of the clustering algorithms, but will also depend on specific reference conditions.
properties of the data such as the nature and amount of experimental noise and the specific nature of underlying patterns that may differ with the biology being studied. The biologist using such methods will want to know: How solid is the membership of a given gene within a proposed expression cluster? How similar or different is one cluster from another? What subset of measurements uncover a difference between two clusters that are otherwise very similar? If there is a hierarchical structure to the data, does that suggest something significant about the regulatory circuitry that generated the hierarchy?

To begin to address these questions, we implemented a framework for comparing and interpreting the results of many clustering algorithms. It includes a mechanism to quantitatively assess and visualize cluster quality by generating receiver operator characteristic (ROC) curves. We also implemented modified normalized mutual information (NMI) and linear assignment metrics (1) to interpret the degree of agreement and disagreement between different clusterings of the same dataset. In working with real and synthetic data, we now appreciate that further modification of the metrics is useful to reward the relative importance of small biologically meaningful clusters and to compare the overall structure in the data, as represented by set of cluster mean vectors.

Thus far, we have implemented and compared: cross-validated expectation-maximization mixture of Gaussian (cv-EM-MoG), a deterministic annealing version of cv-EM-MoG, K-means, phylogenetic clustering (Xcluster), and self organizing maps (SOM).

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Reference


197. Computational analysis of muscle differentiation

Chris Hart, Eric Mjolsness

A very active collaboration has been established between scientists at JPL and members of the Biology Division, including the Wold group. One part of this has led to the development of cellerator, which is a computational tool designed to dramatically facilitate building biological models. Here it is being used to build a simulator for the regulatory machinery that drives the two key developmental switches in muscle development. The first switch converts a multipotential mesodermal precursor cell into a myoblast (committed muscle precursor cell). The second converts this proliferating progenitor into a differentiated myocyte with the molecular and cellular features of skeletal muscle. The MyoD family of transcriptional regulatory proteins are essential for this process; E proteins are critical cofactors through heterodimerization; MEF2 family factors are also important positive regulators and Ids are stoichiometric negative regulators of MyoD and myogenin.

This is a first pass at representing the central control module computationally. Additional relationships in the form of positive and negative feedback loops and additional components may be required. The simulator framework will allow us to probe these possibilities and to guide experimental designs of further perturbations of the pathway. We believe the type of computer-assisted model generation provided by Cellerator, which can efficiently convert this typical genetics/biochemical pathway into a series of ordinary differential equations, will be an important hypothesis-generating tool. These switches in cell state are appear to represent a widespread and potentially general type of signal processing module that has as input a multiplicity of signaling pathways and has as output a irreversible change in cell state (measurable as changed gene expression). Such modules typify metazoan developmental pathways. The pathway is initiated, and transitions are triggered by multiple signaling cascades, frequently involving feedback and amplification. Normally, descriptions of even the simplest networks require manual implementations of dozens to hundreds of differential equations. Cellerator allows the user to specify input in biochemical notation that can then be automatically translated into the corresponding differential equations according to standard mass-action kinetics. Cellerator has recently been used to generate systems containing as many as 100,000 separate biochemical reactions and to simpler but still large groups of reactions such as those describing kinase cascades.

198. New method for assaying gene expression

John Murphy,* Barbara Wold, Mark Davis*

Gene microarrays are proving to be very powerful for large-scale gene expression analysis. However, they have significant shortcomings that we would like to bypass. They generally call for relatively large amounts of input RNA to achieve robust answers, and they are at their best with more abundantly expressed RNAs. They also have dynamic range limits that are considerably narrower than those of the RNA samples to be measured. In all their manifestations so far, they are semiquantitative, even under the best of circumstances. From an entirely different kind of methodology (multiplex single-cell RT-PCR (Cornelison and Wold, 1997) we know that being able to simultaneously monitor multiple genes in single cells gives a different and illuminating view of the gene expression combinatoric "states" that one cannot achieve using pooled cell samples. However the latter assays are thus far quite limited in simultaneous gene number (maximally seven simultaneous genes, thus far), and they also give little information about the quantity of each RNA type in an expressing cell. It is clear that in many biological settings, it would be highly desirable to get good quantitative measurements from just one or a few cells per determination for tens, hundreds, or even thousands of genes simultaneously.

To address this challenge, we are developing an approach that takes advantage of specifically designed families of chemical tags. Oligonucleotides, each designed
to hybridize to a specific RNA species of interest, are synthesized such that each oligo carries a unique covalently attached peptoid (not peptide) tag with a unique mass. At the end of hybridization, chemical methods are used to separate hybridized oligos from those that do not react with a complementary RNA, and then tags are cleaved from their respective oligonucleotides. In these methods, the tags, rather than the RNAs or oligos, are ultimately counted. The tag types being investigated, including families of peptides, have been selected to be optimal for analysis by methods that highly sensitive and also have wide dynamic range. The first method of choice is mass spectrometry. We expect that this approach will permit direct RNA quantitation over several orders of magnitude, and it has the potential to begin with very small input RNA samples. A virtue of the design is that it uses hybridization in which all components are in solution phase, and these reactions are considerably better understood and more efficient than is nucleic acid hybridization in which one member is in solid phase, as is the case for microarrays.

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**Publications**


Molecular Biology and Biochemistry

John Abelson
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Giuseppe Attardi
David Baltimore
Pamela J. Bjorkman
Judith L. Campbell
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Stephen L. Mayo
James H. Strauss
Eukaryotic genes contain introns which must be removed to generate the functional transcript. This is done by the process of splicing in which the intron is clipped out and the ends, the exons, are joined. Pre-mRNA splicing is at its heart likely to be RNA catalyzed but the reaction takes place on a large macromolecular machine, the spliceosome, which contains more than 80 proteins and five snRNAs found in the cell as ribonucleoprotein particles or snRNPs. There are five of these, U1, U2, U4, U5 and U6. Thus the task at hand is two-fold: to understand how the snRNAs interact with the pre-mRNA substrate to form the catalytic structure, and second to understand the role of the proteins. Progress has been made on both of these problems during the past year.

During the past four years Scott Stevens has taken the important step of developing a purification procedure for yeast snRNPs. His procedure involves one affinity step and a sedimentation step. In the process of characterizing the U4/U6•U5 triple snRNP, Scott discovered that at low concentrations of salt a significant portion of the snRNPs are found in the form of a penta snRNP complex containing all five snRNAs. We have purified this particle which contains all five of the spliceosomal snRNAs and 85% of all yeast proteins known to be affiliated with pre-mRNA splicing. This purified particle, or penta-snRNP, is active in pre-mRNA splicing when supplemented with soluble yeast factors, and the particle contains a U1 snRNP which is active in committing pre-mRNA substrates to the splicing pathway. The U4 and U6 snRNAs are base-paired, arguing that this particle is not an active spliceosome or a post-splicing complex of a lariat-intron. Furthermore, it has been shown that the penta-snRNP is likely to be the functional entity in splicing because the individual snRNPs do not reassociate in a mixing experiment. The protein composition of the particle was determined by mass spectrometry microsequencing. It contains all of the previously characterized yeast snRNP-associated proteins and in addition a set of proteins previously known to be involved in splicing but not known to be snRNP associated. This fact alone implicates the penta-snRNP as the functional form of the spliceosome. Another set of proteins found in the particle provides evidence for links between reactions as diverse as RNA turnover, transcription and protein synthesis.

Tracy Johnson has continued with her work on the trans-splicing system to study RNA interactions in the spliceosome. In this system the 5' splice site, synthesized chemically, is joined in trans to the 3' splice site. Though trans-splicing is seen in other organisms such as worms and trypanosomes, it does not take place naturally in yeast and yet provides a very useful tool since one can incorporate powerful crosslinking agents into the synthetic 5' leader which is only 20 nucleotides long. Tracy has found that this process is slow in vitro and this has allowed her to see some novel crosslinks which provide an insight into early spliceosome assembly which have not been seen before in the natural cis-splicing systems which are faster. The most interesting of these is a 5' splice site U4 interaction which had not been seen before. Since this crosslink does not require an intact 3' splice site, it appears that the U4 snRNP interacts with the 5' splice site before the 3' splice site is recognized and fits very well with the apo-spliceosome role of the penta snRNP.

Perhaps the most interesting of the spliceosomal proteins are a set of RNA-dependent ATPases called "DEAD box" proteins. In some cases these proteins have been found to be RNA helicases and are implicated in the RNA rearrangements that occur during spliceosome assembly. Last year, Randy Story for the first time solved the complete structure of a DEAD box protein, in this case from the archaeabacterium M. jannaschii. This structure will be an important tool in understanding the phenotypes of various mutations in conserved regions of the protein and as detailed in Story's report has already suggested modes of RNA interaction. Story is now focusing on a second RNA helicase DpbA, and E. coli protein that has a specific RNA substrate. Efforts are underway to crystallize the protein with its substrate, a fragment of 23S rRNA.

199. Analyzing large spliceosomal RNPs
Scott Stevens

Splicing of pre-mRNA in eukaryotes is performed by the formation of spliceosomes around the precursor RNA. The machinery which performs this task is composed of ribonucleoprotein complexes (RNPs) which are themselves composed of small nuclear RNAs (snRNAs) and tightly associated proteins, as well as protein factors acting in trans. We have comprehensively analyzed a number of RNPs from yeast (including the U5, U6 and U4/U6•U5 snRNPs) and recently have characterized a novel, functional RNP which appears to be a pre-assembled splicing complex, termed the penta-snRNP.

The purified penta-snRNP contains the five phylogenetically conserved spliceosomal snRNAs, and over 60 proteins involved in the pre-mRNA splicing reaction. Among them are the snRNP-associated proteins of the U1, U2 and U4/U6•U5 snRNPs as well as a family of proteins that are known to be splicing related, but
previously not demonstrated to be snRNP-associated. Penta-snRNP is capable of splicing pre-mRNA in an in vitro splicing reaction when complemented with nuclease-treated extract, and forms recognizable splicing complexes on native polyacrylamide gels. In mixing experiments using extracts containing epitope-tagged proteins and differently-sized snRNAs, we demonstrated the participation of the penta-snRNP in pre-mRNA splicing as a functional splicing enzyme.

In human cells, the processing of pre-mRNAs is fundamentally more complicated. Human pre-mRNAs contain multiple introns (yeast pre-mRNAs generally contain only one intron), therefore the splicing machinery is more highly regulated and complex. In an effort to identify all of the proteins associated with the splicing machinery inside human nuclei, we preparativly purified endogenous pre-mRNA splicing complexes from HeLa cells. In addition to the known snRNP-associated factors, all known pre-mRNA-binding hnRNP proteins, other pre-mRNA-binding proteins, all know spliceosome-associated RNA helicase and several novel RNA-interacting proteins were detected.

Further experimentation regarding the assembly of the spliceosome and mechanism of the splicing reaction is being carried out by addressing the three-dimensional structure of the snRNPs and core proteins in the spliceosome.

200. Characterization of 5’ splice site interactions during spliceosome assembly

Tracy L. Johnson

5’ splice site selection is the first step in mRNA splicing. The current model postulates that the spliceosome assembles in a sequenced order of events beginning with U1 binding to the 5’ SS followed by ATP-dependent U2 association with the branch point, and subsequent U4/U6•U5 association with the 5’ SS. These structural rearrangements are driven by eight DExH/D-box RNA helicases, although the precise substrates of these proteins remain unclear.

We have developed a trans-splicing system using S. cerevisiae extracts to study events at the 5’ SS. Using an affinity purification step, even the low-abundance products of splicing and crosslinking can be isolated. This analysis yields a number of results that challenge the current understanding of the order and requirements for 5’ SS recognition.

We identify early, ATP-dependent crosslinks between both the U4/U6 snRNAs and the 5’ SS. The crosslinks, remarkably, do not require either the U1 snRNA or the U2-branch point interactions, which are thought to precede any interaction with the U6 snRNA. We also identify a second ATP-dependent crosslink between the 5’ SS and U6 (downstream of the first crosslink), which persists through the first catalytic step of splicing.

These results suggest that the 5’ splice site is sampled several times early in spliceosome assembly, possibly as part of a “proofreading” activity, to ensure accurate identification of the splice sites. Further, the canonical model of spliceosome assembly appears to be too simplistic, as our results illustrate a remarkable flexibility in the order of interactions established during splice site recognition.

These results raise a larger question, namely, how are these RNA rearrangements mediated? We address this using our crosslinking/trans-splicing system and a variety of extracts derived from yeast strains with mutations in specific splicing proteins. These extracts block splicing at discreet steps along the splicing pathway and allow us to examine which proteins are responsible for mediating the crosslinks that we observe.

The early U4 and U6 crosslinks to the 5’ SS are mediated by one of the DExH/D family proteins, Brt2 (Rss1/Prp44), an essential protein involved in the unwinding of U4 and U6 during spliceosome activation. We also show that the highly conserved splicing protein Prp8 mediates the transitions between the two ATP-dependent U6 crosslinks, and facilitates the U6-5’ SS interaction that persists through the first step.

We are continuing to use a variety of mutant strains to construct a detailed picture of RNA-RNA and RNA-protein interactions that take place during assembly of the spliceosome machinery and during splicing catalysis.

201. Probing the structural and functional role of U6 snRNA in pre-mRNA splicing

Daniel E. Ryan, John Abelson

We are trying to elucidate the roles of some of the key components of the spliceosome. U6 snRNA is the most highly conserved snRNA in the spliceosome and is located at the catalytic core when base paired to the 5’ splice site of the pre-mRNA substrate. We have pursued two complementary lines of study to gain insight into the role of U6 snRNA in splicing. One approach is to develop a more elaborate mutational map of U6 snRNA by incorporating multiple mutations that specifically disrupt or hyperstabilize various helical regions of U6 RNA. Thus, we found that U2-U6 helix II is not required for in vitro splicing, whereas U2-U6 helix I is required at a late stage of spliceosome assembly, just prior to the first catalytic step of splicing. To progress from this stage to splicing requires the action of Prp2 protein, one of the DEAD/H-box RNA helicases required for the various steps of splicing. The possible relationship between the action of Prp2 helicase and the requirement for U2-U6 helix I at the same stage is intriguing.

The other approach to probing the role of U6 in splicing involves generating photoaffinity cross-links between U6 RNA and other spliceosomal RNAs in close proximity (ca. 2 Å). In this way, we have mapped some tertiary physical interactions between U6 and U2 snRNAs. The 5’ end of U6 in U2-U6 helix I was cross-linked to a tetrauridine loop in U2 at the other end of the helix. This crosslink was formed just prior to the first catalytic step of splicing but after the Prp2 step at a late stage of assembly. Interestingly, the same tetrauridine loop of U2 formed cross-links to a tetrauridine sequence in U6, near the U6 3’
stem-loop. This set of U2-U6 cross-links forms prior to the cross-links which span U2-U6 helix I. By generating and mapping cross-links between U6 and U2 snRNAs and the pre-mRNA substrate, we hope to develop a model of the folded RNA structures at the catalytic core of the spliceosome.

202. Structural studies of DEAD-box RNA helicases
Randall M. Story, John Abelson

We have recently determined the structure of a DEAD-box putative RNA helicase from the archaeal hyperthermophile Methanococcus jannaschii (1). Like other helicases, the protein contains two α/β domains, each with a recA-like topology. Unlike other helicases, the protein exists as a dimer in the crystal. Through an interaction that resembles the dimer interface of insulin, the amino terminal domain's seven-strand β-sheet is extended to 14 strands across the two molecules. Motifs conserved in the DEAD-box family cluster in the cleft between domains and many of their functions can be deduced by mutational data and by comparison with other helicase structures. Several lines of evidence suggest that motif III (SAT) may be involved in binding RNA.

Our current efforts focus on crystallization of complexes of the Escherichia coli DEAD-box protein DpbA with small fragments (~30 nucleotides) of 23S rRNA. The ATP hydrolysis activity of DpbA is specifically stimulated by a region of 23S rRNA (2,3). DpbA currently serves as the best model for understanding the specific binding of a DEAD-box protein to its target RNA. The homologous Bacillus subtilis protein YxiN, which binds the same region of 23S rRNA, will also be used for structural studies.

References

203. Interactions between Prp5, Cus2 and U2 snRNA in yeast
Imre Barta, John Abelson

Recent observations have established a functional link between Prp5, Cus2 and U2 snRNA. Interactions between these factors ensure ATP dependence of pre-spliceosome formation in yeast. Featuring characteristic helicase motifs, Prp5 is presumably directly responsible for the hydrolysis of ATP. CUS2 is a non-essential gene that codes for an RRM-type RNA binding protein. A current working model for their action predicts that folding of U2 snRNA is facilitated by Cus2 and ATP hydrolysis by Prp5 allows the addition of correctly assembled U2snRNP to the commitment complex. Cus2 seems to be the center of this regulatory network as its deletion allows ATP-independent pre-spliceosome formation. In the absence of Cus2 the enzymatic activities of Prp5 become dispensable. This phenotype strikingly resembles that of other helicase/RNA binding protein pairs in splicing, namely Sub2/Mud2 and Prp28/U1C. In either case, deletion or mutation of the RNA binding partner renders the helicase dispensable.

Mutating U2 snRNA allows recapitulation of the Cus2 deletion phenotype. We are taking advantage of this observation in trying to determine the residues/structures of U2 snRNA that mediate the Prp5/Cus2 interaction. This in vivo genetic screen should offer further clues to the folding state of U2 snRNA as it enters the spliceosome assembly pathway.

Publications
positive selection of dominant-negative forms of Ras and Mek blocks requirements during thymocyte selection. We have shown the TCR play in the control of lymphocyte differentiation.

Our research interests are focused in elucidating the role context of other receptor-mediated signaling processes.

stimulating antigen, or must be interpreted flexibly in the occupancy of the TCR must either vary with the biochemical signals elicited by ligand identical cells may provoke either death or survival. Hence, the biochemical signals elicited by ligand occupancy of the TCR may result in activation-induced apoptosis (negative selection) of immature thymocytes, or in their survival (positive selection) and further differentiation into either CD4 or CD8 mature T cells. Though the existence of these selective events has been documented extensively, it remains puzzling that signals transmitted through the same receptor in seemingly identical cells may provoke either death or survival. Hence, the biochemical signals elicited by ligand occupancy of the TCR must either vary with the stimulating antigen, or must be interpreted flexibly in the context of other receptor-mediated signaling processes. Our research interests are focused in elucidating the role that different signal transduction pathways downstream of the TCR play in the control of lymphocyte differentiation.

We have focused so far on the signaling requirements during thymocyte selection. We have shown that the Ras/MAPK pathway plays a pivotal and specific role during T-cell development. In particular, expression of dominant-negative forms of Ras and Mek blocks positive selection of αβ T cells, while negative selection is not affected. We have also shown that the lineage commitment of the positively selected T cell is controlled by the intensity of the signal transmitted by the tyrosine kinase Lck. The research projects that we are pursuing build upon these systems and try to understand better the signaling networks that control the behavior of T cells.

204. Role of Ras effectors during thymocyte development

Micheline N. Laurent

The Ras family of GTP-binding proteins is important in the regulation of many different cell functions, including choices made by T cells during their development. Expression of a dominant-negative form of Ras (dnRas) within developing murine thymocytes blocks positive selection. Similar results are obtained when a catalytically-inactive variant of the MAPKK, Mek-1, was expressed in thymocytes. Genetic experiments with a gain-of-function variant of the MAPK Erk (SM) suggest that Erk is the only downstream effector of Mek necessary for positive selection, but additional pathways beside the MAPK cascade play a role downstream of Ras.

To address which other Ras effectors influence positive selection, we are utilizing a series of well-described Ras effector mutants that contain point mutations within the effector loop region of a constitutively active form of Ras. Each of these mutants stimulates only a specific subset of pathways downstream of Ras, thereby providing us with a method to study the effects of activating only particular signaling pathways. Using retroviral-mediated gene transfer, we have introduced the Ras mutants into fetal thymocytes derived from animals expressing the dnRas transgene in which positive selection is significantly reduced. Infected thymocytes are allowed to develop in fetal thymic organ culture (ftoc) for approximately two weeks and subsequently analyzed by flow cytometry for the ability to rescue the effects of a dominant-negative form of Ras.

Mutants that can rescue positive selection in this system will also be examined in 16610 D9 cells, a cell line derived from a thymoma. This system will be used for the biochemical analysis of the mutants’ effects. As the networks that underlie Ras signaling are complex, it is the goal of these experiments to further dissect and clarify the role of various Ras signaling pathways during thymocyte development.

205. Analysis of the role of the kinase suppressor of Ras (KSR) in Ras-mediated T-cell development

Micheline Laurent

The Kinase Suppressor of Ras (KSR) was originally identified in genetic screens for mutations that suppress the effects of activated Ras during vulval, and eye photoreceptor development in C. elegans, and D. melanogaster, respectively. To investigate the role of KSR during murine T-cell development, we over-expressed KSR in the ftoc system and found that this expression was able to inhibit thymocyte development in a
manner similar to that of dnRas. Based on this observation, it is our hypothesis that KSR is involved in transmitting T-cell receptor- and Ras-mediated signals to influence thymocyte development. To test this premise, the structural elements of KSR necessary for transduction of these signals will be identified by generating various KSR mutants containing, or lacking important signaling motifs. Initially, these studies will focus on elements of KSR necessary for its interactions with other molecules involved in Ras signaling as well as domains that may be important for KSR membrane localization. These mutants will be tested in the ftoc system in parallel with the intact KSR molecule to determine those elements important for KSR activity.

Another facet of KSR signaling that we will investigate is whether or not the levels of KSR expression within thymocytes affect Ras signaling. It has been shown in other vertebrate developmental systems that high levels of KSR are able to block Ras signaling while low levels of KSR expression promote Ras signaling, an observation consistent with scaffolding function. To test whether or not this effect holds true during thymocyte development, transgenic mice expressing different amounts of KSR protein within the thymus will be generated to determine if the levels of KSR expression affect potentiation of Ras signaling. These studies will provide insight into the means by which KSR functions in thymocytes, a novel signaling member of the Ras cascade.

206. Characterization of negative regulators of MAPK cascades downstream of the TCR

Susannah D. Barbee, Isaac See

Expression of an activated form of Mek-1, Mek-1(A3,S118,S122) (Mek*), under the control of the lck proximal promoter induces the early formation of thymomas. Analyses of these thymomas showed that the downstream MAPKs Erk-1 and -2 are not activated in Mek* thymocytes, nor can they be activated by stimulation with phorbol esters. However, Erk-1 and -2 can be activated in vitro by Mek-1 following purification, suggesting a specific inhibitory factor may be present in the cell. Likewise, the other MAPK’s SAPK and p38 are not and can not be activated in Mek* thymocytes. Thus the constitutive activation of upstream members of the MAPK cascade likely results in the activation of inhibitory phosphatases that prevent activation of downstream MAPKs. We hope to uncover what phosphatases may play this role and discover how they affect developmental processes.

MAPK can be regulated by members of the family of dual-specificity protein phosphatases (DUSPs). Most of these phosphatases are nuclear molecules regulated at the level of transcription that can dephosphorylate tyrosine, threonine, and serine residues, although some family members may be regulated post-translationally. Using Entrez and BLAST searches, we have identified fourteen phosphatases classified as dual specificity phosphatases (DUSP 1-14) and two additional phosphatases that may be DUSP family members (TS-DSP1 and TS-DSP2): each contains the conserved HCXXRXXG motif and other sequence homology to known dual specificity phosphatases.

We have designed family member-specific PCR primers flanking the conserved DUSP domains with which to characterize the usage of DUSPs in the T lineage. By comparing the expression patterns of these proteins in wild-type C57Bl/6 thymocytes and splenocytes as well as those activated with PMA and Ionomycin (mimicking the crosslinking of TCR), we can begin to understand what DUSPs are important in T cells and how they are regulated. Comparison with DUSP usage in Mek* thymocytes may lend special insight into the role of DUSPs in thymocyte development.

207. Real-time detection of MAP kinase activity using fluorescence resonance energy transfer (FRET)

Harry Green

Previous work from our laboratory has shown that the activation of the MAP kinase (MAPK) signal transduction pathway is necessary for development of mature T cells. We want to understand the mechanisms that regulate MAPK activation downstream the TCR. In particular we would like to know whether the MAPK cascade behaves as an on/off switch, as has been postulated in *Xenopus laevis* oocytes. For these experiments we need to analyze MAPK activity on a single-cell basis. We are developing an assay that can detect MAPK activity on a single-cell level using two-photon confocal microscopy. Cyan Fluorescent Protein (ECFP) and Yellow Fluorescent Protein (EYFP) have been fused together with a short linker sequence between them using cloning techniques, producing a FRET substrate we have termed CLY-1. The linker peptide is a short sequence of amino acids that codes for a MAPK binding domain and phosphorylation site. The binding of MAPK to the linker peptide and subsequent phosphorylation should force apart the ECFP and EYFP, thus disrupting FRET. The ratios obtained through confocal microscopy and flow cytometry should indicate MAPK activity at the single-cell level.

In single NIH-3T3 cells, we have shown CLY-1 produces efficient FRET in its resting state as compared to ECFP/EYFP co-transfected controls. In addition, increasing ECFP:EYFP emission ratios upon MAPK stimulation with phorbol esters indicate diminished energy transfer, correlating MAPK stimulation with a decrease in FRET. Further studies with an inhibitor of the MAPK pathway, PD98059, show no FRET change upon stimulation with phorbol esters, indicating specificity of MAPK for our CLY-1 construct.

Once our assay has been fully established, we will move into different cell lines and use primary cells to confirm the usefulness of this assay system in thymocytes. Using a retroviral packaging system, we will first test CLY-1 in the 16610D9 double-positive thymocyte cell line, which will allow us test this system in a different cell type, as well as work out any possible problems with
imaging. Then we propose functional assays using primary murine thymocytes to study MAP kinase activity during positive selection, giving an indication as to whether or not a threshold for positive selection exists and what that threshold may be.

208. Lck controls CD4/CD8 T-cell lineage commitment
G. Hernandez-Hoyos, J. Alberola-Ila

At the end of maturation of the TCR αβ lineage in the thymus, two different kinds of T cells, CD8+CD4- and CD4+CD8- αβ T cells develop from a pool of CD4+CD8+ double positive (DP) precursor cells. DP cells carrying T-cell receptors (TCR) that recognize peptide in the context of MHC class I differentiate into CD8 T cells, while those recognizing peptide in the context of MHC class II become CD4 T cells. The mechanisms underlying the choice of CD4 or CD8 lineage, and how MHC class recognition, coreceptor expression, and effector function are coordinated are not well understood. Two main models, stochastic and instructive, have tried to explain the developmental link between MHC specificity and coreceptor expression. The stochastic model proposes that commitment to the CD4 or CD8 lineage occurs independently of TCR specificity, and precedes positive selection. Those cells that make the wrong decision are eliminated at a later point. The instructional model proposes that the recognition and coengagement of class I MHC by a specific TCR and CD8 directs the turn-off of CD4, thus generating a CD8 T cell. Recognition and coengagement of class II by a specific TCR and CD4 promotes the down-regulation of CD8, producing a CD4 T cell.

By using transgenic animals that express Lck mutants with increased or decreased activity under the control of the lck distal promoter, we have been able to address directly the putative role of this kinase in CD4/CD8 lineage commitment, something that previous studies using gene disruption or transgenic approaches with promoters that are turned on at earlier stages have failed to address, given the important role of Lck during the β rearrangement checkpoint. Our results demonstrate that there is a quantitative relationship between the intensity of the Lck signal and the lineage commitment of DP thymocytes: thymocytes carrying a class I restricted TCR transgene develop into CD4 T cells when Lck activity is increased. Conversely, thymocytes expressing a class II restricted TCR transgene develop into CD8 T cells when Lck activity is reduced. These "mismatched" cells (class I-restricted CD4 and class II-restricted CD8 T cells) are phenotypically mature, exit the thymus, respond to their cognate peptide, and bear characteristics of bona fide CD4 and CD8 T cells, respectively. These results directly prove that the CD4/CD8 lineage decision reflects quantitative differences in the Lck signal that either CD4 or CD8 bring to the TCR complex, and provide a strong case for an instructive role of Lck signals in determining the divergent maturation programs of CD4 or CD8 T cells.

209. Chemical-genetic analysis of Lck protein tyrosine kinase signaling in T cells
D. Leopoldt

One of the first biochemical events after TCR stimulation is the activation of the src-family protein tyrosine kinase Lck. Studies from many laboratories, including ours, have shown that Lck plays a major role in T-cell development and activation. However, the exact contribution of Lck to many of these processes remains badly defined, due in part to the limitations of the genetic and pharmacological approaches used so far. We are using a recently developed chemical-genetic approach to engineer a mutant Lck protein (Lck-T316G), that is supersensitive to highly specific low-molecular weight kinase inhibitors. These cell-permeable drugs were specifically designed to inhibit Lck-T316G, but not wild-type Lck or any other protein kinase. Their use will allow us to perform a complete or partial functional knock-out of Lck kinase activity at different stages of T-cell development or immune responses.

Using the Cre/loxP-based gene targeting strategy the T316G mutation will be knocked-in exon 8 of the murine lck gene by homologous recombination in embryonic stem (ES) cells. Since the source of genomic DNA utilized in our targeting construct should be from the same strain as that of the target ES cells, we first have screened a 129SVJ mouse library to isolate a genomic lck clone. Two partial clones were isolated and their characterization is currently underway. Clone 7-42 containing the first eight exons can most likely be used for the left (short) arm of the replacement vector after introducing the point mutation by site-directed mutagenesis. A 5 kb fragment of clone 7-111 covering parts of intron 8 and downstream sequences including exon 12 will be used for the right (long) arm of the replacement vector. Pluripotent murine ES cells will be transfected with the linearized targeting vector for homologous recombination. Correctly targeted ES cells will then be used for injection into blastocysts.

Thymocytes and peripheral T cells from wild-type and homozygous T316G mutant Lck mice will be harvested at discrete times during differentiation, treated ex vivo with different doses of selective and potent kinase inhibitors and tested for possible Lck-mediated signal transduction pathways. Generation of these mutant Lck-T316G mice will later allow us to test the hypothesis that Lck activity controls CD4/CD8 lineage commitment, i.e., the decision of a T cell to become either a CD4+ helper T cell or a CD8+ cytotoxic T cell.

210. Signaling molecules in peripheral homeostasis of naive T cells
Eric Tse

The ability of an organism to mount an adaptive immune response to a novel pathogen depends on the maintenance of circulating cohorts of naive lymphocytes in peripheral lymphoid organs, and their numbers are extremely well controlled. Physiologic fluctuations in peripheral T-cell numbers are relatively small when
considering the varied contributions of thymic output, peripheral cell turnover, and massive antigen-induced proliferation; acute lymphopenia induced by chemical or radiological insult also fails to permanently diminish the peripheral T-cell pool as it is replenished by central (thymic) and peripheral homeostatic mechanisms. A number of studies have found that naive, otherwise quiescent, T cells proliferate when transferred into syngeneic, lymphopenic hosts. This homeostatic expansion and the survival of naive T cells have been found to require ligation of the T-cell receptor (TCR) by low-affinity peptides bound to major histocompatibility complexes (pMHC), the same types of ligands required for proper T-cell development in the thymus.

While these studies have examined the extracellular ligands involved in delivering the signal for naive T cell survival and homeostatic proliferation, little is known about the signaling events which relay this signal downstream of the TCR. My studies are addressing the role of the intracellular tyrosine kinase Lck in this context. Preliminary experiments with transgenic T cells expressing constitutively active Lck in the later stages of thymic development and in the periphery (dLGF) have shown that such cells actually undergo fewer cycles of division relative to control cells when adoptively transferred into lymphopenic hosts. Furthermore, stimulation of cells ex vivo with an antibody which crosslinks the signaling chains of the TCR has shown that dLGF cells exhibit a two-day delay in their proliferative response to this stimulation. Whether this impaired proliferation is a result of the transgene expression during development or in the periphery is unclear since it is expressed during both in the transgenic line of mice used. My current efforts are directed at developing a viral system to target transgene expression in peripheral, naive T cells to better address the role of this kinase in peripheral, naive T cells.

211. Helix-loop-helix factors and T-cell lineage commitment

Gretchen Bain*, Cornelis Murre*, J. Alberola-Ila

Recent studies have suggested a role for the transcription factor E-2A in thymocyte maturation. E proteins, which include E-2A, are a class of the ubiquitously-expressed basic helix-loop-helix (bHLH) proteins. A targeted mutation in E-2A has been found to result in a significant increase in the percentage of CD4+ and CD8+ thymocytes and a corresponding decrease in the percentage of their precursors, CD4+CD8+ thymocytes. Interestingly, the increase in CD8+ thymocytes is greater than that of CD4+ thymocytes, suggesting that E-2A may play a role in the choice between the CD4+ and CD8+ lineages. In agreement with this model, our results show that retroviral-mediated overexpression of Id3, a dominant-negative inhibitor of E proteins, results in an increase in the percentage of CD8+ cells and a concomitant decrease in the percentage of CD4+ cells. Furthermore, work of our collaborators at UCSD has shown that Id3 is upregulated in thymocytes after TCR stimulation, and that this upregulation is mediated by Lck and MAPK. We are therefore trying to understand the mechanisms that connect Lck, MAPK and Id3 with the CD4/CD8 lineage choice.

212. Effect of notch signaling on the Ras pathway during positive selection

Gabriela Hernández-Hoyos

Notch is a family of transmembrane receptors of which there are four genes in mammals: Notch-1 to 4. When engaged by its ligand, Notch is cleaved, releasing an intracellular fragment that is translocated to the nucleus, where it activates transcription of various genes. Activation of Notch is involved in different cellular processes including differentiation and cell survival, in a variety of tissues. Recent work from various groups suggests that the Notch cascade is also involved in positive selection of T cells. Overexpression of activated Notch signaling can block CD4 development, or both CD4 and CD8 development, depending on which intracellular domains of the Notch intracellular fragment are overexpressed. Notch signaling appears to block activation of DP thymocytes by dampening the TCR signal. The Ras/MAPK pathway is activated early on during positive selection of T cells and is necessary for this process to occur. Our primary goal is to determine if Notch signaling directly inhibits activation of the Ras/MAPK cascade as it does in vulval development of C. elegans. In this case, phosphorylation of ERK-1,2, which lie at the end of the Ras/MAPK cascade, is inhibited by inducing the expression of specific phosphatases. Our experimental approach involves transducing double-positive cells with activated Notch, and activating the Ras/MAPK cascade to determine if Notch signaling can block Ras activation. We are currently testing four Notch constructs containing the full-length or truncated versions of the intracellular fragment to determine if any of these constructs can block phosphorylation of ERK1,2, and subsequent transcription of EGR-1 and Id-3.

213. Role of phosphatidylinositol 3-kinase in T-cell development

Susannah D. Barbee

Phosphatidylinositol 3-kinase (PI3K) has been implicated in the regulation of proliferation and survival of a number of cell types including lymphocytes. PI3K is responsive to a variety of stimuli in T cells including stimulatory signals (CD3, CD28), inhibitory receptors (CTLA-4), and growth factors and cytokines (IL-2, -4, -7). Given these features, PI3K may play a number of roles in thymocyte development, from regulation of proliferative bursts to positive selection to protection from negative selection.

We have analyzed a strain of mice overexpressing the adaptor-binding domain of p110 (p110ABD). Previously thought to be a dominant negative form of PI3K, we have found that this mutant actually induces constitutive activation of the downstream kinase Akt: by sequestering the regulatory adaptor subunit p85, endogenous p110 exists as a highly unstable but
catalytically active monomer. The thymii of p110ABD animals show an increase in the percentages and numbers the mature single positive thymocyte subsets. BrdU labeling experiments revealed no change in the rate of development of transgenic thymocytes, suggesting that PI3K does not regulate thymocyte proliferation. Thymocytes doubly transgenic for p110ABD and the ovalbumin-specific TCR OT-1 express a single clonal TCR that recognizes the peptide SIINFEKL; co-culture of these thymocytes with an antigen-presenting cell and the SIINFEKL peptide provides an in vitro simulation of negative selection. The p110ABD transgene does not offer statistically significant protection from deletion in these assays. Likewise, endogenous vSAG7-mediated deletion of TCR Vβ6+ thymocytes is unaltered in DBA2/J x p110ABD mice. Although this result is initially surprising given the roles of PI3K and Akt in survival processes, it is confirmed by the unchanged regulation of the downstream survival effector Bad in p110ABD thymocytes.

FACS analysis revealed that the accumulated SP thymocytes are HSAlo, the most mature subset in the thymus. Since neither HSAhi nor HSAlo SP thymocytes proliferate more in p110ABD mice than normal littermates, this accumulation may also reflect a delayed emigration of thymocytes into the periphery. We are currently examining this possibility via two approaches. With an in vitro chemotactic assay, we are measuring the responsiveness of p110ABD thymocytes to known chemotactic agents in the thymus. By co-adaptively transferring p110ABD and C57Bl/6 bone marrow into lethally-irradiated hosts, we can observe parallel cohorts developing in the thymus and measure the kinetics of SP development and emigration.

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mutation were depleted of the majority of their mtDNA by transformants carrying 100% or 0% of the T414G mutation. Evidence on such replicative advantage, mtDNA-less cell advantage of the mutant mtDNA. In order to obtain direct evidence indicating the clonal nature of this phenomenon, the clear evidence of aging-dependent specific point mutations in the mtDNA control region of fibroblasts and skeletal muscles, the clear evidence of the homopolymeric tract D310. This variation appears contrary, they exhibit an extensive variation in the length and muscle-specific aging-related point mutations. On the other hand, lymphocytes and platelets do not exhibit the fibroblast-type of blood cells, in particular granulocytes, with some indication, however, of a decreased occurrence in young and, with higher frequency, in old individuals, of the homopolymeric tract D310. This variation appears consistent with the idea that the mtDNA exhibits two specific mutations at the same critical sites for replication previously found to be targeted by aging-related mutations in fibroblasts, i.e., within the promoter of the primer for mtDNA heavy-strand synthesis and at the main initiation site for this synthesis, but at different nucleotide positions, has dramatically highlighted the non-randomness of this event, pointing to the functional relevance of the mutations. Furthermore, the striking tissue specificity of these mutations strongly suggests the involvement of cell type-specific proteins or cofactors. The evidence for this tissue specificity has been extended in the past year with the discovery that different types of blood cells, in particular granulocytes, lymphocytes and platelets do not exhibit the fibroblast- and muscle-specific aging-related point mutations. On the contrary, they exhibit an extensive variation in the length of the homopolymeric tract D310. This variation appears in young and, with higher frequency, in old individuals, with some indication, however, of a decreased occurrence in centenarians.

As to the mechanism leading to the establishment of aging-dependent specific point mutations in the mtDNA control region of fibroblasts and skeletal muscles, the clear evidence indicating the clonal nature of this phenomenon in the original fibroblasts and in the derived \( \rho^0 \) cell transformants has strongly suggested a replicative advantage of the mutant mtDNA. In order to obtain direct evidence on such replicative advantage, mtDNA-less cell transformants carrying 100% or 0% of the T414G mutation were depleted of the majority of their mtDNA by exposure to a low concentration of ethidium bromide, and then allowed to reestablish their mtDNA complement after removal of the drug. These experiments have shown that the cells containing a residue of mutant mtDNA recovered somewhat faster their full mutant mtDNA complement than the wild-type cells recovered their wild-type complement. These results have clearly indicated that the T414G mutation does not affect negatively the replication capacity of mtDNA, but may have, on the contrary, a stimulating effect on this capacity. If this result is confirmed by further experiments, an interesting hypothesis would be that the aging-dependent mutations discovered in the mtDNA control region may represent a compensatory phenomenon, namely that they may be selected for because they allow the replacement of damaged mtDNA with more intact mtDNA.

In another area, a significant step forward towards the goal of developing therapeutic approaches for mtDNA-linked diseases affecting the first respiratory enzyme, i.e., the NADH dehydrogenase (Complex I), has been made by the demonstration that the complete lack of Complex I activity caused by a nonsense mutation in the gene for ND4, an essential mtDNA-encoded subunit of this enzyme complex, can be fully corrected by the Saccharomyces cerevisiae single polypeptide NADH-quinone oxidoreductase ND11 gene.

Progress has also been made in the analysis of the early mitochondrial involvement in the apoptotic process in human cells. Thus, in both 143B osteoblastoma cells and PC12 pheochromocytoma cells in culture, induced to apoptosis by the protein kinase inhibitor staurosporine, a decrease in overall endogenous respiration rate preceding cytochrome \( c \) release was found to be caspase-independent, insensitive to overexpression of the antiapoptotic Bcl-2 protein and not related to inhibition of protein kinase \( c \). The observation that permeabilization of the osteoblastoma cells with digitonin, an outer mitochondrial membrane-solubilizing detergent, causes a dramatic loss of cytochrome \( c \) and respiration, reversible by exogenous cytochrome \( c \), in cells primed for apoptosis by staurosporine, but not in naïve cells, suggests the possibility that substantial changes in the mitochondrial membranes may be involved in the observed decrease in endogenous respiration prior to cytochrome \( c \) release.

Still in another direction, evidence has been obtained suggesting the possibility that the human mitochondrial transcription termination factor (mTERF) can exist both in a monomeric active form and in a homooligomeric or heterooligomeric inactive form, the transition between the two forms being regulated by phosphorylation-dephosphorylation.
214. Mosaic distribution in vivo and after transfer into mtDNA-less cells of human fibroblast mtDNA carrying the aging-dependent T414G mutation

Yuichi Michikawa, Kenneth Laderman*, Kathleen Richter

Previous work has revealed an aging-dependent large accumulation of a T to G transversion at position 414, within the promoter for the RNA primer for heavy (H)-strand synthesis, in mitochondrial DNA (mtDNA) from human skin fibroblasts1. The mechanisms whereby this mutation appears in a large proportion of normal individuals above 65 years old, and then becomes amplified so as to involve a generally high proportion (up to ~50%) of mtDNA molecules in the cell are unknown. In the present work, fibroblasts from three individuals 78, 80, and 86 years old, which carried originally the T414G mutation in ~24, ~50, and ~23% of their mtDNA, respectively, when grown in vitro for 2 to 2.5 months, underwent a progressive decrease in the proportion of the mutation, down to 0 to 8.6%, which reflected, presumably, outgrowth by wild-type cells. At the same time, three other cultures, from individuals 52 to 71 years old, which lacked the mutation, failed to show its appearance, even after three months of culture. These observations have clearly indicated that the establishment and amplification of the T414G mutation require an in vivo environment. In other experiments, subcloning of fibroblasts revealed, surprisingly, a mosaic distribution of the T414G mutation. This was present, mostly in a homoplasmic or nearly-homoplasmic form (>70%), in a fraction of the original fibroblasts, while it was generally absent in the remainder of the cells. The mosaic distribution of the mutation was maintained, in general with a marked shift to homoplasmy, of the cells. The mosaic distribution of the T414G mutation has significant implications for understanding the mechanism of this unusual phenomenon.

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215. Screening of aging-dependent point mutations in the mtDNA control region from human blood platelets

Jin Zhang, Yuichi Michikawa, Jennifer Fish

Recently, clear evidence has been obtained in our laboratory of a tissue-specific, aging-dependent large accumulation of point mutations in the mitochondrial DNA control region of human fibroblasts (Michikawa et al., 1999) and skeletal muscles (Wang et al., 2000). These observations had suggested that the mtDNA aging-related sequence variation could be used as a marker of biological age. The project described here aims at investigating the occurrence of these aging-dependent mutations in blood cells. We chose to study human blood cell samples since blood cells are the most easily accessible cells for biochemical and genetic analysis in the human body and are considered to be one of the most useful materials to monitor biological age.

In a first study, platelet samples separated from blood of 34 unrelated individuals with an age range from 19y to 79y were analyzed. This material was provided by the National Institute on Aging (Dr. Tamara Harris) and by the Laboratory for Clinical Biochemistry Research of the University of Vermont College of Medicine (Dr. Russell Tracy). MtDNA was extracted from the platelets, and then the tRNA1-DLP3 (nt 21-719), DLP4 (nt 110-271) and DLP6 (nt 260-540) segments in the mtDNA main control region for transcription and replication were PCR amplified, as previously described (1,2). The DLP4 and DLP6 fragments were subjected to first-round DGGE (Denatured Gradient Gel Electrophoresis) analysis in order to investigate overall mutation accumulation. The tRNA1-DLP3 fragment was analyzed by the allele-specific termination of primer extension for the identification and quantification of specific point mutations and was also used as a template for cloning.

The result of the overall mutation accumulation analysis by first-round DGGE showed quite heterogeneous DGGE patterns in the mtDNA from platelets of variously aged individuals. The age distribution of the individuals exhibiting abnormal patterns was different from that observed in skin fibroblast and skeletal muscle mtDNA. After the first-DGGE assays, the samples from four old individuals (two individuals showing multiple bands and two individuals showing normal bands in the first-round DGGE analysis) were selected for further study. For this purpose, the large tRNA1-DLP3 fragments from platelet mtDNA have been cloned in Escherichia coli, and 48 plasmids have been isolated from each source. The plasmid DNAs were then subjected to PCR amplification of DLP6 and DLP4 fragments, and to a second-DGGE step, followed by a sequencing analysis, as previously described. This analysis and the fast screening of the samples by allele-specific primer extension for detection of specific point mutations have been completed. The results indicate an extensive variation in the length of the homopolymeric tract D310, consisting mostly of a polyC sequence. This variation showed little relationship to age, appearing with similar frequency in mtDNA from the individuals 19 to 40 years and in that from individuals 70 to 79 years in age. In addition, the analysis of allele-specific termination of primer extension on the tRNA1-DLP3 fragment revealed the absence or only minimal presence of the mutations that have been frequently observed in skin fibroblast or skeletal muscle mtDNA from elderly individuals.

References
216. Frequency of heteroplasmy in a hypervariable segment of the mtDNA control region in blood cells from variously aged healthy human individuals

Jin Zhang, Yuichi Michikawa, Jennifer Fish

The control region of mitochondrial DNA (mtDNA) that includes the origin of heavy-strand replication and the three origins of transcription (two for the heavy strand and one for the light strand), exhibits a high degree of sequence variability within a single individual or among individuals (Cassandra et al., 2000). The highest degree of polymorphism occurs mainly within two hypervariable segments of the mtDNA control region, HVR1 and HVR2, which have been widely used in evolutionary studies, population genetics, and forensic medicine. A frequent site of sequence variation in the HVR2 region is the homopolymeric tract D310, a sequence of 12 to 18 cytosines interrupted by a T at position 310, which varies in length in the polyC segment from nt 303 to nt 309 preceding the T. The length variation of this polyC tract has been demonstrated to occur not only among individuals but also within an individual (heteroplasmic) (Marchington et al., 1997). However, it remains unclear whether the frequency of heteroplasmy is related to aging.

In the present study, we compared the length of this D310C sequence in mtDNA from human blood cells of variously-aged healthy individuals.

We analyzed 40 lymphocyte-enriched and 50 granulocyte-enriched fractions of blood cells separated from 50 unrelated individuals ranging in age from 27y to 104y and 46 buffy coat fractions separated from blood of 46 unrelated individuals ranging in age from 22y to 103y. This material was provided by the Italian National Research Center on Aging, Ancona, Italy (Dr. Claudio Franceschi) and by the Department of Biology of the University of Calabria, Italy (Dr. Giovanna De Benedictis). All individuals included in this study were free of clinically manifest pathologies. We used total DNA extracted from the blood cell fractions as a template for amplifying a tRNA1-DLP3 (nt 21-719) segment in the mtDNA control region, which contains all of the initiation sites for transcription and replication of mtDNA. After purifying the PCR product, the tRNA1-DLP3 fragment was analyzed by allele-specific termination of primer extension for the identification and quantification of D310C-tract length variation. Our results clearly showed a variable D310C-tract consisting of 7C, 8C, 9C or 10C residues in the mtDNA from blood cells. A highly sensitive analysis of the D310C-tract allowed the detection of length heteroplasmacy in 24 of the 40 lymphocyte samples (60%), in 29 of 50 granulocyte samples (58%) and in 40 of 46 buffy coat samples analyzed (87%). In order to determine whether the frequency of heteroplasmy differed significantly with age, all individuals were divided according to age into three groups: 19-38 year-old, 43-99 year-old, and centenarians. The frequency of heteroplasmy appeared to be lower in the group of younger individuals than in the 43 to 99 years-old individuals, suggesting that heteroplasmy increases with age. Most interestingly, however, the frequency of heteroplasmy showed a tendency to decrease in the centenarians.

Surprisingly, in three cases, the mtDNA from granulocytes and lymphocytes separated from same individual exhibited a totally different pattern of D310C-tract length variation. Two individuals showed a homoplasmic tract length in the mtDNA from granulocytes, but a variable heteroplasmic length in the mtDNA from lymphocytes. By contrast, one individual showed tract length heteroplasmy in the granulocytes, but homoplasmy in the lymphocytes. These results indicate that the frequency of heteroplasmy can also differ across blood fractions.

References


217. Effects of the fibroblast-specific, aging-dependent T414G mutation on the recovery from mtDNA depletion after ethidium bromide treatment

Kathleen Richter, Yuichi Michikawa, Ken Laderman

Recently a large, tissue-specific, aging-dependent accumulation of point mutations in the control region for mitochondrial DNA (mtDNA) was discovered in human skin fibroblasts and muscles (1,2). In fibroblasts the most frequent mutation was a T to G transversion at position 414, within the promoter for the RNA primer of mtDNA heavy (H)-strand synthesis. This mutation was found in 8 of 14 individuals older than 65 years, and was absent in 13 younger individuals (1). It was present in skin but absent in skeletal muscle, heart, lymph node, and spleen (2).

Low levels (~50 ng/ml) of ethidium bromide (EB) have been found to inhibit mtDNA replication, but not nuclear DNA replication, in HeLa cells (3). During recovery from treatment with EB, the mtDNA is gradually replenished, and there is a tendency for the copy number control to be bypassed because there is more than one replication event per cell cycle (3).

In order to obtain some information on the effects of the T414G mutation on mtDNA replication, the approach described above was applied to cells carrying 100% or 0% of the mutation and isolated by mitochondriamediated transfer of the mutation from fibroblasts into the mtDNA-less osteoblastoma cell line 143B.TK.ρ206. Cells were treated with 50 ng/ml EB for three days. The EB was removed and the cells were allowed to recover for five days. Total DNA was extracted on each day, and the levels of mtDNA and nuclear DNA were analyzed. MtDNA from the mutant cells, normalized to nuclear DNA, appeared to recover somewhat faster after EB treatment than mtDNA from the wild-type cells. This suggests that there could be a replicative advantage associated with the T414G mutation. This approach is being applied to other cell lines.
References

218. Detecting single nucleotide mutations using fluorescence-labeled PNA as probes

Yan Wang

We have identified aging-related point mutations in the control region of mitochondrial DNA (mtDNA) from human muscles and fibroblasts. The percentage of those mutations in mtDNA ranged from a few percent up to 50%. It is very important to know the cellular distribution of those mutations, which can help us understand the mechanism of their occurrence. However, there are no established techniques for detecting at the cellular level mtDNA point mutations in tissue sections or in cultured cells. Since changes in Tm are minimal for single base-pair mismatches, it is very difficult to detect point mutations using conventional DNA probes.

Fortunately, the development of the Peptide Nucleic Acid (PNA) technology has provided a possible solution to this problem. Unlike DNA, PNA has a backbone made of peptide bonds. Since the peptide bonds do not have any negative charges, the interaction between complementary strands in PNA:DNA is much stronger than in double-stranded DNA. As a result, the Tm of a PNA:DNA duplex is much higher than that of double-stranded DNA with the same sequence. This particular feature of PNA has made it a promising probe to detect point mutations at the cellular level in tissue sections or cell cultures.

We have used fluorescence-labeled PNA as probes to detect aging-dependent point mutations at mtDNA positions 408, 414 and 189 in muscle sections or cultured cells. The PNA probe can only hybridize to the DNA sequence that matches the probe perfectly, since one nucleotide change will totally abolish the binding. However, since PNA can only be end-labeled, the signal is relatively weak. When we used the probes on muscle sections, the auto-fluorescence in the sample was very high, and we could not distinguish the signal. Further work is required to lower the background or to increase the signal intensity using stronger fluorescent dyes or antibodies against 5PNA labels.

219. Lack of Complex I activity in human cells carrying a mutation in mtDNA-encoded ND4 subunit is corrected by the Saccharomyces cerevisiae NADH-quinone oxidoreductase (NDI1) gene

Yidong Bai, Petr Hájek, Anne Chomyn, Elisa Chan, Byoung Boo Seo+, Akemi Matsuno-Yagi+, Takao Yagi+, Giuseppe Attardi

It is shown here that the gene for the single subunit, rotenone-insensitive and flavone-sensitive internal NADH-quinone oxidoreductase of Saccharomyces cerevisiae (NDI1) can completely restore the NADH dehydrogenase activity in mutant human cells that lack the essential mitochondrial DNA (mtDNA)-encoded subunit ND4. In particular, the NDI1 gene was introduced into the nuclear genome of the human 143B.TK cell line derivative C4T, which carries a homoplasmic frameshift mutation in the ND4 gene. Two transformants, with a low or high level of expression of the exogenous gene, were chosen for a detailed analysis. In these cells, the corresponding protein is localized in mitochondria, its NADH-binding site faces the matrix compartment, as in yeast mitochondria, and, in perfect correlation with its abundance, restores partially or fully NADH-dependent respiration, that is rotenone-insensitive, flavone-sensitive, and antimycin A-sensitive. Thus the yeast enzyme has become coupled to the downstream portion of the human respiratory chain. Furthermore, the P:O ratio with malate/glutamate-dependent respiration in the transformants is approximately two-thirds of that of the wild-type 143B.TK cells, as expected from the lack of proton pumping activity in the yeast enzyme. Finally, while the original mutant cell line C4T fails to grow in medium containing galactose instead of glucose, the high NDI1-expressing transformant has a fully restored capacity to grow in galactose medium. The present observations substantially expand the potential of the yeast NDI1 gene for the therapy of mitochondrial diseases involving complex I deficiency.

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220. Early decrease in respiration, independent of cytochrome c release, in staurosporine-induced apoptosis of 143B osteosarcoma cells

Shili Duan, Petr Hajek, Soo Kyung Shin, Anne Chomyn

We have investigated early mitochondrial events in the apoptotic program occurring in 143B osteosarcoma cells induced to apoptosis by the protein kinase inhibitor staurosporine. We have observed a progressive significant decrease in the endogenous respiration rate in intact cells, which is down to 60% of the control rate after 8 hours' treatment. This contrasts with only a marginal decrease in the TMPD-dependent respiration rate, i.e., the cytochrome c oxidase activity, which, in the same cells, remains at ~93% of the control level.
The decrease in the endogenous respiration rate precedes or overlaps the release of cytochrome c into the cytosol, as determined by confocal immunofluorescence microscopy. We have found, by using specific inhibitors, that the decrease in endogenous respiration is not caspase-dependent, nor is it a result of the opening of the permeability transition pore. Furthermore, overexpression of the gene for the anti-apoptotic Bcl-2 gene, which blocks staurosporine-induced cytochrome c release and apoptosis, does not prevent the decrease in endogenous respiration rate, suggesting that Bcl-2 acts downstream of the perturbation in respiration. Enzyme assays are in progress to determine the cause of the early decrease in respiration rate in apoptotic cells.

Polarography experiments have led to the surprising finding that 143B cells treated with staurosporine for only 4 hours exhibited, after permeabilization with digitonin, a very rapid and dramatic decrease in glutamate/malate- or succinate-dependent respiration rate to less that 20% of the control rate, as well as a nearly complete loss of COX activity. These changes were due to a massive release of cytochrome c, as shown by the observation they were completely reversed by exogenous cytochrome c. Untreated cells showed no loss of cytochrome c after treatment with digitonin. Therefore, in intact cells, the outer mitochondrial membrane undergoes substantial changes long before cytochrome c is released.

221. Early decrease in respiration independent of cytochrome c release and nuclear localization of cytochrome c in PC12 cells undergoing apoptosis after treatment with staurosporine

Elisabetta Ferraro

We investigated the role of mitochondria during apoptosis occurring in PC12 cells upon treatment with staurosporine (STS). Mitochondria are a reservoir of apoptogenic molecules, whose release, during the apoptotic process, is regulated by many Bcl-2 family proteins but is not yet fully understood.

Cytochrome c is one of the molecules released from mitochondria during apoptosis, but very little is known about the effect of its release on respiratory functionality. To unravel the relationship between cytochrome c release and respiratory changes, we measured the O$_2$ consumption rate of apoptotic intact cells and, in particular, we compared the isolated cytochrome c oxidase (COX) activity with the overall endogenous respiration. Our experiments showed that, during the first 2 hours of treatment with STS, the DNP-uncoupled respiration rate of PC12 cells decreased slightly, but significantly, faster than the COX-dependent respiration. The two types of respiration reached, respectively, ~74% and ~87% of the initial rate. Thereafter, they decreased in closer parallelism, reaching, after 9 hours, ~30% and ~42% of the initial rate. Under the reasonable assumption that the loss of COX-dependent respiration results from the release of cytochrome c from mitochondria, the data mentioned above suggest the possibility that, during apoptosis caused by STS, there is an impairment of the respiratory chain which precedes the release of cytochrome c. To corroborate this finding, we tested Bcl-2 overexpressing PC12, cells in which, as demonstrated by immunocytochemistry assay, the cytochrome c release is blocked. Even though cytochrome c is not released, we observed the same early decrease in respiration detected in non-transfected cells. Thereafter, however, there is a diverging behavior. In fact, after 9 hours of incubation, the DNP-uncoupled respiration rate is still about 70% of the initial rate, while the COX-dependent respiration is only mildly affected (it remains at about 90% of the initial rate), as expected from the presumed absence of cytochrome c release due to Bcl-2 overexpression. STS has, therefore, an effect on the overall endogenous respiration which, at least initially, is not related to cytochrome c release.

Finally, we analyzed the localization of cytochrome c by confocal immunofluorescence microscopy, using a triple-staining assay which allowed the simultaneous detection of nuclei, the mitochondrial matrix protein hsp60 and cytochrome c. We observed that STS-treatment promoted the nuclear fragmentation and the redistribution of cytochrome c from mitochondria into the nucleus. According with the accepted theories, our experiments showed that cytochrome c release preceded the nuclear fragmentation. Furthermore, we obtained a kinetic of loss of cytochrome c from mitochondria that correlated very well with the kinetics of COX-activity decrease, and that was than the decrease of the DNP-uncoupled respiration rate. This evidence further supports the finding that there is a decrease in respiration induced by STS which is independent of cytochrome c release. This kind of analysis allowed us to conclude that, after its release from mitochondria, the cytochrome c apparently transfers into the nucleus, as previously reported for some other apoptotic molecules.

We excluded the possibility that the effect of staurosporine on the respiratory chain could be due to the mere inhibition of protein kinase C (PKC) by this drug. In fact, using an analog of STS which is a PKC inhibitor, but not a inducer of apoptosis, we did not observe any of the effects detected before.

222. Analysis of mitochondrial regulation of apoptosis

Petr Hájek, Anne Chomyn

We have completed our study on in vivo changes in respiration accompanying cytochrome c release from mitochondria in Jurkat cells induced to Fas-mediated apoptosis (1). We found 1) that the cytochrome c release was followed by a progressive reduction in the respiratory activity of the last respiratory enzyme, cytochrome c oxidase (COX), and with a little delay, by a decrease in overall endogenous respiration rate, as measured in vivo in the whole cell population, 2) a cellular mosaicism in cytochrome c release and respiration loss, and 3) the occurrence of a rate-limiting step, most probably at the level of the mitochondrial outer membrane, preceding cytochrome c release in already primed cells. In
continuation of our efforts to understand the regulatory functions of mitochondria in apoptosis, the effects of overexpression of anti-apoptotic factor Bcl-2, localized primarily in the mitochondrial outer membrane, were examined. Although the importance of Bcl-2-inhibitable cytochrome c release from mitochondria in the activation of apoptotic proteases is well established, the precise molecular mechanism of the Bcl-2 negative regulation of cytochrome c release from mitochondria is unknown. We have found by immunoblot analysis of cell lysates that Bcl-2-overexpressing osteosarcoma-derived cell lines have a markedly decreased cytochrome c immunoreactivity as compared to the parental cells. The decreased cytochrome c immunoreactivity was also observed by confocal immunofluorescence microscopy in Bcl-2-overexpressing cells, double-labeled with antibodies against cytochrome c and the mitochondrial matrix marker Hsp60. Since two different types of anti-cytochrome c antibodies were used in the experiments described above, i.e., the anti-cytochrome c antibodies that recognize the denatured cytochrome c in immunoblot analysis and anti-cytochrome c antibodies that recognize the native protein, it was concluded, that a conformational change of cytochrome c in Bcl-2-overexpressing cells, that could explain the decreased immunoreactivity, is unlikely. Rather, these results suggested that the cytochrome c protein content is reduced in Bcl-2-overexpressing cells. Northern blot analysis did not reveal, however, any significant decrease in cytochrome c mRNA in Bcl-2-overexpressing cells. In addition, the Bcl-2-overexpressing cells exhibited a normal endogenous respiration rate and a normal excess of COX respiratory activity, which also depends on cytochrome c (2). As expected, staurosporine treatment of Bcl-2-overexpressing cells did not lead to cytochrome c release and apoptosis (2). While it is puzzling why the cytochrome c protein content is lower in Bcl-2-overexpressing cells, an attractive hypothesis can be proposed. It is possible, that one mechanism whereby Bcl-2 protects cells against apoptotic death would involve lowering at the translation or import level the mitochondrial cytochrome c content, but keeping it above a threshold necessary for normal respiration. In support of this, a molar excess of cytochrome c over COX has been shown to occur in certain cell types. Also, the distribution of cytochrome c in different compartments in mitochondria could possibly also play a role in the present observations.

**References**


**223. Structural characterization of unmodified mt tRNAs with mitochondrial and cytosolic methyltransferases from HeLa cells reveals an RNA folding problem**

*Mark Helm*

The increase of genetic information in the past years has led to the identification of a number of genes coding for tRNA modification enzymes, and subsequently, to a surge of new knowledge of structural requirements for tRNA recognition by modification enzymes.

Using this structural knowledge, modification enzymes can be employed as "structural probes," meaning that the extent of modification of a given RNA substrate in an *in vitro* modification experiment is indicative of the degree of resemblance of the RNA substrate with the "ideal" tRNA structure.

Our previous findings indicated that one, and possibly more mitochondrial (mt) tRNAs might be misfolded, unless these tRNAs contain modified bases (1). Hence, to study the modification of such mt tRNA transcripts *in vitro*, we have prepared and characterized protein extracts containing tRNA modification activities from either HeLa cell cytosol or highly purified HeLa cell mitochondria. The initial characterization was carried out using unmodified versions of i) yeast cytosolic tRNA<sup>Phe</sup>, ii) human cytosolic tRNA<sup>Lys1</sup>, and iii) human mt tRNA<sup>8Le</sup>, which were produced by *in vitro* transcription. We have characterized the influence of salt and magnesium ion concentrations on the modification activities using these model substrates. Furthermore, we have compared the retention times of the activities on a gel filtration column, in order to detect differences between otherwise similar activities from mitochondria and the cytosol.

When tested with other transcripts of human mt tRNA genes, namely tRNA<sup>Phe</sup>, tRNA<sup>Leu(CUN)</sup>, and tRNA<sup>Lys</sup>, the mitochondrial extract displayed, among nine different modification activities, a methyltransferase activity specific for position N1A9. This activity methylated position N1A9 in any transcript containing an A9, including human mt tRNA<sup>Lys</sup>. Importantly, this tRNA is not cloverleaf-shaped in its unmodified version (1), but assumes the native cloverleaf structure upon methylation of N1A9. The rate of methylation of this non-cloverleaf-shaped transcript is slow compared to that of cloverleaf-shaped transcripts such as yeast tRNA<sup>Phe</sup> and mt tRNA<sup>Phe</sup>. Therefore it is possible that a small population of the mt tRNA<sup>Lys</sup> transcript exists in cloverleaf-conformation, in a dynamic equilibrium with the non-cloverleaf conformation. The N1A9-methyltransferase might recognize this cloverleaf-shaped population and stabilize it by methylation.

On the other hand, the mitochondrial as well as the cytosolic protein extract failed to modify a number of positions in transcripts of human mt tRNA<sup>Leu(UUR)</sup>, tRNA<sup>Leu(CUN)</sup>, tRNA<sup>Glu</sup>, and tRNA<sup>Gly</sup>, despite the presence of both recognition elements in the tRNAs and corresponding activities in the protein extracts.

We conclude that, probably, in addition to the well described case of mt tRNA<sup>8Le</sup> (1), several other human
mt tRNAs might be misfolded without their modified bases. The mitochondrial protein extract contains an activity that stabilizes the tRNA Lys transcript in its cloverleaf structure, akin to a chaperone. Similar chaperone-like activities for other tRNA transcripts could not be detected in vitro, but might be present in intact mitochondria.

These studies enable us to explore in vitro the influence of pathogenic mt tRNA mutations, to complement earlier in vivo experiments (2).

References


224. Current work on the human mitochondrial transcription termination factor (mTERF) activity

Jordi Asin-Cayuela

The mitochondrial transcription termination factor (mTERF) is a nuclear encoded protein able to promote termination of the rRNA coding region of mitochondrial DNA at the border between the 16S rRNA gene and the tRNA Leu(UUR) gene. The human mTERF cDNA was cloned, sequenced and expressed in an in vitro transcription - translation system, as well as in E. coli and in insect cells transfected with recombinant baculovirus. The mature recombinant protein, while exhibiting the expected specific DNA-binding capacity, is unable to promote transcription termination in an in vitro system, suggesting that a posttranslational modification(s) and/or an additional component(s) is necessary to activate the transcription termination promoting capacity of the protein. This view was further corroborated by the absence of activity shown by a highly purified mTERF from HeLa cells, seemingly indicating that one or some of the proteins that contaminated previous preparations of mTERF from HeLa cells were also necessary for transcription termination activity. In this respect, immunoprecipitation experiments using a mitochondrial lysate showed three proteins co-precipitating with mTERF. Furthermore, gel filtration chromatography of S-100 from a mitochondrial lysate, followed by western blot analysis, clearly showed that mTERF elutes from the column in two peaks, one corresponding to the expected molecular weight of the protein (39 Kda) and the other one at around 100 Kda. Those results came to confirm and expand previous results obtained by centrifugation of S-100 through a glycerol gradient.

On the other hand, ultra-pure mTERF from HeLa cells (lacking termination activity) submitted to gel filtration chromatography, eluted as one peak, with a retention time comparable to the 100 Kda form observed in the S-100. This result might indicate that the 100 Kda peak corresponds to a homopolymer of mTERF. Blue native gel analysis will be performed to estimate the molecular weight of this form of mTERF more accurately, as well as immunoprecipitation experiments of the fractions containing it, in order to identify possible mTERF-binding proteins.

In addition, DNA binding activity assays performed on the fractions derived from the gel filtration chromatography showed that the activity is associated to the 39 Kda form, but not to the 100 Kda form. These results need to be confirmed after purification of the two forms.

Finally, gel filtration chromatography of S-100 in the presence of phosphatase inhibitors showed a change in the ratio of the two forms of mTERF, suggesting that the transition between them is controlled by phosphorylation. This observation agrees with preliminary results from earlier experiments that showed inactivation of mTERF after treatment with soluble calf intestinal alkaline phosphatase. Future experiments will aim at testing of the termination activity after treatment with matrix-bound alkaline phosphatase of the purified forms, as well as at studying the transition between the two forms by phosphorylation-dephosphorylation.

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Publications

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TBK1. We are also examining the pathways of NF-κB one we uncovered involving the novel protein kinase mediating a conditional lethal allele of the abl gene in hematopoiesis and other systems by using genetic methods in cell culture to determine which of the many postulated signaling pathways are critical to the activation process. We are also studying macrophage functions. NF-κB plays in various systems, the function of the abl gene, regulation of T lymphocytes, DNA recombination and repair, and neuronal cell cycle control.

The largest program in the laboratory involves study of the NF-κB transcription factor. Our major tool is a collection of mice in which we have knocked out one or more of the genes encoding proteins that are part of the NF-κB complex or control its activity. Using these mice, we are studying the role of the individual proteins in controlling specific genes in various cell types. A major program involves using fibroblasts of defined genotype. We are also studying macrophage functions. NF-κB is found in high concentrations in neurons and, being present at synapses, is ideally positioned to serve a role as a mediator of synaptic activity. We are examining this possibility using the knockouts and direct study of neuronal and synaptosomal preparations. There are many pathways for activation of NF-κB. We are studying a new one we uncovered involving the novel protein kinase TBK1. We are also examining the pathways of NF-κB and JNK activation using genetic methods in cell culture to determine which of the many postulated signaling pathways are critical to the activation process.

This laboratory has had a long-standing interest in the role of the abl gene in oncogenesis and in normal cellular physiology. We have shown that the closely related arg gene overlaps in its functions with abl, which had confused understanding of abl. The double knockout is an embryonic lethal. We hope to examine the functions of these genes in hematopoiesis and other systems by studying a conditional lethal allele of abl on a background lacking arg. As another approach, we have turned to C. elegans. This organism has only one abl gene, facilitating genetic studies. Our present results suggest that abl acts through an interaction with EPH receptors and we are investigating that possibility.

Another interest in the laboratory has been in immunological tolerance and autoimmunity. In this regard, it is striking that mice lacking IL-2 or a functional IL-2 receptor accumulate activated T cells and develop autoimmunity, suggesting that the dominant role of IL-2 in vivo is to terminate T-cell responses and to maintain tolerance. We are testing this notion and trying to determine the cellular pathways through which IL-2 carries out its various functions.

A further interest of our laboratory is the role of a group of large protein kinases in intracellular signaling processes. One is ATR—we have found that a knockout of the gene encoding this protein leads to very early embryonic lethality. We are investigating ATR in more detail through a conditional allele.

We are interested in the factors that limit the rate of homologous recombination between exogenously introduced DNA and the chromosomal DNA. To study this question we have made cellular substrates that report homologous recombination and have characterized certain variables. We will extend this work by trying to increase rates of site-specific recombination events and to understand the genetic basis of the low rate in somatic cells. We are also investigating whether we can use mobile type II introns for gene transfer in mammalian cells.

Mature neuronal cells do not divide again and thus there are no cell lines representing such cells. In an effort to understand the nature of the replication block in such cells we have attempted to induce them to grow using oncogenes. They will respond by replicating their DNA but they do not go into mitosis. We are attempting to overcome this last block so that cell lines might be made.

**Summary:** Our laboratory currently explores six different areas: the control of NF-κB activation, the role NF-κB plays in various systems, the function of the abl gene, regulation of T lymphocytes, DNA recombination and repair, and neuronal cell cycle control.

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**225. Analysis of TNF and Fas receptor signaling to activation of gene expression**

*Mark Boldin, David Baltimore*

Members of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily play a crucial role in activation, proliferation, survival and death of cells in the immune system. The two prototype death receptors of the family are type I TNF receptor (TNFR1) and Fas/APO-1. The biological functions triggered by these two receptors are characterized by a remarkable duality. Infliction of tissue damage and cell death correlates with activation of tissue repair and expansion. The physiological reason for this duality lies in the ability of these receptors to trigger two kinds of intracellular signaling programs, a proteolytic cell death cascade and a number of kinase cascades leading to activation of gene expression, some of which may protect cells against cytotoxicity. The major transcriptional factors activated in response to TNF and Fas ligand are NF-kB and AP-1 proteins.
Despite significant recent progress in understanding the molecular mechanisms of TNFR1 and Fas receptor signaling towards activation of gene expression, there is still a great deal of confusion and argument with regard to the exact molecular scheme of events. The main cause for much of the disagreement in the field is the use of dominant-negative mutants and overexpression studies in order to elucidate the role of specific players in the pathway. Our rationale is that such approaches should be complemented by genetic studies. Genetic approaches often provide more concise answers to the question of which molecules play an essential role in a given signal transduction pathway. Therefore, in order to identify molecules that play a crucial role in relaying the signals from the TNFR1 and Fas receptors towards NF-κB and AP-1 activation, we are performing genetic screens similar to that developed by George Stark and his colleagues for the analysis of interferon receptor signaling (1). The method is based on creation and subsequent genetic complementation of mutant mammalian cell lines that are defective in cytokine-induced activation of a signal transduction pathway leading to transcriptional activation.

References

226. **Conditional deletion of ATR**

*Eric J. Brown, David Baltimore*

ATM and ATR are mammalian counterparts to a family of high molecular weight kinases conserved in a broad range of species including *S. pombe*, *S. cerevisiae* and *D. melanogaster*. Each of these genes is involved in DNA-damage responses and break into two groups based on homology and function. ATM is most closely related to Tel1, a gene that shares an overlapping role with Mec1 in checkpoint responses to γ-irradiation in *S. cerevisiae*. ATR, on the other hand, is most closely related to Rad3 ( *S. pombe*), Mei-41 ( *D. melanogaster*) and Mec1, each of which is required for checkpoint responses to UV radiation, methyl methanesulfonate, hydroxyurea and δ-irradiation. Disruption of the ATR gene in mice by homologous recombination results in early embryonic lethality and decreased genomic stability. ATR/- embryos develop to the blastocyst stage but arrest prior to gastrulation. Once placed in culture, ATR/- blastocyst cells attempt cell division as assessed by the appearance of mitotic cells but die of apoptosis soon afterwards. Examination of mitotic chromosomes of ATR/- cells indicates that chromosome breaks appear prior to apoptotic cell death. This data is consistent with the hypothesis that early lethality of ATR/- embryos is caused by a loss of genomic integrity. To further examine the role of ATR in genome maintenance, mice expressing a cre/lox conditional allele of ATR have been made. Depletion of ATR from murine embryonic fibroblasts by infection with a lentivirus expressing cre recombinase prevents continued cellular proliferation after approximately three divisions. Future studies will focus on the requirement for ATR in restricting cell cycle progression following DNA damage.

227. **Develop mobile group introns for mammalian gene targeting**

*Huatao Guo, David Baltimore*

Mobile group II introns, found in fungal and plant organelles and in eubacteria, are not only catalytic RNAs, but also retrotransposable elements. In nature, mobile group II introns insert site-specifically into their cognate sites in intronless alleles, a process called intron homing. Intron homing occurs through a target DNA-primed reverse transcription mechanism and is mediated by the intron-encoded, multifunctional protein, which has reverse transcriptase (RT), RNA maturase (splicing) and DNA endonuclease activities. The intron-encoded protein (IEP) first binds to the intron RNA to assist its splicing. After that, the IEP continues to associate with the spliced intron RNA to form a ribonucleoprotein (RNP) particle, which has DNA endonuclease activity. The spliced intron RNA in the RNP particle cleaves the DNA sense strand exactly at the exon junction through a reverse splicing reaction, while the IEP cleaves the antisense strand nine or ten nucleotides (nts) downstream. The cleaved antisense strand is then used as a primer by the IEP to reverse transcribe the inserted intron RNA, which leads to intron integration.

Group II intron endonucleases are highly site-specific and use both the intron RNA and the IEP to recognize an ~31-nt DNA target. A region of about 13-16 nt on this target is recognized primarily by the intron RNA through base pairing interactions. The IEP recognizes only a few key positions flanking the 13-16 nt region and may cause local DNA unwinding, enabling the intron RNA to base pair with the DNA target site for reverse splicing. Recently, by changing the base-pairing residues in the intron RNA, mobile group II intron L1.LtrB has been designed and selected to insert site-specifically into virtually any DNA sequence of *Escherichia coli*. Using an RNP transfection assay, retargeted introns were shown to insert into a plasmid-borne DNA target site in human cultured cells, although inefficiently.

To develop mobile group II introns for efficient mammalian gene targeting, we decided to express the L1.LtrB endonuclease directly in mammalian cells. We have now achieved efficient expression of the L1.LtrB IEP in mammalian cells and are attempting to assemble functional RNP particles *in vivo*. If mobile group II introns can be developed to insert into chromosomal target sites efficiently in mammalian cells, they could have broad applications in genetic engineering, functional genomics, and gene therapy.
228. A genetic analysis of NF-κB function: immunology and gene regulation

Alexander Hoffmann, David Baltimore

The transcription factor NF-κB has been implicated in the regulation of a surprising number of genes that fall into distinct functional categories: inflammatory and immune responses, cell survival and activation. Its activity is regulated in response to a variety of cell stimulatory signals, such as LPS, dsRNA, TNF, cytokines and growth factors. Determining what roles NF-κB has in mammalian physiology and the underlying specificity of gene regulation is the goal of this research project.

NF-κB encompasses five gene products (p50, p52, RelA, c-Rel, RelB) that are capable of homo- and heterodimerization through the shared rel-homology domain (RHD). Cytoplasmic versus nuclear localization of the resulting transcription factors is controlled by a family three 1κB's. Over the past few years, each of these genes has been deleted in mouse knockout strains. While interesting phenotypes have been described, we have documented extensive compensatory mechanisms within the NF-κB/1κB family that complicate the interpretation of NF-κB's physiological functions.

Over the last two years we have generated multiple combination knockouts to extend the genetic analysis. Such knockouts have revealed additional functions for NF-κB in immune activation mechanisms, control of apoptosis and growth. We have generated immortalized fibroblast cell lines from each genetic combination, these lines allow a detailed analysis of NF-κB in gene regulation in response to multiple stimuli. Such studies are being continued in a comprehensive manner utilizing gene chip technology to understand the specificity code for transcriptional control by NFκB/Rel dimers.

NF-κB-dependent gene activation is tightly controlled through a number of mechanisms. One appears to be mediated by the repressive action of RelB-p50/p52 dimers, as cells lacking these family members show hyperactivation of some genes and a lack of temporal control. We are now investigating whether these dimers are recruited to promoters following gene activation.

Another, possibly primary mechanism is nuclear localization of NF-κB that is regulated by the action of three 1κB isoforms. One of these, 1κB-κ, is induced by NF-κB, thereby forming a negative feedback loop. Utilizing cells containing only a single 1κB isoform we have constructed a mathematical model to describe this regulatory system. This model has accurately predicted responses to transient stimulation, and is being used to guide future experimentation.

229. Role of NF-κB in transcription regulation

Thomas Leung, David Baltimore

The transcription factor NF-κB is a dimeric protein that plays an important role in multiple physiologic functions and is activated by a variety of stimuli. The subunits of NF-κB are members of a family of five gene products (p50, p52, p65, cRel, and RelB) which share a rel homology-binding domain (RHD) and are capable of homo- and heterodimerization. NF-κB activates transcription by binding to kB consensus sites. Since each stimulus activates its own profile of genes, our goal is to understand how specificity is generated. The assembly of multiple transcription factors on enhancer sequences, the so-called enhanceosome model, may generate specificity in gene regulation. The possibilities of mixing and pairing enhancer and protein modules allows a tremendous number of gene expression programs to be created and hardwired into the genome.

We observed that NF-κB dependent genes have differential NF-κB protein requirements. RNA was collected from NF-κB knockout 3T3 cells stimulated with TNF, and the RNA levels for NF-κB dependent genes were then assayed. Some genes specifically required p50 for induction, others needed p50 or p52, and in a few, p50 or p52 played no role. When subunit requirements versus κB binding site sequences were compared, no obvious correlation was made. Genes with the same κB site sequence showed different protein requirements and vice versa. Therefore, κB site sequences are not the sole determinant of specificity. Cells were then stimulated with TNF and LPS to see if Rel protein requirements change with stimulus in a single gene. Cox2 was upregulated by TNF or LPS stimulation. However, different Rel protein requirements were needed depending on the stimulus. Cells treated with LPS required p50, while cells treated with TNF could be activated by NF-κB complexes containing p50 or p52.

We are currently exploring the possibility that while the same κB-site-containing enhancer is responsible for the induction of Cox2 by TNF and LPS, different enhanceosomes are formed by each stimulus.

230. Limits to proliferation in mature neurons

Carlos Lois, David Baltimore

Neurons are considered to be terminally differentiated cells that have exited the cell cycle permanently. Whereas cell types such as fibroblasts or glial cells can be stimulated to proliferate when exposed to growth factors, no known extracellular stimulus is sufficient to induce proliferation in mature neurons. However, in addition to growth factors, viral oncogenes can also force quiescent cells into cell cycle reentry. Some of these genes have been extensively studied both in vitro and in transgenic animals; however, due to technical limitations, viral oncogenes have not yet been successfully used to induce proliferation of mature neurons. Recently, new viral vectors have been developed which can deliver genes to quiescent cells in vitro with high efficiency. We are using these vectors to transfer viral oncogenes to mature neurons in vitro and have observed that neurons are induced to reenter the cell cycle. Our experiments indicate that neurons retain the molecular machinery required to replicate their DNA, assemble mitotic spindles, and
condense their chromosomes. However, we have not yet been able to document that neurons forced to reenter the cell cycle can exit from mitosis and give rise to two daughter cells. We want to find the mechanism that limits the ability of neurons to successfully complete the cell cycle. Identifying the hurdles that prevent the proliferation of fully mature neurons will allow us to induce their multiplication and to obtain clonal cell lines with specific functional properties. These clonal lines derived from identified, fully-differentiated cells will provide inroads for the understanding of the development and functioning of neurons and, potentially, a way to replace cells and to restore functions lost to disease or injury in the nervous system.

231  **Arg/Abl signaling in neurons**  
Wange Lu, David Baltimore

The proto-oncogene *abl* is a non-receptor tyrosine kinase. It contains tandem SH3, SH2, catalytic motifs and a large carboxy-terminal fragment with a number of important domains, including a proline-rich motif and an actin-binding domain. Vertebrate Abl family kinases consist of Abl and Arg. Arg differs from Abl at the long C-terminus tail.

In Drosophila, *abl* (D-*abl*) acts within developing neurons to regulate fasciculation and axonal outgrowth. Abl knockout mice display a variety of phenotypes with low penetrance. Arg*−/−* mice develop physically normal, but exhibit many subtle behavioral abnormalities. However, the abl and arg double-knockout mice are embryonic lethal due to a severe neurulation defect. The signaling mechanism of Abl/Arg remains unclear in either system.

To identify the potential signal transduction pathway, we have knocked out the abl gene in another organism, *C. elegans*. The RNAi mutation of *abl* leads to defects similar to Vab-1 and Vab-2, homologues of Eph receptor tyrosine kinases and their ligands respectively. Arg and Eph were found to interact with each other at the overexpression level in HEK cells. Deletion analysis showed that the C-terminus of Arg, which is unique to Arg, is required for binding Eph. This is consistent with the fact that Arg binds to Eph easily, while Abl binds only weakly. Comparison of Eph activity in wild-type and Abl/Arg double-knockout cell lines showed that Abl/Arg is required for Eph activation. Since Arg interacts with Eph specifically, Arg alone can rescue the Eph activity in the Abl/Arg double-knockout cell lines. We are currently comparing Eph activity in primary neurons from wild-type and Arg knockout mice and studying the biological significance of the Eph/Arg interaction.

232.  **Function of neuronal NF-κB**  
Mollie K. Meffert, David Baltimore

Long-lasting alterations of neuronal properties, such as those involved in plasticity and apoptosis, depend upon changes in gene expression. We are exploring the neuronal function of a potent transcription factor, Nuclear Factor kappa B (NF-κB) in mice. NF-κB is the prototype of a family of dimeric transcription factors made from monomers that have Rel regions which bind DNA, interact with each other, and also bind the IκB inhibitors. Although NF-κB has been most thoroughly studied in cells of the immune system, a role for NF-κB in neuronal (and glial) cell function has recently been proposed.

In mature unstimulated hippocampal neurons, we observe a low level of constitutive κB DNA-binding activity. We find that both physiological as well as pathological stimuli can induce κB-binding activity consisting of p50/p65 and p50:p50 dimers (measured by EMSA). Inducible NF-κB resides in the cytoplasm complexed to IκB; cytoplasmic storage enables it to receive upstream signals more directly than transcription factors localized solely to the nucleus and may also allow it to transport signals from distant synaptic stimuli to the nucleus. We constructed a GFP-p65 fusion gene to explore NF-κB localization and translocation in neurons. Our GFP-p65 fusion protein localizes to both the cell body and processes of neurons and undergoes nuclear translocation following stimulation by glutamate or depolarization. Experiments using synaptosomes (isolated nerve terminals) indicate that p50 and p65, as well as IκBα and IκBβ, are found at synapses. While p50:p50 and p50:p65 dimers occur in intact neurons, synapses contain only p65:p50. We have confirmed this finding using a p65 knockout mouse made in our laboratory. While intact neurons from this p65 null mouse contain p50:p50 dimers, no NF-κB or IκB can be detected at synapses. The p65 knockout mouse thus provides an effective system to address the specific functions of synaptic NF-κB. We are currently using this system to examine gene expression controlled by synaptic NF-κB as well as the role of synaptic NF-κB in behavioral and electrophysiological models of plasticity.

233.  **Expression cloning of signaling molecules in NF-κB-inducing pathways**  
Joel L. Pomerantz, Elissa M. Denny, David Baltimore

NF-κB is a transcription factor that functions as a pleiotropic regulator of genes involved in inflammation, the development and function of the immune system, and in anti-apoptotic responses. NF-κB is commonly composed of two subunits, p50 and p65, which are held inactive in the cytoplasm by the binding of an inhibitor molecule, IκB. A variety of extracellular signals activate NF-κB by inducing the targeted destruction of IκB, thereby liberating NF-κB to translocate to the nucleus, bind to specific target sites, and activate a program of gene expression. Signals induce IκB degradation by activating the catalytic activity of a kinase complex (IKK complex) which phosphorylates IκB, targeting IκB for ubiquitination and destruction by the 26S proteasome.

We would like to understand the molecular mechanisms by which multiple stimuli activate NF-κB. How is signaling achieved between an external stimulus
and the activation of the IKK complex? How do disparate stimuli converge upon the IKK complex while maintaining specificity for an elicited response? As a first step toward answering these questions we have developed an expression cloning strategy for the isolation of components of signaling pathways that activate NF-κB. In our strategy, a cDNA expression library is assayed in pools for the ability to activate an NF-κB-responsive reporter when cotransfected into tissue culture cells. Positive pools are tested for specificity using a reporter containing mutated NF-κB binding sites. In addition, positive pools are tested in the presence of a kinase-dead IKKβ subunit to confirm that they activate NF-κB through the activation of the IKK complex. Clones responsible for a particular pool’s activity are purified by sib selection.

Using a human placenta cDNA expression library, we have shown that this strategy can detect and identify molecules of different biochemical types, both catalytic and noncatalytic. We have isolated ligands, receptors, adaptor proteins, kinases, kinase substrates, and a small GTPase. We have isolated molecules with established, pivotal roles in NF-κB activation as well as molecules not previously linked to NF-κB. Currently we are conducting the screen using an expression library from mouse thymus with the hope of isolating molecules that function to regulate NF-κB during the immune response.

234. The regulation of gene targeting in mammalian cells
Matthew Porteus, David Baltimore

Gene targeting is the process by which exogenous DNA replaces homologous sequences in the genome. It occurs at high frequencies in yeast but at low frequencies in mammalian cells (on the order of one in a million cells transfected). Further, in most mammalian cells the rate of homologous integration is much lower than the rate of random integration of exogenous DNA. Thus, in mammalian cells the utility of gene targeting is confounded by both a low absolute rate and low relative rate.

To understand the regulation of gene targeting, we have developed a system to assay for gene targeting in mammalian cells. In this system, we have made stable cell lines in which a mutated marker gene (either LacZ or GFP) has been randomly integrated into the genome under the control of the CMV/Chicken β-Actin promoter. In these stable cell lines, there is no functional expression of the marker gene. These cell lines are transfected with a substrate which can correct the introduced mutation but does not code for a functional gene. If gene targeting occurs, however, then a functional marker is created and the cells can be scored for being GFP or LacZ positive.

Using this system we have found that the rate of gene targeting is increased if the length of homology between the substrate and target is increased and if the amount of substrate introduced into the cell is increased. We have also confirmed that if a double-stranded DNA break is introduced into the targeted locus, the rate of gene targeting is increased over 1000 fold and have found that the rate of gene targeting is directly dependent on the frequency of double-stranded break formation. We have found that the rate of gene targeting is independent of the transcriptional status of the substrate and of the method of transfection.

We are currently using this system to explore the genetic regulation of gene targeting in mammalian cells and the possibility of inducing gene targeting using different viral vectors to introduce the gene targeting substrate.

235. Molecular characterization of CD4+CD25+ regulatory T cells
Xiao-Feng Qin, David Baltimore

Immunological tolerance is a principle feature of the immune system and is intimately related to the discrimination between self and non-self. Research in the past decade, and particularly the more recent studies on humans, has provided firm evidence that tolerance is actively achieved and dominantly maintained by a unique population of CD4+CD25+ regulatory T cells (Tr). However, attempts to characterize Tr cells have been hampered by the lack of exclusive cellular markers, their low abundance inside the body and poor proliferative capacity in vitro. To circumvent these problems, we have developed a high-throughput approach using a combination of magnetic beads plus high speed-multiparameter fluorescent-activated cell sorting (Molto Cytomation) to purify CD4+CD25+ cells from unmanipulated wild-type animals to >99.9%. With this high level of purity, we are able to test various conditions, including multiple IL-2 family of cytokines (IL-4, IL-7, IL-9, IL-15, etc.) and bone marrow-derived DCs, to expand and grow the Tr cells in vitro. The study aims to obtain a large quantity of cells for biochemical and molecular analyses.

One theoretical question about Tr biology is their use of TCR and their antigen specificity. To address this, we have generated hybridoma lines to clone Tr-specific TCR genes. We will also use the hybridoma cells to directly test whether Tr antigen receptors have high affinities to self-peptides in either lymphoid or non-lymphoid tissues. In addition, we have developed a retrovirus-based transgenesis method to introduce TCR genes into BM stem cells or germline. Using RAG-/- mice, we will be able to produce a monoclonal population of T cells that exclusively express the defined Tr antigen receptors. The system will allow us to map the lineage differentiation pathways and to determine whether the cell fate is primarily controlled by the receptor specificity.

Another line of our research on Tr is to identify the molecules responsible for their essential T cell suppression activity. We have devised a signal-peptide-trap cloning strategy to clone Tr-specific surface molecule genes. We will use retrovirus vectors to introduce wild-type or dominant-negative mutants of the candidate genes into conventional and regulatory T-cell lines to test their functional relevance.
Antigen specificity and fate of autoreactive CD4+ T cells

Lili Yang, David Baltimore

The immune system has a remarkable capacity to respond to foreign antigens while maintaining a state of unresponsiveness to self. Breakdown of this self-tolerance results in various autoimmune diseases. Our research focuses on studying the antigen specificity and the fate of autoreactive CD4+ T cells, which are thought to be required for most autoimmune reactions. The current experiments have three aims. The first is to generate and characterize autoreactive CD4+ T-cell hybridoma clones (ARTs) derived from IL-2 and CTLA-4 knockout mice. By sequencing and comparing the TCR to ARTs, we will examine whether there are any conserved structural features amongst each autoreactive TCR repertoire. The second is to analyze the fate of ARTs in vivo. We have developed a novel approach based on retrovirus-mediated gene transfer into bone marrow stem cells to generate TCR transgenics. Using this approach, we will generate TCR transgenic lines from a representative collection of IL-2 and CTLA-4 ko ARTs to follow the fate of these cells in vivo. The third is to define peptide ligands for ARTs. A synthetic peptide combinatorial libraries (SCLs) screening strategy will be used for this purpose. The combination of these approaches should provide valuable insight into the mechanisms of self-tolerance and may suggest novel strategies for therapeutic interventions for autoimmune diseases.

Publications


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Summary: My laboratory is interested in the structure and function of molecules involved in cell surface recognition, particularly those mediating recognition in the immune system (Figure 1). We use a combined approach of x-ray crystallography to determine structures, molecular biological techniques to produce proteins for crystallization and to modify them, and biochemistry to study the properties of the proteins we make. Much of our work has focused upon homologs of class I MHC proteins, which function in many ways that are distinct from their immunological role in peptide presentation to T cells. Our interest in these types of proteins has grown since we solved the structure of the neonatal Fc receptor (FcRn), an MHC homolog that transports immunoglobulin G (IgG). In the FcRn system, we are using structural information to address cell biological issues involving intracellular receptor-ligand trafficking. Our studies of other MHC homologs have expanded our interests to include aspects of iron metabolism (for our work on HFE), cancer (for our work on Zn-α2-glycoprotein; ZAG), and virology (for our work on viral MHC homologs).

Transfer of maternal IgG molecules to the fetus or infant is a mechanism by which mammalian neonates acquire humoral immunity to antigens encountered by the mother. The protein responsible for the transfer of IgG is the MHC class I-related receptor FcRn. MHC class I molecules have no reported function as immunoglobulin receptors; instead they bind and present short peptides to T cells as part of immune surveillance to detect intracellular pathogens. We solved the crystal structures of rat FcRn both alone and complexed with Fc. Our crystallographic and biochemical studies suggest that FcRn dimerizes in response to IgG binding, which may serve as a component of a signal to initiate internalization of FeRn/IgG complexes. We also hypothesize that formation of an oligomeric ribbon of FeRn dimers on adjacent membranes bridged by IgG molecules (Figure 2) is required for FeRn function. We are now interested in understanding the roles of the FeRn dimer and the oligomeric ribbon in IgG transport. We have developed assays to investigate the processes that must both occur for the formation of oligomeric ribbons: (i) dimerization of FeRn, and (ii) bridging of FeRn dimers by Fc. We are in the process of characterizing oligomeric ribbon formation in vitro using biophysical assays, and have extended these studies to real time confocal imaging of cells undergoing FeRn-mediated transcytosis of IgG. We are also beginning structure/function studies of two other Fc receptors that are not MHC homologs: gE/gI, a viral Fc receptor for IgG, and FcγR, a host receptor for IgA.

HFE is a recently discovered class I MHC homolog that is involved in the regulation of iron metabolism, an unexpected function for an MHC-related protein. HFE was discovered when its gene was found to be mutated in patients with the iron overload disease hereditary hemochromatosis. HFE has been linked to iron metabolism with demonstration that it binds to transferrin receptor, the receptor by which cells acquire iron-loaded transferrin. We have solved crystal structures of HFE alone and HFE bound to transferrin receptor. The interaction of HFE with transferrin receptor is a fascinating system to study because we can use crystal structures to answer biochemical, functional, and evolutionary questions that address how binding of HFE interferes with transferrin binding, if conformational changes in the receptor are involved in the binding of either transferrin or HFE, which part of the MHC-like HFE structure binds transferrin receptor, and how the HFE interaction with the receptor compares with interactions of ligands with MHC and MHC-like (e.g., FeRn) proteins. We are expanding our studies to include cell biological investigations of HFE and transferrin receptor intracellular trafficking in transfected cell lines using confocal microscopy and other imaging techniques.

We also study Zn-α2-glycoprotein (ZAG), a soluble MHC class I homolog present in low concentrations in most bodily fluids. ZAG was isolated from blood more than 30 years ago, but it's been a molecule in search of a function for a long time. Recently researchers at Aston University in the U.K. discovered that ZAG is involved in cachexia, a wasting syndrome that can affect people with terminal illnesses. ZAG is responsible for the fat-depletion component of cachexia, since it
stimulates lipid breakdown in adipocytes and reduces fat stores in laboratory animals. We have purified ZAG from serum and completed its crystal structure, which revealed an as-yet unidentified non-peptide compound in the ZAG counterpart of the MHC peptide binding site. A combination of structural studies, computational analysis, and ligand binding experiments will be used for analyzing the function and potential roles of ZAG in lipid catabolism under normal and pathological conditions.

We are also interested in other MHC homologs, including proteins encoded by viruses. Both human and murine cytomegalovirus (HCMV, MCMV) express a relative of MHC class I heavy chains, probably as part of the viral defense mechanism against the mammalian immune system. Our biochemical studies show that the HCMV homolog associates with endogenous peptides resembling those that bind to class I MHC molecules. Current efforts focus upon defining the structure and function of these homologs in order to understand why viruses make them and how they interfere with the host immune system.

Our structural work on class I MHC homologs has elucidated new and unexpected recognition properties of the MHC fold. For FcRn and HFE, the structural and biochemical studies have revealed a similar fold and some common properties, including the assumption that both receptors "lie down" parallel to the membrane when binding ligand, and a sharp pH-dependent affinity transition near neutral pH. In the case of FcRn, we have elucidated the structural basis of its pH-dependent interaction with IgG and will now focus upon cell biological studies of intracellular trafficking, for which the pH-dependent interaction is critical. The pH dependence of the HFE-TfR interaction suggested to us that intracellular trafficking studies of HFE would be interesting, so much of our future efforts on both the FcRn and HFE systems will center around probing their function in a cellular context using imaging techniques. Our functional studies of ZAG are at an earlier stage, since a receptor for ZAG has not been identified and the mechanism by which ZAG promotes lipid degradation is unknown. We are at an even earlier stage in our studies of the viral MHC homologs, in which our primary goal will be to solve crystal structures of one or both viral homologs alone and complexed with their cellular receptors.

In addition to the studies of MHC homologs, we are interested in using structure/function studies to understand bacterial pathogenesis and the innate immune response to bacterial infection. Our efforts in these areas involve the study of the Yersinia pseudotuberculosis protein invasin, the insect immune response protein hemolin, and the mammalian mannose receptor.
Figure 1
Figure 2. The oligomeric ribbon observed in the FcRn/Fc cocrystals as a model for the formation of higher-order oligomers upon binding of IgG to cell surface FcRn. Rcn is gray and Fc is black. (A) Top view of the oligomeric ribbon. FcRn dimers are bridged by Fc molecules. If this network forms under physiological conditions, each FcRn dimer would be associated with a membrane parallel to the plane of the paper: the left-most dimer is associated with a membrane below the plane of the paper, the central dimer with a membrane above the paper, and the right-most dimer is again associated with the membrane below the plane of the paper. (B) Side view, rotated by 90° about the horizontal axis from the view in part A. The FcRn dimers are seen looking down their long axes (vertical in part A).

237. Characterization of the 2:1 complex between the class I MHC-related Fc receptor and its Fc ligand in solution

W. Lance Martin

The neonatal Fc receptor (FcRn) facilitates the transfer of maternal immunoglobulin G (IgG) to offspring and prolongs the half-life of serum IgG. FcRn binds IgG in acidic intracellular vesicles and releases IgG upon exposure to the basic pH of the bloodstream. The low resolution crystal structure of an FcRn/Fc complex revealed FcRn dimers bridged by homodimeric Fc molecules to create an oligomeric array with two receptors per Fc (1), consistent with the 2:1 FcRn/Fc stoichiometry observed in solution (2). Two distinct 2:1 FcRn/Fc complexes were present in the co-crystal structure; a complex containing an FcRn dimer interacting with an Fc, and a complex in which single FcRn molecules are bound to both sides of the Fc homodimer. In order to determine which of the two possible 2:1 FcRn/Fc complexes exists in solution, we generated recombinant Fc molecules with zero, one, and two FcRn binding sites and studied their interactions with a soluble form of rat FcRn. The wild-type Fc with two FcRn binding sites binds two FcRn molecules under all assay conditions and the non-binding Fc with no FcRn binding sites shows no specific binding. The heterodimeric Fc with one FcRn binding site binds one FcRn molecule, suggesting that the 2:1 FcRn/wild-type Fc complex formed in solution consists of single FcRn molecules binding to both sides of Fc rather than an FcRn dimer binding to a single site on Fc.

References
238. **Crystal structure of a complex between the neonatal Fc receptor and a heterodimeric Fc**

*W. Lance Martin, A.P. West, Lu Gan*

The neonatal Fc receptor (FcRn) mediates the passive acquisition of maternal humoral immunity by neonatal and prenatal mammals. In adult mammals, FcRn is also responsible for rescuing IgG from a default catabolic pathway and transfer of IgG from serum into the extravascular space. The receptor performs both of these functions by binding to the IgG ligand at pH 6 - 6.5 and releasing IgG at pH 7.5. The low resolution cocystal structure of FcRn and the Fc fragment of its IgG ligand at pH 6 contained receptor dimers bridged by the ligand in an oligomeric array of repeating FcRn:Fc complexes (the "oligomeric ribbon") (1) (Figure 2). These crystals diffracted to ~3.5 Å along the direction of the FcRn dimer, but to only ~6.5 Å resolution in other directions. In order to prevent formation of the oligomeric ribbon in crystals of an FcRn/Fc complex, we used a heterodimeric version of Fc containing only a single FcRn binding site (see Abstract 237 by W. Lance Martin). Well-ordered crystals were obtained and the structure of the FcRn/Fc complex was solved to 2.8 Å resolution by molecular replacement. The new structure verifies the binding interaction suggested by the low resolution structure but allows a detailed description of the specific residues at the FcRn/Fc interface. For example, the structure reveals three histidines on Fc at the FcRn binding site, each of which interacts with a negatively-charged residue on FcRn. Titration of the histidines near neutrality is predicted to account for the sharp pH-dependent binding between FcRn and Fc. In addition, the structure reveals ordered N-linked carbohydrates on FcRn that contact Fc, rationalizing previous biochemical results suggesting that the nature of carbohydrate attached to FcRn affected its ligand binding interactions (2). Because of the importance of IgG homeostasis to human health we are constructing a human heterodimer Fc to generate a human FcRn/Fc crystal structure.

*Summer research student*

Reference


239. **Generation of mutant Fcs with higher affinity for FcRn**

*W. Lance Martin, Pavel Strop*, Dan Bolon*

The neonatal Fc receptor (FcRn) facilitates the transfer of maternal immunoglobulin G (IgG) to offspring and prolongs the half-life of serum IgG. FcRn binds IgG in acidic intracellular vesicles and releases IgG upon exposure to the basic pH of the bloodstream. Because FcRn binding to IgG prolongs its half-life in serum, we seek to optimize the affinity of Fc for FcRn, which should increase the efficacy of anti-tumor antibodies. This task is especially challenging because the pH dependence of the FcRn/Fc interaction must be maintained to allow FcRn-mediated rescue of IgG from catabolism. To guide the design of Fc mutants we use the 2.8 Å structure of the FcRn/Fc complex (see Abstract 238 by W. Lance Martin, Anthony. P. West, and Lu Gan) to design an Fc that binds more tightly to FcRn. In collaboration with Pavel Strop and Dan Bolon of Steve Mayo’s lab, we are using the force-field algorithm ORBIT to calculate Fc sequences predicted to stabilize Fc in the conformation observed in the Fc/FcRn crystal structure. Mutant Fcs have been expressed in baculovirus-infected insect cells and will be evaluated for binding to FcRn.

*Steve Mayo lab, Caltech*

240. **IgG transcytosis and recycling by FcRn expressed in MDCK cells reveals ligand-induced redistribution**

*T.S. Ramalingam, Scott A. Detmer*

The neonatal Fc receptor (FcRn) transports IgG across epithelial cells to provide maternal antibodies to offspring and recycles serum IgG to protect it from catabolism. FcRn binds IgG at the acidic pH of endosomes and releases IgG at the basic pH of blood. We expressed rat FcRn in polarized MDCK cells, measured transport quantitatively, and localized FcRn by confocal fluorescence imaging. FcRn is predominantly apically distributed in the absence of IgG, but redistributes to basolateral locations upon IgG addition, suggesting that ligand binding induces a signal that stimulates transcytosis. FcRn transcytoses IgG more efficiently in the apical to basolateral than the basolateral to apical direction when IgG is internalized by receptor-mediated endocytosis at acidic pH or by fluid-phase endocytosis at basic pH. The PI 3-kinase inhibitor wortmannin disrupts basolateral recycling and transcytosis in both directions, but reduces apical recycling only minimally. Confocal imaging and quantitative IgG transport studies demonstrate that apically-internalized IgG recycles mainly from wortmannin-insensitive apical early endosomes, whereas FcRn-IgG complexes that transcytose to the basolateral surface pass through downstream wortmannin-sensitive and transferrin-positive compartments. The FcRn-expressing MDCK cells reproduce many aspects of FcRn function in vivo and can therefore be used to investigate the mechanism of FcRn-mediated transcytosis and recycling.

*David Chan laboratory, Caltech*

241. **Neonatal Fc receptor and IgG ligand-induced receptor oligomerization**

*W. Lance Martin, T.S. Ramalingam, Mary Dickinson*

The crystal structure of FcRn complexed with wild-type Fc contains receptor dimers bridged by the ligand in an oligomeric array of repeating FcRn:Fc complexes with an overall 2n:n stoichiometry (the "oligomeric ribbon"; Figure 2). Determining whether the formation of the oligomeric ribbon is important for the receptor's function in vivo is the focus of this research. If the oligomeric ribbon is required, then both FcRn dimer formation and the bridging of FcRn dimers by the Fc must...
occur during FcRn-mediated transport of IgG. Using the low-resolution FcRn/Fc cocystal structure as a guide, we constructed various FcRn and Fc mutants that are predicted to prevent formation of the oligomeric ribbon by either disrupting FcRn dimer formation or by destroying the ability of Fc to bridge between receptor dimers. The Fc mutant that cannot bridge FcRn dimers is a heterodimeric version of Fc (hdFc), composed of a wild-type chain paired with a mutant chain with an altered FcRn binding site (see Abstract 237 by W. Lance Martin). Gel filtration and surface plasmon resonance experiments comparing the interaction of FcRn with hdFc and with wild-type Fc (wtFc) demonstrate that the wtFc employs both FcRn binding sites to bridge FcRn molecules under these conditions. Using a chimeric FcRn protein containing a cytosolic tyrosine kinase domain, we demonstrated that cell-surface FcRn dimerizes in response to binding either wtFc or hdFc. These demonstrations of FcRn dimer formation upon ligand binding and the ability of wtFc to bridge between FcRn molecules constitute independent isolations of each of the interactions required for the oligomeric ribbon under non-crystallographic conditions. To confirm these results in situ, we devised an assay to detect dimerization between a protein fused to enhanced yellow fluorescent protein (EYFP) and another protein fused to enhanced cyan fluorescent protein (ECFP) by fluorescence resonance energy transfer (FRET). To ascertain whether FRET resulting from interactions between CFP and YFP in living cells can be detected, we conducted an experiment using the class II MHC protein HLA-DR, an obligate heterodimer, as a positive control. In collaboration with Dr. Mary Dickinson of Scott Fraser’s laboratory at Caltech, we detected FRET between the two chains of HLA-DR as a peak at the emission wavelength of EYFP and a diminished peak at the emission wavelength of ECFP. Having demonstrated that FRET can be detected when two proteins associate in Cos-7 cells, we will now generate stable MDCK cells expressing FcRn-CPF and FcRn-YFP. With these cells we hope to examine the importance of FcRn dimer formation to its biological activity in transcytosis and recycling of IgG.

*Scott Fraser lab, Caltech

242. Is IgG bridging of FcRn dimers between adjacent membranes required for IgG transcytosis?

T.S. Ramalingam, W. Lance Martin

The neonatal Fc receptor (FcRn) mediates the transport of IgG from ingested milk in the gut to bloodstream of newborn mammals and protects IgG from a default degradative pathway in adult animals. For both functions IgG binds to FcRn at acidic pH (6.0 – 6.5) and is released from the receptor at the basic pH of blood (~7.4). In polarized epithelial cells of the intestine, IgG is absorbed by fluid-phase endocytosis and FcRn binds IgG at the acidic pH of intracellular vesicles. The FcRn-IgG complex is then transcytosed across the cell in these acidic vesicles, which fuse with the basolateral membrane, resulting in exposure of the FcRn-IgG complex to the basic pH of blood and release of the bound IgG. Using transfected MDCK cells expressing wild-type FcRn or an FcRn-GFP fusion protein (see Abstract 240 by T.S. Ramalingam et al.), we have successfully mimicked IgG transcytosis from the apical to basolateral side of the cells as observed in intestinal cells. We previously hypothesized that IgG bridging of FcRn dimers on adjacent membranes to form an "oligomeric ribbon" (Figure 2) is required for transcytosis for IgG. This hypothesis is consistent with confocal imaging studies of FcRn-expressing MDCK cells, in which we show that wild-type Fc is transcytosed from the apical to the basolateral surface, thus faithfully mimicking in vivo FcRn-mediated transport, but that a heterodimeric Fc containing only one functional FcRn binding site is not transcytosed efficiently. These results were further confirmed using radiolabeled wild-type and heterodimeric Fc proteins in a quantitative transcytosis assay. Future efforts will focus upon using time-lapse confocal fluorescence microscopy to create a road map for FcRn-mediated IgG recycling and transcytosis, and to compare the intracellular fates of wild-type Fc and heterodimeric Fc.

243. Structural studies of a herpes simplex virus immunoglobulin G receptor

Elizabeth R. Sprague

The herpes simplex virus (HSV) immunoglobulin G (IgG) receptor is a heterodimer composed of the gE and gI proteins that is found on the surface of the HSV virion or HSV-infected cells. The gE/gI heterodimer binds the Fc portion of IgG, and this interaction has been implicated in several mechanisms of immune evasion, including inhibition of virus neutralization, interference with complement- and cell-mediated lysis pathways, and antibody-bipolar bridging. The interplay between HSV proteins and host immunoregulatory proteins may contribute to the ability of HSV to establish a persistent infection even in the presence of anti-HSV antibodies. The gE protein has also been implicated in the cell-to-cell transmission of HSV. In order to better understand how the gE/gI heterodimer binds the Fc portion of IgG and how this recognition event affects the ability of the host immune system to eradicate the virus, we have undertaken biochemical and crystallographic studies of the gE protein, gE/gI heterodimer, and the gE/gI-Fc complex. The Bjorkman laboratory has previously expressed gE and gI as soluble proteins in Chinese hamster ovary (CHO) cells and characterized the interaction between the gE/gI heterodimer and IgG (1). We have since expressed human IgG Fc in CHO cells and have begun a detailed study of its binding to the gE/gI heterodimer. Crystallization trials of the gE/gI heterodimer alone and complexed with hIgG1 Fc are underway.
Reference

244. Studies of the interaction of FcαR1 with the constant region of IgA1
Andrew B. Herr, Clinton L. White, Edward R. Ballister*

The "first line of defense" against pathogens in humans is the mucosal immune system. Mucosal secretions contain high levels of secretory IgA, which opsonizes pathogens and prevents their adherence to the mucosal epithelium. When the mucosal epithelium is compromised and IgA-coated pathogens invade, the Fc regions of the IgA antibodies are recognized by the IgA-specific receptor FcαR1 and phagocytosed. We are studying the interaction of FcαR1 with Fcα, the constant region of IgA. Using equilibrium gel filtration and analytical ultracentrifugation, we have shown that two FcαR1 molecules interact with a single dimeric Fcα. Biosensor binding experiments show that each binding site has an intrinsic dissociation constant of around 200 nM. The binding has almost no salt-dependence, showing that only 3-5% of the free energy of binding is contributed by electrostatic interactions. However, there is a significant loss in affinity as the pH is dropped from 9.0 to 5.0; these data are consistent with low-pH protonation of a single titratable residue with a pKᵦ near 7.0. Finally, the temperature dependence showed that the free energy of binding can be dissected into a favorable enthalpy term and a slight entropic penalty. Therefore, the binding of FcαR1 to Fcα is primarily composed of hydrophobic and H-bond interactions, with almost no contribution of salt bridges. Furthermore, these data suggest that an unprotonated histidine on FcαR1 binds in a hydrophobic pocket on Fcα. The temperature dependence of binding implies that the interaction is primarily a rigid-body interaction, with little evidence for an induced-fit mechanism or ordering of flexible loops. These studies increase our understanding of how FcαR1 binds to Fcα and lay the groundwork for understanding how this binding event is able to trigger phagocytosis of IgA-opsonized pathogens.
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245. Mutational analysis of TfR reveals overlapping HFE and transferrin binding sites
Anthony P. West, Jr., Anthony M. Giannetti, Andrew B. Herr, Melanie J. Bennett, Jasvinder S. Nangiania*, James R. Pierce**, Leslie P. Weiner†, Peter M. Snow

The transferrin receptor (TfR) binds two proteins critical for iron metabolism: transferrin (Tf) and HFE, the protein mutated in hereditary hemochromatosis. Previous results demonstrated that Tf and HFE compete for binding to TfR(1), suggesting that Tf and HFE bind to the same or an overlapping site on TfR. TfR is a homodimer that binds one Tf per polypeptide chain (2:2 TfR:Tf stoichiometry), whereas both 2:1 and 2:2 TfR:HFE stoichiometries have been observed. In order to more fully characterize the interaction between HFE and TfR, we determined the binding stoichiometry using equilibrium gel filtration and analytical ultracentrifugation. Both techniques indicate that a 2:2 TfR:HFE complex can form at submicromolar concentrations in solution, consistent with the hypothesis that HFE competes for Tf binding to TfR by blocking the Tf binding site rather than by exerting an allosteric effect. To determine whether the Tf and HFE binding sites on TfR overlap, residues at the HFE binding site on TfR were identified from the 2.8 Å resolution HFE-TfR co-crystal structure, then mutated and tested for their effects on HFE and Tf binding. The binding affinities of soluble TfR mutants for HFE and Tf were determined using a surface plasmon resonance assay. Substitutions of five TfR residues at the HFE binding site (L619A, R629A, Y643A, G647A and F650Q) resulted in significant reductions in Tf binding affinity. The findings that both HFE and Tf form 2:2 complexes with TfR and that mutations at the HFE binding site affect Tf binding support a model in which HFE and Tf compete for overlapping binding sites on TfR.
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Reference

246. Expression of ferritin in HuTu-80 cells transfected with the H41D mutant form of the hereditary hemochromatosis protein HFE
Astrid Heikema, T.S. Ramalingam, A.M. Giannetti, Caroline A. Enns*

Hereditary hemochromatosis (HH) is a disorder characterized by excessive deposition of iron in the body that can lead to multi-organ dysfunction if left untreated. The gene that is mutated in HH patients encodes a class I major histocompatibility complex (MHC) related protein called HFE. HFE binds to transferrin receptor, the receptor for iron-loaded transferrin. Most HH patients are homozygous for a C260Y mutation in HFE, in which a cysteine residue at position 260 is changed to a tyrosine residue, disrupting a disulfide bond required for proper folding, cell-surface expression of the protein, and transferrin receptor binding. About 70% of HH patients who are heterozygous for this mutation also carry a second mutation that converts a histidine at position 41 to an aspartate (H41D). This substitution, however, does not prevent proper folding, cell-surface expression, or binding to transferrin receptor. We previously expressed wild-type and mutant forms of HFE in HuTu-80 cells, a cell line derived from human duodenum, in order to assess the effects of the C260Y mutation in HFE. We showed that this expression of wild-type HFE, but not C260Y HFE,
resulted in lower levels of the iron storage protein ferritin. To determine the effects of the H41D substitution, we expressed the H41D form of HFE in HuTu-80 cells. Using confocal microscopy, we demonstrated that both H41D and wild-type HFE are localized in endosomal compartments and at the basolateral surface. To determine if the H41D mutant affects ferritin, we measured ferritin levels in cell lysates using a ferritin ELISA. We found that the H41D HFE-expressing cells had about 7-fold higher levels of ferritin than the cells transfected with wild-type HFE. Further studies to verify this result will be done using soluble forms of wild-type and H41D HFE.

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Reference

247. Structural studies of cytomegalovirus class I MHC homologs
Benjamin E. Willcox, Astrid Heikema, Peter Snow*

Down-regulation of host class I MHC molecules at the surface of infected cells is a strategy that cytomegalovirus (CMV) has evolved in order to evade host cytotoxic T-cell responses. Lack of cell surface class I MHC proteins would be expected to leave infected cells vulnerable to attack by natural killer (NK) cells. However, murine and human CMV each encode proteins (m144 and UL18, respectively) homologous to host class I MHC (~25% amino acid identity), which are thought to facilitate evasion of host NK responses. We are attempting to define how m144 and UL18 mimic class I molecules by determining their crystal structures. Crystallization trials are in progress using protein expressed in Chinese hamster ovary cells, baculovirus, and E. coli. Each homolog heterodimerizes with the class I MHC light chain β2-microglobulin, but only UL18 associates with endogenous peptides.

The host cell ligand for UL18 is Leukocyte Immunoglobulin-like Receptor-1 (LIR-1). LIR-1 belongs to a distinct family of receptor molecules homologous to human FcαR and Killer Inhibitory Receptors, and is expressed mainly on monocytes, B cells, T cells and dendritic cells. LIR-1 and LIR-2 (which also binds UL18, but much more weakly) interact with endogenous class I MHC molecules and transmit inhibitory signals to the cell interior. Previous work in the lab has shown that LIR-1 interacts with the relatively non-polymorphic α3 domain of class I MHC molecules (1), and led to a crystal structure of LIR-1 (2). Current work is focusing on determining the structures of LIR-2, and of complexes of LIR-1 with class I MHC.

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References

248. Characterization and crystallization of r144, a rat cytomegalovirus class I MHC homolog
Astrid Heikema

R144 is a rat cytomegalovirus (RCMV)-encoded class I major histocompatibility complex (MHC) homolog (1). Homologs to mammalian MHC class I genes have been identified within the genomes of two other CMV species: human CMV (HCMV) and murine CMV (MCMV). The genes of HCMV and MCMV encode the MHC class I homologs UL18 and m144, respectively. The sequence of the r144 polypeptide shares 30% and 19% sequence identity with the amino acid sequences of m144 and UL18. Like class I MHC molecules, r144, m144 and UL18 consist of a heavy chain containing three extracellular domains (α1, α2 and α3), which associates with the host light-chain β2-microglobulin (β2m). Unlike MHC class I molecules and UL18, m144 does not bind peptides. This is probably due to a considerable deletion in the α2 domain of the protein, part of the peptide-binding site in MHC class I molecules. R144 also has a deletion in the α2 domain. To determine if r144 binds peptides, soluble r144 was expressed in CHO cells, purified from transfected cell supernatants, then treated with acetic acid to dissociate potential peptides. N-terminal sequencing of r144 acid eluates did not show evidence of peptides, as compared with positive control eluates derived from UL18.

For crystallization trials, bacterially-expressed r144 was refolded together with rat β2m and purified using a size exclusion column. Crystals were obtained using 0.1 M Hepes Na pH 7.5, 10% v/v iso-propanol and 20% w/v polyethylene glycol, which diffracted to ~6 Å. We are in the process of optimizing the growth conditions to obtain better crystals.

Reference
249. Molecular competition for NKG2D: H60 and RAE1 compete for NKG2D with dominance of H60
Christopher A. O’Callaghan, Adelheid Cervenka*, Benjamin E. Wilcox, Lewis L. Lanier*

NKG2D is a C-type lectin-like protein and a potent activating receptor expressed on the surface of natural killer cells, T cells, and macrophages. Mouse NKG2D interacts with two cell-surface ligands related to class I MHC molecules: RAE1 and H60. We have generated soluble recombinant forms of mouse NKG2D, RAE1 and H60. RAE1 and H60 molecules are both stable in the absence of exogenous peptide and in the absence of β2-microglobulin. NKG2D exists as a homodimer. We used soluble recombinant versions of NKG2D, RAE1, and H60 to characterize their interactions using surface plasmon resonance. The molecules were coupled in an oriented fashion to a streptavidin-coated biosensor surface after site-specific biotinylation of the proteins using the enzyme BirA. The recombinant proteins were also shown to be able to bind to the native molecules on the surface of living cells. RAE1 and H60 each bind NKG2D with nanomolar affinities, indicating tighter binding than most cell-surface immune interactions. However, NKG2D binds to H60 with ~25-fold higher affinity than to RAE1. In addition, RAE1 and H60 compete directly for occupancy of NKG2D, thus NKG2D can be occupied by only one ligand at a time. The NKG2D-H60 interaction is more temperature dependent and makes greater use of electrostatic interactions than the NKG2D-RAE1 interaction. The distinct thermodynamic profiles provide insights into the different molecular mechanisms of the binding interactions. Glycosylated and non-glycosylated forms of each of these proteins had similar affinities with their binding partners. The four different RAE1 isoforms were studied and did not differ substantially in their interactions with NKG2D. Both RAE1 and NKG2D have predicted glycosylphosphatidyl inositol membrane anchors. As many pathogens secrete phospholipases, pathogens could release free NKG2D ligands into the environment around them. These free ligands would have a role in the host immune response against the pathogen.

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250. Structural studies of invasin
Zsuzsa A. Hamburger, Kaspar P. Locher*, Adam T. Politzer**, Ralph R. Isberg***

Bacterial pathogens such as Yersinia pseudotuberculosis must bind and enter normally non-phagocytic cells to establish infection. Previous work of Ralph Isberg and colleagues (Tufts University) showed that the protein responsible for mediating uptake of the bacterium is a 986-residue outer membrane protein called invasin. Invasin binds to several members of the β1 integrin family, presumably activating a reorganization of the host cytoskeleton to form pseudopods that envelop the bacterium. Integrin binding has been localized to the extracellular region of invasin (Inv497) comprised by the COOH-terminal 497 residues. In order to gain insight into the mechanism of bacterial pathogenesis, we previously solved the 2.3 Å crystal structure of Inv497 (1). Currently we are interested in determining the structure of the NH2-terminal ~500 residues of invasin, which are important for outer membrane localization and for presentation of the integrin-binding region of invasin. This region of invasin is thought to reside in the outer membrane and as the transmembrane regions of outer membrane proteins of known structures are β-barrels, we believe that this region of invasin also forms a β-barrel. We have expressed this region of invasin as inclusion bodies and have successfully refolded the protein as indicated by circular dichroism and analytical ultracentrifugation studies. Currently we are working on crystallizing this membrane protein by screening detergents and setting up crystallization trials.

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Reference

251. In search of the ligand of Methuselah, a Drosophila GPCR associated with extended lifespan
Anthony P. West, Jr., Laurent Seroude, Lynda L. Llamas, Seymour Benzer

The Drosophila mutant methuselah was identified from a screen for single gene mutations that extended average life-span. The methuselah mutant has a 35% increase in average life-span and increased resistance to several forms of stress (1). The protein affected by this mutation appears to be a G protein-coupled receptor (GPCR), though the upstream and downstream pathways that Methuselah (Mth) connects are unknown. Mth has a large extracellular N-terminal domain, which may be the ligand-binding domain of the receptor by analogy to related GPCRs, whose isolated extracellular domains function in ligand binding. We have determined the 2.3 Å crystal structure of the Mth ectodomain (2). The structure did not closely resemble any other protein and thus did not suggest what the natural ligand might be. Nevertheless, the ectodomain structure reveals a potential ligand-binding site, in a shallow groove between two flexible domains.

We now plan to identify the natural ligand and G-protein effector of Mth, which should help us to understand how Mth affects Drosophila lifespan. The basic plan is a biochemical approach: Drosophila extracts will be applied to cells expressing full-length Mth, the system will be varied until Mth activation is observed, and the extract will be fractionated until the ligand can be
identified. We are exploring two assay systems that have been used to identify the ligands of other orphan GPCRs: (i) *Xenopus* oocytes injected with receptor mRNA, with electrophysiological monitoring of receptor activation, and (ii) transfected mammalian cells, where GPCR activation can be followed by increases in intracellular calcium. Coexpression of Mth with a ‘universal G protein’ provides a method to follow Mth activation without assumptions concerning its normal signaling pathway. We have raised monoclonal antibodies against the Mth ectodomain for immunoprecipitation of Mth from *Drosophila* membranes. G proteins, or other interacting proteins, may coimmunoprecipitate with Mth and identifying these proteins should assist in developing the Mth assay.

**Reference**


### 252. Structural studies of huntingtin exon 1 complexed with an Fab that binds poly-glutamine

Melanie J. Bennett, Kathryn E. Huey-Tubman

Huntington's disease (HD) is a neurodegenerative disorder caused by expansion of CAG repeats that encode a poly glutamine (poly-Gln) stretch in the huntingtin protein. Huntingtin (htt) contains 16-35 glutamines in normal individuals and ≥ 39 glutamines in HD patients. We have been studying the N-terminal fragment of huntingtin (htt exon 1) alone and in complex with an Fab derived from a monoclonal antibody that recognizes expanded (pathogenic) glutamine repeats. This work will provide insight into the structure of exon 1 and its poly glutamine tract and the mechanism by which expanded glutamine tracts are specifically recognized by antibodies. We have expressed and purified histidine-tagged thioredoxin fusion proteins containing huntingtin exon 1 with glutamine repeats of different lengths (16Q, 25Q, 39Q and 46Q). Thus, we have a set of exon 1 fusion proteins that includes non-pathogenic repeats (16Q and 25Q), a borderline repeat (39Q) and a pathogenic repeat (46Q). The other protein species that we have generated for our studies is a proteolytically cleaved anti-poly-Gln Fab fragment. The Fab fragment is derived from MW1, an antibody that preferentially binds mutant (glutamine-expanded) huntingtin on Western blots (Dr. Jan Ko and Dr. Paul Patterson, Caltech). We are presently using biophysical and biochemical techniques to investigate the structures of htt exon 1 constructs with various glutamine repeat lengths, as well as their interactions with the anti-poly-Gln Fab fragment.

### Publications


**Professor:** Judith L. Campbell  
**Visiting Associates:** Laura Hoopes\(^1\)  
**Member of the Professional Staff:** Martin Budd, Piotr Polaczek  
**Research Fellows:** Susanna Boronat, Rajiv Dua\(^2\), Shaune Edwards, Osamu Imamura, Caroline Li, Tao Wei  
**Graduate Students:** Won-chae Choe, Isabelle Lesur  
**Research and Laboratory Staff:** Jules Chen, Ping Yang  

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**Summary:** Our laboratory studies DNA replication, using yeast as the primary experimental paradigm. An increasingly pervasive and novel theme of the DNA replication field is genomic stability, and accordingly, the point of departure for our studies is currently genomic stability. During cell proliferation, DNA replication and segregation must occur with high fidelity. One level of control is exerted by confining DNA replication to the S phase of the cell cycle and has been the subject of intense investigation for the past ten years. However, in addition, surveillance mechanisms, called checkpoints, are tightly coupled to the replication process and cause cell cycle arrest or delay to repair damage and allow collapsed replication forks to reassemble. Failure of these checkpoints in humans results in genome instability and predisposition to cancer and is therefore of interest to both basic scientists and cancer researchers.

Our studies on DNA polymerases have unexpectedly and increasingly led us into the area of checkpoints and genome stability. We focus on DNA polymerase epsilon, one of the three major polymerases that moves the replication fork. We and others have shown that this polymerase is a major signaling enzyme in the S-phase checkpoint that retards fork movement to allow repair of endogenous and exogenous DNA damage being encountered by the replication fork. In attempting to elucidate the signaling mechanism we have found that the essential function of polymerase epsilon is not its catalytic function, but rather, a role in controlling the architecture of the fork. Based on discoveries in the lab in the last year, we are proposing that the most important function of the essential region of the polymerase is to interact with a newly discovered DNA polymerase, polymerase kappa, which is required to link DNA replication to sister chromatid cohesion. We have identified polymerase epsilon mutants that show premature sister chromatid separation and are testing the model that polymerase epsilon and polymerase kappa participate in a polymerase switch when the replication fork encounters a site where cohesion between the two sister chromatids is formed. Demonstrating the mechanism of the polymerase switch is of fundamental importance. During the last year it has become apparent that there are at least 16 DNA polymerases in the eukaryotic cell. What is their function? It is to preserve the fidelity of genome duplication and chromosome transmission. In addition to the link to sister chromatid cohesion and sister separation at anaphase, that we are studying, the three major polymerases at the replication fork collaborate with at least eight other polymerases – mutagenic and non-mutagenic base excision repair polymerases and translesion synthesis polymerases.

A second aspect of genomic stability that we study has to do with the contribution of genomic instability to the process of cellular aging. Increasing efforts have been made in recent years to develop systems to detect the genetic program of cellular aging. It has also recently been realized that, due to endogenous DNA damage, such as that caused by reactive oxygen species arising due to normal metabolism, that DNA replication is not a continuous process. Replication forks stall at sites of damage and replication fork collapse leads to a hyper-recombinogenic state that results in amplifications, translocations and other genomic rearrangements. We have found that mutations affecting the replicative DNA2 helicase cause a shortening of the yeast life span. This led us to test other DNA replication and postreplication repair mutants for similar effects on life span. Every replication mutant tested showed decreased life span. We have therefore proposed that a major cause of replicative senescence is collapse of DNA replication forks and resulting genomic instability. We are testing this model using the Dna2 helicase as a starting point. Interestingly, Dna2 is a helicase-nuclease of the same superfamily as the human Werner syndrome gene product, and therefore studying aging in yeast may have important implications for human aging and cancer studies.

253. **A gene cassette for magnetotaxis?**  
Elizabeth Bertani

Last fall, the total genome sequencing of two magnetotactic bacteria, *Magnetospirillum magnetotacticum*, strain MS-1, and a magnetococcus, MC-1, was carried out by the Department of Energy (http://www.jgi.doe.gov), with our assistance. Both of these organisms are magnetotactic because they produce structures called magnetosomes—single-domain crystals of magnetite enclosed in protein-lipid membranes. We have compared the genomes of these two otherwise unrelated strains for genes that might be common to magnetotaxis by using sequences of MS-1 to search either the MC-1 genome (for magnetotactic bacteria) or Genbank (for non-magnetotactic bacteria).

MS-1 has three genes that have been considered to be important for magnetite synthesis: *bfr*, which encodes an iron storage protein; *magA*, related to the potassium efflux pump gene, *kefB*; and *mam22*, which was isolated by reverse genetics of a magnetosomal protein. According to the search, MC-1 has neither a *bfr* nor a *magA* gene, although it does have an analogue of *mam22*. In addition, the MC-1 and MS-1 *mam22* genes show more similarity to
each other than to other related genes in Genbank. Most interesting, however, is that two of the three ORFs (open reading frames) upstream of mam22, are also more conserved between MC-1 and MS-1, as compared to other organisms. One of these ORFs encodes a Zn/Cd pump that could possibly pump Fe.

Our tentative proposal is that this region contains genes that are essential for magnetite synthesis and that may be conserved in all magnetotactic bacteria, perhaps organized into some sort of magnetic cassette that can be transferred between strains.

254. Role of Cdc6p in cell cycle progression

Susanna Boronat, Jules Chen, Judith L. Campbell

Initiation of eukaryotic DNA replication depends on the origin recognition complex (ORC) which is bound to chromatin at all times during the cell cycle in Saccharomyces cerevisiae, and on Cdc6p and Mcm proteins which associate with chromatin during the G1 phase of the cell cycle. Cdc6p is synthesized at the end of mitosis, binds to the chromosomally bound ORC, and in turn enables the subsequent binding of Mcm proteins at the replication origins during G1 to form pre-replicative complexes (preRCs). During late G1-phase, preRCs are activated by Cld5p/Cdc28 and Dbp4p/Cdc7p protein kinases leading to the onset of S phase. At this stage, the preRCs are lost from origins and cannot reassemble until passage through the next mitosis, since the Cld/Cdc28 kinases block preRC formation. In addition to its role in forming the preRC, Cdc6 also plays crucial roles in preventing reinitiation at the replication origins and thus limiting replication initiation to once per cell cycle. The levels of Cdc6p fluctuate during cell cycle: Cdc6p is synthesized at the end of mitosis, and is subject to degradation at the onset of S phase.

Cdc6p contains six potential target sites for the Cdc28 protein kinase, three at the N-terminus and three at the C-terminus. The mutation of all these sites, a subset of them, or just one site at C-terminus, into non-phosphorylatable sites results in the stabilization of Cdc6p. The overexpression of the particular allele in which one of the phosphorylation sites at C-terminus has been mutated to a non-phosphorylatable site (Cdc6-F), results in a dominant negative effect in a variety of different yeast backgrounds. This dominant negative effect is defined by a strong delay in the G2/M phase of the cell cycle. We have analyzed this delay by monitoring the levels of Pds1p and Cld2p in classical arrest and release experiments. The levels of these proteins oscillate during the cell cycle at particular stages and, therefore, are very accurate markers to monitor the progression of the cell cycle. Cells overexpressing Cdc6-F show a very slow kinetics of Pds1p degradation at the metaphase to anaphase transition and the levels of Cld2p do not decrease during the time of the experiment. Cells overexpressing wt Cdc6p or other phosphorylation site mutants show a much milder delay. To investigate the mechanism of the G2/M delay, we have performed chromatin binding experiments to monitor the presence or absence of Cdc6p bound to chromatin throughout the cell cycle. We have found that Cdc6-F, expressed under the control of its own CDC6 promoter, remains bound to chromatin after the G1/S transition, at times in which wt Cdc6p is barely detectable on chromatin.

We are currently investigating the possibility that Cdc6-F overexpression results in the activation of one of the cell cycle checkpoints that halt the cell cycle in mitosis when previous events in the cell cycle have been perturbed. The morphogenetic checkpoint monitors the actin cytoskeleton and when this is perturbed cells arrest at mitosis by the inhibitory phosphorylation of Cdc28 mediated by the Swe1p kinase. We have constructed swe1D strains that overexpress Cdc6-F, but we have not found any suppression in the delay of Pds1p degradation. In a similar approach, we have disrupted the RAD24 gene, involved in the DNA replication checkpoint, but the kinetics of Pds1p degradation is identical to the one in wild-type strains overexpressing Cdc6-F. At the present time we are considering the possibility that Cdc6-F overexpression activates the spindle checkpoint. Cells with perturbed spindle pole bodies or defective mitotic spindles arrest at the metaphase to anaphase transition by inhibiting the degradation of Pds1p. This arrest is mediated by the MAD1-3 and BUB1-3 genes. We are on the way of constructing yeast strains defective in those genes to see if they are able to suppress the dominant negative effect of Cdc6-F overexpression.

255. Role of subcellular localization in the regulation of Cdc6p

Susanna Boronat, Jules Chen, Judith L. Campbell

The replication protein Cdc6p is synthesized at the end of mitosis, binds to the chromosomally-bound ORC, and in turn enables the subsequent binding of Mcm proteins at the replication origins during G1 to form pre-replicative complexes (preRCs). During late G1-phase, preRCs are activated by Cld5p/Cdc28 and Dbp4p/Cdc7p protein kinases leading to the onset of S phase. At this stage, the preRCs are lost from origins and cannot reassemble until passage through the next mitosis, since the Cld/Cdc28 kinases block preRC formation. The levels of Cdc6p fluctuate during cell cycle: Cdc6p is synthesized at the end of mitosis, and is subject to degradation at the onset of S phase.

Cdc6p contains six potential target sites for the Cdc28 protein kinase, three at the N-terminus and three at the C-terminus. The mutation of all these sites, a subset of them, or just one site at C-terminus, into non-phosphorylatable sites results in the stabilization of Cdc6p. Previous data in our laboratory obtained by Kathy Luo and Suzanne Elsasser had shown that the nuclear localization of Cdc6p was important for its degradation: mutant Cdc6p with defective nuclear localization signal was very stable and localized both in the nucleus and the cytoplasm. On the other hand, it has recently been shown that cell cycle specific phosphorylation of Cdc6p from human and Xenopus is important for localization. Since we have never been able to observe Cdc6p in the
cytoplasm, we hypothesized that Cdc6p degradation could take place in the cytoplasm once Cdc6p has performed its preRC role in the nucleus. According to this hypothesis, stable phosphorylation site mutants would be more stable due to a defective nuclear export.

To verify this hypothesis we have analyzed the stability of wt Cdc6p and several phosphorylation site mutants in a wild-type background and in an otherwise isogenic strain defective in the nuclear export gene CRM1. We have not found any difference in the stability of the different phosphorylation site mutants between the two strains. However, we have found an increased stability of wt Cdc6p in the crm1 strain. When we have analyzed the cell cycle dependence of this stabilization, we have found it to be more significant in HU arrested cells (S phase) than in nocodazole arrested cells (G2/M phase). These results have prompted us to search a nuclear export sequence (NES) in Cdc6p. We have found a L/I-rich region at C-terminus that we have shown to work properly as a nuclear export sequence in a reporter assay where he have looked at the subcellular localization of a GFP molecule fused to the SV40 NLS and the CDC6-NES. In addition, when we have analyzed the stability of Cdc6p mutated at this NES, we have found it to be more stable than wt Cdc6p. Other data showing the role of nuclear export in Cdc6p regulation is that Cdc6-GFP nuclear staining is stronger in wild-type strains than in crm1 strains.

At the present time we are investigating the role of Msn5p, another protein involved in nuclear export, in the regulation of Cdc6p.

256. DNA2 is required for the normal life span of Saccharomyces cerevisiae
Laura Hoppes, Martin Budd, Judith Campbell

Aging in yeast is characterized by a number of phenotypic changes, including increasing sterility, cell enlargement, fragmentation of the nucleolus, and production of extrachromosomal rDNA circles. DNA2 gene has been found of be essential for normal life span in yeast. Four different dna2 mutants had average life spans from five to nine generations, while wild-type life span average was 24 generations. We found normal life spans for overproducers of Dna2p. Mutants of genes interacting with Dna2p, such as rad27 and ctf4, also exhibited short life spans. DNA2 belongs to the post-replicative DNA repair pathway by epistatic analysis, and found that several other mutants affecting genes in this pathway such as rad6 and rad18 had short life spans as well. Interestingly, deletion of all rDNA and replacement with a multicopy plasmid did not prevent aging, but led to a shorter life span. The phenotype for the aging dna2 strain was strikingly similar to that of sgs1. Early sterility, nucleolar fragmentation, and accumulation of extrachromosomal rDNA circles were found. However, dna2 sgs1 double mutants are synthetically lethal. Synthetic lethality of dna2 was also observed with srs2, a helicase which suppresses recombination and is synthetically lethal with sgs1. When an additional copy of the SIR2 gene is integrated into a dna2-1 strain the average life span the strain does not change, however a set of long-lived survivors emerges from the population, suggesting the cells are dying from two different mechanisms. The result is similar to dna2-2 fob1 strains, in which the average life span of dna2-2 fob1 strains is similar to dna2-2 strains, however in the double mutant long-lived survivors emerge. dna2-2 mutants are synthetically lethal with rrm3 mutants. rrm3 mutants exhibit replication blocks in the rDNA locus suggesting one role of dna2 protein is repairing broken and stalled replication forks.

257. Physical and genetic mapping of the M. magnetotacticum genome
Khristie Phillips1, Rachel Gray1, Julie Weko, Elizabeth Bertani

The bacterium, Magnetospirillum magnetotacticum, strain MS-1, converts iron into single-domain crystals of the iron mineral magnetite. The crystals remain within the organism, organized in long chains that retain their north/south orientation. As a result, MS-1 is sensitive to the earth’s magnetic field and is attracted to a magnet placed on the wall of a culture vessel. Like most magnetite-producing strains so far described, MS-1 has been classified as belonging to the α-subgroup of the proteobacteria. Some members of this group are photosynthetic. In these cases, most of the genes involved in photosynthesis are found to be clustered in superoperons, occupying about 1% of the genome. This observation suggested the possibility that the genes involved in magnetite synthesis might be similarly clustered. The potential for clustering, together with a need for information about physical structure, made a characterization of the genome with respect to size, structure and gene distribution desirable. This is the first time a physical or genetic map of a magnetotactic organism has been reported.

Pulsed-field gel analysis of MS-1 DNA indicates that the genome is a single, circular structure of about 4.3 Mb. A few genes have been identified by sequence similarity. They include putative genes involved in DNA (dnaA), RNA (rpoA) and protein synthesis (rpl6, rrm), as well as cell division (ftsH); a gene encoding a subunit of the protein involved in dissimilatory nitrate reduction (napA); and two nifL analogs, encoding components of a nitrogen-fixation system. In addition, five (stp) out of the twenty-seven genes appear to be components of sensory transduction systems. These genes have been localized and arranged in a map with dnaA, indicating the presumed origin of replication. There are at least two rRNA operons. In addition, rRNA genes are found on a 40 kb, possibly extrachromosomal, structure. The genes thought to be involved in magnetite synthesis, bfr and magA, are located in the same 17% of the genome and are undoubtedly closer than that.

1Undergraduates, California Institute of Technology
The nuclease activity of yeast Dna2 protein is essential in vivo
Martin Budd, Judith Campbell
Saccharomyces cerevisiae Dna2 protein is required for DNA replication and repair and is associated with multiple biochemical activities: DNA-dependent ATPase, DNA helicase, and DNA nuclease. To investigate which of these activities is important for the cellular functions of Dna2, we have identified separation of function mutations that selectively inactivate the helicase or nuclease. We describe the effect of six such mutations on ATPase, helicase, and nuclease after purification of the mutant proteins from yeast. A mutation in the Walker A box in the C-terminal third of the protein inactivates ATPase, helicase, but not nuclease. A mutation in the N-terminal domain (amino acid 504) results in significantly decreased ATPase, helicase, and nuclease. Two mutations in the recB homology domain, (AA 657 and 675) abolish nuclease but not helicase, or ATPase. An additional mutation in the putative nuclease site AA 640 reduces nuclease in the absence of ATP and abolishes nuclease but not helicase in the presence of ATP. The mutation of AA 693 also reduces nuclease activity in the absence of ATP and reduces it further in the presence of ATP. ATP is a negative regulator of the nuclease activity of Dna2p. None of the nuclease-deficient mutants complement the temperature-sensitive growth defect of a dna2-1 strain. The nuclease-defective mutant AA 640 did not complement the repair-deficient phenotype of a dna2-2 sgs1 mutant strain. The helicase, ATPase, and nuclease activities of Dna2 are required for its essential function in replication and repair.

Subunit interactions within the Saccharomyces cerevisiae DNA polymerase epsilon (pol ε) complex — Demonstration of a dimeric pol ε complex
Rajiv Dua, Shanhe Edwards, Daniel L. Levy, Judith L. Campbell
Saccharomyces cerevisiae DNA polymerase epsilon (pol ε) is essential for chromosomal replication. A major form of pol ε purified from yeast consists of at least four subunits: Pol2p, Dpb2p, Dpb3p, and Dpb4p. Pol2p is the catalytic subunit. The precise roles of the other subunits is not known. We have investigated the protein/protein interactions between these polypeptides by using expression of individual subunits in baculovirus-infected Sf9 insect cells and by using the yeast two-hybrid assay. The essential subunits, Pol2p and Dpb2p, interact directly in the absence of the other two subunits, and the C-terminal half of Pol2p, the only essential portion of Pol2p, is sufficient for interaction with Dpb2p. Dpb3p and Dpb4p, non-essential subunits, also interact directly with each other in the absence of the other two subunits. We proposed that Pol2p/Dpb2p and Dpb3p/Dpb4p complexes interact with each other and document several interactions between individual members of the two respective complexes. The study also presented a biochemical evidence to support the proposal that pol ε may be dimeric in vivo. Gel filtration of the Pol2p/Dpb2p complexes reveals a novel heterotetrameric form, consisting of two heterodimers of Pol2p/Dpb2p. Dpb2p, but not Pol2p, exists as a homodimer, and thus the Pol2p dimerization may be mediated by Dpb2p. The pol2-E and pol2-F interzinc finger mutations that cause replication defects in vivo (1) weaken the interaction between Pol2p and Dpb2p and also reduce dimerization of Pol2p. This suggests, but does not prove, that dimerization may also occur in vivo and be essential for DNA replication.

Reference

Role of Cdc6p phosphorylation in cell cycle progression
Susanna Boronat, Jules Chen, Judith L. Campbell
Initiation of eukaryotic DNA replication depends on the origin recognition complex (ORC) which is bound to chromatin at all times during the cell cycle in Saccharomyces cerevisiae, and on Cdc6p and Mcm proteins which associate with chromatin during the G1 phase of the cell cycle. Cdc6p is synthesized at the end of mitosis, binds to the chromosomally bound ORC, and in turn enables the subsequent binding of Mcm proteins at the replication origins during G1 to form pre-replicative complexes (preRCs). During late G1 phase, preRCs are activated by Cib5,6/Cdc28 and Dbf4p/Cdc7p protein kinases leading to the onset of S phase. At this stage, the preRCs are lost from origins and cannot reassemble until passage through the next mitosis, since the Clb/Cdc28 kinases block preRC formation. In addition to its role in forming the preRC, Cdc6 also plays crucial roles in preventing reinitiation at the replication origins and thus limiting replication initiation to once per cell cycle. The levels of Cdc6p fluctuate during cell cycle: Cdc6p is synthesized at the end of mitosis, and is subject to degradation at the onset of S phase.

Cdc6p contains six potential target sites for the Cdc28 protein kinase, three at the N-terminus and three at its C-terminus. Cdc6p interacts and forms a tight complex with Cib5/Cdc28, and the N-terminal domain is important for this interaction. We recently reported that one role for phosphorylation of Cdc6p is to target it for polyubiquitination and degradation. We showed that single-site phosphorylation mutants, singly or in combination, were more stable than the wild-type protein. This increased stability could be correlated with decreased interaction with Cdc4p and ubiquitination. However, one of the stabilized mutant containing a single phosphorylation site mutation at the C-terminus, seemed to escape this correlation since it could interact with Cdc4p and was partially ubiquitinated in vitro. Interestingly, the overexpression of this particular allele results in a dominant negative effect in a variety of different yeast strains. We are currently investigating the basis for this
dominant negative effect. Our preliminary results show that cells overexpressing this mutant version of Cdc6p arrest at the G2/M phase of the cell cycle. However, this terminal arrest phenotype is heterogeneous, since some cells appear to have undergone nuclear division, whereas others contain a single nucleus. We are constructing yeast strains that will allow us to follow the migration of the chromatinids and therefore ascertain whether in mutant cells that undergo nuclear division, the separation of chromatids is normal or takes place in an aberrant way. We are also performing cell cycle arrest and release experiments to analyze the ability of this mutant allele to bind to chromatin, and recruit Mcm proteins, at the G1 phase, as well as its ability to allow the formation of a postreplicative complex at the onset of S phase.

261. Functions of Dna2p at the telomeres

Wonchae Choe, Judith L. Campbell

Saccharomyces DNA2 encodes a single-stranded DNA stimulated ATPase and DNA helicase that is required for chromosomal replication. Strains with a deletion of the gene are not viable. Dna2 proteins are very required for chromosomal replication. Strains with a DNA stimulated ATPase and DNA helicase that is normal or takes place in an aberrant way. We are also performing cell cycle arrest and release experiments to analyze the ability of this mutant allele to bind to chromatin, and recruit Mcm proteins, at the G1 phase, as well as its ability to allow the formation of a postreplicative complex at the onset of S phase.

Our cell cycle analysis of Dna2p showed that Dna2 is at the elongation stage of DNA replication, we also monitored Dna2 localization as a function of the cell cycle. Our cycle analysis of Dna2p showed that Dna2 is at telomeres in G1 and G2/M, but Dna2p delocalized through the nucleus in S phase. Our initial model was that Dna2 might be stored at the telomeres and recruited to function at replication fork at replication fork and double-stranded break. Moreover, based on recent results, indicating that the finding that when Dna2 was overexpressed, telomeres was shortened, implying that Dna2 might play a role in telomere metabolism, for example, telomere replication. Further studies are directed of determining the role of Dna2p in telomere metabolism.

Reference

262. In vivo and in vitro studies on the effects of synthetic pyrrole-imidazole polyamide ligands on the function of Escherichia coli origin of DNA replication

Piotr Polaczek, Zheng-Yun Zhan1, Peter Dervan1, Judith Campbell

Sequence-specific, minor groove binding ligands have been shown to specifically interfere with the transcription machinery in several systems tested. So far, these compounds have not been tested as inhibitors of DNA replication. We have attempted to test the synthetic polyamides as inhibitors of DNA replication in E. coli both in vivo and in vitro. We have designed the polyamides to bind to the IHF binding site since it is known that IHF protein binds exclusively in the minor groove. We assume that targeting the IHF site will affect the architecture of the origin and thereby prevent proper initiation complex assembly.

Our results to date are as follows. First, in vitro footprinting shows specific binding of the polyamides to the IHF site and the AT-rich 13-mer region (site of DNA unwinding prior to initiation of replication). Second, in vivo, a cytotoxic effect is observed in cells that have been permeabilized, for instance, in hypotonic solutions (Tris buffer). The polyamides inhibit not only DNA, but also RNA synthesis (at micromolar concentrations). RNA inhibition in vivo may be due to the high concentrations of polyamides used and relatively low selectivity of the polyamides for the target site (recognition of other AT-rich regions). We reasoned that testing the polyamides in a DNA replication system dependent on oriC might give us more meaningful answers. Indeed, in vitro (in a crude extract), total inhibition of oriC-dependent DNA replication is achieved at nanomolar concentrations of polyamides.

The question we would like to address is exactly what stage of DNA replication is being inhibited in vitro (inhibition of IHF binding, 13-mer unwinding, or something else?). Testing the polyamides in an oriC system reconstituted from purified proteins may provide us with the answer.

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Publications


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Summary: Diverse biological systems—including viruses, cells, and organelles—are enclosed within lipid membranes that normally serve to compartmentalize the systems and distinguish their contents from the environment. Under certain circumstances, however, these membrane-bound systems can undergo membrane fusion events, in which the lipids of two compartments fuse and the internal contents ultimately mix. In general, these fusion events are multi-step processes, involving recognition of two membrane surfaces, membrane apposition, and finally, lipid and content mixing. Such membrane fusion events are central to many fundamental cellular processes, including entry of enveloped viruses into cells during infection, entry of sperm into an egg during fertilization, and fusion of organelles during protein trafficking and organelle biogenesis. Using cell biological, biophysical, and genetic approaches, our lab is studying the mechanisms through which these membrane fusion events occur.

Our major research focus is on the fusion of mitochondria. Mitochondria are dynamic organelles that undergo cycles of homotypic fusion and fission. Such membrane fusion and fission events play an important role in controlling organelle number, subcellular distribution, morphology, and ATP production. In some cells, fusion of numerous mitochondria into a well-organized reticulum is thought to enable transmission of mitochondrial membrane potential, thereby facilitating ATP generation to active regions of the cell. In some cases, mitochondrial fusion is developmentally regulated; for example, during insect spermatogenesis, the mitochondria of immature spermatids aggregate and fuse into giant mitochondria. Such regulated mitochondrial fusion is likely required in order to accommodate the changing metabolic state of the cell. Recent experiments in flies and budding yeast have demonstrated that developmentally regulated fusion of mitochondria is necessary for respiratory function and have identified the mitochondrial GTPase, Fzo, as an essential player. In addition, genetic studies in yeast and C. elegans have identified a second GTPase, Dnm-1, that plays a central role in fission of mitochondria.

Although the importance of mitochondrial fusion in lower eukaryotes is well documented, its role during mammalian development remains unclear. Accordingly, we have placed a major emphasis on addressing this issue through the analysis of mice deficient in homologs of Fzo (see abstracts by H. Chen). This work should reveal the role of mitochondrial fusion in tissue development and bioenergetics. Furthermore, these studies may also lead to mouse models of human mitochondrial diseases, a diverse group of diseases characterized by defective mitochondrial function.

Genetic studies have uncovered Fzo as a central molecule in the fusion of mitochondria, but its mechanism of action is unknown. It may act as a truly fusogenic molecule, analogous to viral envelope proteins that directly mediate membrane fusion. Alternatively, it may act as a regulatory protein, directing assembly of a fusion machinery in a GTP-dependent manner. We are currently performing extensive structure-function analyses of Fzo to address these issues (see abstracts by S. Detmer and E. Griffin). We have also begun attempts to genetically and biochemically characterize proteins that interact with Fzo in order to identify other proteins involved in mitochondrial fusion (see abstract by E. Griffin). A full understanding of the biochemical basis of mitochondrial fusion will require in vitro systems, and an approach towards such an assay is underway (see abstract by T. Rosen).

In a second line of research, we are continuing our investigations of membrane fusion by viral envelope proteins. Previous structural studies have shown that the gp41 envelope protein of the human immunodeficiency virus (HIV) undergoes a transition to a six-helix bundle structure that is essential for fusion. A class of HIV entry inhibitors are synthetic peptides that inhibit formation of this six-helix structure. We are currently using these structural insights to develop novel inhibitors of HIV infection.

The study of HIV inhibitors also provides the opportunity to dissect HIV-mediated membrane fusion. We are testing whether particular peptide inhibitors bind to fusion intermediates of gp41 (T. Koshiba). In addition, we would like to define sequential steps in the fusion pathway. We are setting up screens to identify mutants of the HIV envelope protein that are partially defective and thereby trap fusion intermediates (T. Suntoke). Finally, we have begun a biochemical analysis fusion mediated by the murine retrovirus, Moloney Murine Leukemia virus (A. Hamburger).

263. Analysis of mitochondrial fusion during mouse development

Hsiuchen Chen

Mice contain two Fzo homologs, termed Mfzo1 and Mfzo2, that are highly similar to yeast and Drosophila Fzo. Mfzo1 is broadly expressed, while Mfzo2 is primarily expressed in skeletal muscle and heart. As expected for proteins likely to play a role in mitochondrial fusion, both Mfzo1 and Mfzo2 localize to mitochondria.

To determine the role of mitochondrial fusion in mammalian development, we are analyzing the phenotype of mice deficient in the two homologs of Fzo. We constructed gene-replacement vectors for mfzo1 and mfzo2 using the neomycin gene for positive selection and the
diphtheria toxin subunit A gene for negative selection. The \textit{mfzo1} targeted allele should produce a truncated protein of 86 residues, out of 741 residues in the wild-type protein; the \textit{mfzo2} targeted allele should produce a truncated protein of 107 residues out of 757 residues. In addition to the premature stop codon, each of the resulting genomic loci has a substitution that removes the G-1 and G-2 motifs of the GTPase domain and replaces them with the neomycin expression cassette. Because of this genomic deletion, it is impossible to produce Mfzo with GTPase activity, even in the unlikely event that aberrant splicing circumvents the stop codon. Genetic analyses in \textit{Drosophila} and \textit{S. cerevisiae}, as well as our own studies in murine cells (abstract by S. Detmer), demonstrate that mutations in the GTPase region disrupt Fzo function. Therefore, the targeting constructs described here should generate null alleles upon homologous recombination with the endogenous genomic loci.

For both Mfzo1 and Mfzo2, we have generated two independent lines of mice and are currently performing phenotypic analysis. Homozygous Mfzo1-deficient mice show embryonic lethality, with loss of embryos at embryonic day 8.5-10.5. We are performing extensive histological and biochemical analysis of homzygous embryos, particularly of skeletal muscle, the central nervous system, cardiac muscle, and the endocrine system. These are the organs and tissues which display clinical manifestations of mitochondrial disease in humans, presumably because they have the greatest need for respiratory function. Significantly, cell lines derived from these mutant mice show dramatic defects in mitochondrial morphology.

### 264. Structure-function analysis of murine Fzo

\textit{Scott Detmer}

At present, we have almost no understanding of how Fzo functions from a mechanistic perspective. It may act as a directly fusogenic molecule, using energy from GTP hydrolysis or coiled-coil formation to appose membranes and mediate fusion. In such a model, it would play a role analogous to viral fusion proteins during viral entry or SNARE complexes during intracellular membrane trafficking. These proteins contain helical hairpins that are thought to appose lipid membranes. Alternatively, Fzo may act as a regulatory protein that assembles a separate fusion machinery in a GTP-dependent manner. In either case, it is likely that the study of Fzo will yield novel insights into membrane fusion, since mitochondrial fusion is a unique process that fuses two sets of double membranes.

To better understand the role of Mfzo1 in mitochondrial fusion, we are performing a systematic structure/function analysis. Cells derived from \textit{mfzo1}-deficient mice provide an ideal experiment system for such an analysis. Whereas wild-type cells have reticulated and tubular mitochondria, the mitochondria of cells lacking Mfzo1 are small, round, and individual. Transient transfection of epitoped-tagged Mfzo1 into the \textit{mfzo1}-deficient cell line completely restores wild-type mitochondrial morphology, as assessed by green fluorescent protein (GFP) targeted to the mitochondrial matrix. We have used this system to test Mfzo1 mutants for their ability to localize to the mitochondria and to restore wild-type mitochondrial morphology.

Mfzo1 is a 86 kDa protein and contains an N-terminal GTPase and two predicted coiled-coil domains separated by two transmembrane domains. Our initial mutagenesis has focused on the GTPase and putative coiled-coils. Two distinct point mutant that are predicted to prevent GTP binding localize to the mitochondria but fail to rescue morphology, strongly suggesting that a functional GTPase domain is required for mitochondrial fusion. Missense mutations creating proline residues in both the N- and C-terminal predicted coiled-coils affects mitochondrial morphology without affecting the localization. Because prolines are known to disrupt the helical nature of coiled-coils and thus their interactions with other domains, this suggests that these regions may indeed be functioning as coiled-coils. We will use this system to further study structure-function relationships of Mfzo1 with additional GTPase mutations, various deletion mutants, and to test for Mfzo1 intramolecular interactions with protein fragment complementation.

### 265. Structure-function analysis of yeast Fzo

\textit{Erik Griffin}

As a first step towards understanding the mechanism of Fzo1 action, it is necessary to identify critical functional domains in Fzo. We are developing cell-based assays that will allow us to systematically evaluate the functional role played by various protein motifs within mammalian and yeast Fzo. Structure-function analysis of yeast Fzo1p is possible using a plasmid shuffling technique. In this system, a \textit{URA3}-containing plasmid expressing wild-type Fzo1p "covers" a deletion in the chromosomal \textit{FZO1} gene (\textit{fzo1Δ}). This strategy allows \textit{fzo1Δ} cells to grow on plates lacking a fermentable carbon source and prevents the irreversible loss of mtDNA that normally occurs in yeast cells deficient in Fzo1p. To test the role of the GTPase domain, the heptad repeats, and the transmembrane regions, we have designed expression plasmids to express a variety of mutant Fzo1p molecules. These plasmids will be transformed into the yeast strain, and loss of the wild-type Fzo1p (\textit{URA3}-containing) plasmid will be selected using 5-fluoroorotic acid (5-FOA). With this plasmid shuffle, the wild-type plasmid is exchanged for a mutant one, and the ability of mutant constructs to rescue the \textit{fzo1Δ} mutation can be assessed by growth on plates lacking a fermentable carbon source as well as by evaluation of mitochondrial morphology. We have identified several loss-of-function alleles of Fzo1, will exploit these alleles to identify interacting genes.
266. **Identification of Fzo interacting proteins**

*Erik Griffin*

We are using a "double tagging" method, designed in the Deshaies lab (see research abstracts of Ray Deshaies), to identify proteins that specifically interact with Fzo1p. We have generated yeast in which the C-terminal sequences of Fzo1p are fused in-frame to a His8 tag, two TEV (tobacco etch virus) proteolysis sites, and a 9X Myc epitope tag. Using an anti-Myc antibody, we can readily immunoprecipitate Fzo1p. Fzo1p and associated proteins are released by cleavage with recombinant TEV protease. TEV is a reliable, site-specific protease that interacts with a seven-residue recognition site, minimizing the possibility that it may cleave Fzo1p-associated proteins. Proteins eluted by TEV cleavage are bound to nickel-NTA beads for a second round of purification. Fzo1p and associated proteins eluted from the nickel-NTA beads will be analyzed by SDS-PAGE and autoradiography. Comparison of elution profiles from wild-type cells and Fzo1p-His8/TEV/Myc-tagged cells should reveal candidate Fzo1p-associated proteins. Since Fzo1p is a GTPase, we will also run parallel purifications in the presence of guanosine analogs. Analogous strategies have been critical in identifying proteins specifically bound to the GTP-bound form of Rab proteins involved in vesicle trafficking. Once we have identified promising bands using 35S-labeled cells, we will scale up reactions and excise proteins from silver-stained SDS-PAGE gels. These bands will be identified using N-terminal sequencing and MALDI (matrix-assisted laser desorption ionization mass spectrometry) peptide mapping. Since the yeast genome has been sequenced, this approach will lead to rapid identification of the associated proteins.

267. **An in vitro assay for mitochondrial fusion**

*Tobias Rosen*

Previous membrane fusion analyses, such as that of the yeast vacuole, have relied on robust *in vitro* assays to describe the molecular interactions involved. Likewise, we are trying to develop a cell-free system that recapitulates the fusion of mitochondria observed *in vivo*. Such an assay could be exploited both to analyze the main features of this process as well as to identify novel molecular components. We expect the biochemical features of mitochondrial fusion to be unique, because unlike vesicle or viral fusion, four separate bilayers must fuse.

An assay for mitochondrial fusion has been designed based on the rationale that if mitochondria fuse *in vitro*, the soluble contents of their matrices will mix. To provide a biochemical readout of this post-fusion mixing, two strains of yeast have been constructed. One strain expresses a mitochondrially-targeted TEV protease while the other strain produces a mitochondrially-targeted GFP-Myc fusion protein with a TEV cleavage site. By physically combining purified mitochondria from each strain, and then assaying for cleavage of the substrate by Western blotting, a measurement of mitochondrial fusion can be provided. Preliminary results suggest that a fraction of the mitochondria in such an assay may be fusing, and experiments involving yeast strains expressing temperature-sensitive alleles of mitofusion-related genes are in progress.
Our design approach utilizes a "compute and build" strategy that is based on the physical/chemical principles governing protein stability and catalytic mechanism. For an initial test, we used the catalytically inert 108-residue E. coli thioredoxin as a scaffold, the histidine-mediated nucleophilic hydrolysis of p-nitrophenyl acetate (PNPA) as a model reaction, and the ORBIT protein design software to compute sequences. We modeled a high energy state (HES) of the nucleophilic attack of a histidine side chain on PNPA. In our model, chemical similarity between PNPA and the HES provided for substrate affinity, and catalytic orientation of the histidine side chain and transition state stabilization provided for catalytic rate enhancement. An active site scan was performed to rank different positions for the designed catalytic histidine within the protein scaffold. Individually, each position in the protein was modeled as the histidine-HES complex and other spatially near positions were optimized as either their wild-type identity or alanine. Limited combinatorial complexity made this calculation very fast and allowed each position in the protein to be scanned as the catalytic residue. Ranking of models from the active site scan based upon substrate recognition and active site accessibility identified two promising catalytic positions and surrounding active site mutations required for substrate binding. Experimentally, both candidate protozymes demonstrated catalytic activity significantly above background. One of the proteins, PZD2, displayed "burst" phase kinetics at high substrate concentrations, consistent with the formation of a stable enzyme intermediate. The kinetic parameters of PZD2 are comparable to early catalytic antibodies. But, unlike catalytic antibody design, our design procedure is independent of fold, suggesting a possible mechanism for examining the relationship between a protein fold and its adaptive potential.

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269. The importance of polar residues in protein cores

Daniel N. Bolon1, Stephen L. Mayo

Most globular proteins contain a core of hydrophobic residues that are inaccessible to solvent in the folded state. In general, polar residues in the core are thermodynamically unfavorable except when they are able to form intramolecular hydrogen bonds. Compared to hydrophobic interactions, polar interactions are more directional in character and may aid in fold specificity. In a survey of 263 globular protein structures, we found a strong positive correlation between protein size and the number of polar residues at core positions. In order to probe the importance of buried polar residues, we experimentally tested the effects of hydrophobic mutations at the five polar core residues in E. coli thioredoxin, using our ORBIT protein design software to predict the optimal hydrophobic mutations. The combined five-fold mutant protein (IAALV) is less stable than wild type and has an unfolding transition that is substantially less cooperative. NMR spectra as well as amide deuterium exchange indicate that IAALV is likely sampling a number of low energy structures in the folded state, suggesting that polar residues in the core are important for specifying a well-folded native structure.

Three slightly overlapping categories suffice to explain most core polar residues: active sites, thermodynamic stability, and structural specificity. Active sites use polar residues to perform chemistry and are often buried in clefts. Polar residues can increase thermodynamic stability when they form intramolecular hydrogen bonds to polar atoms that are inaccessible to solvent. The directional nature of polar interactions as
well as their hydrophobic aversion allows core polar residues to determine a fold in a more specific manner than hydrophobic residues alone.

The relatively high conservation of hydrophobic core residues in sequence alignments clearly indicates the structural importance of core residues in terms of the hydrophobic effect and packing specificity. Consistent with this observation, we observe small proteins in our structural database survey with no polar residues at core positions. As the size of a protein increases, the probability of having polar main-chain atoms that require a core polar residue to satisfy their hydrogen bonding potential also increases. Similarly, the number of folds that bury substantial hydrophobic surface area increases with protein size. In larger proteins, polar residues at core positions likely aid in satisfying the hydrogen bond potential of main-chain polar groups and help to energetically distinguish a single well-folded structure from alternative structures.

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270. Designing a conformation switch in proteins using metal binding

Deepshikha Datta, Stephen L. Mayo

Our goal is to design a sequence capable of switching conformation between two completely different structures – the fold adopted by Protein G (PG) and that seen in engrailed homeodomain (SC1). We plan to achieve this by using metal binding as an external force to direct the sequence towards the α-helical (SC1) conformation over the β-sheet (PG) conformation. We therefore designed metal binding sites in SC1 that are placed in a way that they stabilize the helical conformation. His-X3-His metal-binding sites that have a geometry known to chelate metals on helical surfaces were engineered into different regions of the protein surface and the resultant proteins were tested for improved stability in the presence of nickel. Maximum gain in stability was observed when metal binding occurred near the C and N termini of the protein. We therefore chose these positions for our metal binding sites.

To design a sequence capable of adopting both conformations, we used a strategy of first determining positions on both proteins that are highly tolerant to substitutions to common residues. The tolerance of protein structures to mutations can be calculated using a mean-field approach to identify the high entropy positions. Sites with low entropy are intolerant to mutations and are classified as conserved residues, whereas positions with high entropy are tolerant to mutations; these high entropy positions were therefore selected for design. The combined tolerance of the two structures to common mutations was extracted from the combined probabilities of the paired positions calculated using mean-field theory. The high entropy positions are being designed using our protein design algorithm, ORBIT, to select for common amino acids and consequently, to bring the two conformations closer in sequence space. This is achieved by linking these positions in the optimization calculations to maintain sequence symmetry. The protein sequences selected in the initial calculations are now being made and tested experimentally.

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271. Improving tools used in computational protein design

Geoffrey Hom, Stephen L. Mayo

We are working on four different computational design projects. The first project is an attempt to improve our existing electrostatics force field that is based on Coulomb's law; a similar force field which also includes a term for charge/dipole interactions may have more predictive power. The second project is an advanced version of the split dead-end elimination (DEE) algorithm we use for finding the global minimum energy conformation (GMEC) of an energetic landscape. The third project is the full redesign of a 110-amino-acid region of the cyclin-box fold; because of the size of the problem, approximate search algorithms (rather than the DEE algorithm) will be used. The fourth project is the development of a new, approximate algorithm that will hopefully be faster than advanced DEE algorithms and more accurate than existing approximate algorithms such as Monte Carlo.

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272. Design of de novo protein dimers

Po-ssu Huang, Stephen L. Mayo

We are exploring the possibility of using our ORBIT protein design software to drive two protein monomers to form a dimer. Novel protein dimers were simulated using two copies of the protein G backbone; the two backbones were docked so that the surface complementarity between them was optimized. This dimer backbone structure was then used for subsequent sidechain design. Amino acid positions that became buried upon docking (the monomer-monomer interface) and residues in the immediate vicinity were included in the design. ORBIT was used to select the set of sidechains that would be the most energetically favorable for the dimer configuration. This set of protein sequences was then produced and tested using NMR and analytical ultracentrifugation to determine whether dimers were actually formed as designed. Initial NMR data is consistent with dimer formation. Dimer orientation will be determined upon completion of NMR peak assignments.

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273. Design and validation of DNA-binding proteins: Initial experiments with engrailed homeodomain

Shira L. Jacobson-Rogers, Stephen L. Mayo, Peter B. Dervan

We have completed the computational design of two engrailed homeodomain surface mutants. In both mutants, DNA-binding residues have not been altered. One surface mutant (unbiased) has a melting temperature of 45.9°C, comparable to that of wild-type engrailed
homeodomain, which has a melting temperature of 48.7°C. The second mutant (biased), whose design restricts residues in order to account for helix macrodipoles, exhibits a melting temperature of 64.5°C. Gel-shift experiments were used to assay the proteins for DNA-binding activity. Wild-type homeodomain experiments agree reasonably well with literature values, with a $K_d$ of $6.7(\pm 1.8) \times 10^{-8}$ M. The unbiased mutant exhibits a $K_d$ of roughly $7 \times 10^{-6}$ M, while the biased mutant exhibits a $K_d$ of roughly $7 \times 10^{-7}$ M. We propose that the primary reason for loss of binding affinity is a loss of positive electrostatic potential on the DNA-binding face of the engrailed homeodomain mutants. Proposed force-field developments to increase accuracy in developing DNA-binding proteins include increased accuracy in recognizing intermolecular electrostatic interactions, as well as explicit inclusion of water-mediated hydrogen bonds. Both developments will seek to address issues commonly encountered in specific binding of DNA.

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274. Evaluation of the energetic contribution of an ionic network to $\beta$-sheet stability

Kirsten S. Lassila, Stephen L. Mayo

The role of surface electrostatic interactions in promoting protein stability is not fully understood. Networks of charged amino acids are observed on the surfaces of hyperthermophilic proteins and have been proposed to offer an energetic advantage over single ion pairs due to the reduced entropic cost of fixing additional residues. Although stabilizing interaction energies have been reported for ionic networks in $\alpha$-helical systems, side-chain electrostatic interactions have not been as well characterized for $\beta$-sheet surfaces. We used double mutant cycles to evaluate the interaction energy of a three-residue ionic network constructed on the $\beta$-sheet surface of protein G. The two individual ion pairs were each stabilizing by around 0.6 kcal/mol. However, the ionic triad did not appear to enhance protein G stability any more than the simple sum of the individual pairs.

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275. Protein engineering: Novel protein/protein docking by design

John J. Love, Stephen L. Mayo

The overall protein engineering goal of this project is to redesign small monomeric proteins such that they self-assemble into complexes of predefined, specific structure. The small proteins will then be redesigned to bind, with high affinity, to specific protein targets. The immediate design goal is to drive Protein G (a small, well-behaved, monomeric protein) to bind to itself with high affinity and specificity.

The first step in driving de novo protein self-assembly is the computational docking of the two molecules together in the predefined orientation. To this end we have modified an established docking algorithm borrowed from the field of native protein docking, the

Geometric Recognition Algorithm (GRA). The GRA treats the molecules as rigid bodies and rigorously assesses the interfacial surface complementarity as a function of translational and rotational position. This process is computationally intensive yet has been rendered tractable by exploiting the Fourier Correlation Theorem.

Upon obtaining the optimal intermolecular atomic coordinates, the two molecules are treated as one and a suite of highly developed protein design algorithms (which utilize advanced molecular mechanics force fields) is used to computationally repack the interfacial side chains in a manner analogous to the cores of well-folded proteins.

We have successfully developed the above computational algorithms and have obtained mutant sequences, of favorable relative energy, for three dimer complexes. Molecular biology and biochemistry were used to introduce the mutations and physically generate the three complexes.

The extent of docking was initially assessed with analytical ultracentrifugation. Due to the combination of the low molecular weight of the complexes and the current analytical ultracentrifuge optics, the binding parameters obtained are inconclusive yet initially indicated weak binding (~300 $\mu$M) for the first complex tested. The second complex exhibited no measurable binding. For the third complex, one of the monomers was significantly destabilized and therefore unattainable by conventional means (i.e., bacterial T7 expression). The gene for the destabilized monomer has since been cloned into a system developed specifically for unstable protein expression (i.e., ubiquitin fusion system). Using this system we have greatly increased expression levels and will soon assess binding affinity for the third complex.

Because of the inconclusiveness of the analytical ultracentrifuge results, we decided to measure binding affinity and specificity with heteronuclear 2D- and 3D-NMR methods. To this end we have produced $^{15}$N-labeled monomer A ($^{15}$N-A) from complex one and have collected 2D-HSQC spectra of $^{15}$N-A alone and in complex with unlabeled monomer B. We have also collected 3D-TOSY and NOESY HSQC spectra of $^{15}$N-A alone and are currently in the process of analyzing the data to ascertain the degree of binding specificity.

276. Double mutant cycle study of cation-π interactions

Jessica Mao, Shannon A. Marshall, Stephen L. Mayo

Cation-π interactions are the favorable electrostatic interactions between a positive charge and the partial negative charge of the quadrupole of an aromatic ring. In proteins, it is the interaction between a cationic side chain (Lys or Arg) and an aromatic side chain (Trp, Phe, or Tyr). While cation-π interactions have been observed in numerous proteins, and theoretical calculations have proven the interaction energy to be comparable to salt bridges in aqueous media (1), to date there has been no experimental data reported that quantifies the interaction energy of a cation-π pair. In this study, we used the
double mutant cycle to calculate the amount of energy a cation-π interacting pair contributes. We chose the engrailed homeodomain as the scaffold and the Arg-Trp pair as a representative of cation-π interactions since it is the most commonly occurring pair (2). We mutated positions 9 and 13 on an α-helix on the surface of the protein to either alanines or arginine and tryptophan respectively to make the four mutants that comprise a double mutant cycle. Initial results suggest that charged residues on the same helix need to be mutated to neutral ones in order to isolate the cation-π interaction.

References

277. An electrostatic model for protein design calculations
Shannon A. Marshall1, Stephen L. Mayo

Electrostatic interactions are often critical determinants of protein structure and function. Computational protein design algorithms typically use fast methods based on Coulomb’s law and/or geometry-dependent hydrogen bond terms to model electrostatic interactions. These methods fail to accurately account for desolvation of polar protein groups and solvent screening of Coulombic interactions, which strongly attenuate electrostatic interactions in proteins. Continuum models of electrostatics based on the finite difference Poisson-Boltzmann method (FDPB) have substantially better predictive power, but are far too slow for problems with high combinatorial complexity. To date, the role of electrostatics in protein design has not been the subject of extensive testing and so the consequences of using a highly approximate model of electrostatics in design force fields are not well understood.

We are using experimental and computational methods to determine the effect of electrostatic interactions on protein stability, assess the accuracy of the ORBIT force field, and develop and test new strategies for modeling electrostatics. Electrostatic interactions were found to significantly affect the stability of designed variants of the Drosophila engrailed homeodomain. Using the current force field, we designed a 25-fold mutant with moderate stability similar to the wild-type protein. Incorporating two classes of electrostatic interactions using simple rules yields a nine-fold mutant of the initial design that is over three kcal mol⁻¹ more stable than the first designed variant. The simple electrostatic model used in the ORBIT force field is unable to predict the experimentally determined stabilities of the designed variants. The current method overestimates the energetic contribution of side chain - side chain interactions, underestimates the importance of side chain - backbone interactions, and neglects the effects of desolvation. We have developed a new strategy for modeling electrostatics in protein design problems that utilizes one- and two-body decomposable FDPB methods. Computational results suggest that this method has the accuracy and speed required for design calculations; experimental tests are planned.

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278. Backbone design for proteins
Joseph J. Plecs, Stephen L. Mayo

Our research in this area is directed at the design of protein backbones. While the Mayo Group has been very successful in stabilizing existing protein structures and in designing new ones using the dead-end elimination algorithm (DEE) to redesign protein side chains, we are still restricted to using backbones from known protein structures. The ability to explore variations in protein backbones would greatly expand the power of protein design. For instance, protein molecules are known to shift their backbones as part of ligand binding or catalysis; improvements in our ability to design these processes into proteins would therefore be very useful.

Redesigning the backbone itself presents an array of new challenges. DEE cannot be applied directly to backbone design because one of DEE’s fundamental principles, the pairwise energy function, no longer applies to a protein molecule if the backbone is allowed to vary. Other problems are inherent to backbone design, such as the construction of favorable loops to connect units of secondary structure and the arrangement of helices and sheets with respect to one another to form a stable fold. I am presently addressing these problems by combining DEE with parametrized-backbone and energy-minimization methods. Experimental verification of the calculations is underway.

279. Structure determination of designed protein G core variants
Scott A. Ross, Catherine A. Sarisky1, Stephen L. Mayo

Experimental validation of calculated structures is a central feature of our approach to protein design. In this instance, we solved the solution structures of two computationally designed core variants of the β1 domain of streptococcal protein G (Gβ1) by 1H NMR methods to assess the robustness of amino acid sequence selection by the ORBIT protein design package under changes in protein backbone specification. One variant has mutations at three of ten core positions and corresponds to minimal perturbations of the native Gβ1 backbone. The other, with mutations at six of ten positions, was calculated for a backbone in which the separation between Gβ1’s α-helix and β-sheet was increased by 15% relative to native Gβ1. Exchange broadening of some resonances and the complete absence of others in spectra of the six-fold mutant bespeak conformational heterogeneity in this protein. The NMR data were sufficiently abundant, however, to generate structures of similar, moderately high quality for both variants. Both proteins adopt backbone structures similar to their target folds. Moreover, the
sequence selection algorithm successfully predicted all core $\chi_1$ angles in both variants, five of six $\chi_2$ angles in the three-fold mutant and four of seven $\chi_2$ angles in the six-fold mutant. We conclude that ORBIT calculates sequences that fold specifically to a geometry close to the template, even when the template is moderately perturbed relative to a naturally occurring structure. There are apparently limits to the size of acceptable perturbations: in this study, the larger perturbation led to undesired dynamic behavior.

280. A novel method for calculating rotamer surface areas: Implications for protein design
Premal S. Shah¹, Stephen L. Mayo

We have developed a method for calculating the exact multibody surface area of a rotamer in the context of our protein design algorithm. The new method, termed type 4 solvation, uses a Monte Carlo search algorithm to generate approximate structures from which exact rotamer surface areas are determined and applied to our solvation scoring function.

The effectiveness of type 4 solvation was experimentally tested by optimizing the boundary positions of the $\beta$ domain of protein G (G$\beta$1) and the engrailed homeodomain (EH). We observed that sequences generated using type 4 solvation contained a high ratio of polar to non-polar residues in the boundary, resulting in proteins with little or no stability. We therefore decided to impose a fixed binary pattern (eight nonpolar residues and four polar residues were required in the boundary) during our optimizations. The resulting proteins were stabilized relative to wild type and were comparable in stability to proteins generated using our current surface calculation (type 2 solvation). We are currently working on a way of ensuring a more favorable polar/hydrophobic balance in our boundary calculations without having to invoke a fixed binary pattern. This is being tested by decreasing the penalty for nonpolar surface area exposure that is included in our solvation potential. Sequences generated using this approach will contain more hydrophobic residues in the boundary and should be more stable.

The type 4 solvation calculation has also provided a significant decrease in computational time during the dead-end elimination (DEE) segment of our protein design calculations. DEE is used to evaluate rotamer energies and arrive at a global minimum energy conformation (GMEC). Decreases in time ranging from eight to 400-fold are observed. This increase in computational efficiency has the potential for allowing larger, previously intractable calculations, such as full sequence designs, to be performed.

281. Computational redesign of the calmodulin receptor
Julia M. Shifman, Stephen L. Mayo

Recent advances in computational techniques have allowed researchers to select amino acid sequences that will fold into proteins with a desired three-dimensional structure. Moreover, it remains a challenge to supply novel proteins with real function such as enzyme catalysis or signal transduction. The theoretical understanding of protein-ligand binding interactions is a very important yet difficult step towards the design of such molecules. Our goal is to redesign a naturally existing receptor in order to alter its binding specificity. For this purpose we have chosen calmodulin (CaM), a small, ubiquitous Ca$^{2+}$-binding protein that can bind very tightly to at least 30 functionally and structurally diverse targets. Optimization of the CaM binding interface is performed using a discrete set of amino acid rotamers and a very fast sequence selection algorithm based on the Dead-End Elimination theorem. Starting with a crystal structure of CaM complexed with smMLCK, a new CaM sequence is selected to improve CaM-smMLCK interactions. Computationally designed CaM molecules are constructed and tested for binding to a set of five targets. In the first generation of design only core positions in contact with the target were included. The calculation yielded an 8-fold CaM mutant that was well-folded and showed a slight increase in binding affinity to smMLCK and some increase in binding specificity. The second round of CaM optimizations included boundary and surface positions surrounding the target peptide on top of the 8-fold core mutant. Seven additional boundary and surface mutations predicted by the calculation altered the binding specificity of the CaM molecule. However, the CaM affinity to smMLCK was compromised. Our results on CaM redesign suggest that while hydrophobic residues in the CaM binding interface ensure tight binding affinity to the targets, hydrophilic residues, mostly glutamates, control specificity of binding. We are currently working on improvements in the calculation methods that will allow us to design a boundary and surface CaM mutant with enhanced binding affinity to smMLCK.

282. Computationally guided evolutionary protein design
Christopher A. Voigt¹, Stephen L. Mayo, Zhen-Gang Wang², Frances H. Arnold³

We are developing new strategies in the design of proteins by combining two powerful but largely separate design methodologies: that of rational computational design and that of directed in vitro evolutionary design. The central goal of this research is to develop computer-aided methods for generating mutant libraries in directed evolution with targeted diversity. The research has two primary focuses: (1) to computationally predict the location of residues for site mutagenesis that are most likely to lead to functional proteins; and (2) to computationally predict the optimal crossover locations and segment sizes, and the best sets of parental genes for
recombination. State-of-the-art computational protein structure design tools are used to provide rapid prescreening to eliminate sequences that would destabilize the folded structure of a specific protein, thus reducing the search space that needs to be traversed in directed evolution. This approach will greatly accelerate the directed evolutionary design processes and will allow us to make efficient use of the rapidly growing database of protein structures and sequences in developing new or improved proteins for industrial catalysis, pharmaceuticals, and antibodies.

To model point mutagenesis, we simulated directed evolution using a simple statistical description of the fitness landscape. We found that beneficial mutations tend to occur at amino acid positions that are tolerant to substitutions, in the limit of small libraries and low mutation rates. This observation was extended to analyze directed evolution data by applying mean-field theory to a structure-based computational model to calculate each residue's structural tolerance. We found that thermostabilizing and activity-increasing mutations accumulated during the experimental directed evolution of subtilisin E, T4 lysozyme, and antibody 4420 are strongly biased towards highly tolerant sites identified using this computational approach. This method can be used to predict positions where mutations are likely to lead to improvement of specific protein properties.

Further, we constructed a computational method to determine the locations of crossovers that lead to functional hybrid proteins during in vitro recombination. Borrowing a concept from the schema theory of genetic algorithms, our approach assumes that crossovers resulting in functional proteins are those that least disrupt structural integrity. We examined schema disruption in four shuffling experiments: recombination of β-lactamases, glycaminide ribonucleotide transformylases, and two P450 experiments. The locations of crossovers that are predicted by this approach strongly correlate with boundaries between schemata. This method can be used to predict sites for protein shuffling and to screen sequence databases to determine optimal sets of starting sequences for evolution by recombination.

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Publications
Ethel Wilson & Robert Bowles Professor: James H. Strauss
Senior Research Associate: Ellen G. Strauss
Postdoctoral Scholar: Jeroen Corver
Visitor: Ellen Marion Westerhout
Research Laboratory Staff: Edith M. Lenches
Undergraduates: R. Aaron Robison, Trisha Sando, Kayla Smith

Summary: We are interested in two groups of animal viruses, the alphaviruses and the flaviviruses. These viruses contain an RNA genome of about 11,000 nucleotides and are enveloped, having a lipid envelope that surrounds an icosahedral nucleocapsid. Both alphaviruses and flaviviruses are vectored by mosquitoes and infect both mosquitoes and a wide range of vertebrates. We wish to understand the replication of alphaviruses and flaviviruses at a molecular level and to study the evolution of these two virus groups and more broadly the evolution of all RNA viruses. This year all of the abstracts listed below involve flaviviruses.

The flaviviruses contain about 70 members, most of which are important human pathogens causing hundreds of millions of cases of human disease annually. Diseases caused by the different viruses include yellow fever, dengue fever, and encephalitis. West Nile virus, which appeared two years ago for the first time on the East Coast of the United States, is a member of this family. The genome RNA of about 10,700 nucleotides is the only viral message RNA and is translated into a long polyprotein that is cleaved to give both structural proteins required for virus replication and nonstructural proteins required for RNA replication.

Our studies have been greatly aided by the development of cDNA clones that allow us to express all or parts of the viral genome. In the case of alphaviruses we have constructed complete cDNA clones of several viruses, including Sindbis, Ross River, and Semliki Forest viruses, from which infectious virus can be recovered after transfection of susceptible cells. Similar full-length clones have been derived for the flaviviruses, but they are in general unstable and possess "poison sequences" that limit their replication. However, using an existing full-length clone of yellow fever 17D virus as a backbone we have been able to make a number of chimeric cDNA clones that incorporate various genetic elements from either dengue or hepatitis C viruses. These constructs have also been used for mutagenesis of the dimer interface in the flavivirus E protein, and studies on the role of RNA cyclization in flavivirus replication.

Recently, we have devoted considerable effort towards producing flavivirus preparations of sufficient purity and homogeneity for examination by cryoelectron microscopy. A number of such preparations have been sent our collaborators at Purdue University for analysis, including mature dengue virus and immature dengue virus containing prM. These studies, which comprise the first reconstruction of a flavivirus, have shown dengue to possess a unique structure with elements of icosahedral symmetry, but only quasi-equivalent placements of the protein subunits.

283. Mutagenesis of the proposed dimer interface of the YF E protein

Ellen Westerhout

A flavivirus virion consists of an isometric nucleocapsid containing the positive-stranded RNA genome complexed with the capsid protein (C). The surrounding bilayer contains the small membrane protein (M) and the envelope glycoprotein (E). The uncleaved precursor of M, prM, has a protein E-protective function during transportation and maturation of the virion and is required for proper folding of the E protein. The E protein is involved in receptor-binding and membrane fusion. A truncated form of E, missing the transmembrane and stem region, can be secreted by cells in a soluble form (sE) when co-expressed with prM.

Through X-ray crystallography, the atomic structure of the flavivirus tick-borne encephalitis (TBE) E protein was found to consist of a central β-barrel domain (I) connecting an Ig-like domain (III) to a dimerization domain (II). The E proteins form head-to-tail dimers positioned parallel to the viral membrane.

However, the biological significance of this dimer has never been shown. We therefore mutated amino acids, located at the dimer interface, which are proposed to be involved in dimerization. Subsequently these mutant sE were examined for their capability to form dimers.

Plasmid constructs containing the yellow fever virus (YF) prM and E genes were prepared and soluble forms of E from the wild type and several mutants were expressed in Cos-7 cells. These soluble proteins are being treated with chemical crosslinkers, followed by SDS-PAGE analysis of the E proteins, to determine whether they are capable of dimer formation. In this way we hope to identify those amino acids crucial for dimerization and ultimately determine whether interference with dimer formation prevents flavivirus replication and assembly.

284. Assembly of flaviviruses

Jeroen Corver, Edith Lenches

Flaviviruses are spherical particles that consist of a nucleocapsid that is enveloped by a lipid bilayer in which the E (envelope) and M (membrane) glycoproteins are anchored. It has been shown that assembly of flaviviruses involves interactions between E and prM, the precursor of M, and prM and the capsid protein (C).

The interactions between the proteins E and (pr)M are critical for the formation of viral particles.
Previously, we showed that swapping of one of these genes between yellow fever virus (YFV) and dengue-2 virus (Den-2) resulted in non-viable viruses. Only viruses with both membrane proteins from the same flavivirus placed in a heterologous background are able to grow. To examine why chimeras with only E, prM or parts thereof are non-viable, BHK cells were transfected with the in vitro synthesized infectious RNA of the wild-type YFV, the E chimera (YFV/Den E), or the pr/E chimera (YFV/Den pr/E). Radiolabeling and SDS-PAGE analysis of immunoprecipitated proteins of cell lysates showed that the YFV E protein could be detected in wt-transfected cells. In contrast, the presence of the Den-2 E protein could not be demonstrated in the cells transfected with YFV/Den E RNA, suggesting that the Den-2 E is not stable in a YFV context. However, the additional presence of the pr protein in another construct (YFV/Den pr/E) did result in the detection of the Den-2 E protein, indicating that the Den E protein needs at least the pr part of prM for its stability.

285. Construction of a bicistronic YFV genome

Jeroen Corver

The yellow fever virus (YFV) vaccine has been used for a long time and is known as very safe and reliable. It was initially produced by numerous passages through African green monkey cells, mouse cells, and chicken embryo fibroblasts. We have cloned a full-length cDNA copy of the RNA genome of this vaccine strain into a BAC vector. The genome has one large open reading frame (ORF). It has been shown for the flavivirus Kunjin that it is possible to add an extra ORF into the genome, by making use of an Internal Ribosome Entry Site (IRES). We have constructed a yellow fever virus clone with a second ORF introduced in its 3' untranslated end (UTR). This second ORF encodes the green fluorescent protein (GFP) and is driven by the internal ribosome entry site that was cloned upstream of the gene. This bicistronic virus was shown to be infectious and to express GFP, albeit at low levels. The GFP fluorescence was not observed in infected cells, but expression of GFP was confirmed by Western blot analysis of infected cell lysates. Currently, we are trying to optimize the expression of GFP, so it will be visible in the infected cells.

These result show that the YFV genome can be used for expression of heterologous proteins, without losing its infectivity.

286. Construction of a yellow fever virus replicon

Jeroen Corver, Trisha Sando*, R. Aaron Robison*

A YFV replicon was constructed by deleting the prM, E, and all but the first 20 amino acids of the capsid gene (C20 replicon). The remaining RNA sequence was able to replicate itself; once transfected into BHK cells, as demonstrated by immunofluorescent staining. Cotransfection of this C20 RNA with the full-length wild-type RNA resulted in packaging of full-length RNA, as expected, but also of the C20 RNA, as shown by RT-PCR analysis of virus particles secreted by transfected cells. This shows that the C20 RNA can compete with full-length RNA for packaging by the virus capsid and envelope proteins and thus that it contains the packaging signal, the sequence that is recognized by the capsid protein. Currently, we are deleting additional parts of the C20 clone in order to find the exact location of the packaging signal of YFV.

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287. Cyclization of the YFV RNA genome

Jeroen Corver, Kayla Smith*, Edith Lenches

Previously, it has been shown that the flavivirus capsid (C) gene contains a sequence that is complementary to a sequence in the 3' untranslated region (UTR). This suggests that a circular RNA intermediate might occur during the replication of the YFV genome. Analysis of the sequence of YFV showed that the proposed sequence in the capsid gene lies within the first 20 amino acids of the capsid gene. This is consistent with the fact that our C20 replicon (see previous abstract) was able to replicate. Moreover, partial deletion of the proposed cyclization sequence resulted in a lack of replication. Currently, we are introducing a frame shift upstream of the cyclization signal to check the importance of the translated aminoacid sequence. In addition, we will change the nucleotide sequence while preserving the aminoacid sequence. We will also introduce compensatory mutations in the 3' end that will restore the ability of the 5' and 3' ends to hybridize.

Since YFV is a positive-stranded RNA virus, its replication includes the synthesis of a negative strand that is subsequently used as a template for new positive strands. We will examine which step in the replication (negative-strand synthesis or positive-strand synthesis) is blocked in several of the mutants in order to determine exactly when a circular RNA intermediate is necessary during replication.

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288. Structure of dengue 2 virus


The first near atomic structure of a flavivirus has been determined by using a combination of cryo-electron microscopy and fitting of the known structure of glycoprotein E into the electron density map. The virus core, within a lipid bilayer derived from the host cell, has a less ordered structure than the external, icosahedral scaffold of 90 glycoprotein E dimers. The three E monomers per icosahedral asymmetric unit do not have quasi-equivalent symmetric environments. Difference maps indicate the location of the small membrane protein M relative to the overlaying scaffold of E dimers. The position of M implies that the cleavage of the precursor membrane protein (prM) activates flavivirus particles to
permit fusion during virus entry in a manner similar to the cleavage of PE2 in alphaviruses. Extension of this comparison suggests that these virus groups employ a similar fusion mechanism in which the distal β-barrels of domain II of the glycoprotein E are inserted into the cellular membrane.

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289. Production of immature flavivirus


Flaviviruses contain a membrane envelope in which two glycoproteins are anchored, the E (envelope) and M (membrane) protein. During assembly of the virus in the endoplasmic reticulum, a precursor of M called prM interacts with the E protein to form a heterodimer. Once the nucleocapsid buds into the ER, immature virus particles, containing prM, are present. The particles are then transported to the cell surface through the Golgi network. In the trans Golgi, a mildly acidic pH triggers a conformational change in the prM protein that makes a furin protease site accessible. Just before release of the virus particle, furin cleaves the prM protein to yield mature, M-containing particles.

The E protein is involved in low-pH-dependent membrane fusion to initiate the infection of new cells. The exposure to low pH in the trans Golgi during exocytosis could trigger a premature conformational change and expose the fusion peptide of the E protein. The presence of prM protects the E-protein from low pH and prevents this premature conformational change. Once the virus has passed the low-pH stage in the secretion pathway of the cell, the pr part of prM can be cleaved.

By addition of 20 mM ammonium chloride to the cell medium, we were able to neutralize cellular low-pH compartments, like the endosome and the trans Golgi, and thus inhibit the low-pH-induced conformational change in prM that is required for the furin cleavage, and could in that way produce prM-containing particles. We harvested these immature dengue-2 particles and purified them. SDS-PAGE analysis confirmed the presence of prM. Cryo-EM analysis revealed a spectacular difference in the conformation of prM-containing particles from that of the mature virus which was described in the previous abstract. In contrast to the relatively flat surface of mature Den-2 particles, these prM-containing particles had "spikes" protruding from the surface. This reconstruction shows that in addition to the presence of pr, the orientation of the E protein in prM-containing particles is dramatically altered from its conformation in mature virions. Currently, we are attempting to treat these prM particles with low pH and analyze the conformational changes by cryo-EM. Also, we will try to reactivate these immature particles by treating them with low pH and subsequently with purified furin.

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290. Crystallization of SIN nsP3

Jeroen Corver, Anthony P. West*

We have cloned the Sindbis virus non-structural protein 3 (nsP3) in the pet23a bacterial expression vector. Initial expression experiments using the IPTG inducible promotor, showed that SIN nsP3 was produced as an insoluble protein at levels of 0.5-1 mg/l of medium. Purification of inclusion bodies was possible, but refolding of the protein resulted in precipitation of insoluble aggregates. To obtain soluble well-folded protein, salt-inducible E. coli cells (BL-21 SI) were used. With this method of induction we were able to control the level of expression and soluble protein was produced that could be purified by Ni-columns (the protein has a C-terminal His-tag). In this way, we were able to purify 300 µg of nsP3 up to 90% purity from 1 l of induced E. coli cells. We will induce a large volume of cells in order to obtain enough protein for crystallization.

The function of the SIN nsP3 has not yet been determined. However, there are indications that the protein is somehow involved in replication of the virus. Sequence comparisons among alphaviruses have shown that the protein contains two domains. The conserved N-terminal domain has been shown to vary in size among alphaviruses from 322 to 329 aminoacids and the C-terminal, non-conserved domain from 134-246 residues. The two domains may well have two separate functions in the replication of the virus. Since the function of the protein is not known, determination of the crystal structure of the conserved domain of SIN nsP3 may help us understand its function.

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Publications


Cellular, Molecular, and Developmental Neurobiology

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Summary: There are currently three major areas of investigation in this laboratory: the development of the nervous system; the development of the circulatory system; and the functional neuroanatomy of fear.

Our studies of neural development focus on the control of differentiation in the vertebrate peripheral nervous system, which derives from the neural crest. We have isolated and characterized stem-like progenitor cells exhibiting multipotency and self-renewal capacity, and are investigating the control of the fate of these cells by both cell-extrinsic and cell-intrinsic factors. Our experimental approaches include in vitro clonal analysis, in vivo transplantation, and loss- and gain-of-function genetic manipulations in mouse and chick embryos. Subtractive hybridization and microarray analysis are being used to identify genes that distinguish stem and progenitor cells at various stages of development. We are also investigating the molecular basis of the specification of sensory neuron identity, connectivity and function.

Our studies of the circulatory system stem from our serendipitous discovery that arteries and veins are genetically distinct from the earliest stages of angiogenesis. Arteries express the transmembrane ligand ephrinB2, and veins express one of its receptors, EphB4. Gene-targeting studies have indicated that reciprocal ephrinB2-EphB4 signaling is essential for proper cardiovascular development, and may mediate bidirectional communication between arteries and veins. Our current studies include a further analysis of ephrinB2-EphB4 function in angiogenesis, using cell type-specific gene targeting; and experiments to understand the developmental origins of vessel identity. We are also exploring the existence and function of reciprocal interactions between the developing nervous system and circulatory system.

A new initiative in the lab has begun to move from the area of nervous system development to adult neural function. We are interested in developing and applying novel molecular biological tools to map and manipulate the neural circuits involved in innate behaviors. Our initial studies have focused on innate fear as a behavioral system. We are developing novel behavioral paradigms to study defensive responses to unconditioned unimodal sensory stimuli, and are using immediate early genes to map the brain regions that are active in these paradigms. Efforts to identify genes expressed in these regions, both constitutively and in an activity-dependent manner, are also underway, in parallel with the development of new methods to trace axonal connections and, in collaboration with the Lester laboratory, to reversibly silence neuronal activity. Together these tools should permit the mapping and functional manipulation of the neural circuits underlying various forms of fear.

291. Functional roles of Hey genes in the neuron versus glial lineage decision

Gloria B. Choi

The goal of the project is to understand the cellular and molecular mechanisms that control the choice between neuronal and glial fates in the developing peripheral nervous system. More specifically, I am studying the function and regulation of Hey1 and Hey3, two transcription factors belonging to the family of Hairy and Enhancer-of-Split related genes, in the neural crest stem cell (NCSC) culture system.

It has been previously shown in our lab that the transient activation of Notch signaling endows NCSCs with a glial fate and with the irreversible loss of neurogenic capacity and these consequences are cell-heritable. Hey genes are good candidates to be the downstream effectors of the Notch signaling and to maintain its effect in the NCSCs. They are expressed in the peripheral nervous system, including dorsal root ganglia and autonomic ganglia. Moreover, studies from other laboratories and ours have demonstrated that their expression is positively regulated by the constitutive activation of Notch. I am in the process of investigating whether Hey1 or/and Hey3 recapitulate all or some of the effects of Notch activation on NCSCs to restrict them from neuronal fate and to promote them to glial fate.

Another question raised by the observation that the Notch signaling instructively influences the fate of neural crest cells to glial lineage is whether the effect of the Notch activation is primarily positive (i.e., to promote a given fate), negative (i.e., to inhibit alternative fates), or
both. Several lines of evidence argue against the idea that the Notch activation simply blocks neurogenesis and leads to glial differentiation by default. A more direct way of testing Notch as positive regulator of glial differentiation would be to examine its downstream transcription effectors that recapitulate all or some of its functional aspects. In this regard, I am investigating whether Hey 1 and Hey3 work as activators, repressors, or both.

292. Functional cloning of candidate genes for neurogenic capacity
Kenji Orimoto

Neural crest stem cells (NCSCs) can differentiate into neurons, Schwann cell and myofibroblast in response to BMP2, GGF2/Delta, and TGF-Bs. NCSCs are postulated to possess neurogenic, gliogenic, and myofibrogenic capacities as well as self renewal capacity.

Mash1 plays an essential role in the genesis of autonomic neurons. In response to BMP2, NCSCs can express Mash1 and later differentiate into neurons. Gain-of-function assays have demonstrated that misexpression of Mash1 in NCSCs can evoke overt neurogenesis with the help of BMP2. Although NCSCs express Mash1 at mRNA level, it is still undetectable at protein level and is also not strong enough to evoke overt neurogenesis. BMP2 signaling appears to be involved in accelerating Mash1 induction at transcriptional and/or post-transcriptional level. At the transcriptional level, presumptive positive regulatory loop might be involved. The functional threshold could be postulated between the expression level of Mash1 and the initiation of neurogenesis. When the expression of Mash1 exceeds the threshold, overt neurogenesis appears to progress.

The molecules that could drive Mash1-positive regulatory loop initiated by BMP2 are supposed to play a central role in neurogenesis of autonomic neurons. Neurogenic capacity is defined as the ability of gene to activate Mash1-positive regulatory loop, leading to overt neurogenesis in response to BMP2. To isolate the candidate genes for neurogenic capacity, cDNA subtraction was performed between sciatic NCSCs and Schwannomyofibroblastic progenitors. Based on the results of sequence analyses, candidate genes are functionally assorted into the following categories: (1) cell fate determination; (2) chromatin remodeling; (3) cell cycle machinery and; (4) signal transduction.

To examine the spatiotemporal regulation of expression, in situ hybridization will be employed. To make sure of their functional competence of cell fate regulation, misexpression experiments including loss-of-function and gain-of-function assays would also be conducted. The candidate genes are further analyzed to see their functional integration into Delta/Notch signaling cascade since the treatment of NCSCs with Delta-Fc can efficiently eliminate neurogenic capacity.

293. A diverse family of GPCRs expressed on specific subsets of nociceptive sensory neurons
Xinzhong Dong, Mark Zylka

To study pain pathways at the molecular level, a subtractive hybridization technique was used to screen for genes expressed specifically in nociceptive neurons, which mediate pain sensation. One gene isolated from the screen is a G-protein-coupled receptor (GPCR) with characteristic seven transmembrane domains. Screening of cDNA and genomic library and searching of genomic database using the founding GPCR gene led to identification of additional 40 closely-related genes. These genes define a novel GPCR family related to Mas1, called mrgs (mas related genes).

A subset, including mrgAs and mrgD, is specifically expressed in small-diameter sensory neurons, which include cutaneous nociceptors. Expression of these mrgs is further restricted to a subpopulation of these neurons that is isolocetin B4 and Substance P, and which does not express the vanilloid (capsaicin) receptor VR1. The expression of mrgs thus reveals an unexpected degree of molecular diversity among these somatosensory neurons.

MrgA1 and A4 can be specifically activated in heterologous cells by RFamide neuropeptides such as NPFF and NPAF, which also produce analgesic effects when injected intraspinally. These data, and the specificity of expression of mrgAs and mrgD, suggest that these receptors may be involved in sensory functions that include the sensation or modulation of pain.

Publication

294. Specification of arterial and venous endothelial cells
Dong Hun Shin

The transmembrane ligand ephrinB2 and its receptor tyrosine kinase EphB4 are molecular markers of embryonic arterial and venous endothelial cells (ECs), respectively, and are essential for angiogenesis. In addition, several other genes are differentially expressed between arterial and venous ECs.

These molecular differences between arterial and venous ECs raise the issue of the specification of arterial and venous ECs. We assume that transcription factors differentially expressed between arterial and venous ECs will play important roles in EC specification. We used purified umbilical arterial and venous ECs to isolate differentially expressed genes and carried out subtraction and differential screening. We isolated several arterial specific genes and are investigating the functions of the isolated genes in the vascular system. Even though we want to isolate transcription factors involved in the specification, other differentially expressed genes will help
us to understand the functional differences between arterial and venous ECs.

**Publication**

**295. The development of neural circuits between sensory neurons and the spinal cord**
*Emma-Louise Dormand*

In order to repair injury to the spinal cord, it is necessary to ensure that the regenerating nerves connect to their correct targets to form functional neural circuits. Understanding the development of neural circuits in the embryo can provide clues as to how the spinal cord might be successfully repaired in the adult. A useful model system for studying neuronal connectivity is the development of sensory neurons and their connections to specific targets in the spinal cord. Subtypes of sensory neuron convey different sensations such as pain, temperature, touch and muscle position. The cell bodies of the sensory neurons lie in the dorsal root ganglia and they project axons peripherally to the skin and muscle, and centrally into the spinal cord.

A family of cadherin-related neuronal receptors (CNRs) has been discovered that is expressed in the mouse brain. The diversity and expression of the CNRs suggest a role in the development of neuronal circuits in the brain (Kohumura et al., 1998). I have found that the CNRs are also expressed by sensory neurons in the mouse, as well as by sympathetic neurons, motorneurons and certain laminae in the spinal cord. The expression of different CNRs does not appear to be restricted to subsets of sensory neurons, that are commonly classified by the expression of different markers or the sensation conveyed by the neuron. I have isolated CNR cDNA from the chick and found that it has a similar expression pattern to that in the mouse. In order to investigate the functional role of the CNRs during development of neuronal circuitry, I have developed a series of truncated CNR proteins that are expected either to have a positive or negative effect on CNR signaling. I am using retrovirus to express these proteins in the chick embryo to determine the function of the CNRs during the development of neuronal connectivity between sensory neurons and the spinal cord.

**296. Transsynaptic tract tracing and genetic ablation of pain circuitry**
*Mark J. Zylka*

For this project, we will attempt to develop a genetic method that combines the activity-inducible *c-fos* promoter, Cre/loxP recombinase, and traditional tract tracers to label active neurons and circuits. Since traditional tract tracers do not spread far in a circuit, we also will attempt to develop a non-viral method for labeling entire multisynaptic circuits and for studying their function. This method incorporates the Cre/loxP recombinase with proteins that pass between neurons to generate a Transynaptic Recombinase Chain Reaction (TRCR). The chain reaction is initiated by supplying Cre to a functionally or genetically defined neural circuit.

We will use these new tools to study pain-sensing (nociceptive) circuitry. We have decided to focus on pain circuitry for several reasons: (1) pain sensing neurons and circuitry are found in a restricted part of the nervous system—the dorsal root ganglia; (2) there are a number of well-documented stimuli that induce pain "behaviors" in model animals (examples of stimuli include noxious heat, chemicals or wounding); (3) traditional tract tracing and neuroanatomical studies have provided a general picture of how the pain circuitry is wired peripherally and centrally, and most importantly; (4) we have identified a large family of *Mrg* G protein-coupled receptors (Mas-related gene GPCR's) that are expressed only in restricted subsets of pain-sensing neurons.

These GPCR’s represent unique molecular markers for subsets of nociceptive neurons. We will use these *Mrg* genomic loci to target tract tracing molecules (such as the technology described above) and toxins to these nociceptive circuits. These various lines of mice will allow us to: (1) trace circuitry in the periphery and in the spinal cord; (2) transsynaptically identify pain circuitry in the central nervous system; (3) study pain behaviors in the presence and absence of the neurons (via conditionally killing or silencing the cells).

Ultimately, these studies will provide us with new knowledge about the types of painful stimuli that *Mrg*-containing neurons sense as well as how *Mrg*-expressing circuits are wired at the systems level.

**Publication**

**297. Blood vessel remodeling through communication with peripheral nerves/Schwann cells**
*Yosuke Mukoyama*

Our outstanding question is whether there is essential cross talk between developing blood vessels and peripheral nerves/Schwann cells. We have shown that nerves/Schwann cells associate preferentially with arterial vessels at E15.5, whereas there is no association between them at E13.5. These data raise the possibility that angiogenic remodeling events that occur during these stages may be related to blood vessel and nerve/Schwann cells interactions. To address the possibility, we genetically eliminated nerves/Schwann cells in the limb skin using NGN1/NGN2 double homozygous mutants. It exhibits not only the absence of nerves/Schwann cells but also a defect in arterial marker induction and incomplete
angiogenic remodeling. In erbB3 homozygous mutants which have a severe defect in Schwann cell development, it exhibits not only disorganizing nerves but also no association between blood vessel and nerves, defective arterial marker induction and incomplete angiogenic remodeling. Although Sema3A homozygous mutants also show disorganized nerves, blood vessels associate with the disorganized nerves/Schwann cells and develop arterial marker induction as normal. The analysis of these mutants raises two questions that we will explore further. (1) Do Schwann cells play a role in promoting the blood vessel and nerves/Schwann cells association, arterial marker induction and angiogenic remodeling? (2) Do nerves that Schwann cells support structurally and functionally play a role in promoting them? To answer these questions, we have established a co-culture system between nerves/Schwann cells and endothelial cells to see whether nerves or Schwann cells promote arterial marker induction.

298. Eph family kinases in angiogenesis

Sebastian S. Gerety

We are studying the role of Eph receptor tyrosine kinases (RTKs) in embryonic angiogenesis. There is growing evidence of an important role for these molecules in angiogenesis. The knockout mouse of the ephrinB2 ligand dies in utero with severe malformations of the vasculature in the yolk sac, trunk and developing brain, as well as an enlarged heart. In the yolk sac, although the primary capillary plexus forms, the subsequent remodeling of this plexus normally seen does not occur in the ephrinB2 homozygous mutant. This lack of remodeling is the predominant feature of the phenotype in all tissues examined. Interestingly, within the vasculature the expression of ephrinB2 is restricted to the arterial endothelial cells. EPH-B4, an RTK that binds ephrinB2, is expressed exclusively on venous endothelial cells. The phenotype of the EPH-B4 knockout is identical to that of the EphrinB2 ligand. Because the expression of EphrinB2 occurs not only on endothelial cells lining the vessels but also in the mesenchyme surrounding developing vessels, it has not been clear how important endothelial EphrinB2 versus mesenchymal EphrinB2 is for angiogenesis. We have generated a conditional knockout allele of the EphrinB2 ligand. Using tissue-specific transgenic mouse cre lines, we will be able to specifically knock it out in a subset of tissues. This should enable us to determine whether endothelial or mesenchymal EphrinB2 is required for angiogenesis.

Publications


299. c-fos expression in the mouse brain during delay and trace fear conditioning

C.J. Han1*, L. van Tright, R. Mongeau, C. Koch1

Patterns of neuronal activity in the brains of male C57BL/6N mice during fear delay and trace conditioning were compared by examining expression of c-fos mRNA. Trace conditioning is different from delay conditioning in that there is a temporal gap between the end of the conditioned stimulus (CS) and the start of the unconditioned stimulus (US). It has been shown in humans that trace, but not delay, conditioning requires awareness of the CS-US contingency and is dependent on the integrity of the hippocampus (Clark and Squire, 1998).

Mice were housed in the training chambers overnight and fear conditioned on the second day (6 x 16-sec 2k-Hz sine-wave tone followed by a 2-sec 0.5-mA footshocks). The temporal gap between the CS and the US in the trace group was 18 sec, while the CS immediately preceded the US in the delay group. Some mice were sacrificed 30 minutes after training for quantification of c-fos mRNA (an indicator of neuronal activity). In situ hybridization was carried out using a cRNA digoxygenin-labeled probe detected by an alkaline phosphatase conjugated antibody. Other mice were tested with the tone the next day in a different context to confirm the efficacy of the trace and delay conditioning. Preliminary analyses indicated more c-fos mRNA expression in the dorsal lateral septum during trace than delay conditioning. Electrophysiological studies in mice suggest that the septum, which modulates hippocampal activity, might be important in predicting if and when a US occurs.

1Computation and Neural Systems, Caltech

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300. Olig2 and neuron-glia switch

Qiao Zhou, Gloria Choi

In the vertebrate CNS, both neurons and glial cells have been shown to arise from common progenitor cells residing in the ventricular zone. The production of neurons and glia, however, does not proceed in parallel: neurons are born before glia cells. Until recently, there are few clues as to what causes this neuron to glial production switch.

We have been focusing on a bHLH transcription factor, Olig2, which is expressed in both motoneurons and oligodendroglia. It has been well documented that a specific ventral domain of the spinal cord ventricular zone sequentially generates motoneurons and oligodendrocytes. Work from Tom Jessell’s lab showed that ectopic Olig2 derepresses Neurogenins (Ngn2), a group of proneural
bHLH genes, and promotes the motoneuron fate. Our mis-expression studies using electroporation in chick embryos revealed that if Ngns are downregulated, i.e., by Notch signaling, then Olig2 gene will instead promote oligodendrocyte precursor formation. Olig2 by itself, however, is insufficient for the maturation of the oligodendrocyte precursors. Another homeobox transcription factor, Nkx2.2, is needed in cooperation with Olig2 for the maturation step. The proneural capacity of the Ngn3 is apparently dominant over the gliogenic capacity of Olig2: forced expression of Ngn3 in the endogenous Olig2 domain abolished the in vivo generation of oligodendrocytes. Together, these data suggest that Olig2, via cooperation with different transcription factors, directs the progenitor cells from producing motoneurons to switch to producing oligodendrocytes. We are now in the process of examining the phenotype of Olig2 "Olig1" double knockout mice to further test this hypothesis.

Publication

301. Determination of peripheral neuronal subtype identity by MASH1 and neurogenins
Liching Lo
Evidence from Drosophila and vertebrates suggested that bHLH proteins are necessary for the development of different subclasses of neurons. Drosophila bHLH proteins, such asachaete-scute and atonal, are expressed in the precursors of different neurons. These so called proneural genes have two distinct functions: a "neural precursor selection" function and a "neuronal subtype determining" function. In vertebrates, Mash-1 and neurogenins are also specifically expressed in neuronal precursors. However, it is not clear whether these bHLH proteins also have coupled these two functions. In NCSCs (neural crest stem cells), both MASH1 and neurogenins can promote neurogenesis in accordance with their neural precursor selection function. However, neurogenins and MASH1 can only promote autonomic fate in NCSCs cells. To investigate if neurogenins have a neuronal subtype determining function in cells more primitive than NCSCs, I have used the E10.5 rat dissociated neural tube culture (dNT) and a plZRS-based retroviral system to overexpress MASH1 and neurogenins in these cells. By using the newly developed anti-Sox10 antibodies as a neural crest specific marker, many of these dNT cells respond to BMP2 and become neural crest cells. More strikingly, many of them turn on sensory specific markers, such as Brn3a, an effect not seen in the NCSCs. We have found that different concentrations of BMP2 also influences sensory vs. autonomic fate in dNT culture. In dNT cells, neurogenins, but not MASH1, enhance sensory fate at lower BMP2 concentrations. These data suggest neurogenins have a identity-determining function in certain cell contexts. At higher BMP2 concentrations, neurogenins, like MASH1, can promote an autonomic fate in the dNT cells. Why do cells need both MASH1 and neurogenins if neurogenins can promote both sensory and autonomic fates? One possibility is that neurogenins interfere with cell proliferation and MASH1 does not. In vivo, autonomic neurons proliferate longer and become postmitotic later than sensory neurons. Our BrdU labeling with the NGN2 infected dNT cells seems to support that.

302. Testing the developmental potential of a specific subset of embryonic precursor cells
Limor Gabay
Recent studies show that adult stem cells derived from different organs have a wider differentiation potential than the types of cells present in their original tissue. For example, neural stem cells derived from adult brain were shown to give rise, when injected into chick embryos, to cells of all germ layers (Clarke et al., 2000). It was also shown that bone marrow stem cells which were thought to give rise only to hematopoetic lineages, can generate cells in the brain which express proteins which are typical of neurons (Mezey et al., 2000).

These findings may imply that stem cells maintain a generalized differentiation potential and that which cell type they will actually produce depends on environmental signals. It also implies that there may be common precursors to different cell types. I am testing the above hypothesis by checking the developmental potential of a specific subset of progenitors. These cells are abundant in early mice embryos and express on their surface the EGF receptor Flk1. Most of these cells have been shown to become endothelial cells. However, Flk-1 is also expressed by early hematopoetic stem cells, and combined with the results of Mezey et al., (2000), this suggest that Flk1-positive cells may have the potential to differentiate also into neurons. My aim is to check whether the Flk1+ precursor cells differentiate into neurons. To this end I am using chick transplantation assays. I am isolating the Flk1 positive cells from Rosa26 mice embryos using the FACS machine and directly afterwards injecting them into day two chick embryos. Following three days incubation the injected embryos are stained with X-Gal and antibodies to neural and endothelial markers in order to determine the location and lineages of the mouse cells.

References
304. Cloning of genes involved in fear sensitization
Walter Lerchner, Laurent VanTrigt

Fear sensitization refers to a process where the sensitivity to an aversive stimulus is changed because of a past fearful experience unrelated to the stimulus. In this case naive mice and mice previously sensitized by a series of footshocks are exposed to an aversive ultrasound. Naive mice predominantly respond with instances of flight and very little freezing, while sensitized mice respond with fewer instances of flight and higher amount of freezing (Raymond Mongeau, Gabriel Miller and David J. Anderson, unpublished). Expression of the immediate early gene c-fos showed differential activation of neurons in the Ventrolateral Septum (LSV) area in sensitized mice when exposed to the ultrasound (Raymond Mongeau, Gabriel Miller and David J. Anderson, unpublished). mRNA of the LSV, as well as the Dorsolateral Septum (LSD) and an unrelated brain area were extracted using Laser Capture Microdissection of frozen sections.

Two different strategies are being employed to screen for genes differentially expressed in the LSV. Linear amplification in combinations with hybridization of Affimetrix oligonucleotide arrays will yield results for differential expression for known genes and ESTs. Differential PCR amplification by the Clontech PCR select kit should in addition yield genes that are not yet identified. Potential candidates will be tested for coinexpression with c-fos in response to ultrasound exposure in sensitized mice. The long-term goal is to use the expression domains of these genes to genetically ablate expressing neurons and test for behavioral alteration in the process of sensitization.

305. Neural correlates of mouse fear behavior in response to an innately aversive stimulus
R. Mongeau, G.A. Miller

Graeff et al. (1996) claimed that anticipatory anxiety might inhibit panic-like fear reactions. We have developed a paradigm for predictably altering fear motor outputs in response to specific manipulations: exposure to conditions known to produce anticipatory "anxiety" in rodents, such as a previous "traumatic" event or placement in an unfamiliar cage, increase freezing but decrease panic-like flight to a US.

A group of male C57Bl/6N mice was sensitized with footshocks (no association with any auditory stimulus), while another group was not. The next day, mice were exposed to an ultrasound stimulus that unconditionally elicits fear. Sensitized mice displayed enhanced freezing and decreased flight reactions to this US compared to naive mice. This switch was enhanced by placing mice in a new cage. To map the neural substrates underlying this behavioral alteration, we have carried out an analysis of c-fos mRNA (an indicator of neural activity) using in situ hybridization with single-cell resolution. Widespread brain c-fos activation occurred in mice sacrificed after, but not before, the US onset. Unbiased stereology indicates different patterns of US-induced neural activation between groups. For example, increased freezing in sensitized mice is associated with an increase in c-fos expression in the lateral septum ventral and the medial preoptic nucleus of the hypothalamus, while, conversely, increased flight in naive mice is linked with increased activity in the cingulate cortex and the medial amygdala. In contrast, there was no group difference in brain areas involved in the behavioral task, but not directly linked with fear, such as the dorsal thalamus and the auditory cortex. Our goal is to expand such correlations to neurally define the behavioral switch.

306. Identification of amygdala-enriched genes by microarray technology
Mariela Zirlinger

Microarray technology represents a potentially powerful method for identifying cell type and regionally-restricted genes expressed in the brain. We have combined a microarray analysis of differential gene expression among five selected brain regions, including the amygdala, cerebellum, hippocampus, olfactory bulb and periaqueductal gray, with in situ hybridization. On average, 0.3% of the 34,000 genes interrogated were highly enriched in each of the five regions, relative to the others. In situ hybridization performed on a subset of amygdala-enriched genes confirmed in most cases the overall region specificity predicted by the microarray data, and identified additional sites of brain expression not examined on the microarrays. Strikingly, the majority of these genes exhibited boundaries of expression within the
amygdala corresponding to cytoarchitectonically defined subnuclei. These results define a new set of molecular markers for amygdaloid subnuclei, and provide tools to genetically dissect their functional roles in different emotional behaviors.

Additionally, we are currently comparing gene expression patterns among different amygdala subnuclei using laser capture microdissection to finely dissect the initial tissue. It will be interesting to know how the enriched genes identified in this fashion differ from the ones identified by our previous analysis, and whether we can pinpoint further genes with restricted domains of expression in distinct amygdala subnuclei.


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Graduate Student: Brian Zid

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Summary: Our group uses Drosophila as a model system in which to identify and isolate genes homologous to humans, using the fly for experimental analysis of their basic functions. The data on the fly and human genomes, with their impressive degree of homology, lend support to this strategy.

The single-gene approach is a direct and rapid method of screening for mutants with enhanced longevity. For instance, the mutant, methuselah, which extends the average lifespan of Drosophila by some 30%, also provides increased resistance to different stresses, including heat, starvation, and the oxidative damage induced by paraquat. We have undertaken a screening program for mutants that are resistant to stress, as a molecular approach to the identification of genes that are induced or repressed by different forms of stress, which may identify regulatory pathways relevant to aging. Caloric restriction is a well-known method of extending lifespan in various organisms, and we are investigating that in Drosophila, as well as the possible role of steroid hormones. We are developing biomarkers to monitor the progress of aging during lifetime. And we have shown that fly lifespan can be extended by simple feeding of a drug.

Flies, like humans, suffer from a variety of hereditary neurological diseases. One approach has been to isolate mutants with shortened lifespan, thus identifying genes whose functions are needed to maintain the integrity of the nervous system against late-onset deterioration. This has led to a series of mutants such as drop-dead, spongecake, eggroll and bubblegum, the names describing structural changes that appear with aging in the mutant brains, as seen by electron microscopy. The hereditary defects in some of these mutant brains resemble those seen in various age-related diseases in humans. For instance, spongecake has inflated axon terminals, as in Creutzfeldt-Jakob disease. In bubblegum, there is visual system degeneration associated with an excess of very long-chain fatty acids, as in human adrenoleukodystrophy, and we have shown that the degeneration can be prevented by feeding the mutant with one of the ingredients of "Lorenzo's oil."

At least eight hereditary neurological diseases in humans are associated with the presence in the causative genes of expanded stretches of triplet nucleotide repeats. The consequently coded polypeptides create nuclear inclusions which apparently underlie neuronal degeneration. We have reconstituted this phenomenon in the compound eye of the fly and searched for genes whose expression can counteract the toxic effects of polyglutamine repeats. Several such suppressor genes have been cloned, and their action verified in transgenic flies.

Our group is also investigating nociception in Drosophila, which bears much resemblance to human pain. Mutants such as painless represent an entry into a molecular genetic analysis of this phenomenon.

307. Dissection of the aging process with genetic biomarkers
Laurent Seroude, Seymour Benzer

It is desirable to identify molecular changes that can be used as indicators of the "aging state" of an organism at any time during its life cycle, i.e., biomarkers of aging. One approach, initiated by Helfand (1), is the enhancer-trap technique, in which an artificial exogenous genetic construct is expressed under the control of natural endogenous genetic regulatory elements (2). We have used a construct encoding the yeast transcription factor GAL4, which can be monitored and visualized by various reporters (3). That provides a range of analytical tools, since GAL4 can be used to direct the expression of any desirable gene to examine its consequences on the aging process. We established a collection of ~500 GAL4 enhancer-trap Drosophila lines. To determine their temporal expression patterns, these lines are crossed with a reporter line expressing the bacterial enzyme β-galactosidase (lacZ) under the control of a GAL4-dependent promoter (UAS-lacZ).

The progeny from such crosses were tested at different ages by a biochemical assay on homogenates of whole flies to quantify β-galactosidase activity, which reflects the activity of the particular regulatory element. At least 80% of the enhancers tested changed with age. By cluster analysis, one can define several groups according to their general trends of expression. We examined the tissue localization of enhancer activity at different ages by serial sections of the entire fly. Apparently, a well-controlled and orderly pattern of gene expression in various tissues takes place during aging. This raises the question of whether gene expression directs the aging process or aging causes the changes in expression.
We initiated a molecular and genetic analysis of several enhancer trap insertions. Cloning of the genomic DNA around the insertion leads to identification of the endogenous gene that is normally under the control of the enhancer. By making the GAL4 insertion homozygous, or by generating an imprecise excision, we examine the effects of modifying the expression of this gene. Analysis of DNA sequences around the enhancer trap insertion reveals potential regulatory elements responsible for age-dependent transcription.

References

308. Role of molecular chaperones in aging
Horng-Dar Wang, Pankaj Kapahi, Parsa Kazemi-Esfarjani, Seymour Benzer

Enhanced resistance to environmental stresses has been hypothesized to slow down the rate of aging. Hence, to find longevity genes, we screened for ones that respond to the multiple stresses paraquat, starvation, and heat. Several genes coding for heat-shock proteins were found to respond to all three different stresses. One of them, hsp70, had been reported to extend Drosophila lifespan when overexpressed (1). We tested two others, hsp26 and hsp27, in transgenic flies, using the yeast UAS/GAL4 system. RT-PCR analysis confirmed that the transgenic flies, when crossed with GAL4 driver flies, did overexpress those genes, and the flies showed a 30-50% extension in median lifespan, as well as elevated resistance to all three stresses.

We also tested other chaperones that were discovered in our screen for activity in suppressing polyglutamine toxicity, namely, HSP40/HDJ1 and dTPR2, which contain J domains (2). These also extended lifespan by around 30-40%. The results support the hypothesis that damaged proteins play a role in aging, and that overexpression of molecular chaperones can extend lifespan.

It is often true that extended lifespan is associated with reduced fecundity. In Hsp26 and Hsp27 transgenic flies there was, indeed, a 35-60% reduction in fecundity. Moreover, overexpression of HSP40/HDJ1 and dTPR2 at high levels drastically shortened lifespan and caused some embryonic lethality. These results suggest that while slight increases in chaperone activity may be beneficial, beyond a certain level they may be detrimental. Normal levels of chaperones may be set so that they do not compromise reproduction and survival.

References

309. Life extension in Drosophila by feeding a drug
Hyung-Lyun Kang*, Seymour Benzer, Kyung-Tai Min*

We report that feeding Drosophila throughout adulthood with sodium-4 phenylbutyrate (PBA) can significantly increase lifespan, without diminution of locomotor vigor, resistance to stress, or reproductive ability. Treatment for a limited period, either early or late in adult life, is also effective. Flies fed PBA show a global increase in histone acetylation as well as a dramatically altered pattern of gene expression, including induction or repression of numerous genes. The delay in aging may result from the altered physiological state.

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310. Down regulation of the ecdysone pathway increases Drosophila lifespan and stress resistance
Anne F. Simon, Cindy Shih, Monica Dus, Seymour Benzer

Steroid hormones are known to be involved in Drosophila development. However, little information is available concerning their role during adulthood, aside from oogenesis. Using mutant alleles of genes involved in the ecdysone pathway, we found that partial inhibition of this pathway results in increased lifespan and stress resistance.

Most mutants of the ecdysone pathway are blocked in metamorphosis and never reach adulthood. We therefore utilized heterozygous null recessive and thermosensitive alleles. We first examined two mutant alleles of the ecdysone receptor, EcR\textsuperscript{f5596} and EcR\textsuperscript{f6637} (Carney and Bender, 2000). When heterozygous, these mutants display increased resistance to starvation, and extended longevity (30 to 50%). Similar results were observed with mutant heterozygous alleles of genes involved in the biosynthesis of ecdysone: a weak allele of the dare gene, dare\textsuperscript{1} (Freeman et al., 1999) and DTS-3, a conditional mutant (Holden et al., 1986).

Further studies on EcR\textsuperscript{f5596} showed that there was no trade-off between fertility and longevity. These results suggest that there is a steroid endocrine control of longevity in adult Drosophila. Further studies will include the localization of the target tissues and analysis of the fertility and vigor of these flies by their performance in various behavior paradigms.

References
311. Isolation and characterization of genes that extend lifespan in Drosophila melanogaster upon overexpression in the nervous system

Pankaj Kapahi, Brian Zid, Seymour Benzer

Understanding the mechanisms of aging remains a biological mystery. Using Drosophila melanogaster as a model system, we propose to identify and characterize genes that are important in extending lifespan and preventing neurodegeneration. Overexpression of superoxide dismutase in the motor neurons has been shown to extend lifespan (1). In addition, overexpression of molecular chaperones like Hsp70 can also extend lifespan (2). To identify novel genes that can extend lifespan, a P element-mediated longevity screen was undertaken using the Gal4-UAS system to overexpress genes in the nervous system. This screen identified seven genes that extend lifespan by at least 25%. The lifespan extension also is observed by overexpressing these genes ubiquitously in the body. We have confirmed that the P elements are in the 5′ regions of these genes, and in the correct orientation to overexpress them. One of the genes is Sir2, a histone deacetylase which has been shown to extend lifespan in S. cerevisiae and C. elegans upon overexpression (3,4). Putative functions associated with other identified genes include metabolism, chromatin remodeling, protein misfolding, and DNA repair. Others are of no currently known function. We are constructing transgenic flies to confirm whether they are responsible for the lifespan extension.

References

312. Neurodegeneration and lifespan extension genes in Drosophila

Pankaj Kapahi, Emily Wang, Seymour Benzer

A gain-of-function screen was performed to isolate novel genes involved in neurodegeneration. We hypothesized that some genes that cause neurodegeneration and early death upon overexpression might lead to an extension in lifespan if underexpressed. UAS-containing P element (EP) lines generated as part of the Berkeley Drosophila Genome Project (BDGP) were crossed with a strain expressing Gal4 in the central and peripheral nervous systems. The progeny from 1500 crosses were then assessed for lifespan at 25°C.

One hundred strains that showed lifespans of shorter than 4 weeks (normal lifespan is around 11 weeks) were examined for neurodegeneration by electron microscopy.

To test whether reductions in expression of the corresponding genes would cause extension in lifespan, we tested these strains as heterozygotes for the EP-element. Several different long-lived lines were thus obtained. The identities of these genes are being determined using the Berkeley Drosophila gene database. One strain found from this screen was INDY, a previously identified long-lived mutant encoding a sodium dicarboxylate transporter (1). In addition, seven similarly long-lived strains were obtained. Double heterozygotes for these mutants will be tested for synergistic effects on lifespan.

Reference

313. Isolation of Drosophila mutants with altered sensitivity to hyperoxia

David W. Walker, Seymour Benzer

The idea that aging is caused by oxidative stress is the most enduring mechanistic theory of aging. To identify and characterize components of the oxygen defense pathway in Drosophila, we have screened a collection of 800 UAS-containing P-element (EP) insertion lines for altered sensitivity to 100% oxygen. Since wild-type control lines survive for only 7-8 days under hyperoxia (1,2), this approach facilitates the rapid molecular cloning of key molecules involved in oxygen defense. We observed that the long-lived methuselah mutant (3) displays ~30% increased survival under hyperoxia, and have isolated, in addition, a set of mutants (~20) that display similar or greater increases in resistance. A subset of these mutants also display increased survival under normoxic conditions. We are currently using genetic and molecular tools to determine which genes are responsible for life extension in these mutant lines.

We have also identified a separate class of mutants that display hypersensitivity to hyperoxia. In these cases, the disrupted gene is necessary to cope with oxidative stress. Using this approach, we have identified genes involved in DNA replication and repair, and in antioxidant defense, as well as genes of previously uncharacterized function. We are utilizing the GAL4/UAS system to test these genes for overexpression phenotypes. Overexpression of a subset of these genes leads to both resistance to hyperoxia and extension of lifespan. Future work will focus on understanding the mechanisms of action of the various mutants, thus providing a greater understanding of the biochemical and physiological basis of the aging process.

References
314. Role of the Drosophila P38a MAP kinase gene in aging
Ted Brummel, Laurent Seroude, Yi-Jyun Lin, Seymour Benzer

As a result of a screen for lines with increased longevity, we have identified a mutant line, LYT 106, with a 40% increase in mean lifespan in animals heterozygous for the mutation. Interestingly, this mutation is lethal when homozygous. Using the lethality as a marker, we mapped the mutation to the 95E region. Sequence analysis of genes within the region led to the identification of a point mutation within the P38A gene, a MAP kinase, resulting in a histidine to tyrosine change within a highly conserved region of the protein. We also identified two P element insertions approximately 500 bases upstream of the transcription start of the P38 gene, which will be mobilized to obtain new mutations in the P38 gene. Through the use of kinase assays, we are measuring the P38 kinase activity in the various lines, and are experimenting with specific P38 inhibitors to see if pharmacological intervention can reproduce the longevity phenotype.

315. Dissociation of longevity from stress resistance
Ted Brummel, Seymour Benzer

Towards understanding the mechanisms involved in regulating the aging process, we are attempting to identify genes critical for long life. Two different screening strategies were used. The first was to screen directly for lines which lived longer than wild-type flies. The second involved screening indirectly for phenotypes often correlated with increased longevity. Since many long-living mutants are more resistant to environmental stress and/or have delayed reproductive potential, we screened independently for these phenotypes and for stress resistance.

Using EMS as a mutagen, the screen for delayed reproductive potential yielded 30 lines, of which only three were long-lived. The screen for stress resistance yielded 40 lines, of which only three showed significant increase in longevity. Paradoxically, some stress-resistant lines were found to have shortened lifespan, and some long-lived mutants had only normal resistance to stress. Reliance on stress resistance to obtain long-lived mutations is therefore inefficient; direct screening for long-living mutants is preferable. These observations raise intriguing questions concerning parallel and independent pathways for resistance to stress and lifespan, which may be unraveled by molecular analysis.

To identify the key genes, we will compare global changes in gene expression in long-living mutants to those that occur in stress-resistant mutants vs. wild type. This may define three categories, genes that affect stress-resistance, ones that affect longevity, and others affecting both. Preliminary studies using chip technology support the feasibility of such an approach, since only a small subset (~3%) of genes are differentially expressed (by differences of more than three-fold) in several different backgrounds tested.

316. Forward genetic screening for long-lived flies with multiple stresses
Horng-Dar Wang, Seymour Benzer

Since screening for resistance to a single form of stress often fails to yield mutants with extended longevity, we performed a screen for mutants resistant to both oxidative stress (paraquat) and starvation. The mutant methuselah was used for comparison, since it has a 30% increased lifespan and better resistance to both paraquat and starvation, and we looked for flies with similar or greater survival rate when exposed to both stresses. The screening is rapid, taking about three days. We screened 261 lines from the EP collection on the 2nd chromosome, and 390 on the X chromosome. Twenty-five candidates were found among the 2nd chromosome insertion mutants, and 44 candidates from the X chromosome screening. Interestingly, ten of the 25 turned up independently in David Walker's hyperoxia screening, and 20 of the 44 lines in a paraquat screening by Ted Brummel. Therefore screening with multiple stresses may uncover some resistant mutants other than by single stress screening. The lifespan measurements of the multiple stress-resistant mutant flies are in progress.

317. Jumpy, a Drosophila age-dependent enhancer-trap expression in muscle, identifies a tyrosine phosphatase related to the myotubular myopathy family
Annemarie Selaya, Laurent Seroude, Seymour Benzer

The jumpy enhancer-trap has a complex temporal pattern of expression, increasing up to mid-life and subsequently decreasing. Examination of the expression localization within the fly shows that it is restricted to muscle tissue. These observations indicate that muscles are subjected to specific molecular changes during aging. Analysis of the sequence surrounding the P-element insertion revealed its location inside the LD39930 gene (previously identified by the Berkeley Drosophila Genome Project). This gene encodes for a putative tyrosine phosphatase, an enzyme that removes a phosphate group from a substrate by hydrolysis. Since jumpy expression is restricted to muscle tissue, the enzyme presumably acts on a substrate within muscles.

A GenBank search identified a human version of this phosphatase (GeneBank accession number AY007098, p-value = 2e-71) related to the myotubular myopathy (MTM) family, a group of phosphatases. Mutations in the human MTM1 gene are associated with myotubular myopathy, a severe muscle disorder characterized by muscle weakness and severe hypotonia (1). Since the jumpy and MTM proteins only share the tyrosine phosphatase active site consensus sequence (I/V)HCXAGXXRS(T/R)G and a FSG motif, jumpy defines a new family of muscle phosphatases.

This finding raised the question of the biological function of the jumpy family. Removing the gene and examining the consequences will enable us to resolve this issue.
318. Genetic analysis of pain

W. Dan Tracey Jr., Seymour Benzer

We are using the fruitfly Drosophila melanogaster as a model system to understand the molecular basis of pain. Drosophila larvae respond behaviorally to noxious stimuli in a highly reproducible and stereotypic fashion. A normal, undisturbed larva moves through its environment with a rhythmic, peristaltic motion. When touched lightly with an innocuous probe, the larva briefly pauses, then resumes motion. However, when touched with a probe heated to an unpleasant temperature, the larva responds by vigorously rolling in a corkscrew-type motion. We are using this stereotyped behavior to perform genetic screens with the goal of identifying mutations which are insensitive or sensitized to noxious stimuli. One such Drosophila mutant, insensitive to noxious heat, we have named painless. Larvae mutant for painless show a delayed response to the noxious heat stimulus; with a stimulus which causes wild-type larvae to respond in less than one second, painless mutant larvae require on average six seconds of stimulation to generate the response. The painless phenotype is caused by insertion of a P-transposable element, which has allowed us to identify the affected gene. The predicted painless protein is a member of the transient receptor potential family of ion channels and is distantly related to the vanilloid receptor, a molecule recently implicated in vertebrate pain pathways (Caterina et al., 1997). In addition to painless, we have identified nearly fifty mutations which cause insensitivity to noxious heat. To determine which of these mutants may function in the same molecular pathway as painless, we are performing epistasis experiments by constructing lines doubly mutant for painless and the other genes.

Reference

319. Suppression of polyglutamine toxicity by a Drosophila homologue of myeloid leukemia factor 1

Parsa Kazemi-Esfarjani, Seymour Benzer

In Huntington's disease and several other hereditary neurodegenerative disorders, abnormally long polyglutamine tracts within specific proteins are the major contributing factors to selective neuronal toxicity. By a genetic screen in Drosophila, devised to uncover genes that suppress polyglutamine toxicity, we discovered a Drosophila homologue of human myeloid leukemia factor 1 (MLF1). Expression of the Drosophila homologue (dMLF) ameliorates the toxicity of polyglutamine expressed in the eye and in the central nervous system. In the retina, the ectopically-expressed, epitope-tagged protein colocalized with polyglutamine aggregates which, together with other recent observations, suggests that dMLF suppresses toxicity by sequestering polyglutamine and/or its aggregates.

Publications
Professor: William J. Dreyer  
Research Scientist: Janet Roman-Dreyer  
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**Summary:** Experimental embryologists can observe single cells as they migrate and join other cells during the sculpturing of an embryo. The specificity and precision of these events is truly extraordinary. Migrating cells coalesce to create the heart. Cells originating in the neural crest migrate and continue to divide as they contribute to the shaping of teeth – and their innervation. The genetic programs responsible are so extraordinary that the upper and lower sets of teeth fit together, and even more amazingly the teeth (and indeed all other parts) of identical twins are -- well -- nearly identical.

Half a century ago Caltech’s Roger Sperry showed that the growth cones of regenerating nerves have very great specificity. His results and the other examples of the specificity of migrating cells are so remarkable that even today some scientists find it hard to believe that the cell surface molecular codes are that precise. Those of us who have searched for genes and molecules with specificity adequate to account for the observed biological phenomena have made progress but have not found the answers.

We now believe that we have identified some of the genes and molecules that provide the required very high specificity on cell surfaces and thus we have begun to uncover the genetic and molecular basis of the cell surface address codes indicated by the biological phenomena mentioned above.

The new genomic databases that have become available during the past few years have allowed us to carry out research that could not be done previously in our local laboratories. Accordingly, we have made extensive use of genomic techniques and the databases to obtain the new results. We feel that we have now benefited greatly from investments made in previous decades at Caltech to develop the microchemical instruments used in the genome projects.

Recently, we have begun to examine the possibility that genetic machinery, evolutionarily related to two types of genetic switches that are known to be used in the developing immune system, are also used in other developmental systems. We have written genomic software ("Tapestry") to aid in this project. In addition, experiments have been initiated in collaboration with Scott Fraser and David Koos in the Beckman Institute Image Center to explore the role of genetic switches in development.

**320. Olfactant receptors have a dual function and may play a central role in cellular dressing for many types of organogenesis**  
*William Dreyer*

Studies in our laboratory as well as many others have succeeded in identifying a large number of cell surface molecules that play a role in cellular assembly and seem to function much like the country codes, area codes and prefixes of a telephone dialing system. However, the predicted highly specific final part of the code, equivalent to the last four digits of a unique phone number, has eluded us until very recently when we uncovered a new type of candidate code molecule: the odorant receptors. These serpentine receptors (they pass through the cell membrane seven times) are represented by thousands of genes in the human and mouse genomes. There are more than 1000 members of odorant receptor related families in *C. elegans* – more than the total number of cells in that organism. Recent research has shown that the odorant receptors not only detect odors, a relatively common type of function for a protein molecule, but also play an extremely sophisticated role in axonal targeting as axonal processes extend from the olfactory epithelium to highly specific points (glomeruli) on topological maps on the olfactory bulb. Because these receptors are capable of such a high targeting specificity they bear the hallmarks of the proposed area code molecules that we believe aid in the assembly of most tissues. It therefore seemed appropriate to ask if they might be expressed in other parts of the developing embryo (and adult) as expected for such molecular codes. A search of the genome and literature databases revealed a remarkable number of examples of these genes expressed in tissues other than the olfactory system. The widespread expression in numerous organ systems of molecules related to the olfactory receptor family obviously supports the hypothesis that such receptors perform functions other than the recognition of olfactants. Since receptors of this type play a dual role as receptors for small molecules in the olfactory epithelium and as cell surface addressing molecules that aid in the assembly of the olfactory bulb of the brain, one obvious notion is that they may also play an assembly role in other parts of the brain and embryo and are the much-sought-after key cell surface molecules used in assembling embryonic tissues.

**References**


Homophilic interactions of olfactory receptors can explain otherwise perplexing experimental observations

William Dreyer

Scientists using the two-photon confocal microscopes in the Beckman Institute Biological Imaging Center (headed by Scott Fraser) have been able to observe olfactory axons as their growth cones migrate to the brain (see references below). I have been fortunate to be able to observe this work and have used it, together with studies from other labs, to draw the following conclusions: 1) Axons that express one specific receptor have a strong tendency to fasciculate (bond) to themselves to the exclusion of the 1,000 or so axons that display other receptors; 2) As a specific axon homes in on its target glomerulus, the growth cones sometimes move to the correct target after first going near a neighboring glomerulus (see below re heterophilic interactions); 3) When all olfactory axons are labeled (with olfactory marker protein) it can be seen that thick bundles of axons emerge from each adult glomerulus without obvious mixing with other fascicles.

These observations argue strongly that each of the 1,000 or so olfactory receptors is capable of recognizing itself with highly specific homophilic interactions. Since these receptors are expressed on cells of essentially all tissue types, homophilic interactions would add high specificity to cell-cell interactions throughout the organism. While the proposed homophilic interactions seem highly likely based on the experimental evidence mentioned above, heterophilic interactions with very similar receptors on neighboring cells could provide a "gradient of receptor affinities," a potentially powerful mechanism for the assembly of organisms (see Dreyer, 1998; Dreyer and Roman Dreyer, 1999).

References


Publications

leads to influx of calcium and activation of CaMKII. CaMKII can phosphorylate synGAP, and may regulate its location or activity at synapses. It is well known that disruption of the CaMKII gene leads to derangement of synaptic regulation. We have recently used homologous recombination in embryonic stem cells to create mutant mice in which the synGAP protein is deleted, and are using the mutant mice to establish when and how the synGAP protein is functionally significant.

We found that a second signaling complex in the PSD forms around the tail of the PDZ-domain protein, densin-180. This high affinity ternary complex contains CaMKII and the actin-binding protein α-actinin. Densin-180 is the founding member of a family of proteins now termed LAP proteins (LRR-domain and PDZ-domain containing proteins). The phenotypes of mutants in LAP proteins in C. elegans and Drosophila indicate that these proteins play important roles in directing the polarized localization of receptors and other proteins. We are using several strategies to gain insight into the functional role of densin-180 at the synapse.

In conjunction with our biochemical and cell biological experiments, we are using computer simulations to study the kinetics and interactions of signaling pathways in the tiny postsynaptic spine. The complex signaling machinery at the synapse integrates a variety of signaling influences and determines the "set-point" of synaptic strength at individual synapses. Many of the important signaling cascades are triggered by calcium influx into the postsynaptic spine, and result in phosphorylation of proteins that eventually leads to changes in synaptic strength. Our near-term goal is to create simulations that will generate testable predictions about the flow of protein phosphorylation events in excitatory spines and dendrites, under conditions of calcium influx that cause changes in synaptic strength. Our ultimate goal is to create simulations that will illuminate our understanding of biochemical information coding at different types of synapses in the brain. We have initiated a collaboration with scientists at the Salk Institute to build computer simulations of the flux of protein phosphorylation events in spines in the hippocampus. We will use the program MCell to implement stochastic simulation methods that accurately model the position and behavior of immobilized signaling molecules within the spine. Predictions arising from the simulations will be tested experimentally by measuring the time course and spatial distribution of phosphorylation of CaMKII and other phosphorylated synaptic molecules under a variety of physiological conditions. This effort will lay the groundwork for understanding the behavior of highly interconnected signaling pathways at the synapse.

References
protein kinase II (CaMKII) in the signaling cascade that mediates changes in synaptic strength has been well established.

The major molecules forming the signaling machinery at the postsynaptic density of dendritic spines have now been characterized by our lab and others. In collaboration with scientists at the Salk Institute, we are using stochastic computer simulation techniques to better understand the interactions of the several signaling pathways at the postsynaptic site, as well as adaptive changes in the signaling machinery that underlie synaptic plasticity. To build realistic simulations, it is necessary to determine the relative stoichiometries of the molecules whose function will be simulated. We have, therefore, used quantitative Western blot analysis of subcellular fractions of brain, including synaptosomes and the postsynaptic density fraction, to estimate the relative numbers of the subunits of the NMDA receptor, PSD-95, CaM kinase II, synGAP, and other signaling molecules at the synapse. We have normalized the relative numbers by the numbers of glutamate receptors estimated at individual spines from glutamate receptor currents, obtaining estimates of the protein composition of the signaling apparatus at individual spines. These numbers will place important boundary values on the numbers of each molecule that we will incorporate into our simulations.

324. Kinetics of activation of CaMKII in vivo

Vladan Lucic, Gabriela Greif*

CaM kinase II (CaMKII) has been shown to be a crucial component of several important neural protein phosphorylation pathways. In particular, it is concentrated beneath the postsynaptic membrane of excitatory synapses in the cortex and hippocampus and is a major target of the calcium ion flowing through the activated NMDA receptor. In order to include it in simulations of signal transduction in the spine, we must construct a kinetic scheme that captures the essential features of activation of CaMKII by Ca²⁺ and calmodulin. Activation of CaMKII is not simple. It involves the binding of four calcium ions to individual calmodulin molecules and the association of calmodulin with a specific site on each CaMKII subunit that leads to activation of the catalytic domain. The CaMKII holoenzyme is a dodecamer of individual catalytic subunits held together in two parallel rings of six by the association domains of each subunit. When calmodulin binds to two adjacent subunits in one of the rings, one of the two subunits is autophosphorylated at a specific site that causes the subunit to remain activated even after the calcium concentration falls. When a subunit has been autophosphorylated, its activity ceases only when it is dephosphorylated by cellular phosphatases. We have measured autophosphorylation of CaMKII at a variety of concentrations of calcium and calmodulin and are constructing a kinetic model of activation that will fit this data. The resulting model will be incorporated into a simulation of synaptic signal transduction in MCell.

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325. The role of the intracellular domain of the NMDA-type glutamate receptor

Leslie Schenker, Rolf Sprengel*, Peter Seeburg*, Mary Kennedy

The NMDA-type glutamate receptor in the central nervous system is composed of NR1 subunits of approximately 110 kDal and combinations of four NR2 subunits of approximately 160 to 180 kDal. The larger size of the NR2 subunits reflects the presence of carboxyl terminal tails of about 300 amino acids that extend into the cytosol. The cytosolic tails of the NR2 subunits associate with intracellular signaling machinery that regulates the strength of synaptic transmission. The principal NR2 subunits in the hippocampus and cerebral cortex are NR2A and NR2B. Mutant mice lacking the intracellular C-terminal tails of the NR2A (NR2AΔC) and NR2B (NR2BΔC) subunits were created in the Seeburg lab to study the role of these domains in receptor function in vivo (Sprengel et al., 1998). NR2BΔC mice die shortly after birth, whereas NR2AΔC mice are viable, but show defects in synaptic transmission. Our recent collaborative studies with NR2AΔC mutants established that the intracellular C-terminal of the NR2A subunit plays an important role in localizing the NR2A subunit at functional synapses in mouse hippocampal neurons (Steigerwald et al., 2000). This year we have succeeded in breeding mice that produce embryos lacking the tails of both NR2A and NR2B subunits. We are now studying dissociated neuronal cultures from NR2A/B mutants and from the double-truncation mutants to establish the roles of the cytosolic tails of each of the NR2 subunits in the organization of postsynaptic proteins.

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References


326. Variability of calmodulin activation in response to calcium influx in spines

Stijn Cassenaer, Vladan Lucic

Our collaborators at the Salk Institute, Kevin Franks, Tom Bartol, and Terry Sejnowski, have constructed a model of calcium influx into a glutamatergic spine in a pyramidal neuron in the CNS. The model was constructed in MCell and incorporates an idealized spine shape, as well as densities, distributions, and kinetic behaviors from the literature of NMDA receptors, voltage-dependent sodium, potassium, and calcium channels, ATP-dependent calcium pumps in the plasma membrane and dendritic endoplasmic reticulum, and calmodulin concentrated in the vicinity of the postsynaptic density. The model includes arbitrarily defined "calcium-buffers" derived by Koester and Sakmann (1) in their study of changes in calcium concentration in spines upon synaptic activation. The model also includes the calcium-sensing fluorophore, calcium green, which was used in the experiments to which the model was tuned. The simulation is driven by changes in voltage simulated in the program "Neuron" and imported into MCell.

Upon examination of the kinetics of formation of the various calcium bound "states" of calmodulin as simulated in this model, we noticed that the peak amounts of the active "four-bound" state of calmodulin varied dramatically among individual runs of the same simulation, reflecting a large stochastic variation in the behavior of calmodulin in the spine. For each simulated run, MCell places molecules randomly on specified surfaces at specified densities. In addition, calcium movements are simulated randomly from NMDA receptor "point sources." Using a different random number seed. To isolate the source of large stochastic variation in calmodulin behavior, we systematically held individual variables constant from run to run. We found that the source of the large variation was the random placement of voltage-dependent calcium channels in the spine head. Experimental studies in the literature estimate that individual spines contain from 0 to 10 or more voltage-activated calcium channels. Calcium flux through these channels is more synchronous than that through NMDA receptors, which "flicker" open and shut over a few hundred milliseconds. The presence of several calcium channels in the spine head, apparently can lead to very large local increases in calcium that succeed in producing local high concentrations of four-bound calmodulin. These data suggest that local calcium-regulated signal transduction might be quite different in spines that contain more than one or two voltage-dependent calcium channels, as compared to those containing none or a few. Thus, this characteristic could define synaptic types with distinct signaling behavior.

Reference


327. Densin associates specifically with CaMKII holoenzymes containing only the α-subunit

Randy Walikonis, Joan Jeng

CaMKII is a key regulator of synaptic strength at glutamatergic synapses and is required for activity-dependent long-term potentiation. It is concentrated in the postsynaptic density through highly dynamic protein associations. Activation of synapses by glutamate triggers movement of CaMKII from dendritic shafts into spines.
Two molecules in the PSD can act as docking sites for CaMKII; NR2B, the cytosolic tail of which binds CaMKII, and densin, a transmembrane PSD protein identified recently in our lab. Densin comprises a large, highly glycosylated extracellular domain (see accompanying abstract by Jeng), a single transmembrane domain, and a relatively short intracellular domain that ends in a PDZ domain. Last year we found that the intracellular portion of densin forms a ternary complex with CaMKII and the actin-associated protein, α-actinin. This year, we discovered that the interaction of densin with CaMKII is highly specific for the α-subunit of CaMKII. CaMKII is a dodecameric holoenzyme composed of α- and β-subunits assembled randomly as they are synthesized. In adult brain approximately three times more α-subunits are synthesized than β-subunits. Thus, on average, each holoenzyme contains about nine α-subunits and three β-subunits. However, the composition of the holoenzymes is not uniform, and about 10% of the holoenzymes formed in the cell somas would be expected to contain all α-subunits. However, the message encoding the α-subunits is shipped out into dendrites where activity can stimulate dendritic synthesis of α-subunit. In contrast, the message encoding the β-subunit is confined to the cell soma. Thus, newly synthesized holoenzymes formed in the dendrite in response to neuronal activity would be expected to contain only α-subunits.

We have shown that the intracellular portion of densin associates only with CaMKII holoenzymes that do not contain β-subunits. In other words, only all α-subunit containing holoenzymes form complexes with densin. Thus, densin may preferentially bind CaMKII holoenzymes synthesized in dendrites in response to neural activity. This finding may shed light on the specificity of remodeling of synapses during activity-dependent synaptic plasticity.

328. Actin depolymerization in spines during induction of LTP in hippocampal slices increases diffusion of proteins from shaft into spines

Yannan Ouyang

Long-term potentiation (LTP) is the most extensively studied synaptic model for learning and memory. Some forms of LTP appear to involve structural modification of postsynaptic spines. Examples of such modification may include insertion of new AMPA receptors and translocation of CaMKII from the dendritic shaft into spines.

We have investigated changes in the cytoskeletal protein, actin, produced by tetanic stimulation, using the immunocytochemical method we described previously (Ouyang et al., 1999). Actin is concentrated in dendritic spines and serves as the major component of the spine cytoskeleton. After induction of LTP by tetanus in synapses of the stratum radiatum of area CA1, we found that staining of F-actin by fluorescent phalloidin was decreased over a wide area of synaptic neuropil, indicating depolymerization of actin filaments. This year we found that actin polymers may help to control access of proteins to spines, and the depolymerization of actin following tetanic stimulation may permit diffusion of large proteins from the shaft into spines. First, we found that stabilization of F-actin by treatment with the actin polymer stabilizing drug, jasplakinolide, significantly decreases movement of GFP-labeled CaMKII into spines following treatment of transfected hippocampal neurons with glutamate. Second, we loaded hippocampal neurons with fluorescently-labeled 3 kDal dextran through a patch pipette. In control neurons, labeled dextran diffused into about 35% of spines after 15 minutes. In neurons treated with jasplakinolide, dextran diffused into 22% of spines after 15 minutes. The results were analyzed by researchers who were blind to the specific treatment, and were statistically significant. Thus, polymerized actin may help to form a barrier to diffusion of large molecules into spines, and its depolymerization following tetanic stimulation may help to permit diffusion of new proteins into activated spines.

References


329. SynGAP deletion mutants

Luis Vazquez, Hong-Jung Chen, Pat Manzerra, Susan Catalano *

SynGAP is a synaptic Ras GTPase-activating (GAP) protein identified by us in the rat forebrain postsynaptic density fraction (1). SynGAP is highly localized to synapses in hippocampal neurons in culture. It associates with the PDZ domains of the scaffold protein PSD-95 and is present in a protein complex containing PSD-95 and the NR2 subunits of the NMDA receptor. We have hypothesized that, by catalyzing inactivation of Ras, synGAP down regulates signaling through various growth factor receptors at synapses. To gain more insight into the functions of synGAP in vivo, we employed homologous recombination in mouse ES cells to generate mutant mice that do not express synGAP. Mice that are homozygous for the deletion die about three days after birth. Their brains have normal cellular histology, although they are smaller on average than brains from three-day old wild-type mice. The barrel fields of the somatosensory cortex form normally in homozygous mutants indicating that synapse formation is not grossly disrupted. Mice heterozygous for the mutation grow normally and breed. Primary neuronal cultures prepared from homozygote embryos at E17 live normally in culture for three to four weeks. We are comparing signal transduction through the NMDA receptor and through various growth factor receptors in dissociated neuronal cultures from WT and mutant mice.

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330. Formation of densin-CaMKII complexes in vivo

Eugenia Khorosheva, Randy Walikonis, Wei Dang

We have shown that the intracellular portion of the transmembrane postsynaptic density protein, densin, forms a tight ternary complex with α-actinin and CaMKII in vitro. CaMKIIα binds to densin in a segment between densin’s transmembrane domain and the PDZ domain. The COOH-terminal portion of α-actinin binds to the PDZ domain of densin. Finally, actinin binds directly to the catalytic domain of CaMKII. We have hypothesized that this complex might participate in localization of CaMKII to the postsynaptic density. To test whether this complex can assemble stably in vivo, we are expressing the three proteins alone and in combination in the non-neuronal tissue culture cell line, HEK293. We have created stable cell lines expressing densin and CaMKII, and we are now making expression vectors encoding fusion proteins between various isoforms of GFP and CaMKII, densin, or actinin. We will transfet these constructs into cells and use two-photon confocal fluorescence imaging combined with FRET (fluorescence resonance energy transfer) to examine formation of the ternary complex under different circumstances in living cells.

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Publications


**Bren Professor of Biology:** Henry A. Lester  
**Norman Chandler Professor Emeritus of Chemical Biology:** Norman Davidson  
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**Summary:** We continue our work on ion channels, receptors, and transporters. We have continued to analyze knock-in mice generated in our laboratory for two ligand-gated channels, the nicotinic α4 receptor and the serotonin 5-HT3 receptor. The former has generated interesting insights into neurodegenerative disease and epilepsy. The latter has generated insights into murine urologic syndrome.  

We continue our joint work with the Dougherty group, in Caltech’s Chemistry Division, on aspects of ion channel structure-function. We have brought novel techniques to these studies, including mass spectrometry and fluorescence. We work on unnatural amino-acid mutagenesis and attempt to extend this method to mammalian cells. We also work on aspects of intracellular signaling, in particular RGS proteins and GIRK channels.  

Our work continues on quantitative aspects of transporter function, primarily measured with fluorescence and with knock-in mice.  

The subgroup led by Norman Davidson works on aspects of synaptic plasticity, particularly those that depend on A kinase stimulation.  

Our group’s home page has additional up-to-date information and images. It’s at http://www.cco.caltech.edu/~lester.  

### 331. Generation of mutant mice with milder gain of function properties of the α4-containing nicotinic acetylcholine receptor  
**Cesar Labarca, Purnima Deshpande**  
We have generated knock-in mice with a Leu9Ser mutation in the α4 subunit of the neuronal nicotinic acetylcholine receptor (AChR). This mutation gives the α4β2 AChR a higher sensitivity to agonist which results in a gain of function of the receptor. Three phenotypes were produced that differ in the magnitude of this gain of function, depending on the number of mutated alleles and the presence of the neomycin cassette, which markedly reduces the expression of the gene. Only the mice with the mildest gain-of-function phenotype, heterozygous for the mutation and containing the neomycin cassette, survive beyond the first day after birth and reproduce normally.  

We are now trying to get a broader range of useful phenotypes by generating knock-in mice with a Leu9Ala mutation. This mutation, as tested in oocytes, produces a milder gain of function (about 7-fold) than the Leu9Ser mutation (about 35-fold). We have several clone lines of embryonic stem cells containing the Leu9Ala mutation within the α4 subunit gene. We have neo-intact lines, still containing the neomycin cassette, and also neo-deleted lines in which the neomycin cassette was removed by re-electroporating the cells with a cDNA construct expressing Cre recombinase. These cell lines have been injected into blastocysts and implanted into pseudopregnant females, giving rise to a number of chimeric animals that will be tested for germline transmission when they are sexually mature.  

Embryonic stem cell clone lines with exon 5 of the α4 subunit of the AChR gene removed have also been generated to produce α4 subunit knock-out mice. Crossing these animals with our mutant knock-in mice will allow us to generate additional genotypes that will expand the range of gain of function phenotypes.  

### 332. Nicotine responses of knock-in mice carrying hypersensitive α4 nicotinic receptor  
**Carlos Fonck, Imad Damaj, Raad Nashmi, Purnima Deshpande, Barbara Bowers, Jeanne Wehner, Johannes Schwarz, Cesar Labarca**  
We have confirmed the gain of function properties of the mutated α4 containing nicotinic acetylcholine receptors of our Leu9Ser knock-in mice. These mice displayed a heightened pharmacological sensitivity to nicotine as an analgesic and to nicotine-
induced seizures. Animals heterozygous (hets) for the α4 mutation and their wild-type littermates (WT) were evaluated in their response to nicotine using the tail flick and hot plate nociceptive behavioral assays. Mice were tested two hours before, and five minutes after a single subcutaneous nicotine injection, and the latency to avoidance of heat-based nociceptive stimuli was recorded. Nicotine increased the latency of the pain avoidance response in hets at 0.05 to 0.5 mg/kg, and in WT at 0.5 to 2 mg/kg. Hets displayed a 5.3 fold lower ED50 than WT. The specific nicotine binding site blocker mecamylamine (1 mg/kg) almost completely abolished the nicotine effects in both hets and WT. WT mice displayed a dose-dependent increase in response times to nicotine injections when evaluated on the tail flick assay. Interestingly, their heterozygous littermates were unaffected by nicotine injections at 0.05 to 0.5 mg/kg.

Hets, but not WT, displayed Straub tail and seizures with a dose-dependent decrease in latency at nicotine doses ranging from 1 mg/kg to 2 mg/kg. Seizures were observed in WT at nicotine concentrations of 10 mg/kg or higher. These data support the gain of nicotinic receptor function in our knock-in mice, the importance of the α4 subunit in mediating nicotine analgesia in the supraspinal responses measured in the hot plate, the minimal α4 modulation of the primarily spinal reflex-dominated pathway assessed by the tail flick assay, and the possible involvement of gain-of-function α4 receptors in epilepsy. The hypersensitive α4 knock-in mouse is a promising animal model for the acute effects of nicotine. Future experiments will focus on electroencephalographic recordings from α4 mice treated with nicotine, and the electrophysiological characterization of nicotine effects on slices derived from the hippocampus and frontal cortex of the mutated α4-mouse line.

333. **Calcium imaging of nAChR responses in cultured hippocampal neurons using fura-2/AM**

   A.L. Mundy

Neuronal nicotinic acetylcholine receptors (nAChRs) are composed mainly of the α4β2 and α7 subtypes, and are expressed in a number of areas in the brain, including both the hippocampus and midbrain. Intracellular calcium levels were assessed using fura-2-loaded hippocampal cells from rat brain cultures. Stimulation of the cells with 100 μM nicotine caused an increase in the intracellular calcium concentration in approximately 40% of the cells observed. Cells incubated for seven days in 100 nM nicotine had a slightly increased response to 100 mM nicotine application. Knock-in mice with hypersensitive α4β2 receptors were generated in this lab; calcium imaging will be used to investigate the response to nicotine stimulation in cells from the substantia nigra in both mouse brain slices and cultures.

Mice with a GAT1-GFP fusion have also been generated, and the GFP fluorescence is localized to GABAergic interneurons. It has been suggested that acute nicotinic stimulation of hippocampal interneurons causes the activation of α4β2 nAChRs and the desensitisation of α7 nAChRs. Fura-2-loaded hippocampal cells from the GAT1-GFP fusion mice will be investigated.

334. **α4β2 neuronal nicotinic receptor trafficking in nicotine addiction**

   Raad Nashmi, Cesar Labarca, Carlos Fonck

Chronic nicotine exposure, as achieved by smoking, leads to activation, subsequent long-lasting desensitization, and upregulation of nicotinic acetylcholine receptors (nAChRs). Heteromeric α4β2 nAChRs have a high affinity to nicotine and form the major subtype of nAChRs in the brain. Protein kinase C (PKC), cAMP-dependent protein kinase (PKA) and protein tyrosine kinase (PTK) are known to modulate nAChR desensitization. The main focus of the project will be to examine the trafficking of α4β2 nAChRs during desensitization and its modulation by phosphorylation.

Patch clamp recordings of nAChR whole cell currents and cell capacitance changes will be measured in dopaminergic mesencephalic neurons during brief test pulses of acetylcholine (ACh). Desensitization and recovery of the nAChR current will be measured before, during and up to 40 min following a prolonged (30 min) incubation of ACh or nicotine.

The electrophysiology data will be correlated with real-time fluorescent imaging data to examine cellular trafficking of nAChRs during desensitization and its recovery. α4-yellow fluorescent fusion protein (YFP) and β2-cyan fluorescent fusion protein (CFP) constructs will be produced in order to examine with fluorescent microscopy the spatial intracellular trafficking of nAChRs.

Activators and inhibitors of PKA, PKC and PTK, and site-directed mutagenesis at predicted phosphorylation sites in the α4 subunit will be used to examine the effect of phosphorylation of the α4 subunit in the desensitization and trafficking of the α4β2 nAChR.

A detailed cellular and molecular mechanism underlying nicotine addiction is not clearly understood. Imaging studies in conjunction with electrophysiology will help to delineate how altered receptor trafficking during prolonged nicotine exposure can result in changes in nAChR function and ultimately neuronal excitability.

335. **Selective silencing of mammalian neurons: Strategies using chloride channels**

   Eric Slimko, Raymond Mongeau, David Anderson

Selectively reducing the excitability of glutamatergic neurons will (1) allow for the creation of animal models of human neurological disorders and (2) provide insight into possible treatments for conditions such as epilepsy, stroke, and excitotoxin poisoning. Because there are no pharmacological agents that target subsets of glutamatergic cells, we are focusing on genetic approaches. Our strategy is to express invertebrate
Ivermectin-sensitive chloride channels (C. elegans GluCl $\alpha$ and $\beta$) under tissue-specific promoters and activate them with the drug to produce the silencing effect through a Cl-conductance. Ivermectin is an anthelmintic used to suppress a variety of parasitic infections in both humans and animals. Our goal is to construct in vitro models that mimic conditions that can be obtained in transgenic mice and in human gene therapy. We have constructed a three-cistron Sindbis virus that expresses the $\alpha$ and $\beta$ subunits of GluCl along with EGFP. Using this construct, we have shown a dramatic reduction in the excitability of hippocampal neurons in vitro upon incubation with very low (500 pM) concentrations of ivermectin. Although ivermectin can have a variety of toxic non-specific effects, we believe concentrations this low will be well tolerated in the brains of mice. We have moved to performing stereotaxic injections of this virus into mouse brain, with the goal of doing behavioral experiments. Infected neurons in vivo seem to retain normal morphology for at least a week post infection. However, at this time, we have found that the efficiency of the in vivo infection is rather low (approximately 25%). We are continuing to pursue viral injection strategies, as well as the generation of transgenic mice with this gene.

336. Cation-$\pi$ binding-site in the 5HT$_3$ receptor

*Darren L. Beene, Dennis A. Dougherty*

As in other members of the superfamily of ligand-gated ion channels, aromatic amino acids are important components of the agonist binding site in the 5-hydroxytryptamine$_3$, receptor (5HT$_3$,R). This motif suggests that a cation-$\pi$ interaction is involved in agonist binding. Sequence alignment shows Trp 183 to be the homologue of Trp 149 in the nicotinic acetylcholine receptor, which has previously been shown to be the cation-$\pi$ binding site for acetylcholine. Using in vivo nonsense-suppression methods, we incorporated a series of progressively fluorinated Trp derivatives at Trp 183 of the 5HT$_3$,R. The EC$_{50}$ values recorded for the mutant receptors show a compelling correlation with *ab initio* quantum mechanical predictions of the cation-$\pi$ binding ability for the fluorinated derivatives. This finding indicates that the 5HT$_3$,R binds 5-HT through a cation-$\pi$ interaction, and that upon binding the primary ammonium group of 5-HT makes van der Waals contact with the indole sidechain of Trp 183.

337. State-dependent structural rearrangements in AChR

*John F. Leite, Michael Blanton*, *Dennis Dougherty*

In the absence of crystal structures at atomic resolution in the resting and open state, we must rely on biochemical and biophysical techniques to identify changes in structural conformation that lead to agonist-induced channel activation. In this study, we utilize photoreactive probes [benzophenone and various 3-(trifluoromethyl)-3-(m-iiodophenyl) diazirine derivatives] to probe state-dependent global changes in topology. Two-electrode voltage clamping of *Xenopus* oocytes expressing AChR expressing AChR is used to coordinate photoactivation with channel state. Furthermore, we use mass spectrometry (MS) to identify sites of photoincorporation. Also, we are refining this technique to probe-specific domains of AChR by scanning cysteine accessibility mutagenesis using a heterobifunctional cross-linking reagent, benzophenone-methane-thiosulfonate. This latter technique not only allows us to identify channel state-dependent changes in structure, but also assigns an upper limit to the relative distance between crosslinked residues. In this manner, we have identified a region within the N-terminal domain (residues 173-180), which possibly moves closer to the membrane periphery upon channel activation. In addition, we have identified potentially novel sites of membrane-association that may refine the current topological model for the nicotinic receptor superfamily of ligand-gated ion channels.

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338. Spectroscopic mapping of agonist-induced conformational changes in the nicotinic acetylcholine receptor

*David S. Dahan, Edward Hawrot*, *Arthur Karlin*, *Dennis A. Dougherty*

The nicotinic acetylcholine receptor (nAChR) is a pentameric, cation-selective channel that opens transiently when the receptor binds ACh. Although the nAChR is the most thoroughly studied neurotransmitter-gated ion channel, the detailed structural mechanisms underlying the opening of the channel remain unknown. Since fluorophores can serve as versatile probes of protein structure and dynamics, our objective is to combine site-specific fluorescent labeling with voltage clamping to monitor the conformational changes that accompany channel gating. By site-directed mutagenesis we introduced into the $\beta$-subunit cysteine-substitutions at either L268C (17') or A272C (19'). These substitutions, which are towards the extracellular end of the channel-lining M2 transmembrane domain, were expressed in *Xenopus* oocytes and reacted with membrane impermeable, sulfhydryl-reactive methanethiosulfonate sulfo-rhodamine (MTSR). MTSR modification of the mutants occurred only in the presence of acetylcholine and resulted in fluorescence signals that were approximately three-fold greater than those seen from control oocytes expressing wild-type nAChR. Conformational changes in the protein will be monitored via changes in the fluorescence intensity. We anticipate that these studies will lead to the development of more detailed conformational models of nAChR gating.

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The tethered agonist approach to mapping ion-channel proteins - Toward a structural model for the agonist binding site of the nicotinic acetylcholine receptor

Lintong Li, Wenge Zhong1, Niki Zacharias, Caroline Gibbs2, Dennis A. Dougherty1

The integral membrane proteins of neurons and other excitable cells are generally resistant to high resolution structural tools. Structure-function studies, especially those enhanced by the nonsense suppression methodology for unnatural amino acid incorporation, constitute one of the most powerful probes of ion channels and related structures. The nonsense suppression methodology can also be used to incorporate functional side chains designed to deliver novel structural probes to membrane proteins. In this vein, we sought to generalize a potentially powerful tool - the tethered agonist approach - for mapping the agonist binding site of ligand-gated ion channels.

Using the in vivo nonsense suppression method for unnatural amino acid incorporation, a series of tethered quaternary ammonium derivatives of tyrosine have been incorporated into the nicotinic acetylcholine receptor. At three sites a constitutively active receptor results, but the pattern of activation as a function of chain length is different. At position \( \alpha_{149} \), there is a clear preference for a three-carbon tether, while at position \( \alpha_{93} \) tethers of two to five carbons are comparably effective. At position \( \gamma_{55}/\delta_{57} \) all tethers except the shortest one can activate the receptor. Based on these and other data, a model for the receptor binding site can be developed by analogy to the acetylcholine esterase crystal structure.

Through the use of nonsense suppression techniques, the tethered agonist approach has been made into a general tool for probing receptor structures. When applied to the nicotinic receptor, the method places new restrictions on developing models for the agonist binding site.

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Incorporation and pH modulation of secondary and tertiary tethered agonists in the binding site of the nicotinic acetylcholine receptor

E. James Petersson, Dennis A. Dougherty

The cation-π interaction of ammonium agonists with \( \alpha_{Trp149} \) of the nicotinic acetylcholine receptor (nAChR) is crucial in opening the channel (Zhong et al., 1998). A series of tethered quaternary ammonium derivatives of tyrosine (TyrO\(_n\)Q, tether length: \( n = 2 \)-5) have previously been incorporated into the nAChR using the in vivo nonsense suppression method (Li et al., 2001). These experiments yielded constitutively active (self-gating) receptors. We have attempted to incorporate secondary and tertiary amine tethered agonists to give receptors whose constitutive activity can be modulated by pH. Lowering the pH is expected to protonate the tethered amine, giving it a positive charge and allowing it to reversibly activate the receptor. Though efforts to incorporate a tertiary amine have failed thus far, a secondary tethered amine, TyrO3S, has been successfully incorporated and preliminary data indicate that its constitutive activity shows a strong pH dependence. The pKa of the tethered agonist in the binding site appears to be 5.5 or lower, substantially different than the secondary amine's pKa in solution (~10).

Figure 1. Top: Acetylcholine positioned with quaternary ammonium group over six-membered ring of Trp149. Middle: TyrO3Q, quaternary tethered agonist. Bottom: TyrO3S, secondary tethered agonist.

Efforts towards unnatural amino acid incorporation by nonsense suppression in mammalian cells

Sarah L. Monahan, Dennis A. Dougherty

The use of unnatural amino acids to study protein structure-function relationships in vivo has now been well established in Xenopus oocytes, enabling a wide range of studies of neuronal ion channels. However, the power of the methodology would be greatly enhanced by developing techniques to allow unnatural amino acid incorporation in mammalian cell lines. This would enable the study of proteins of mammalian origin in a more relevant expression system, as well as more complex cellular signaling pathways.

Unnatural amino acid incorporation into proteins is achieved by nonsense suppression. In Xenopus oocytes, the technique requires microinjection of an in vitro transcribed amber suppressor tRNA that is chemically aminoacylated with the unnatural amino acid, and mRNA with an amber stop codon at a position of interest for a given protein. Since only one molecule of protein is made for every molecule of aminoacylated tRNA delivered, a large quantity of tRNA must be delivered to each cell.
This proves to be a challenge in developing a mammalian cell expression system.

Currently, electroporation is being used to deliver the required materials to CHO cells. By co-electroporation of a human serine amber suppressor tRNA (HSAS) that is aminoacylated by the endogenous CHO cell serine synthetase, and DNA encoding for a Ser29TAG mutant EGFP, we have demonstrated that (1) electroporation successfully delivers both tRNA and message to CHO cells and (2) nonsense suppression is achieved with the exogenous tRNA. We are now in the process of optimizing this technique to deliver tRNAs chemically aminoacylated with unnatural amino acids.

342. Analysis of accelerated activation and deactivation kinetics of GIRK channels produced by RGS proteins
A. Kovoor

The key step in G-protein signal termination occurs when GTPase activity of the Gα subunit hydrolyses bound GTP to GDP. Gα subunits stimulate the GTPase activity of G-protein subunits Gai and q. As expected, Gα proteins increased the deactivation rate of G-protein gated GIRK channels following activation by Gαi-coupled receptors. Gα proteins also increased the observed GIRK activation rates but did not change the equilibrium activation levels. We reproduced these results with Gαi, Gα4, Gα7, Gα9, with constructs consisting solely of the Gα domain and with a Gαi-specific mutant. Gα point mutants designed to specifically lose GTPase stimulating activity had no effect on GIRK kinetics or equilibrium activation. Activation of GIRKs by Gαi coupled β2-adrenergic receptors was unaffected by expression of Gα. Accelerated activation kinetics (increased $1/\tau_{on}$) are a necessary consequence of the accelerated deactivation kinetics ($1/\tau_{off}$), because from a simple single exponential model $1/\tau_{on} = 1/\tau_{off}$, linear with $1/\tau_{off}$. What was surprising was that the increased $1/\tau_{off}$ did not lead to decreased steady-state activation, since the predicted fraction of open channels, $f = 1/\tau_{on}/(1/\tau_{on} + 1/\tau_{off})$. An explanation was that Gα proteins increased the on-rate, $1/\tau_{on}$, to compensate for increasing the off-rate, $1/\tau_{off}$ (e.g., GDP-GTP exchange rate enhanced to compensate for increased rate of GDP hydrolysis). However, when we calculated $1/\tau_{off}(1/\tau_{on} - 1/\tau_{off})$ from the measured values for $1/\tau_{on}$ and $1/\tau_{off}$, we found that RGS proteins did not significantly increase on-rates suggesting novel unanticipated mechanisms of GIRK regulation by RGS.

343. Knock-in mice carrying GABA transporter (mGAT1)-GFP fusion protein: Method for absolute mGAT1 quantification
C.-S. Chiu, C. Labarca, P. Deshpande, S. Barghishoon, N. Davidson, S. Quake

We have created a knock-in mouse line whose mGAT1 has been replaced by an mGAT1-GFP fusion, allowing both (a) absolute quantification of mGAT1 in the nervous system and (b) dynamic tracking of GAT1 in response to various perturbations.

To identify an appropriate fusion construct, both N-terminal and C-terminal GFP fusions of mGAT1 were constructed and tested by transfecting into HEK 293T cells. A construct comprising mGAT1, a 12-residue c-terminal spacer, followed by GFP, localizes to the plasma membrane. The mGAT1-GFP has EC50 and Vmax values indistinguishable from those of mGAT1, indicating mGAT1-GFP has similar transport activity as mGAT1.

The knock-in mouse line, created by exon replacement, carries this mGAT1-GFP fusion construct. Our emerging results indicate that mGAT1 is expressed in axons and pre-synaptic terminals, suggesting that GABA can be cleared from extracellular space in the pre-synaptic and the extra-synaptic regions. Using the GFP-bead calibration technique (Chiu et al. (2001) J. Neurosci. Methods, 105:55-63), we are refining initial data suggesting 1000-5000 mGAT1-GFP/µm² in synapses of the molecular layer of cerebellum. We also observe green fluorescent GABAergic interneurons in dissociated hippocampal cultures from the knock-in mouse embryo.

Absolute quantification of mGAT1 will be important for understanding the role of transporters in synaptic transmission. The knock-in approach can be broadly applied to measuring absolute density of membrane proteins and to dynamic tracking of such proteins expressed at native cells and native locations.

Division of Engineering and Applied Science

344. Molecular mechanism of drug interactions with serotonin transporters
M. Li, R. Farley

Previous work shows that various psychostimulants, antidepressants, and local anesthetics block mammalian serotonin transporters. We tested several amine compounds on rSERT and hSERT expressed in oocytes. In voltage-jump experiments, we measured effects on both the hyperpolarization-activated transient current and fluorescence changes due to covalently-linked tetramethylrhodamine. At micromolar concentrations of the drugs, the mechanisms of inhibition vary and appear to depend on the charge state of the blocker molecules. Cocaine, desipramine, and lidocaine inhibit SERT in a partially use-dependent and voltage-dependent fashion, similar to open-state blockade of ion channels. On the other hand, fluoxetine and the quaternary derivative of lidocaine, QX222, display little use-dependent block of SERT. Results support the idea that the modulated-receptor scheme of ion channel blockade is useful for neurotransmitter transporters.

Department of Physiology, USC School of Medicine, Los Angeles, CA
345. High-speed electrophysiology

George Shapovalov

The major limitation on the time course of electrophysiological recording is set by the electrical noise. Compared with other ion channels, bacterial MscL channels have larger conductance—in the range of a few nS at a typical salt concentration. This allows us to work toward higher recording bandwidth while maintaining reasonable signal-to-noise ratio. MscL channels show many conductive substrates and complex kinetics, which are observed as fast transitions between the main conductive state and additional substrates. Therefore it is beneficial to achieve higher temporal resolution that will allow us to resolve fast transitions and to build more detailed kinetic models for MscL. Additionally, it has not yet been possible to resolve the time course of the transitions between the closed and open state for any channel. Perhaps achieving time resolution in the range 100kHz to 1MHz will help to resolve openings or closings of single channels. We have modified the Axopatch 200B patch amplifier and have now recorded E. coli mscL activity at the bandwidth of 70 kHz, achieving a time resolution of 6 µs. It was possible to resolve flickering of the channel between substrates and actually to register a few datapoints per substrate (with typical dwell times of 10-30 µs). This bandwidth already corresponds to the fastest electrophysiological measurements reported in the literature.

It became necessary at this stage to address the problem of electrical interference with characteristic frequencies of ~120 kHz, produced by a variety of electrical equipment present in the building. We proceeded by building a well-conducting aluminum shed around the setup. The amplifier is fed by an external power supply, and DC voltages enter the shielded area and around the setup. The amplifier is fed by an external power supply, and DC voltages enter the shielded area and no electromagnetic pickup occurs before the signal is amplified to macroscopic values. This allows a quieter recording system, and we are now determining the temporal resolution.

346. Electrophysiological characterization of gain-of-function mutants of the M. tuberculosis MscL channel

George Shapovalov, Don Elmore, Josh Maurer

A number of point mutants in Tb-MscL have been produced and characterized biochemically by the Rees and Dougherty groups. A significant fraction of these mutants have a gain-of-function phenotype. In ongoing work, the electrophysiological activity of Tb-MscL, E. coli MscL and Tb-MscL mutants expressed in giant round cells (spheroplasts) is analyzed. Typically MscL channels have large open channel current and require large tensions for activation. For example when expressed in spheroplasts, the E. coli MscL channel typically opens at twice the tension necessary to open MscS channels of E. coli, while Tb-mscL requires even larger tension to open. The Tb-mscL D108N-E104Q double mutant has an activation threshold similar to that of E. coli MscS. MscL channels (unlike MscS) exhibit complex kinetics and have many conductive substrates.

This behavior appears to be common for E. coli-, Tb-MscL channels and its mutants. Hidden Markov modeling will be used to prepare idealized traces from noisy data, followed by maximum likelihood analysis in order to determine kinetic properties such as rate constants and their dependence on membrane tension.

347. cAMP-mediated long-term potentiation and long-term depression at CA3-CA1 synapses of organotypic hippocampal slice cultures

Tzu-ping Yu, Norman Davidson

Previously we reported that bath application of the cAMP analog, Sp-cAMPS, induced two distinct phases of long-term potentiation (LTP): a protein synthesis-independent early phase of LTP and a protein synthesis-dependent late phase of LTP. Both phases require activation of PKA. We also observed a novel form of long-term depression (LTD) induced by concurrent application of Sp-cAMPS and the GABAA receptor antagonist picrotoxin. In the present study, we further investigate the mechanisms underlying the cAMP-mediated synaptic plasticity at CA3-CA1 synapses.

Spatently connected CA3 and CA1 cells of organotypic hippocampal slice cultures were impaled by sharp electrodes. Excitatory postsynaptic potentials of a CA1 cell were evoked by single action potentials of a CA3 cell. The GABA<sub>A</sub> receptor antagonist picrotoxin was applied to slices at different time points after Sp-cAMPS was perfused. Sp-cAMPS-induced potentiation could be reversed to depression when picrotoxin was applied within 30 minutes after perfusion of Sp-cAMPS. However, picrotoxin applied one hour after perfusion of Sp-cAMPS did not have any effect on Sp-cAMPS-induced synaptic potentiation. These results suggest that a critical time period may be required for picrotoxin to modulate and determine the direction of cAMP-mediated synaptic plasticity. In order to investigate whether cAMP-mediated LTP and LTD could be induced at the same CA3-CA1 synapses, Sp-cAMPS and picrotoxin were applied after LTP was induced by Sp-cAMPS applied alone. The result showed that once LTP was induced by Sp-cAMPS, the subsequent application of Sp-cAMPS and picrotoxin no longer had any effect on synaptic potentiation. We are currently testing whether LTD induced by co-application of Sp-cAMPS and picrotoxin could be reversed to LTP by a subsequent application of Sp-cAMPS.

Publications


Nadeau, H. and Lester, H.A. NRSF causes cAMP-sensitive suppression of sodium current in cultured hippocampal neurons. Submitted for publication.


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Summary: Much of the research in this laboratory involves the study of neuroimmune interactions. Using knockout (KO) mice and over-expression in vivo with adenoviral vectors, we are exploring the role of neuopoietic cytokines in synaptic activity and plasticity, PNS and CNS injury and repair, stroke, epilepsy, Alzheimer’s disease, pain and inflammation. We are also investigating a mouse model of mental illness based on the known risk factor of maternal influenza infection. Additional projects include the cloning and characterization of new genes associated with axon outgrowth, and the use of a growth factor receptor antagonist to inhibit melanoma tumor growth in culture and in a mouse tumor model in vivo.

Cytokines are diffusible, intercellular messengers that were originally studied in the immune system. Our group has contributed to the discovery of a new family that we have termed the neuopoietic cytokines, because of their action in both the nervous and hematopoietic/immune systems. We have demonstrated that one of these cytokines, leukemia inhibitory factor (LIF), can coordinate the neuronal, glial and immune reactions to injury. Using both delivery of LIF in vivo and examination of the consequences of knocking out the LIF gene in mice, we find that this cytokine has a powerful regulatory effect on the inflammatory cascade, both within and outside the nervous system. We are exploring the implications of these findings in the study of animal models of nerve and spinal cord injury and regeneration, stroke, epilepsy and inflammatory pain. We find, for instance, that LIF is a critical regulator of astrocyte activation following stroke or seizure, and that this cytokine also regulates inflammatory cell infiltration, neuronal death, gene expression, and the production of new neurons from stem cells following injury. These results highlight this cytokine as an important therapeutic target. We are also examining the role of related cytokines in a transgenic mouse model of Alzheimer’s disease as well as in several types of cancers. Moreover, in a mouse model using human melanoma tumors, we find that an antagonist of the endothelin B receptor is an effective treatment.

Cytokines may also be involved in the modulation of connections during the synaptic plasticity that underlies learning and memory. We are examining the role of LIF and related cytokines in hippocampal long-term potentiation in slices and in vivo. Results in the latter preparation suggest an involvement of immune cells in LTP. This work involves KO mice, electrophysiology, and delivery of recombinant proteins or the genes that code for them.

Cytokines involvement in a new model for mental illness is also being investigated. This mouse model is based on epidemiological findings that maternal viral infection can increase the likelihood of schizophrenia or autism in the offspring. We are using behavioral, neuropathological and brain imaging methods to investigate the effects of maternal influenza infection on fetal brain development and altered behavior in adult offspring.

We are utilizing intracellular antibody expression to block the toxicity of mutant huntingtin, the protein that causes Huntington’s disease (HD). We have produced single-chain antibodies (SCFVs) that bind to various domains of huntingtin, and these can either exacerbate or alleviate huntingtin toxicity in cultured cells. Work has begun on delivering these SCFVs using viral vectors in a mouse model of HD.

348. Pathogenesis of Huntington’s disease

Ali Khoshnan, Jan Ko, Paul H. Patterson

Huntington’s Disease (HD) is caused by expansion of a polyglutamine stretch in the N-terminus of the protein, huntingtin (Htt). Expression of this mutant Htt results in selective death of neurons in the striatum and the cortex. Large nuclear aggregates and amyloid-like complexes containing Htt are observed in post-mortem tissues. Elucidating the mechanism of neuronal death caused by mutant Htt and devising molecular strategies for blocking its toxic effects are the goals of our research. We generated eight monoclonal antibodies (mAbs), which recognize the expanded polyglutamine domain, the polyproline domains, or the C-terminus of exon-1 of Htt. We have cloned the complementary DNAs of the antigen binding regions of several of these mAbs. Intracellular expression of the mAb (MW7) targeted to the polyproline domains of Htt reduces cell death caused by expression of mutant Htt in cultured cells. In contrast, expression of two mAbs recognizing the polyglutamine domain exacerbates Htt toxicity. We are now asking if intracellular expression of MW7 can similarly prevent neuronal death in the mouse striatum caused by expression of expanded repeat Htt.

A related project involves the cell signaling pathways affected by mutant Htt. Several transcription factors and signaling molecules including p53, CREB, and NF-kB interact with the N-terminus of Htt. Our preliminary data indicates that up-regulation of some components of the NF-kB pathway accelerate aggregation
and nuclear localization of Htt. We are using the anti-Htt mAbs to perturb binding of these transcription factors to Htt to aid in identifying the normal and pathological functions of Htt.

349. Endothelin receptor B and melanoma  
Garrett Heffner¹, Paul H. Patterson

Like embryonic melanocyte precursors, malignant melanoma cells are relatively undifferentiated and proliferate rapidly. We therefore asked whether the receptor for the growth factor endothelin may play a role in melanoma growth as it does in melanocyte development. We find that the selective endothelin receptor B (ETRB) antagonist BQ788 can increase pigmentation and dendritic morphology of eight human and one murine melanoma cell line in culture, while decreasing their growth. In contrast, the ETRA antagonist BQ123 does not have these effects, although these cells express both ETRA and ETRB mRNA. In addition, BQ788 has no effect on a non-melanoma cell line, and a selective agonist of the ETRB completely blocks the effects of BQ788 on the melanoma cells. Injection of BQ788 into implanted human melanoma cells in immuno-compromised, nude mice also reduces tumor growth. Moreover, when BQ788 is injected systemically, half of the treated mice show a complete arrest of tumor growth. Histological examination of tumor sections suggests that the inhibitor increases melanoma cell death. We are now testing BQ788 in a model of metastasis in which murine melanoma cells are injected into the mouse tail vein and form tumors in the lungs. We are also examining potential effects of BQ788 on tumor angiogenesis, and searching for novel ETRB transcripts. ¹Caltech undergrad

350. The role of LIF and MMPs in spinal cord injury  
Romulo de Castro Jr., Limin Shi, Nora Tu¹

We utilize a complete transection of the adult mouse spinal cord at the thoracic level to study regeneration in the CNS. We find that ascending axons, most likely primary sensory afferents that contain calcitonin gene related peptide (CGRP), extend rostrally through the lesion despite of the presence of a glial scar. On the other hand, descending serotonergic axons stop and form terminal bulb-like structures at the glial scar. Thus, we can examine the role of various molecules in a regenerating system, exemplified by primary sensory afferents, and in a non-regenerating system, exemplified by the descending serotonergic tract.

Leukemia inhibitory factor (LIF) is up-regulated following injury in the PNS and CNS, where it regulates glial, neuronal and inflammatory cell responses. To examine the role of LIF in spinal cord regeneration, we are comparing the growth of CGRP- and serotonin-positive axons in LIF knockout and wild-type mice. We are also evaluating the influence of another neutopoietic cytokine, IL-6, to see if there is redundancy in cytokine function.

Metalloproteinases (MMPs) and their endogenous inhibitors are important in the maintenance and function of the extracellular matrix (ECM). Axon regeneration following injury is influenced by ECM proteins, and some of these molecules (e.g., proteoglycans) are degraded by MMPs. Thus, expression MMPs and their inhibitors after injury may influence nerve regeneration. We find up-regulation of MMP-9 at 1-2 days, and MMP-2 at 1-2 weeks after injury, indicating a potential role in the initiation of the nerve regeneration response as well as in secondary tissue damage. Immunoreactivity for MMP-9 in the injured spinal cord is diffuse, while MMP-2 staining is strong in vascular structures. Although LIF can regulate the activities of MMPs and their inhibitors in cultured cells, we found no difference in MMP-2 or -9 expression between LIF KO and WT mice in the injured spinal cord. ¹Caltech undergrad student

351. LIF stimulates cell death and cell division in the brain  
Jing Han, Paul H. Patterson

Endogenous LIF is critical for neurogenesis in the injured olfactory epithelium, and LIF delivered by an adenoviral vector stimulates cell death as well as BrdU labeling of olfactory neurons in the normal adult olfactory epithelium (Bauer et al., abstract). Moreover, there are indications from our ischemia (Schwarz abstract) and seizure (Rasika abstract) models that endogenous LIF is required for neuronal apoptosis and neurogenesis in the injured striatum and hippocampus. To extend these findings to sites of ongoing neurogenesis in the normal adult mouse brain, we are investigating the effects of viral delivery of LIF to the hippocampus and lateral ventricles. Compared to controls injected with LacZ adenoviral vector, LIF adenoviral vector injection strongly stimulates BrdU labeling in a number of brain areas, including the hippocampus, the subventricular zone and the rostral migrating stream of newly generated neuroblasts.

352. A neuronal RhoGAP homologue is involved in axon growth  
Jian Zhong, Jing Han

Attempting to clone genes involved in nerve growth factor (NGF)-induced neurite extension, we cultured dorsal root ganglion (DRG) explants in the presence of either NGF or NT3. In the NGF-treated cultures, neurites grew rapidly and fasciculated into large bundles, while NT3-induced neurite growth was less robust, and little fasciculation was observed. Using subtraction methods, cDNAs expressed exclusively in NGF-treated explants were enriched, cloned and sequenced. We identified several known genes, a few of which have previously been reported as genes expressed in TrkA-positive DRG neurons. We also found more than 100 novel gene fragments. One of these, designated AN-1, has stretches of significant homology with the GTPase-activating protein RhoGAP. RhoGAP participates in the control of the Rho family small G-proteins, which have been shown to regulate the actin cytoskeleton as well as
neurite outgrowth. A search of EST databases as well as RT-PCR analysis suggests that at least 13 further homologues of AN-1 exist. In situ hybridization shows that AN-1 mRNA is selectively and highly expressed in embryonic DRG neurons, spinal motor neurons, and sympathetic neurons. AN-1 mRNA reaches its highest expression level in the DRG at E11, when the first sensory axons extend to form peripheral connections. This suggests that AN-1 may be involved in sensory axon outgrowth. In perturbation studies, over-expression of AN1 in PC12 cells induces neurite outgrowth. Furthermore, in cultured E13.5 DRG neurons, expression of a derivative of AN1 with its RhoGAP domain deleted, inhibits NGF-induced axon outgrowth. In a search for AN1-interacting proteins, we find that AN1 is co-immunoprecipitated with RhoA (RhoA63L) but not with RhoG (RhoG61L), Rac (Rac161L) or Cdc42 (Cdc4261L). Deletion of the RhoGAP domain in AN1 eliminates this binding. Several potential interacting partners of AN-1 have also been identified in a yeast two-hybrid screen.

353. Developing a viral model for mental illness
Limin Shi, Paul H. Patterson
Behavioral and neuropathological observations indicate that schizophrenia and autism may originate in defective brain development during fetal life. While there is a strong genetic component in these disorders, it is clear that environmental factors can play a role as well. For instance, influenza infection of the mother at the appropriate stage of pregnancy can increase the likelihood of the child becoming schizophrenic. Given this background, and the need for animal models that incorporate known risk factors, we are exploring the utility of a mouse model involving maternal influenza infection. We are asking whether such infection leads to disrupted brain development in the offspring, and whether such changes resemble findings in human mental illness. Like schizophrenic and autistic patients, C57Bl/6 and BALB/c mice born to infected mothers display deficits in prepulse inhibition (PPI) in the acoustic startle response, a measure of sensory gating. These mice also display highly abnormal responses to acute administration of anti-psychotic (clozapine and chlorpromazine) and psychomimetic (ketamine) drugs. Moreover, these mice are deficient in exploratory behavior in both open field and novel object tests, and they are deficient in social interaction compared to mice born to sham-infected mothers. Thus, using tests that reveal abnormalities in people with schizophrenia or autism, we find that maternal viral infection has a profound effect on the behavior of adult offspring. It is likely that this effect is due to the maternal immune response rather than to viral infection of the fetus because evoking an anti-viral type of immune response in the absence of virus also leads to PPI deficits in the offspring. For comparison to human MRI results with patients, we are now studying neuronal activity in the brains of these mice using fluorodeoxyglucose imaging.

354. LIF mediates apoptosis and neurogenesis in lesioned primary olfactory neurons
Sylvian Bauer¹, Jing Han, Sowmyalakshmi Rasika, Emmanuel Moyse¹, Paul H. Patterson
Sensory neurons in the olfactory epithelium project to the olfactory bulb (OB). These neurons undergo spontaneous apoptosis and are renewed throughout adult life by the proliferation and differentiation of local neuronal progenitors, the globose basal cells (GBC). Removing the target of the olfactory neurons by ablation of the olfactory bulb (OBX) leads to a wave of neuronal apoptosis in the olfactory epithelium that is followed by a wave of GBC proliferation and differentiation. Various growth factors and cytokines, including leukemia inhibitory factor (LIF), stimulate olfactory neurogenesis in vitro. We find that LIF mRNA is up-regulated in olfactory epithelial neurons by OBX. To examine the role of this cytokine, LIF knockout (KO) mice were subjected to OBX and injected with bromodeoxyuridine (BrdU), a cell division marker. Apoptosis and neurogenesis were quantified in olfactory epithelial sections by TUNEL and BrdU labeling, respectively. We find that, although neurogenesis is strongly stimulated by injury in the WT, there is no increase in the LIF KO. Cell death also appears to be deficient in LIF KO mice, although this difference does not reach statistical significance. Furthermore, administration of an adenoviral vector expressing LIF to the normal adult olfactory epithelium mimics the changes in apoptosis and neurogenesis induced by OBX. These results indicate that LIF is a necessary and sufficient regulator of neurogenesis in this paradigm.
¹CNRS, Villeurbanne, France

355. The role of LIF in the cellular and molecular sequelae of seizure
Sowmyalakshmi Rasika, Joanna Jankowsky
Epilepsy, a disease that affects about 1% of the world's population, is characterized by periodic and unpredictable seizures, which can lead to long-term functional impairment of brain function. Both in humans and in experimental animals, seizures can lead to neuronal death, astrocyte activation and axonal sprouting in the hippocampus. There is also an enhanced generation of new neurons in rodents that may contribute to synaptic reorganization. Seizure induction further results in changes in the regulation of a variety of immediate early genes as well as neuropeptides and neurotransmitters. Prior work in this laboratory revealed that seizure induced by electroconvulsive shock (ECS) in mice or pilocarpine injection in rats induces the expression of leukemia inhibitory factor (LIF). This is of interest because LIF is known, after injury and in culture, to control the expression of some of the same markers that are up-regulated by seizure. Indeed, compared to wild-type mice, we found that LIF knockout (KO) mice display very little astrocyte activation following seizure. Preliminary results indicate that the expression of neuropeptide Y, a critical anti-epileptic and neuroprotective peptide, is elevated by seizure in the LIF KO hippocampus, relative to WT.
Consistent with this, the number of cells undergoing apoptosis after seizure in the pyramidal layer of the hippocampus also appears to be lower in the KO animals. However, galanin, another neuroprotective peptide, appears to be lower in the LIF KO both before and after seizure. Modulating LIF production or action after seizure might therefore have potential therapeutic value.

356. The role of LIF in focal cerebral ischemia

Sigrid C. Schwarz

The inflammatory response is a prominent feature in a variety of brain injuries. Our laboratory has studied this reaction in models of mechanical lesions, epilepsy and neurodegenerative disease. The present study examines the effects of focal cerebral ischemia in wild-type (WT) mice and mice deficient in leukemia inhibitory factor (LIF). This lesion is induced by local heat coagulation of the medial cerebral artery on one side of the brain. We find that the inflammatory reaction, as assayed by astrogial activation, is much less pronounced in LIF knockout (KO) mice, as we previously found with seizure and mechanical lesion. Surprisingly, there also appears to be a significant increase of neuronal survival in the dorsolateral striatum in the absence of LIF. Moreover, TUNEL staining reveals markedly more apoptosis in WT than in LIF KO mice, suggesting that LIF is a positive effector in the intercellular death pathway. This cytokine may also be involved in cell proliferation in the ischemic paradigm, as BrdU incorporation is more pronounced in WT than in LIF KO mice following injury. Both the apoptosis and BrdU results are consistent with our findings in injured primary olfactory neurons.

Publications

de Pablo, F., Banner, L.R. and Patterson, P.H. (2000) IGF-1 expression is decreased in LIF-deficient mice after peripheral nerve injury. NeuroReport 11:1365-1368.


We are also examining the role of the cadherins family of cell adhesion molecules in synaptic plasticity. Several labs have shown that cadherins are localized to synapses in the hippocampus. We have recently shown that function-blocking cadherin antibodies or peptides can prevent long-term potentiation, without interfering with basal synaptic transmission. These effects are sensitive to the concentration of extracellular calcium; high concentrations of calcium protect LTP from blockade. These results suggest that cadherin bonds may be sensitive to local fluxes in extracellular calcium imposed by action potential activity. We are now examining the molecular mechanisms by which cadherins influence synaptic strength and the involvement of cadherins in the formation and maintenance of synapses, using fluorescence resonance energy transfer.

We are also interested in how information is processed in the brain. In particular, how does the circuitry of the hippocampus gate or influence information flow? Information reaches the CA1 region of the hippocampus via the conventional trisynaptic loop as well as via a direct input from entorhinal cortex. We are examining how these two inputs interact and influence the output of CA1 pyramidal neurons. Lastly, we are interested in human hippocampal function. In particular, we are conducting multi-unit recordings from human hippocampus in vivo during learning and memory tasks to determine whether changes in spike activity are correlated with behavior.

### 357. Abundant GFP expression and LTP in hippocampal acute slices by in vivo injection of Sindbis virus

**Massimo D’Apuzzo, Georgia Mandolesi, Gerald Reis**

Virus-mediated gene transfer into neurons is a powerful tool for the analysis of neuronal structure and function. Recombinant Sindbis virus has been previously used to study protein function in hippocampal neuron cultures as well as in hippocampal organotypic slice cultures. Nevertheless, some concern still exists about the physiological relevance of these cultured preparations. Acute hippocampal slices are a widely used preparation for the study of synaptic transmission, but currently, recombinant gene delivery is usually achieved only through time-consuming transgenic techniques. In this study, we show that a subregion of the CA1 area in acute hippocampal slices can be specifically altered to express a gene of interest. A Sindbis virus vector carrying an Enhanced Green Fluorescent Protein (EGFP) reporter was injected in vivo into the hippocampus of adult rats. After 18 hours, rats were sacrificed and acute hippocampal slices, infected in the CA1 field, were analyzed morphologically and electrophysiologically. Infected slices showed healthy and stable electrophysiological responses as well as long-term potentiation (LTP). In addition, infected pyramidal cells were readily recognized in living slices by two-photon imaging. Specifically, the introduction of an EGFP-actin fusion protein greatly...
enhanced the detection of fine processes and dendritic spines. We propose this technique as an efficient tool for studying gene function in adult hippocampal neurons.

358. Local protein synthesis and synapse specificity

**W. Bryan Smith**

Our recent work demonstrating the capacity for protein synthesis in the dendritic compartment of hippocampal neurons provides a plausible mechanism for achieving and maintaining specificity during synaptic enhancement. Using a membrane-anchored, destabilized variant of GFP, we are currently investigating the spatial specificity of BDNF-induced local protein synthesis in the dendrites of cultured hippocampal pyramidal cells. Our data indicate limited diffusion of this reporter within the cell, although a precise diffusion coefficient has yet to be determined. In order to restrict exogenous BDNF delivery to a small region, we have developed a local perfusion technique permitting BDNF application to a 10 micron region of a single dendrite. Preliminary data indicate that a majority of the BDNF-induced GFP synthesis in dendrites occurs within 15 microns of the site of BDNF application. With this system we can investigate the spatial precision of BDNF-induced local protein synthesis. Additionally, we will use whole cell electrophysiology to investigate the correlation between sites of increased local protein synthesis and synaptic potentiation.

359. Contributions of synaptic activity to dendritic protein synthesis in cultured hippocampal neurons

**G. Aakalu**

We are interested in studying the effect of native synaptic activity on dendritic protein synthesis in cultured hippocampal neurons. We make use of a diffusion limited GFP-based dendritic protein synthesis reporter that contains the 5' and 3' UTRs of CaMKIIα (Aakalu et al., 2001). This reporter's rate of synthesis can be stimulated by the application of BDNF. However, neurons transfected with this construct also express significant amounts of the reporter in the absence of any exogenous stimulus. Indeed, under control (unstimulated) conditions regions of high expression of the reporter are in the vicinity of synaptic markers. We wish to investigate the possibility that the reporter's expression may be modulated by intrinsic synaptic activity and the extent to which activity modulates the reporter's localization near synapses. To do so, we are employing a variety of pharmacological agents that block synaptic activity and then assessing the effects on the synthesis of our reporter in dendrites.

Our current data indicate that there is no difference in the overall level of dendritic reporter translation in neurons whose activity has been silenced when compared to controls. However, this result may be due to our inability to detect small differences in levels of synthesis. We plan to utilize a new version of the reporter system, with lower levels of basal synthesis, to further study this question.

We are also interested in the mechanisms by which BDNF is capable of stimulating dendritic protein synthesis. In particular, we wish to study if the mechanism by which BDNF increases dendritic protein synthesis involves increasing the levels of electrical activity at the synapses. Therefore, we will analyze the effects of BDNF in the presence and absence of pharmacological agents that block synaptic activity on the levels of dendritic reporter synthesis.

360. Studying dendritic protein synthesis in rat hippocampal slices and living animals

**Changan Jiang**

Local protein synthesis in dendrites has been postulated to contribute to activity-dependent and site-specific long-term synaptic modifications. As a first step to test this hypothesis, we have developed a high-fidelity dendritic protein synthesis reporter. In this reporter construct, we fused a destabilized GFP (dEGFP) cDNA with a canonical myristoylation signal and flanked the fusion gene with the 5' and 3' untranslated regions (UTRs) of CaMKIIα gene. These UTRs are involved in the dendritic targeting and translational regulation of CaMKIIα mRNA. By expressing this reporter in cultured hippocampal neurons, we found that the myristoylated dEGFP only show limited diffusion, which enables it to faithfully report the synthesis of the endogenous CaMKIIα protein in the dendrites. To study dendritic protein synthesis in a more physiological context, such as in hippocampal slices and in living animals, we have generated transgenic animals carrying the reporter construct under the control of CaMKIIα promoter or a tetracycline-sensitive promoter. These animals will allow us to express our reporter in a neuron-specific and temporal-specific manner. Further experiments will address whether new protein is synthesized in the dendrites of hippocampal neurons when they are stimulated to induce long-term potentiation and when the animals are trained to form long-term memories.

361. Identification of dendritic mRNA in rat hippocampal neurons

**Changan Jiang**

Messenger RNAs (mRNAs) are present in the dendrites of hippocampal neurons. Their translation in response to synaptic stimulation may underlie long-term changes in synaptic strength. To understand the functions of the locally-synthesized proteins in synaptic plasticity, we are developing a cell culture system to identify mRNAs in dendrites. Hippocampal neurons were isolated from new-born pups and cultured on a porous membrane, which allows the dendrites to extend away from the cell body to the opposite side of the membrane. Next, the cell bodies were removed with a cotton applicator and the dendrites were used for mRNA isolation. One major limitation with this system is that the isolated dendrites are contaminated with glial cells. Although required for the growth and development of neurons, glial cells can also pass through the pores on the membrane. Currently, we are taking two
approaches to solve this problem. First, we are using a subtractive hybridization technique to isolate dendrite-specific cDNAs from the dendrite-glia mixture. Second, we are using an immuno-panning technique to purify neurons and culture them in glia-conditioned medium. This approach may enable us to isolate pure dendrites.

362. Staufen in dendritic RNA targeting  
*Shaojun Tang, Nalini Colaco*

mRNAs are present in neuronal dendrites, and likely serve as templates for local protein synthesis at synaptic sites during the expression of long-term synaptic plasticity. To understand the mechanisms underlying RNA targeting into dendrites, we study the function of a rat Staufen protein, whose *Drosophila* counterpart is known to play an important role in asymmetric RNA distribution in *Drosophila* oocytes and neuroblasts. Our previous work showed the distribution of Staufen protein in dendrites and co-localization of Staufen with dendritic RNAs, indicating a role of this protein in dendritic RNA targeting. Consistent with this notion, over-expression of wild-type Staufen results in an increase of dendritic RNA contents in cultured hippocampal neurons, whereas the expression of a Staufen mutant protein, which contains the RNA-binding domains but lacks the C-terminal region responsible for dendritic distribution, leads to a decrease of dendritic RNA. These data suggest that Staufen is necessary and sufficient for dendritic RNA targeting. It is becoming clear that RNAs are usually transported in a RNP complex containing translational machinery. To assess the potential role of Staufen protein in dendritic targeting of ribosome-containing RNP particles, we examined if the Staufen protein is a component of such particles. Preliminary results indicate that EGFP-Staufen signals highly overlap with ribosomes revealed by immunostaining, suggesting the co-localization of Staufen protein and ribosomes in the same complex. We are currently investigating the effect of over-expression of wild-type and mutant Staufen protein on patterns of ribosome-containing RNP particles in dendrites.

363. Regulation of Staufen protein distribution by synaptic activity in the hippocampal slice  
*Shaojun Tang, Nalini Colaco*

The Staufen protein is expressed in the hippocampus of adult rats. Within the hippocampal formation, the Staufen protein is abundant in the cell body layer and is present at lower levels in the dendrites. Previous work in our lab and others indicates a role for Staufen in dendritic RNA targeting. It has been hypothesized that synaptic activity may direct synaptic RNA targeting and initiate local protein synthesis at the synaptic regions during the expression of long-term potentiation (LTP). To assess the potential role of Staufen in activity-induced RNA distribution, we are currently examining Staufen distribution in dendrites after LTP expression. To this end, we use tetanic stimulation paradigms to induce LTP expression in the stratum radiatum of area CA1 in hippocampal slices. Slices that have expressed early or late-phase LTP are collected for immuno-staining experiments using an anti-Staufen antibody. Staufen protein signals in the experimental and control stratum radiatum regions will be quantified to determine the effect of LTP expression on dendritic distribution of this protein.

364. Translational control in synaptic plasticity  
*Chiranjib Dasgupta*

Local protein synthesis in dendrites may contribute to late-phase long-term potentiation (L-LTP) and synaptic plasticity. We are exploring the mechanism(s) that may control dendritic protein synthesis. Several studies in *Drosophila melanogaster* (Dm) and *Xenopus laevis* (Xl) indicate that translational control plays a major role in regulation of gene expression; regulated gene expression dictates organismal pattern formation and might also contribute to synaptic plasticity. *Pumilio* (Pum) and Cleavage and Polyadenylation Specificity Factor (CPSF) are two genes whose products are known to regulate translation. In fruitfly, Pum, along with Nanos and Brain Tumor repress translation of hunchback mRNA for abdominal pattern formation. CPSF160 is the principal subunit of the polyadenylation complex that control translation. We are investigating if these proteins might play a regulatory role in dendritic protein synthesis.

As a first step, we have recently cloned from rat hippocampal cDNA library a 1.27 kb rat Pum2 fragment that encodes a 349 amino acid stretch containing an intact Pu motif. The Pu motif contains the amino acids essential for mRNA binding. The rat Pum2-Puf domain has 78% homology with Dm Pum-Puf, 90% with Xl Pum-Puf, 97% homology with mouse Pum2-Puf and 98% homology with *Homo sapiens* Pum2-Puf. Like its homologues it has eight imperfect repeats that are involved in RNA binding. This domain thus represents the rat Puf homology domain. Since the Puf domain of Dm-Pum is sufficient for translational repression, we are in the process of overexpressing a rPuf-YFP fusion protein in cultured neurons and organotypic slices to address its effects on dendritic protein synthesis and LTP. (If necessary, 3' UTR of CamKIIα will be fused to the gene segment of interest for specific dendritic targeting.) Experiments are also underway to isolate the full-length rPum2 clone. Further, we would like to evaluate and understand the role of the domain N-terminal to the Puf motif. We are also in the process of making monoclonal antibodies against the rPuf domain to analyze the somato-dendritic distribution of rPum2 protein. Similar experiments are also proposed with CPSF160. Eventually, we would like to create mutants of rPum2 RNA binding domain and CPSF160 to evaluate if they may act in dominant negative fashion.
365. Probing the necessity of dendritic protein synthesis during synaptic plasticity

*Girish Aakalu, Carlo Quinonez*

It has been previously shown that protein synthesis is required for LTP to persist for over 1-hour (Frey et al., 1988). Whether the newly synthesized proteins come from the soma or the dendrites has not been determined. To explore the necessity of dendritic protein synthesis, one would have to inhibit protein synthesis in the dendrites of neurons in a hippocampal slice while maintaining normal somatic protein synthesis and an intact route for protein transport from the soma to the dendrites. If late phase LTP were abolished under these conditions, it would demonstrate the insufficiency of somatic protein synthesis and the necessity of dendritic protein synthesis in sustaining late-phase LTP. In order to inhibit protein synthesis exclusively in the dendrites, we have developed a photo-caged anisomycin compound. The cage consists of a p-nitrobenzylcarbamate group which can be removed through exposure to UV light. Therefore, the area of protein synthesis inhibition can be restricted to a small region defined by the limits of UV light exposure.

We have tested the compound’s effectiveness using cultured BHK cells and *in vitro* translation systems. These tests indicate that the compound does not affect protein synthesis in its caged form and can effectively inhibit protein synthesis when uncaged. We will now use this compound and test the spatial limits of protein synthesis inhibition in cultured hippocampal neurons using a focused UV light source and our dendritic protein synthesis reporter. Future experiments will be conducted in hippocampal slices where the cell body or synaptic layers can be selectively photoalyzed to specifically inhibit protein synthesis in each compartment. These experiments will elucidate the roles of somatic and/or dendritic protein synthesis during LTP with both spatial and temporal precision.

366. 4E-BP1, S6K1, and S6K2 are not necessary for normal synaptic transmission or synaptic plasticity in area CA1 of the hippocampus

*Gerald F. Reis, Mario Pende, Nahum Sonenberg*

We used electrophysiology to study the role of 4E-BP1, S6K1, and S6K2 in synaptic transmission and long-term potentiation (LTP). These proteins were knocked out in mice to yield the following homozgyous knockout (ko) strains: 4E-BP1, S6K1, and S6K1/S6K2. We prepared 500 µm-thick transverse hippocampal slices from young animals (30 to 70 days old) using a vibratome and standard dissection techniques. After allowing the slices to recover for at least 2 hours, we transferred single slices to a heated recording chamber (28 ± 1°C). Slices were completely submerged in artificial cerebral spinal fluid (ACSF) and constantly perfused at a flow rate of at least 165 mL/hr. We used a two-pathway recording paradigm to obtain field excitatory postsynaptic potentials (fEPSPs) in stratum pyramidale of hippocampal area CA1. This approach gave us an internal control while testing pre- and postsynaptic properties of synaptic transmission.

We examined input-output relationships, paired-pulse facilitation (PPF), and both the short (1 hour) and long (4 hour) phases of LTP. Surprisingly, we found no difference between experimental and control groups in any of the conditions tested. Our results were as follows. For 4E-BP1: (i) Input-output relationship: an ANOVA indicated no significant difference between control and ko (n = 9, 10 for ko and control, respectively); (ii) PPF (n = 8 for ko; n = 13 for control): 25 ms inter-stimulus interval (ISI): ko: 132.25 ± 2.67%; control: 139.00 ± 6.14%; 50 ms ISI: ko: 140.25 ± 2.78%; control: 147.08 ± 4.09%; 100 ms ISI: ko: 137.38 ± 2.99%; control = 140.69 ± 2.64%; (iii) short-phase LTP mean percent of baseline @ 60 minutes: ko = 177.2 ± 10.9% (n = 7); control = 170.3 ± 8.7 (n = 9); not significant (ns); (iv) late-phase LTP mean percent of baseline @ 240 minutes: ko = 161.04 ± 18.82% (n = 6); control = 150.72 ± 11.06%; ns. For S6K1: (i) Input-output relationship: an ANOVA indicated no significant difference between control and ko (n = 8 for both groups); (ii) PPF (n = 18 for ko; n = 25 for control): 25 ms inter-stimulus interval (ISI): ko: 142.2 ± 4.2%; control: 140.8 ± 3.9%; 50 ms ISI: ko: 140.4 ± 3.6%; control: 140.2 ± 3.2%; 100 ms ISI: ko: 133.6 ± 2.2%; control = 135.5 ± 2.7%; (iii) short-phase LTP: ko = 197.5 ± 14.7% (n = 11); control = 185.4 ± 10.9 (n = 11); ns; (iv) late-phase LTP: ko = 164.66 ± 4.81% (n = 10); control = 155.26 ± 9.48% (n = 10); ns. For S6K1/S6K2: (i) Input-output relationship: an ANOVA indicated no significant difference between control and ko (n = 8 for both groups); (ii) PPF (n = 6 for ko; n = 8 for control): 25 ms ISI: ko: 148.3 ± 4.2% (n = 6); control = 150.2 ± 13.1% (n = 7); 50 ms ISI: ko = 138.5 ± 3.4% (n = 6); control = 146.6 ± 6.8% (n = 7); 100 ms ISI: ko = 135.7 ± 3.4% (n = 6); control = 131.6 ± 5.7 (n = 7); (iii) short-phase LTP: ko = 173.1 ± 9.6% (n = 4); control = 162.1 ± 12.0% (n = 7); ns; (iv) late-phase LTP: ko = 168.6 ± 7.7% (n = 5); control = 157.8 ± 10.6% (n = 6); ns. These results lead us to conclude that 4E-BP1, S6K1, and S6K2 are not essential for synaptic transmission in area CA1 of the hippocampus. In addition, they are not important for either the induction or maintenance of short- or long-phase LTP.

367. CPEB is not necessary for long-term potentiation in hippocampal area CA1 of mouse

*Gerald F. Reis, Joel Richter*

The cytoplasmic polyadenylation element binding protein (CPEB) acts as an important regulator of messenger RNA (mRNA) translation. It modulates translational repression and mRNA localization, thereby making it an attractive candidate for study in our laboratory. Using electrophysiology and a homozygous knockout mouse line, we studied the role of CPEB on synaptic transmission and long-term potentiation. As described above, we prepared 500 µm-thick transverse hippocampal slices from young animals (30 to 70 days old) using a vibratome and standard dissection techniques. After allowing the slices to recover for at least 2 hours, we transferred single slices to a heated recording chamber (28
Slices were completely submersed in artificial cerebral spinal fluid (ACSF) and constantly perfused at a flow rate of at least 165 mL/hr. We used a two-pathway recording paradigm to obtain field excitatory postsynaptic potentials (fEPSPs) in stratum pyramidale of hippocampal area CA1. In most cases, we recorded for 8 hours after the induction of long-term potentiation (LTP). Interestingly, both experimental and control groups displayed the same amount of potentiation. Our results were as follows. At 4 hours post-induction: control: 148.1 ± 7.5% (n = 5); ko: 144.0 ± 6.5% (n = 6); ns. At 8 hours post-induction: control: 130.3 ± 10.9% (n = 3); ko: 127.1 ± 7.6% (n = 6); ns. We also tested input-output relationships and paired-pulse facilitation (PPF) in these slices. Although at the present we do not have enough data to perform a meaningful statistical analysis, the two groups appear indistinguishable in preliminary experiments. These data lead us to conclude that CPEB is not necessary for the induction and maintenance of LTP in hippocampal area CA1 of young mice. In addition, it does not appear as if CPEB plays an important role in basic synaptic transmission. Further experimentation should help us clarify this issue.

368. A potential role for ubiquitin-mediated protein degradation in AMPA receptor recycling and synaptic plasticity

Gentry Patrick, Baris Bingol, Gerald Reis, Holli Weld

The availability of proteins likely plays an important role in synaptic function and plasticity. Protein synthesis has been clearly shown to play a role in synaptic plasticity; however, little is known about the potential role of protein degradation. We have examined the localization of ubiquitin in hippocampal neurons. Ubiquitin was present in the soma, dendrites, and occasionally in dendritic spines of cultured hippocampal neurons. We therefore asked whether ubiquitin-mediated protein degradation plays a role in synaptic plasticity. In other cell types, proteasome activity has been shown to regulate agonist-induced internalization of receptors. Thus, we asked if blocking proteasome activity changed the pattern of agonist-induced GluR1 internalization. In agreement with published work, short-term treatment with AMPA induced an average 8- to 10-fold increase in internalized GluR1. In contrast, brief (5-15 min) pre-treatments of the cultured neurons with the proteasome inhibitor MG132 dramatically reduced the amount of GluR1 internalization induced by AMPA. MG132 did not affect the GluR1 surface receptor population prior to AMPA treatment. We are currently pursuing the identity of the degraded protein(s) involved in GluR1 trafficking. In the second set of experiments, we asked whether blocking ubiquitin-mediated degradation affects LTP in hippocampal slices. Slices treated with MG132 exhibited significantly diminished late-phase LTP (measured 7.5 hours after induction) when compared to control slices (mean percent of baseline: control: 144.1%, n = 7; MG132: 122.6%, n = 4 ). Taken together, these results suggest that ubiquitin-mediated protein degradation may play an important role in synaptic function.

369. Visualization of cadherin-cadherin association in neurons.

Eric Mosser

Classic cadherins, in particular N- and E-cadherins, are expressed and localized at synaptic sites of the adult rat hippocampus and involved in LTP. Cadherins exhibit Ca$^{2+}$-dependent adhesion: the removal of Ca$^{2+}$ from the extracellular solution results in a loss of adhesion. Thus, it is possible that changes in extracellular Ca$^{2+}$ associated with synaptic activity may alter cadherin-cadherin interactions. We are attempting to visualize cadherin-cadherin associations at hippocampal synapses. This will enable us to determine if synaptic activity and plasticity affects cadherin dynamics and synaptic structures.

To visualize cadherins in living cells, we have successfully expressed E-cadherin with either the ECFP or EYFP green fluorescent protein (GFP) variants inserted on a flexible linker in a non-structural loop on the extracellular domain in mouse L cells. The ECFP and EYFP constructs can be expressed in different populations of L cells, which are known to express no or very low levels of cadherins. ECFP can act as a fluorescence resonance energy transfer (FRET) donor for EYFP and FRET will be used to visualize the homophilic interactions between cadherins on adjacent cells. Sindbis viral vectors are being prepared for expression of these constructs in neurons. Cadherin containing one GFP variant will be expressed in presynaptic neurons while cadherin containing the other GFP variant will be expressed in postsynaptic neurons. FRET will then be used to detect these exogenous cadherins and their homophilic interactions between pre- and postsynaptic cells in differing conditions. In particular, we will examine the effects of synaptic activity and varying extracellular calcium concentrations on cadherin-cadherin dynamics.

370. β-catenin dynamics

Sachiko Murase

One possible mechanism for long-term synaptic plasticity in the adult brain is to change synaptic structure in response to neural activity. Cell adhesion molecules (CAMs), which are thought to play important roles for morphological changes, have been shown to be involved both in induction and maintenance of long-term potentiation (LTP), a type of synaptic plasticity thought to be important for memory formation. Recent studies have demonstrated that cadherins are present at synaptic sites in hippocampal neurons. In addition, cadherins are involved in LTP induction (Tang et al., 1998) and late-phase LTP-dependent morphology changes (Bozdaji et al., 2000).

To investigate how neural activity affects cadherin function, we visualized β-catenin. β-catenin mediates the interaction between cadherin and actin-filaments and regulates cadherin adhesion activity.
We expressed an EGFP-β-catenin fusion protein in hippocampal neurons via Sindbis virus infection. We monitored β-catenin localization within dendrites and spines before and after a period of depolarization (using KCl). We found that β-catenin moves into spines from dendritic shafts upon KCl stimulation. The application of Cytochalasin D, an actin polymerization inhibitor did not mimic or block the KCl-induced distribution change, indicating that the movement of β-catenin does not rely on actin filaments. It is known that the phosphorylation state of β-catenin can regulate its association with cadherins. We found that the effect of KCl stimulation can be mimicked by the application of genistein, a tyrosine kinase inhibitor, and can be blocked by the application of orthovanadate, a tyrosine phosphatase inhibitor. We tested the involvement of tyrosine 654 in β-catenin by mutating it to a glutamine (Y654E), which should mimic the attachment of a phosphate group. This EGFP-labeled Y654E β-catenin mutant exhibited no redistribution change either by KCl or by genistein. However, the involvement of other tyrosine residues is suggested because a phosphorylation blocking mutation at the same site (Y654F) did not block the redistribution induced by KCl and genistein. The serine/threonine kinase CDK5 has been reported to regulate the affinity of β-catenin for cadherins. It is thought that CDK5 activity promotes dissociation of β-catenin from cadherins possibly by promoting the tyrosine phosphorylation of β-catenin. Interestingly, the application of roscovitine, a specific inhibitor of CDK5 mimics KCl stimulation, and this effect can be blocked by orthovanadate. In fact, biochemical assays showed that the activity of CDK5, which is known to decrease the cadherin-β-catenin affinity, dramatically decreased upon KCl stimulation in hippocampal neurons. Both Y654E and Y654F mutants show no distribution change by roscovitine. These results suggest that CDK5 regulates cadherin-β-catenin affinity in an activity-dependent manner, and that Tyr-654 of β-catenin is the target residue for the CDK5-mediated regulation. What ramifications does the β-catenin redistribution have for cadherin function? The co-immunoprecipitation of cadherin and β-catenin using anti-cadherin antibody showed that the endogenous β-catenin/cadherin ratio increases after KCl stimulation. Given that association with β-catenin promotes adhesion, these results suggest that depolarization-induced redistribution of β-catenin may result in greater cadherin-mediated adhesion at synaptic sites.

References

371. Short- and long-term modulation of hippocampal CA1 synaptic activity and plasticity by the direct entorhinal input
Miguel Remondes
The temporoammonic (TA) pathway originates in layer III of the entorhinal cortex, ultimately bringing direct cortical input to the stratum lacunosum moleculare (SLM) of the CA1 and CA3 regions. SLM is rich in both excitatory and inhibitory synapses, both of which are driven by activity in the TA pathway. As such, this pathway is likely a major modulator of synaptic transmission in area CA1, ultimately gating the output from the hippocampus back to the cortex. We have used the rat hippocampal slice preparation to ask whether the TA pathway is itself plastic and whether it can modulate CA1 synaptic activity.

We have developed a technique for inducing LTP in the TA pathway in the absence of pharmacological inhibitors. This LTP was long-lasting (~ 3 hrs) and sensitive to inhibition of NMDA receptors. We previously showed that burst stimulation in the TA pathway can block the spiking of pyramidal cells in response to suprathreshold Schaffer collateral (SC) stimulation (Dvorak and Schuman, 1998). We now report that this "spike blocking" can be significantly potentiated or depressed, by LTP or LTD, respectively, at the TA–SLM synapses. Previous work has also shown (Colbert, 1998) that when TA stimulation precedes and overlaps with TBS of the SC pathway, it diminishes the levels of SC-LTP achieved. Here, we show that this effect is also significantly modulated by long-term plasticity in the TA pathway. Induction of LTP at the TA synapse can increase the net inhibition of SC LTP, whereas LTD decreases the inhibition. Taken together, these results indicate that the TA pathway not only provides a temporal gating of the output from CA1 pyramidal cells, but also modulates it through LTP and LTD of the excitatory synapses onto SLM dendrites.

372. Human neural activity during learning and memory
Jessica Edwards, Miguel Remondes, Adam Mamela
While learning and memory are widely studied in a variety of systems, it is still rare to be able to examine these behaviors at the single cell level in humans. Working with a group of epileptic patients, we are able to record from individual neurons in alert and learning humans. Patients suffering from medically intractable epilepsy are resistant to drug therapies that are traditionally used for seizure control. Resection of the epileptogenic focus provides seizure relief. To localize the area for surgery, patients are implanted with up to twenty electrodes, including microwire, hybrid electrodes in the hippocampus and amygdala. The duration of the medical procedures allows us to monitor the electrical activity of cells in the hippocampus and amygdala for up to one week. Using a battery of neuropsychological tests, we are able to examine rapid learning and declarative memory. Tests we
are currently using include three versions of the Recognition Memory Task: Faces, Objects and Words; a Continuous Visual Memory Task; Verbal Paired Associates and a variation of the Taylor picture task. We also use a virtual Water Maze, a joystick-operated simulation of the Morris Water Maze task, to test object-cued place memory. We hope to add several emotional memory tasks as well as a version of the memory game "Concentration" in the upcoming months. Currently, we are beginning to analyze data that may demonstrate a direct relationship between hippocampal activity and memory formation in humans. Further, we hope to examine the correlation between local field potentials (EEG) and single-unit activity.

**Publications**


The double RPTPs are likely to directly couple cell recognition and axon guidance during embryonic development. RPTPs are selectively expressed on CNS axons and growth cones. In the 1990s, we showed that four receptor-linked protein tyrosine phosphatases (RPTPs) are involved in growth cones, presynaptic terminals, and their postsynaptic partners.

**Summary:** Our overall focus is on the molecular mechanisms of axon guidance and synaptogenesis in *Drosophila*. Our approach combines genetics, molecular biology, biochemistry, and cell biology. We are especially interested in cell-surface and signal-transduction proteins that function in growth cones, presynaptic terminals, and their postsynaptic partners.

**Receptor tyrosine phosphatases and axon guidance:**

**Genetics of receptor tyrosine phosphatases.** In the 1990s, we showed that four receptor-linked protein tyrosine phosphatases (RPTPs) are selectively expressed on CNS axons and growth cones in the *Drosophila* embryo, and that these RPTPs regulate motor and CNS axon guidance during embryonic development. RPTPs are likely to directly couple cell recognition via their extracellular domains to control of tyrosine phosphorylation via their cytoplasmic enzymatic domains. The extracellular regions of the fly RPTPs all contain immunoglobulin-like (Ig) and/or fibronectin type III (FN3) domains, which are usually involved in recognition of cell-surface or extracellular matrix ligands. Their cytoplasmic regions contain either one or two PTP enzymatic domains.

Our recent genetic studies on the RPTPs have focused on DPTP10D and a new RPTP we identified, DPTP52F. Double mutants lacking both DPTP10D and DPTP69D have a unique and highly penetrant phenotype in which longitudinal axons are routed across the midline of the embryo (Sun et al. 2000 Development 127:801). The double *Rptp* combination interacts with *robo*, *slit*, and *comm*, a set of mutations affecting a pathway controlling repulsion of longitudinal axons from the midline. DPTP10D and/or DPTP69D may be positive regulators of signaling through the Robo family of receptors.

*Ptp*10D mutations also affect motor axon guidance when they are combined with mutations in the other *Rptp* genes. We have performed a detailed characterization of the genetic interactions among all four RPTPs. We find that each growth cone guidance decision in the neuromuscular system has a requirement for a unique subset of RPTPs. In some cases, the RPTPs work together, so that defects are only observed when two or more are removed. In other cases, however, phenotypes produced by removal of one RPTP are suppressed when a second RPTP is also absent. Our results provide evidence for three types of relationships among the RPTPs: partial redundancy; collaboration; and competition (Sun et al. 2001 Mol. Cell. Neurosci. 17:274-291).

By analyzing genomic sequences, we identified a new RPTP, DPTP52F, which contains five FN3 repeats and a single PTP domain and is selectively expressed in the CNS. We examined the role of DPTP52F by inhibiting its expression using dsRNA (RNAi) injection into embryos and by identifying EMS mutations in its gene. *Ptp52F* single mutants cause motor and CNS axon guidance phenotypes, and *Ptp52F* interacts genetically with *Ptp69D*, *Dlar*, and *Ptp10D* (Schindelholz et al., Development, in press. (see Abstract 373)).

**Searching for RPTP substrates and interacting proteins.** We are taking several approaches to address this problem. It is difficult to identify PTP substrates biochemically because PTPs usually do not display strong specificity in vitro. We previously identified a potential DPTP10D substrate, Gp150, using biochemistry (Fashena and Zinn, Mol. Cell. Biol. (1997) 17:6859); but subsequent genetic studies (Q. Sun, unpublished) indicated that Gp150 was unlikely to be important for axon guidance in the embryonic CNS.

One current approach is to perform yeast two-hybrid screens with 'substrate-trap' versions of DPTP10D, DPTP69D, and DPTP99A. We introduced a constitutively activated chicken Src tyrosine kinase into the yeast together with the PTP 'bait' constructs, in the hope that it would phosphorylate relevant substrate fusion proteins made from cDNA library plasmids. The screen should also recover non-substrate interacting proteins that may form complexes with the RPTPs in vivo. We have identified several classes of clones whose interactions with the substrate-trap RPTPs are dependent on coexpression of the tyrosine kinase, suggesting that they may be substrates. We are currently analyzing these further to determine which ones are likely to be of interest for the future (see Abstract 374).

We have also purified a substrate-trap DPTP69D protein from embryos in which its overexpression causes axon guidance phenotypes (this line was made by Paul Garrity’s group at MIT). The implication of the phenotype is that the substrate-trap protein is sequestering proteins that participate in guidance in vivo; accordingly, it will be of interest to determine what might be complexed to this protein in overexpression embryos. We hope to analyze the proteins in the putative DPTP69D complex by cutting bands from a gel and determining portions of their sequences. These experiments are being done together with Dr. Peter Snow, the Director of the Protein Expression Facility at Caltech (see Abstract 375).
Searching for RPTP ligands, and development of a new method for identification of Drosophila cell-surface proteins. We have conducted several mammalian COS cell expression screens to attempt to identify ligands and/or coreceptors for the fly neural RPTPs, so far without success. This has been a major problem in the RPTP field; indeed, in vivo ligands for RPTPs have not been identified in any system. In order to understand how RPTPs regulate axon guidance, it is essential to know when and where they engage ligands, and how ligand binding affects enzymatic activity and/or localization. Our current approach is to screen for ligands through the development of a novel method for expressing the entire repertoire of fly cell-surface proteins on the surfaces of transfected mammalian cells. Cells expressing this collection of cell-surface proteins can be screened using RPTP extracellular domain fusion proteins in order to attempt to identify ligands. We will also generate monoclonal antibodies (mAbs) to these cell-surface proteins, and we have developed an approach which should allow us to directly clone the genes encoding the proteins recognized by these mAbs without any biochemical purification or screening of expression libraries. These genes and mAbs should be of interest in understanding axon guidance as well as other processes that occur during fly development.

Screens for genes controlling axon guidance and synaptogenesis: An overexpression/misexpression screen for genes that regulate axon guidance and synaptogenesis in the larval neuromuscular system. The 32 motor neurons in each hemisegment of a Drosophila embryo innervate 30 muscle fibers, and each motor axon extends along a stereotyped route and always targets the same fiber. Motor growth cones reach their muscle targets during late embryogenesis and then gradually mature into presynaptic terminals. These synapses continue to expand and change as the larva grows, because their strengths must be matched to the sizes of the muscle fibers they drive. The pattern of Type I neuromuscular junction (NMJ) synapses in the third instar larva is simple and highly stereotyped, with boutons restricted to specific locations on each muscle fiber.

Although these features should make the third-instar NMJ pattern an ideal system in which to perform genetic screens to identify molecules involved in the assembly of synaptic circuits, there are problems in the execution of such screens. Anatomical screens typically require dissection and antibody staining of larvae from each mutant line, which is very time-consuming. Also, due to 'genetic redundancy', single loss-of-function (LOF) mutations in genes involved in axon guidance or synaptogenesis often do not produce strong neuromuscular phenotypes.

Because of the problems associated with forward genetic screens, most of the molecules that are known to participate in assembly of the larval NMJ pattern have been defined by reverse genetics, in which embryos or larvae bearing LOF mutations that are already known to affect a protein of interest are examined by antibody staining of motor axons. This is the approach we have taken for the RPTPs. Many interesting insights have emerged from reverse genetic studies, but these experiments are inherently limited by the theories that motivate them. One only studies a particular gene after first developing the idea that it might be involved in a process of interest. Unexpected findings are more likely to emerge from forward screens in which the genome is randomly mutagenized and mutations producing a specific phenotype are characterized.

In order to perform a forward screen for axon guidance and synaptogenesis phenotypes that avoids the problems with LOF screens of dissected animals described above, we devised and executed a gain-of-function (GOF) screen of live larvae (Kraut et al. (2001) Current Biology 11:417-430 (see Abstract 376). We used the EP transposable element to drive high-level expression of genes in motor neurons. This overexpression strategy was chosen in order to sensitize the screen and minimize the problem of genetic redundancy. Work on transcriptional regulators, neural cell-surface proteins, and signaling molecules has shown that pan-neuronal overexpression can produce strong effects on motor axon guidance and/or synaptogenesis, even when LOF mutations produce only weak phenotypes that would be missed in a screen. Genetic redundancy may allow growth cones to identify their targets and differentiate into synapses in the absence of a pathway involved in response to a specific adhesive or repulsive cue. An excess of signaling through a response pathway, however, can upset local balances between attraction and repulsion and cause errors in pathfinding and/or synaptogenesis.

It is important to note that overexpression/misexpression screens are inherently risky, in that high-level expression of a gene in a particular cell type may produce a phenotype even when the gene is not normally involved in development of that cell type. We have thus developed strategies for rapidly sorting isolated genes to identify those that participate in neuromuscular development in wild-type animals.

The EP system employs a P element containing 14 copies of the UAS element that is responsive to the yeast transcription factor GAL4. If an EP element is inserted in the appropriate orientation 5' to a gene, one can direct high-level transcription of that gene in a particular cell type by crossing the EP line to a 'driver' line in which GAL4 is expressed in that cell type. We screened a set of 2293 EP lines for which the insertion site sequences have been determined by the Berkeley Drosophila Genome Project (BDGP). EP-driven expression from these BDGP lines can be effectively used for gene identification, because the UAS element functions as part of a promoter, but does not work as an enhancer. Each EP line was crossed to a driver line containing a pan-neuronal (Elav) GAL4 source and a UAS-GFP construct. In the F1 progeny from such crosses, transcription from the EP is driven in neurons, and these neurons also contain large amounts of GFP. Because the GFP readily enters axons and presynaptic terminals, we can visualize individual
boutons in every Type I synapse of the neuromuscular system through the translucent cuticle of live F1 larvae.

Our screen identified 41 'known genes' (those with published mutant alleles) and 35 'new genes' for which high-level neuronal expression produces axonal/synaptic phenotypes. We assembled published phenotypic data on the 'known genes', and examined larval neuromuscular LOF phenotypes for some of them ourselves. These results showed that at least 3/4 of the 'known genes' are important for nervous system development or function in wild-type flies. An analysis of homology relationships displayed by the 'known gene' and 'new gene' sets suggests that most of the 'new genes' will also have neural LOF phenotypes. The products encoded by the 76 genes identified in our screen include kinases, protein and lipid phosphatases, GTPases, guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), ATPases, cell-surface receptors, RNA-binding proteins, transcriptional regulators, and a variety of other molecules likely to be involved in protein trafficking, modification, and degradation.

Having identified such a large number of 'new genes', we needed to devise strategies to rapidly select those that were likely to be important for CNS/neuromuscular development in wild-type animals. We ordered cDNA clones for the 'new genes' (34/35 have at least one EST match), and conducted in situ hybridization experiments to identify those expressed in the wild-type CNS (and confirm overexpression in EP x driver embryos). 17/20 'new genes' examined thus far are expressed in the wild-type CNS.

To begin to determine whether the 'new genes' have neural LOF phenotypes, we homozygosed their EP elements, then examined motor axons and synapses in mutant larvae by antibody staining. P elements located in the 5' ends of genes often disrupt promoters and thus reduce gene expression. Many EPs are homozygous lethal, suggesting that they do affect expression of adjacent genes. For 'new genes' where no phenotype was observed for the homozygous EP insertion, we began generating LOF phenotypes using RNAi methods. In Drosophila, RNAi is usually performed by injecting dsRNAs for a portion of an mRNA into syncitial blastoderm embryos, allowing these to develop, then examining phenotypes in embryos or hatched larvae (see Schmid et al. (2001) Trends in Neuroscience, in press). To streamline the process of searching for RNAi phenotypes, we developed a protocol in which we inject dsRNAs for each gene into Elav-GAL4, UAS-GFP embryos, then pick hatched larvae that contain coinjected blue dye in their guts and allow them to develop to third instar. We can then directly examine the neuromuscular system of these live larvae as we did in the original screen. This procedure eliminates nonspecific phenotypes resulting from injection damage, because damaged embryos usually do not hatch, and those larvae that do hatch do not feed and die quickly. Since we do not have to dissect and stain the larvae with antibodies, we can examine a large number of hemisegments in order to quantitatively assess phenotypes. Thus, even if an observed phenotype is of low penetrance, we can still detect and evaluate it.

We have initiated further study of a number of genes identified in the screen. Two of these are defined by existing mutations. Robo2 is a cell-surface protein that mediates growth-cone repulsion from sources of its ligand Slit. We find that Robo2 overexpression changes sensory neuron fates in the peripheral nervous system (PNS), and LOF mutations in Robo family genes also affect PNS cell fates (R. Kraut, unpublished). Pumilio (Pum) encodes an RNA-binding protein that shuts down translation of specific mRNAs by binding to their 3' untranslated regions. We found that Pum is expressed in a small subset of CNS neurons, and pum LOF mutations alter motor axon guidance in the embryo. Pum protein is also localized to NMJs in third instar larvae (see Abstract 377).

'New' genes identified by the screen include a number of molecules involved in protein trafficking in neurons. We have investigated two of these thus far. Beach is a huge protein that is closely related to the human protein whose loss causes Chediak-Higashi syndrome, a lethal disease affecting lysosomes and related organelles (see Abstract 378). Gartenzwerg (Garz) is a Sec7-related GEF that is orthologous to a plant GEF that regulates cell polarity during development (see Abstract 379).

An AAA-ATPase that regulates axon guidance across the midline. We also performed an EP screen in which we generated 5000 new EP lines and crossed these to the Elav-GAL4 driver, selecting those that produced driver-dependent. One EP, T32, produces a phenotype in which the CNS is narrowed and axons abnormally cross the midline. This EP is inserted immediately upstream of a gene encoding an AAA-ATPase that is the ortholog of human spastin, a gene mutated in the disease autosomal dominant spastic hemiplegia. AAA-ATPases are a class of proteins that form multimeric complexes that regulate a variety of protein trafficking events within the cell, including vesicle sorting, protein degradation, and microtubule dynamics. There are about 30 such proteins encoded in the fly genome. We made LOF mutations in the T32 (spastin) gene, and found that these produce weak motor axon phenotypes. T32-LOF homozygotes are viable but display abnormal behaviors. Our current focus is on examining T32’s role in axon guidance via genetic interaction studies and yeast two-hybrid screens to identify the other components of the pathways in which it acts (see Abstract 380).

Screening for genes involved in targeting of motor axons to embryonic muscles olfactory receptor axons to the antennal lobe. We are devising screens to identify genes which, when expressed in all muscle fibers, perturb targeting of specific motor axons without altering the muscles themselves (see Abstract 381). We are also using conceptually similar techniques to examine targeting of olfactory receptor axons to the glomeruli of the antennal lobe (see Abstract 381). These screens are still in the
conceptual stage, because we need to build transgenic flies carrying novel gene expression systems in order to carry them out. We expect to initiate the screens themselves within a year.

373. Regulation of CNS and motor axon guidance in *Drosophila* by the receptor tyrosine phosphatase DPTP52F

Benno Schindelholz, Matthias Knirr, Rahul Warrior¹, Kai Zinn

Receptor-linked protein tyrosine phosphatases (RPTPs) regulate axon guidance and synaptogenesis in *Drosophila* embryos and larvae. Here, we describe DPTP52F, the sixth RPTP to be discovered in *Drosophila*. Our genomic analysis indicates that there are likely to be no additional RPTPs encoded in the fly genome. Five of the six *Drosophila* RPTPs have *C. elegans* counterparts, and three of the six are also orthologous to human RPTP subfamilies. DPTP52F, however, has no clear orthologs in other organisms. The DPTP52F extracellular domain contains five fibronectin Type III repeats, and it has a single phosphatase domain. DPTP52F is selectively expressed in the CNS of late embryos, as are DPTP10D, DLAR, DPTP69D, and DPTP99A. To define developmental roles of DPTP52F, we used RNA interference (RNAi)-induced phenotypes as a guide to identify Ptp52F alleles among a collection of EMS-induced lethal mutations. *Ptp52F* single-mutant embryos have axon guidance phenotypes affecting CNS longitudinal tracts. This phenotype is suppressed in *Dla* *Ptp52F* double mutants, indicating that DPTP52F and DLAR interact competitively in regulating CNS axon guidance decisions. *Ptp52F* single mutations also cause motor axon phenotypes that selectively affect the SNa nerve. DPTP52F, DPTP10D, and DPTP69D have partially redundant roles in regulation of guidance decisions made by axons within the ISN and ISNb motor nerves (Schindelholtz et al., Development, in press).

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374. Two-hybrid screens for RPTP substrates and interacting proteins

Lakshmi Bugga

*Drosophila* RPTPs are selectively localized to central nervous system axons in the embryo. Genetic analysis of growth cone guidance choice points in *Drosophila* identified RPTPs as key determinants of axon pathfinding behavior. Within the growth cone, phophotyrosine is enriched at the tips of filopodia suggesting tyrosine phosphorylation as a key means of transducing and integrating guidance information. To understand fully the function and regulation of RPTPs, it will be necessary to identify the physiological substrates of the individual RPTPs. To isolate RPTPs in a complex with their target substrates, I am using substrate-trap yeast two-hybrid system. I am looking for substrates that interact with *Drosophila* neuronal RPTPs-10D, 69D, 52F and 99A. I am also looking for interactions that require tyrosine phosphorylation. As yeast does not contain tyrosine kinases, we introduced tyrosine kinase, Src, into the yeast.

Two-hybrid systems exploit the fact that transcription factors are comprised of two domains, a DNA binding domain (DBD) and an activation domain (AD). We constructed three separate hybrid proteins, GAL4 DBD/RPTP and GAL4/src as our bait proteins and GAL4AD/cDNA library as the prey protein. The bait (with or without src) and prey hybrid proteins are transformed into separate yeast mating strains (A and alpha) that contain three reporter genes-adenine, histidine, and LacZ, and also an auxotrophic marker. Positive interactions are detected by selecting on plates lacking the auxotrophic marker and screening for reporter expression.

My screen with RPTP10D with Src resulted in eleven positive clones, out of which seven clones interact with a protein called "CG12533," which has Calponin Homology (CH) domain. The CH-domain is a superfamily of actin-binding domains found in both cytoskeletal proteins and signal-transduction proteins. With 99A and also Src 99A I got eight positive clones, out of which three clones interacted with a known protein-CSP (Cysteine String Protein), a J-domain chaperone protein involved in neurotransmitter release. I am currently looking for more interactions and also investigating the requirement of tyrosine phosphorylation (Src dependence) and biochemical interactions with CG12533 and CSP.

375. Examination of genetic interactions displayed by the *Ptp52F* gene

Amuradha Ratnaparkhi

Receptor tyrosine phosphatases have been shown to be involved in axon guidance in *Drosophila*. However, the ligands that trigger signaling through these receptors have not yet been identified. The receptor-linked tyrosine phosphatase DPTP52F is the sixth and most recently identified phosphatase in *Drosophila*. Molecular characterization of this gene by Benno Schindelholz, a former post-doc in the lab, showed that in addition to its expression in the embryonic CNS, DPTP52 is also expressed in cells that invaginate through the ventral furrow during gastrulation. The early expression of DPTP52F in these cells is similar in its pattern of expression to the gene *fog*, which, has been shown to coordinate cell shape changes in the invaginating cells. *fog* encodes a putative secreted protein that is known to function upstream of a G protein-coupled receptor *concertina*.

Phenotypic analysis of *Ptp52F* mutants show flattening of the ventral furrow similar to that observed in *fog* mutants. Since the phenotypes of the two mutants appear similar we are interested in testing if *Fog* functions as a ligand for DPTP52F. To test this, we will be examining for genetic interactions between *fog* and *Ptp52F* mutants. These experiments are currently being done.
376. A gain-of-function screen for genes controlling motor axon guidance and synaptogenesis in the Drosophila larval neuromuscular system
Rachel Kraut, Kaushiki Menon

In the neuromuscular system of the Drosophila larva, motor neurons make genetically-defined synaptic connections with muscle fibers. We generated high-level expression of genes in these motor neurons by crossing 2293 GAL4-driven 'EP' element lines with known insertion site sequences to lines containing a pan-neuronal GAL4 source and UAS-green fluorescent protein elements. This allowed visualization of every synapse in the neuromuscular system in live larvae.

We identified 114 EPs that generate axon guidance and/or synaptogenesis phenotypes in F1 EP x driver larvae. Analysis of genomic regions adjacent to these EPs defined 76 genes that exhibit neuromuscular gain-of-function phenotypes. Forty-one of these ('pc genes') have published mutant alleles; the other 35 ('new genes') have not yet been characterized genetically. To assess the roles of the 'pc genes', we surveyed published data on their phenotypes and expression patterns. We also examined loss-of-function mutants ourselves, identifying new guidance and synaptogenesis phenotypes for eight genes. At least 3/4 of the 'pc genes' are important for nervous system development and/or function in wild-type flies.

The 'pc genes', the 'new genes', and a set of previously analyzed genes with phenotypes in the Adh region display similar patterns of homology to sequences in other species and have equivalent EST representations. Most of the 'new genes' are expressed in the central nervous system of wild-type embryos. We infer from these results that most 'new genes' will also have nervous system loss-of-function phenotypes. The proteins encoded by the 76 identified genes include GTPase regulators, vesicle trafficking proteins, kinases, and RNA-binding proteins.

377. 3rd chromosome genes emerging from the gain-of-function screen
Kaushiki Menon

I am characterizing the functions of three genes that I had identified from the screen. One of them is a known gene, pumilio, and the other two are novel genes, Drosophila pharbin and wts-like kinase. pumilio is an RNA-binding protein, well-known for its role in the formation of posterior segments early in embryogenesis. It binds to nanos and brat proteins to inhibit translation of hunchback, an anterior determination gene. In the embryonic CNS, I have found that pumilio mRNA and protein are expressed in the nuclei of select cells near the midline and in the lateral bipolar dendritic (LBD) neuron in the periphery. LBD neuron is part of the transverse nerve. In mutant alleles, pathfinding defects affecting the transverse and the intersegmental nerves are observed. These defects are being further analyzed. I am also examining if nanos and brat mutant alleles show defects in the CNS.

Wts-like kinase is a serine-threonine kinase. It is most similar to the wts/lats tumour suppressor gene in the kinase domain. This gene is interesting since it has dual expression pattern like pumilio. It is expressed in the pole cells early in development and then later on in the CNS. In hypomorphs of this gene, I have found defects in migration of the pole cells to the gonads, as stained by the vasa antibody. The loss-of-function phenotypes in the CNS is being determined.

Pharbin is an inositol polyphosphate phosphatase. It has been cloned in rats and overexpression of this gene in fibroblasts produce dendritic extensions. In Drosophila, this gene is expressed strongly only in the embryonic CNS. I am currently determining its function in the CNS using RNAi and by generating P-element excision lines. The loss-of-functions of all these genes will be analyzed using antibody staining of mutant embryos and larvae.

378. Analysis of the functions of BEACH domain proteins during nervous system development
R. Kraut, V. Nesterova

The family of BEACH proteins was originally defined by the cloning of the human gene mutated in Chediak-Higashi syndrome (CHS), a lethal recessive disorder. The CHS ortholog is mutated in the beige mouse. A region of 280 amino acids (aa) near the C terminus of Beige/CHS was found to be related to segments of a few other proteins, and this region was denoted the BEACH (Beige and Chediak-Higashi) domain. Beige/CHS is a large protein (>3500 aa) whose homology to other proteins is confined to the BEACH domain and one or more WD40 repeats C-terminal to it. The fly genome encodes five BEACH/WD40 proteins. The protein (Beached) encoded by the gene identified in our screen is the ortholog of uncharacterized human and C. elegans genes. Unlike any other BEACH domain proteins, each of these three contains a C-terminal FYVE domain. FYVE domains bind to the lipid second messenger phosphatidylinositol-3-phosphate (PtdIns-3-P).

beached mRNA is normally expressed in a subset of cells in the CNS. Neuronal overexpression of beached from EP2299 generates a unique branching phenotype in which the motor axon networks resemble dead trees. Bulges form at the junctions between the axons and their synaptic branches, and the synaptic branches are thinner than normal. The phenotype suggests that the flow of cytoplasm and/or membrane between the axons and synapses has been altered in some manner. We did not observe a similar phenotype for any other EP line. We have analyzed beached LOF phenotypes using RNAi. beached RNAi produces highly penetrant axon guidance and synaptogenesis phenotypes that primarily affect ISNb motor axon branches. We are currently making P-element excision mutations that should eliminate beached function.
379. Regulation of synapse formation by the Sec7-related guanine nucleotide exchange factor Gartenzwerg (Garz)
R. Kraut

Our screen identified seven genes (six of which are 'new') encoding GEFs, GAPs, or small GTPases. These include RhoL, the Rho-GEF pebble, p50Rho-GAP, the Sec7-family GEF garz, two genes with RCC GEF domains, and one with a tuberin-like Rap-GAP domain. The Rho family GTPases (Rho, Rac, and Cdc42) regulate cytoskeletal structure, and have been extensively studied in the context of growth cone guidance. We were interested in the Sec7-family GEF identified in our screen because this class of GEFs has not been previously examined in the context of neural development. This GEF was also of special interest because it is the ortholog of the Arabidopsis gene gnom (hence the choice of name gartenzwerg, which is German for garden gnome). gnom plants have developmental defects in cell polarity. The PIN1 protein, which is an auxin efflux carrier, is normally expressed in the entire cell membrane during early embryogenesis, but then becomes localized to one face of each cell type (the basal boundary of inner cells, and the apical boundary of epidermal cells). This relocalization of PIN1 is blocked in gnom mutants, so that PIN1 becomes expressed in a random pattern during late embryogenesis.

Garz protein is predicted to be 2040 aa in length. gart mRNA is normally expressed in the CNS, and also elsewhere in the embryo. EP2028 is inserted 295 nt upstream of the predicted ATG, probably within the gart 5' UTR. Neuronal overexpression of garz from EP2028 generates strong ISNb pathfinding defects and ectopic ISNb synaptic branches. EP2028 is homozygous lethal and lethal over a deficiency (Df) for the region. EP2028/EP2028 and EP2028/Df animals (henceforth called garz mutants) usually die as first or second instars, but some survive until third instar. Second-and third-instar garz larvae have greatly reduced synapses that do not extend normally across the muscle surface.

380. Spastin, the fly ortholog of a human spasticity gene
Nina Tang Sherwood, Qi Sun

In an earlier EP screen, we identified a line, EPT32, that drives overexpression of a gene we have denoted as spastin. When EPT32 is crossed to sebraus-GAL4, which is expressed in neural precursors and neurons, a dramatic phenotype is observed in which the commissures are completely fused and the CNS axon array is narrowed. The longitudinal tracts are missing or greatly reduced.

A paper describing the cloning of the gene (SPG4) mutated in the most common form of ADSP appeared at the end of 1999. SPG4 encodes a 616 aa AAA ATPase protein called Spastin. ADSP is a member of a group of diseases called hereditary spastic paraplegias (SPGs), in which patients exhibit progressive spasticity that primarily affects the lower limbs.

Our fly D-spastin sequence is very similar to the human/mouse Spastin sequence across the entire length of the Spastin protein. The data clearly show that D-spastin is the fly ortholog of mammalian Spastin. Beyond this relationship, the D-spastin and Spastin sequences provides few clues to their functions. Spastins fall within the 'meiotic' subgroup of AAA proteins. The only member of this group for which a biochemical activity has been identified is katanin-60, which severs microtubules.

We generated three imprecise excision mutations from EPT32 that delete the N-terminal 111 aa (spastin(102)), and 251 aa (spastin(77) and spastin(28)), respectively, of the D-spastin coding sequence. All three are viable, but young adult flies are slow-moving and most cannot fly. We are currently examining whether these flies have a short lifespan and/or exhibit an unusually rapid decline in function with age. The CNS and motor axon tracts of most spastin embryos appear relatively normal when stained with mAb mAb BP102. We do, however, observe some defects at a low penetrance (<10% of hemisegments): breaks and thinning of the longitudinal tracts, and occasional crossovers of the ISN motor pathway between segments.

381. Analyzing olfactory sensory neurons in Drosophila
Anna Salazar

Due to the recent elucidation of a family of odorant receptors in Drosophila melanogaster, further efforts to gain insight into the topic of olfaction have progressed into studies of Drosophila, whose genetic capabilities make it a model organism for this type of study. Olfactory sensory neurons in Drosophila are housed in sensory hairs, called sensilla, which are located in the two olfactory organs of the adult fly, the third antennal segment and the maxillary palp. It has been shown that the axons of antennal sensory neurons expressing a particular olfactory 7-transmembrane receptor all project to a single glomerulus in the antennal lobe. We have obtained a line from Dr. Leslie Vosshall in which an olfactory receptor promoter drives GAL4. The same strain also contains a UAS-GFP that is presynaptically localized. In adults from this line, GFP is observed within a single large glomerulus on each side of the antennal lobe. We are also making olfactory receptor promoter-GAL4 transgenic lines with other promoters. We have crossed these GAL4 lines to a subset of the BDGP EP collection that has been shown by our lab to possess axon guidance problems and started screening for EPs that cause mistargeting of the GFP signal within the antennal lobe. Several lines with potential targeting difficulties have been discovered and we plan on choosing several of these genes for more in-depth studies that may involve excision of the EP elements to make LOF mutations. We are also developing a technique based on recent RNAi findings, whereby a specific gene can be silenced through the creation of dsRNAi constructs. This method is being used to silence specific olfactory receptors to address the role of the receptor itself in correct glomerular formation, and also to...
silence the genes obtained through the screen in which LOF mutants are not viable as adults. Furthermore, the question of activity dependence in the formation of the glomeruli has been addressed through the crossing of a UAS-tetanus toxin line with the Dor 25-Gal4; UAS-GFP line and looking for correct glomerular targeting. Initial findings are being further studied using a different GFP line to more closely analyze the projections of the axons and through the study of different genes that may be involved in the activity of olfactory sensory neurons.

Publications
Integrative Neurobiology

John M. Allman
Richard A. Andersen
Christof Koch
Masakazu Konishi
Gilles Laurent
Shinsuke Shimojo
SPINDLE CELLS COULD BE RELEVANT TO THE PATHOGENESIS OF PSYCHIATRIC OR LEARNING DISORDERS SINCE THERE IS EVIDENCE THAT OTHER POPULATIONS OF POSTNATALLY-GENERATED NEURONS ARE HEAVILY INFLUENCED BY ENVIRONMENTAL FACTORS. FOR EXAMPLE, THE POSTNATALLY-GENERATED NEURONS IN THE DENTATE GYRUS OF THE HIPPOCAMPUS ARE VULNERABLE TO MANY STRESS-RELATED EVENTS AND THEIR SURVIVAL CAN BE ENHANCED BY ENRICHED ENVIRONMENTS, PHYSICAL ACTIVITY AND SEROTONIN (JACOBS ET AL., 2000). IF THE SURVIVAL OF THE SPINDLE CELLS IS SIMILARLY INFLUENCED BY ENVIRONMENTAL CONDITIONS DURING INFANCY, IT IS CONCEivable THAT POSITIVE ENVIRONMENTS COULD FAVOR SPINDLE CELL SURVIVAL AND ENHANCE MENTAL FUNCTIONING LATER IN LIFE, AND THAT UNFAVORABLE ENVIRONMENTS COULD LEAD TO THE DEATH OF SPINDLE CELLS AND INCREASED VULNERABILITY TO PSYCHIATRIC OR LEARNING DISORDERS.

SUMMARY: OUR INTERESTS LIE IN THE EVOLUTIONARY AND ONTOGENETIC DEVELOPMENT OF BRAIN. WE ARE CONTINUING TO EXAMINE THE FUNCTION AND DEVELOPMENTAL TIMELINE OF ANTERIOR CINGULATE CORTEX, PARTICULARLY THE UNIQUELY HOMONOID ANTERIOR CINGULATE SPINDLE CELLS. WE ARE ALSO PURSUING SEVERAL LINES OF RESEARCH TO DETERMINE EVOLUTIONARY PRESSURES AND SCALING RELATIONSHIPS IN THE BRAINS OF PRIMATE SPECIES. FINALLY, WE ARE CONTINUING RESEARCH ON THE VISUAL SYSTEM OF PRIMATES WITH AN EYE TOWARD THE EARLY HISTORY OF THE PRIMATES. WE HAVE ATTEMPTED TO DETERMINE THE ROLE VISION PLAYED IN THE DEVELOPMENT AND ENCEPHALIZATION OF THE EARLY PRIMATES.

382. SPINDLE CELLS MAY MIGRATE POSTNATALLY INTO ANTERIOR CINGULATE CORTEX

John Allman, Cyndi Shannon-Weickert, Karli Watson

The spindle cells are a population of large bipolar neurons in anterior cingulate cortex that are unique to humans, chimpanzees, gorillas and orangutans and are not present in other mammals. The anterior cingulate cortex functions in focused problem solving, error recognition and self-awareness. Dysfunctions of anterior cingulate cortex are implicated in most of the major neuropsychiatric illnesses. Based on the ontogenetic series of human brains at the National Museum of Health and Science, the spindle cells are not discernible at birth, but rather appear to migrate into anterior cingulate cortex beginning two months after birth. It is possible that they are present earlier in development and only change their shape, but there are several reasons to suspect that they are migrating into the anterior cingulate cortex. In human infants, the spindle cells often appear in pairs and sometimes in vertical chains of three or four neurons, which suggest that they might be tracking an anatomical or chemical path. Sometimes the spindle cells in infants have long undulating processes that resemble flagella. We have found in human infants that the spindle cells are labeled with antibodies to the protein doublecortin, which functions in the motility of migratory neurons. Finally there are probable neuroblasts in the nearby subependymal zone lining the anterior horn of third ventricle (Shannon-Weickert et al., 2000). The late development of the spindle cells could be relevant to the pathogenesis of psychiatric disorders since there is evidence that other populations of postnattally-generated neurons are heavily influenced by environmental factors. For example, the postnattally-generated neurons in the dentate gyrus of the hippocampus are vulnerable to many stress-related events and their survival can be enhanced by enriched environments, physical activity and serotonin (Jacobs et al., 2000). If the survival of the spindle cells is similarly influenced by environmental conditions during infancy, it is conceivable that positive environments could favor spindle cell survival and enhance mental functioning later in life, and that unfavorable environments could lead to the death of spindle cells and increased vulnerability to psychiatric or learning disorders.

References
neurons are smaller in the dwarf lemur than in the other primates or the cat and unlike these other animals the peripheral cells are smaller than the central cells. The peripheral neurons are only slightly larger than the central cells in the tarsier and bush baby, but the peripheral cells are much larger in the anthropoid primates and the cat. This implies that these sets of large peripheral neurons evolved independently in anthropoid primates and cats. Since neuron size may be related to axon diameter and conduction velocity, the presence of these large peripheral neurons points to the existence of a system for the rapid transmission of information from the visual field periphery. This system is present in anthropoid primates and cats but is not present in dwarf lemurs and only weakly developed in tarsiers and bush babies. Such a system would serve to alert the animal to novel stimuli in the peripheral visual field and enable the animal to fixate rapidly on them, which would obviously enhance survival.

These results may have implications for our understanding of the evolutionary history of the visual system in primates to the extent that dwarf lemurs may constitute a model for the primitive condition in primates. If this model is correct, it implies that early primates had very limited acuity. I have suggested elsewhere that the forward direction of the eyes in primates was an adaptation to enhance retinal image quality for praxic space directly in front of the animal under nocturnal conditions. The apparently poor acuity in dwarf lemurs is not consistent with this model and suggests that the eyes are directed forward for some other reason, perhaps to enhance binocular summation under low light conditions. Alternatively it is conceivable that dwarf lemurs have lost acuity relative to ancestral primates, although it is hard to imagine a loss of acuity in this nocturnal group but not in bush babies or tarsiers. Finally, in our microelectrode study of the topographic organization of visual cortex in dwarf lemurs, we found evidence for a large number of cortical visual areas including V1, V2, DL, MT, and several others. This implies that the basic cortical organization found in other primates is also present in dwarf lemurs and further suggests that this organization may have arisen in evolution before the emergence of retinal specializations that support high acuity.

384. The spindle cell: A neuron unique to humans and great apes
Karli Watson, John Allman

Humans are natural problem solvers: they use past experience to gauge the probability of a particular outcome and plan their actions accordingly. I study a region of the brain that is particularly relevant to this kind of adaptive behavior.

The anterior cingulate cortex (ACC) curves around the front part of the corpus callosum on the medial plane of each hemisphere. We know from EEG and functional imaging studies that one subregion of the ACC is activated by tasks which elicit a strong emotion, while another subregion is activated during cognitively difficult tasks which require focused concentration. This and other evidence suggests that the ACC acts as an interface between emotion and cognition. This integration allows the organism to select the most appropriate behavior for a particular situation.

One neuronal subtype in the ACC, the spindle cell, is unique to the greatest of the primate problem solvers: the humans and great apes. My goal is to characterize spindle cells by describing their dendritic morphology, local axonal connections, and molecular makeup. To do this it is necessary to develop various techniques for use on post-mortem human tissue. One promising method is diolistic labeling, in which tiny (approx. one micron) tungsten beads are coated with lipophilic dye and shot with a gene gun into a slice of brain. The dye integrates into the lipid bilayer of the cell membrane and, with time, diffuses out to the distal dendrites and some length along the axon. The neuron can then be imaged with confocal or two-photon imaging.

385. A fossil anthropoid primate has olfactory bulbs intermediate in size between simians and prosimians
John Allman, Eliot Bush, David Dubowitz, Miriam Scadeng, Elwyn Simons

We have used a CAT scan to analyze the braincase of a Parapithecus grangeri fossil from the Fayum deposit in Egypt. This technique has allowed us to distinguish the fossilized bone of the braincase from the sandstone matrix within. Parapithecus grangeri was a basal anthropoid primate in the early oligocene, 32 million years ago. This fossil gives us a glimpse of the state of the brain in early anthropoid evolution. One general trend in anthropoid evolution has been a reduction in the size of the olfactory bulbs relative to the rest of the brain. We have measured the size of the olfactory bulbs and the whole brain of this fossil. Our results show that the olfactory bulbs of Parapithecus were of intermediate size between modern anthropoids and prosimians.

386. Reconstructions from the braincase of a recently extinct sub-fossil lemur
John Allman, Eliot Bush, David Dubowitz, Miriam Scadeng, Elwyn Simons

We have CAT-scanned a subfossil Archeolemur, a recently extinct Malagasy primate. We plan to scan a whole series of these subfossils. Though there are surviving highly-encephalized haplorhines, we have no surviving examples of large-brained strepsirhines. The large Archeolemur can give us an independent view of what happens to a primate brain when it enlarges during evolution. We will analyze a number of features of the brain based on the braincase. We can identify the olfactory bulb, and based on the sulcal and gyral patterns we can demarcate the different lobes of the hemispheres. In addition, because of the quality of these subfossils, we can measure several cranial nerves based on the sizes of various fossae visible in the scans. We will compare all of these features with the characteristics of living lemurs.
allowing us to learn something about the effect body size on the evolution of lemur brains.

387. **Reconstructions of primate neocortex from histological sections**

*Eliot Bush, Stephen Shepherd, John Allman*

When closely-related species have brains of different sizes, is the structure of neocortex also different? Do different neocortical areas scale consistently with overall brain size, or do they vary in ways that cannot be predicted from overall brain size? To address these questions we are making measurements of surface area, volume, and cell density in neocortical areas of closely-related primates that differ greatly in brain size. We have collected a set of high-resolution digital images from the Comparative Mammalian Brain collection at the University of Wisconsin. We are using cytoarchitectonic and electrophysiological data to demarcate and measure various cortical areas on three-dimensional reconstructions of these scans. We also plan to make stereological measurements of cell density in a number of areas including V1, MT, and Brodmann Area 10. Comparing these various measurements across many species, we hope to be able to separate the effects of size and selection pressure in primate brain evolution.

388. **Brain scaling: A comparison of rodent olfactory bulbs**

*Florian Merkle, John Allman*

An important but unanswered question in comparative neuroscience is: “How does brain size fundamentally scale as a function of body size?” The scaling of biological systems is allometric, that is it follows certain well-established power laws. Through empirical studies, investigators have uncovered scaling relationships that generally have good predictive power. Unfortunately, the predictive accuracy of these relationships disintegrates for the brain, an inherently plastic organ that is sensitive to environmental and evolutionary pressures. To avoid such confounds, we have chosen to study the rodent olfactory bulb. The olfactory bulb is a highly conserved brain structure, particularly in rodents, whose primary sensory modality is olfaction. Furthermore, rodents are an excellent choice because they constitute the largest mammalian order and span over three orders of magnitude in body size.

We are reconstructing three-dimensional volumes from serial thionin-stained sections to determine the volume of the olfactory bulb and glomeruli as well as the number of glomeruli in each olfactory bulb. By comparing these data to the total brain and body size in a number of species, we hope to discover a relationship that is relatively independent of confounding factors. This baseline scaling relationship would permit more accurate interpretation of observed discrepancies between predicted and observed brain size, leading to a more complete understanding of brain evolution.

Our initial observations suggest that the larger rodents, such as the beaver and capybara, may have more glomeruli than their smaller cousins. This might be due to the ability of physically larger animals to express a larger repertoire of olfactory receptor genes. Alternatively, it may represent a computationally or architecturally more efficient way of mapping a large number of primary olfactory receptor neurons onto the olfactory bulb. To explore this issue, we plan to analyze the olfactory bulbs of several strains of genetically similar mice that appear to differ only in brain size (up to 40%).

**Publications**


Recently we have begun a project attempting to develop a neural prosthesis for paralyzed patients. This prosthetic system will be designed to record the electrical activity of nerve cells in the posterior parietal cortex of patients with paralysis, enabling them to operate external devices with their movement thoughts.

Our laboratory also examines the coordinate frames of spatial maps in cortical areas of the parietal cortex coding movement intentions. Recently, we have discovered that plans to reach are coded in the coordinates of the eye. This is a particularly interesting finding because it means the reach plan at this stage is still rather primitive, coding the plan in a visual coordinate frame rather than the fine details of torques and forces for making the movement. We have also discovered that when the animal plans a limb movement to a sound, this movement is still coded in the coordinates of the eye. This finding indicates that vision predominates in terms of spatial programming in primates.

Another major effort of our lab is to examine the neural basis of motion perception. One series of experiments is determining how optic flow signals and efference copy signals regarding eye movements are combined in order to perceive the direction of heading during self-motion. These experiments are helping us understand how we navigate as we move through the world. A second line of investigation asks how motion information is used to construct the three dimensional shape of objects. We asked monkeys to tell us which way they perceived an ambiguous object rotating. We found an area of the brain where the neural activity changed according to what the monkey perceived, even though he was always seeing the same stimulus. In other experiments we have been examining how we rotate mental images of objects in our minds, so-called mental rotation. In the posterior parietal cortex we find that these rotations are made in a retinal coordinate frame, and not an object based coordinate frame, and the mental image of the object rotates through this retinotopic map.

We have successfully recorded functional magnetic resonance signals following visual activation of the monkey brain. This finding is important since this type of experiment is done routinely in humans and monitors the changes in blood flow during different cognitive and motor tasks. However, a direct correlation of brain activity with blood flow cannot be achieved in humans, but can in monkeys. Thus the correlation of cellular recording and computer to surf the internet. Currently we are testing whether intentional signals can be deciphered by recording the activity of single cells in the reach region of healthy monkeys. We have found that we can predict where an animal plans to reach in a task in which he is instructed to plan to reach in one of two directions. We provided feedback to the animal regarding this prediction, and the animal quickly learned that he could receive his juice reward at the end of each trial by simply thinking about reaching in the correct direction without actually reaching. We are currently expanding this research to record from multiple cells using arrays of implanted electrodes. This approach will allow us to increase the number of potential reach directions that can be decoded. If these multicellular recording experiments are successful, in principle this approach could be used to record from the posterior parietal cortex of patients with paralysis, enabling them to operate external devices with their movement thoughts.

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Summary: While the concept of artificial intelligence has received a great deal of attention in the popular press, the actual determination of the neural basis of intelligence and behavior has proven to be a very difficult problem for neuroscientists. Our behaviors are dictated by our intentions, but we have only recently begun to understand how the brain forms intentions to act. The posterior parietal cortex is situated between the sensory and the movement regions of the cerebral cortex and serves as a bridge from sensation to action. We have found that an anatomical map of intentions exists within this area, with one part devoted to planning eye movements and another part to planning arm movements. The action plans in the arm movement area exist in a cognitive form, specifying the goal of the intended movement rather than particular signals to various muscle groups.

Recently we have begun a project attempting to develop a neural prosthesis for paralyzed patients. This prosthetic system will be designed to record the electrical activity of nerve cells in the posterior parietal cortex of paralyzed patients, interpret the patients’ intentions from these neural signals using computer algorithms, and convert the “decoded” intentions into electrical control signals to operate external devices such as a robot arm or a computer to surf the internet.
389. **Neural correlates for the representation of head position in the posterior parietal cortex**

Brian D. Corneil, Richard A. Andersen

Sensory-motor studies of the nervous system have, over the past 50 years, focused primarily upon either sensory or motor aspects. Relatively little work has been devoted to the neural events intervening between sensation and action, as the coding of the sensory and motor systems needed description before progressing to intervening events. During these intermediate stages, a plan to make a movement must be formed; knowing how movement plans are represented and developed can tell us much about the brain's mechanisms to generate accurate movements. This project utilizes the gaze shifting system as a model for sensory-motor transformations, specifically examining how sensory information from neck muscles is used within the lateral intraparietal area (area LIP) of the posterior parietal cortex. Accurate gaze shifts, which are rapid and coordinated movements of the eye and head, hinge on a sense of head position relative to the body; this sense of head proprioception is thought to arise from the many spindles within neck muscles. We propose to manipulate head proprioception via mechanical vibration. Such a manipulation should induce a change in the perceived position of the head, which will be assessed through a variety of gaze shifting tasks and correlated to neural activity in area LIP. This technique will provide a direct means to assess the mechanisms by which sensory information from neck muscles affects the planning of gaze shifts within area LIP. It is likely that similar mechanisms will influence other types accurate motor actions, such as reaching, in other regions of the posterior parietal cortex.

390. **The effects of saccadic adaptation on the representation of motor intention in LIP and decoding motor intention during natural behaviors**

Bradley Greger, Richard A. Andersen

Using a saccadic adaptation task, plasticity in the neural representation of motor intention in LIP is under investigation. Two monkeys have been trained to make direct and memory guided saccades from a central fixation point to peripheral target points displayed on a tangent screen. The saccadic target disappears at the beginning of the saccade, covertly shifts its position, and then reappears in its new location prior the end of the saccade. The shift in target location is undetectable, however the resulting motor error, i.e. that the image of the target is not on the fovea, is clearly changed. Within a block of trials, the shift in target location is always a fixed distance and direction from the initial target location. Over several hundred trial monkeys adapt to the constant shift in target location and saccade directly to the shifted target, not to the initial target. There is a re-mapping of the expression of motor intention. Electrophysiological recordings are being used to test for a neural correlate of this re-mapping in LIP. Specifically, the response field of a neuron will be mapped using the delayed saccade task. The monkey will then undergo the saccadic adaptation paradigm. Following saccadic adaptation the response field will again be mapped using the memory saccade task. Post-adaptation changes in the shape and/or location of response fields will confirm LIP as a site of plasticity and may provide insights in to the mechanism of adaptation.

A second line of inquiry is the study of motor intention in the parietal lobe during natural eye and arm movements. In order to study the representation of eye movement intention in the parietal lobe during natural eye and arm movements. In order to study the representation of eye movement intention in the parietal lobe during natural eye and arm movements. In order to study the representation of eye movement intention in the parietal lobe during natural eye and arm movements. In order to study the representation of eye movement intention in the parietal lobe during natural eye and arm movements. In order to study the representation of eye movement intention in the parietal lobe during natural eye and arm movements. In order to study the representation of eye movement intention in the parietal lobe during natural eye and arm movements.

391. **Spectral properties of neuronal spiking activity**

Bijan Pesaran, Partha P. Mitra, Richard A. Andersen

How to characterize the temporal patterning of neural activity during behavior is a problem of broad interest in systems neuroscience. An accumulating body of work shows that patterns in spiking are modulated by behavior and that such correlations form dynamic receptive fields which may be suitable for neural coding. Here we address two statistical issues of central importance to this line of work: 1) What statistical quantities are suitable for detecting correlations in neural data during behavior? and 2) What classes of stochastic process models explain experimental observations? We present a set of measures based on the spectrum, including the interval spectrum, the weiner entropy, and the spectral derivative. We show these measures have better statistical properties than the correlation function, which is in common use, and we apply them to analyze cortical spiking activity in behaving monkeys. Stochastic models of correlated spike trains can be used to investigate the underlying activity. Many studies have compared refractoriness, oscillations, or bursting by assuming a Poisson or renewal process model for the activity. We show that the simplifying assumptions these processes use, can be inconsistent with the data. We then present models
of spiking activity using hybrid (point-continuous) stochastic processes. These models feature a spiking neuron whose probability of firing is dependent on a continuous process with gamma band temporal structure which could represent synaptic inputs to the cell.

392. Neural activity in the posterior parietal cortex during decision processes for generating visually-guided eye and arm movements in the monkey

Hansjoerg Scherberger, Richard Andersen

The decision to pick a particular visual stimulus as a target for motor action involves a network of cortical areas. The target choice depends on external and internal variables including the type of movement (e.g., arm or eye movement) and the stimulus location in space. We asked whether the decision process is reflected in the neural activity of cells in the posterior parietal cortex (PPC).

Monkeys were trained to visually fixate red lights on a board and to reach out and touch buttons on that board when illuminated by a green LED light. Using a double stimulation (DS) paradigm, two green targets appeared 16° bilateral to a fixation point, and the monkey had to choose one of the targets and immediately reach to it in order to receive a juice reward. Left and right target choices were made equally frequent by introducing stimulus onset asynchrony and a staircase procedure. Single neurons were recorded while the animal was performing this task.

Preliminary results from recordings in the medial intraparietal (MIP) area in PPC showed a correlation between the firing rate of individual cells and the reach choice. The firing rate was significantly larger when the choice of the animal was in the cell’s preferred direction, than when the choice was in the opposite direction, even though the visual stimulation was virtually identical.

This result indicates that the decision process is reflected in the neuronal activity and suggests that the PPC is participating in the decision process for target selection.

393. The auditory space represented in the temporo-parietal auditory association cortex (Tpt)

Shau-Ming Wu, Richard Andersen

When a bird sings we perceive the source of the song and the image of the bird as spatially coincident. The multimodal perception of space occurs so effortlessly that we do not recognize what a formidable task it is to bring auditory and visual stimuli into the same reference frame. Results from our previous research shows that the brain successfully achieves the bimodal synthesis by aligning auditory spatial information with visual information in a common eye-centered reference frame in posterior parietal cortex (PPC). The planning of a saccade to sound and the planning of a manual reach to sound are both coded in an eye-centered reference frame in PPC.

However, it has been well known that the location of a sound is computed based on head-centered information in the early stages of the auditory pathway. A coordinate transformation from a head-centered reference frame to an eye-centered one likely occurs as auditory signals ascend from early auditory areas to parietal areas. Temporo-parietal auditory association cortex (Tpt) receives major auditory inputs from primary auditory cortex and sends projections to PPC. It very likely plays an important role in this coordinate transformation.

We record the neural activities in the Tpt in an awake monkey. The animal is trained to look at one of a few visual targets and we map the auditory receptive field of a sampled Tpt cell for each eye-position. We recorded from about four dozen spatially-tuned auditory cells in Tpt. Our preliminary data analysis concludes that 1. Auditory space is coded in a head-centered reference frame in the Tpt. 2. The auditory receptive fields of the cells are gain-modulated by eye-position. These results suggest that Tpt may be intermediate between cells in the primary auditory cortex and PPC in the process of coordinate transformation. The gain modulation by eye position may be the mechanism for making this transformation.

394. FMRI of visual motion and visual motion after effect in the awake behaving macaque

David J. Dubowitz, Kyle A. Bernheim, Richard A. Andersen

The use of a macaque for functional magnetic resonance imaging of cortical activation during a cognitive task requires extensive behavioral training. We have had success in training a macaque to remain motionless and fixate for prolonged periods of time, allowing true awake behaving primate fMRI studies. In particular, we were able to assess the distribution of cortical areas sensitive to visual motion and visual motion after effect. The macaque was trained to lie motionless within a conventional MRI scanner and fixate on a point at the center of a 22° visual stimulus. Eye position during training and within the MRI scanner was maintained to within a 4° deviation for the duration of the stimulus presentation and was monitored using an infrared eye tracking system, custom modified for use during MRI.

FMRI used both BOLD and iron oxide (CBV-sensitive) techniques. Functional imaging of the entire brain was acquired using an EPI sequence repeated every 2000ms (isotropic voxel volume 64 uL). This dataset was post processed with AFNI software and projected on a MPRAGE anatomical image (interpolated voxel volume 0.25 uL). Visual stimuli included expanding, contracting and stationary concentric circles. Length of signal acquisition and stimulus presentation ranged in excess of 3 minutes and 30 seconds, respectively.
395. Cognitive control signals for prosthetic systems
Daniella Meeker, Krishna V. Shenoy, Shiyan Cao, Bijan Pesaran, Hans Scherberger, Murray Jarvis, Christopher A. Buneo, Aaron P. Batista, Sohaib A. Kureshi, Partha P. Mitra, Joel W. Burdick, Richard A. Andersen

The feasibility of using signals from the brains of paralyzed patients to control prostheses has been demonstrated by several groups using peri-movement signals from multiple single cells in non-human primates. However, the use of neural signals for prosthetic control without concurrent movement remains to be demonstrated. We sequentially recorded single unit activity from macaque PRR neurons, located at the visual-motor interface, during a delayed reaching task and used the cognitive signals from the delay period to determine the presence and goal of movement plans. We also determined when the plan was to be executed, and distinguished saccade and reach plans, using both the local-field and action potentials. Using a multi-state dynamic model that limits the variables estimated from the neural activity, the internal state (when and where to reach) could be predicted. With a maximum likelihood classification of spikes in a sliding 250 ms window as the input to a finite state machine, we predicted both when and to where (which of eight targets) a reach occurred with 89%, 69% and 48% accuracy, using 41, 16, and eight neurons, respectively. These results suggest that high-level control signals can be estimated from relatively few neurons/electrodes. Furthermore, by relegating the subsequent and more detailed control functions to engineered systems, viable prosthetic systems may require relatively few neurons/electrodes.

396. Reference frames for reach planning
Chris Buneo, Richard Andersen

We have been exploring the reference frames for reach-related activity in two parietal areas: the parietal reach region (PRR), located posterior and medial to LIP, and area 5, an adjacent parietal region known to be active during reaching. Single-cell recordings were obtained from two monkeys trained to perform an instructed-delay-reaching task. Eye position and initial hand location were varied in four, randomly interleaved, subtasks. Reaches were made to an average of eight locations on a two-dimensional grid of push buttons. In PRR we found that neurons encode the location of reach targets with respect to the input to a finite state machine, we predicted both when and to where (which of eight targets) a reach occurred with 89%, 69% and 48% accuracy, using 41, 16, and eight neurons, respectively. These results suggest that high-level control signals can be estimated from relatively few neurons/electrodes. Furthermore, by relegating the subsequent and more detailed control functions to engineered systems, viable prosthetic systems may require relatively few neurons/electrodes.

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We have also been exploring the existence of temporal coding in area 5 and PRR. The spectrum of the single unit firing rates in PRR and area 5 was studied during the delay period that followed the presentation of an instructional stimulus. We found strong differences in the intrinsic dynamics of the two areas. Area 5 had a population spectrum that contained no structure beyond suppression at low frequencies. In contrast, the population spectrum of PRR contained spatially tuned broad band oscillatory activity that peaked at 25Hz (+/- 5Hz) and extended up to 60Hz (+/- 10Hz). On a single cell level the spectra were significantly different from the spectrum of a Poisson process in approximately 1/3 of cells and were spatially tuned for the planned movement direction. The peak frequency of the broad band activity in PRR was unrelated to the firing rate of the neurons but showed the same spatial tuning. This activity is an example of a temporal code for the direction of movement.

397. What does it mean to read a temporal code?
Partha P. Mitra, Bijan Pesaran, Hemant Bokil, Richard A. Andersen

In previous studies, we have shown that spectrograms and associated quantities provide a robust measure of temporal structure in neuronal activity recorded from macaque area LIP during a working memory task. We have extended these studies to provide a robust definition of "temporal codes" in neuronal activity in general. The relevant concepts were developed through numerical simulations and analysis of multielectrode recordings from LIP and decoding algorithms for neuromotor prosthetics. We found that the spectrographic representation can be made independent of choice of time window, and is in general capable of handling arbitrary non-stationarities in the data. A dynamical model of underlying cognitive states assisted in decoding the neuronal activity in the neurophysiological experiments being studied.

Publications


transmit this information? Analytical work, backed up by complex are single nerve cells? What is the code used to mechanisms underlying neuronal computations? How of individual neurons? What are the biophysical limit signal detection or signal reconstruction at the level noise, noise due to synaptic background firing, and so on) extent do neuronal noise sources (thermal noise, channel published by Oxford University Press in 1999. To what Information Processing in Single Neurons summarized in a textbook, program called "Biophysics of Computation" studies how the biophysics and microanatomy of neurons subserve understanding the existence and precision of the temporal code used by nerve cells to transmit information.

Understanding complex information-processing tasks, in particular the action of selective, visual attention (both saliency-driven bottom-up as well as task-dependent, top-down form) requires a firm grasp of how the problems can be solved at the "computational" level, and how the resulting algorithms can be implemented onto the known architecture of the visual cortex and associated subcortical areas. We use analytical methods, coupled with detailed computer simulations of the appropriate circuitry in the primate visual systems, to study how these neuronal networks give rise to spatial and motion perception and how they are involved in the control of selective visual (focal) attention. Researchers in our laboratory study visual perception in the presence and (near)-absence of selective visual attention as well as our ability to classify and distinguish two-dimensional visual patterns using psychophysical techniques in normal subjects. We are complementing some of these studies using noninvasive fMRI imaging under the identical stimulus protocols. Our laboratory is collaborating with Dr. Itzhak Fried at UCLA in recording single units from multiple electrodes in the medial temporal cortex of awake patients during visual perception, rivalry, and imagery. We continue to grapple with the problem of consciousness and its neuronal correlate in the mammalian cortex and associated structures. We are collaborating with Professors David Anderson (Caltech) and Henry Lester (Caltech) and Michael Fanselow (UCLA) to develop a rodent model of consciousness based on associative fear conditioning.

Because both animals and machines are faced with similar problems when perceiving their environment, strategies devised over the last 500 million years by evolution can help us design better machines. We are exploiting our understanding of early vision in flies by fabricating electronic circuits that implement these tasks in real time. Using analog cMOS VLSI circuit technology, we are designing sensors that determine acceleration (using Coriolis force) and that are fabricated using micro-electronic and mechanical structures (MEMS) technology. Our aim is to endow small flying systems with single chip navigational abilities using both vision and vestibular inputs.
398. Working memory tasks interfere with aversive conditioning in humans

Ronald M. Carter, Christof Koch

Previous studies of human conditioning implicated awareness of CS/US relationship as a prerequisite for conditioning. Clark and Squire (1998) showed that awareness of the CS/US relationship was necessary for eye-blink conditioning in a trace, but not a delay, paradigm. Using a differential aversive conditioning protocol, with auditory CSs and a shock US, we evaluated subjects' abilities to be delay or trace conditioned by distracting them via a working memory task. CSs were 1s long, USs were 250 ms, and the trace period was 3 s. During conditioning there were 76 trials; 4 habituation, 48 acquisition (20 CS+ with US, 4 CS+ only, 24 CS-) and 24 extinction. Concurrent with attempted differential conditioning, subjects were visually presented with a series of random numbers between 1 and 4 and asked to press a key when the current number matched either the previous number (1-back) or the number presented before the previous number (2-back). Key pressures and response times were recorded. Preliminary results indicate subjects can be conditioned using this protocol (trace and delay p<0.01, n=3). If subjects perform a '1-back' task during this procedure there is significant delay conditioning (p<0.05, n=4) but no significant trace conditioning (n=4). When conditioning was attempted using the same protocol while subjects performed a '2-back' task no significant conditioning was found using delay or trace paradigms (n=3). These results indicate a working memory task can interfere with both delay and trace aversive conditioning in humans. They also show that an increase in working memory load (an increase in remembered number stream length from 1 to 2) interferes with aversive delay conditioning.

399. Stimulus encoding and feature extraction by multiple neurons using the electric fish as a model

R. Krahe1, G. Kreiman, F. Gabbiani2, C. Koch, W. Metzner3

Are highly correlated responses of nearby neurons within topographic sensory maps merely a sign of redundant information transmission or do they carry relevant information? To tackle this problem we recorded simultaneously from pairs of electrosensory pyramidal cells (PCs) in a somatotopic map in the electrosensory lateral line lobe of the weakly electric fish, Eigenmannia, while randomly modulating the amplitude of a mimic of the animal's electric field. Previous work had shown that single PCs encode the stimulus time course only poorly. Instead, they extract upstrokes and downstrokes in stimulus amplitude by firing short bursts of spikes which reliably indicate the presence of behaviorally relevant stimulus features. Extending these approaches to pairs of PCs with overlapping receptive fields, we found that: (1) PC-pairs exhibit strong correlations on a time scale of several tens of milliseconds, mainly due to time-locking of spikes to the stimulus and not to common synaptic input.

(2) This was corroborated by Neurobiotin-labeling of primary afferent fibers, yielding an estimated divergence of one afferent fiber onto only 3-8 PCs. (3) In a feature-extraction task, PC-pairs perform significantly better than single PCs. Thus, our results demonstrate that while the occurrence of stimulus features can be reliably indicated by spike bursts of single PCs, this reliability significantly increases by considering stimulus-induced coincident activity across multiple neurons, i.e., by evaluating "distributed bursts" of spikes.

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Reference

400. Single-neuron responses in humans during binocular rivalry and flash suppression

Gabriel Kreiman, Izhak Fried1, Christof Koch

If different stimuli are presented to the two retinas, perception alternates between them in a random fashion (binocular rivalry). A related phenomenon called flash suppression consists of the perceptual suppression of one stimulus that was previously presented monocularly to one eye upon flashing a new stimulus to the other eye. It has been reported that there are neurons in high visual areas in monkeys that modulate their firing depending on what the animal perceives. We investigated the neuronal correlates of these perceptual alternations in humans at the single-neuron level. Subjects were patients with pharmacologically intractable epilepsy implanted with depth electrodes to localize the seizure focus for possible surgical resection. We studied the activity of neurons in the entorhinal cortex, amygdala, hippocampus and parahippocampal gyrus while subjects reported what they saw during binocular rivalry and flash suppression. We observed neurons that fired selectively in response to complex visual stimuli. Most (>60%) of these neurons also fired selectively when the effective stimulus was perceived during the flash suppression paradigm, but not when it was perceptually suppressed by flashing an ineffective stimulus. That is, in two situations with identical visual stimulation, the activity of most neurons followed the perception, significantly enhancing their firing only when the subject saw the effective stimulus. Our results suggest a correlate at the single-neuron level to the seemingly random alterations in these bistable perceptual phenomena in the medial temporal lobe of the human brain.

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401. Synchrony between single neurons in the human brain during sleep
Gabriel Kreiman, Richard J. Staba1, Charles L. Wilson1, Izhak Fried1

In order to study the neuronal mechanisms occurring during sleep, we directly compared the spontaneous electrophysiological activity of ensembles of single neurons in the human temporal and frontal lobes while subjects were awake, during slow wave sleep (SWS) and during rapid eye movement (REM) sleep. Subjects were patients with pharmacologically intractable epilepsy who were implanted with depth electrodes to localize the seizure focus for possible surgical resection. The number of electrodes, as well as their position, was based exclusively on clinical criteria. Electroencephalographic (EEG), electro-oculogram (EOG) and chin electromyogram (EMG) activity were concurrently monitored and used to classify the different sleep stages. We recorded from a total of 141 neurons mostly located in the hippocampus and entorhinal cortex. We observed a significant increase in the number and strength of synchronous discharge among pairs and triplets of neurons during slow wave sleep compared to the other two states. The proportion of pairs of neurons that showed significant synchronous firing was 11.3%, 20.9%, and 8.0% during wake, SWS and REM sleep states respectively. For triplets, the proportions were 2.0%, 8.4% and 3.1% respectively. Such synchronous activity could provide an important physiological mechanism for the transfer of information from the hippocampus to neocortex and the modification of neuronal synaptic strength during sleep.

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References

402. Processing capacity in the human visual system
Rufin Van Rullen, Christof Koch

Of all the objects entering our retina at a given time, only a subset will gain sufficient attentional resources to participate in the aware visual representation of the scene presented. The number of objects accessing short-term visual memory might be even smaller. Finally, it is not known to what extent "ignored" objects (those that do not enter visual awareness) will be processed or recognized. By combining free recall, forced-choice recognition and visual priming paradigms for the same natural visual scenes, and the same subjects, we were able to estimate these numbers, and provide insights as to the fate of objects that are not explicitly recognized in a single fixation. When presented for 250 ms with a scene containing ten distinct objects, human observers can remember around four objects with full confidence. If forced to guess a further number of objects, they can reliably report around two and up to four more objects above chance level. These numbers depend on various factors such as target object size, eccentricity, or familiarity. Finally, even the objects that the subjects consistently failed to report elicited a significant negative priming effect when presented in a subsequent task, suggesting that their identity was explicitly represented in high-level cortical areas of the visual system, before the corresponding neural activity was suppressed during attentional selection. These results indicate that there is no absolute capacity limitation at early stages of object recognition. Rather, attention is given the opportunity to select among high-level object representations which ones are relevant enough to enter visual awareness and short-term memory.

403. Recognizing the gist of a visual scene: possible perceptual and neural mechanisms
Christoph Rasche, Christof Koch

We try to understand the basics of human image processing from a gist recognition perspective. Because the gist is only a subset of the image's information, we think that it is extracted with help of interpretation (feedback). In a perceptual section we list possible mechanism that the interpretation process uses to determine the gist: in addition to the commonly known local-to-global mechanism, there are likely to be also global-to-local, coarse and fine, as well as foreground and background mechanisms. In a neural section we firstly summarize feedback connections that can possibly be involved in gist recognition. Secondly, we propose that the perceptual mechanisms are spread all over the cortex and that visual cortical computation occurs distributive rather than hierarchical.

Reference

404. Swift and flexible neuronal computation through spatial spike-pattern transformations performed in the analog domain
Christoph Rasche, Fidel Santamaria

We observe that neural processing of any cognition or action occurs at a very high speed, widely distributed in the brain, in computational maps and at low spike frequency rates. From these observations we derive a theory on neuronal computation in which map computation occurs computational maps exclusively in the
affected category selective visual responses. We examined investigated how the specific demands of the visual task parahippocampal gyrus, respond selectively to categories structures, such as the amygdala, hippocampus, and 406. Task demand changes the visual category performance dropped significantly in the concurrent task. With our subjects’ performance on each was not correct in (a). We found that when performing both tasks concurrently, our subjects performed around 90% correct in (b) were instructed to prioritize performance on the central categorize the photograph, as containing an animal or not, by releasing a button as fast as they could. The tasks were performed both separately and simultaneously—by first releasing the button and then pressing a key. Subjects were instructed to prioritize performance on the central task. Our subjects performed around 90% correct in (b) and SOA was adjusted to be in the range of 210 ms - 250 ms so that they performed asymptotically at around 80% correct in (a). We found that when performing both tasks concurrently, our subjects’ performance on each was not significantly different from (a) and (b). In a control experiment, where the natural scene was substituted with a stimulus known to require attention, our subjects' performance dropped significantly in the concurrent task. Our findings strongly suggest that ultra-rapid visual categorization of scenes does not require attention.

References

405. Do animals pop-out? Role of attention in ultra-rapid visual categorization
Fei Fei Li1, Ruﬁn Van Rullen, Pietro Perona, Christof Koch
Humans quickly detect animals in images of natural scenes. Naive subjects are as fast and accurate as practiced observers that are familiar with the stimuli. What is the role of attention in this task? We explored this issue using a concurrent-task paradigm. Subjects were shown displays containing both a central arrangement of five similar letters (T,L) followed by a mask, and, simultaneously, the color photograph of a natural scene brieﬂy flashed in a random position at 6° eccentricity (same photographs as in). Five subjects performed two tasks: (a) classify the central letter display as ’same’ or ’different’ by pressing either one of two keys; (b) categorize the photograph, as containing an animal or not, by releasing a button as fast as they could. The tasks were performed both separately and simultaneously—by first releasing the button and then pressing a key. Subjects were instructed to prioritize performance on the central task. Our subjects performed around 90% correct in (b) and SOA was adjusted to be in the range of 210 ms - 250 ms so that they performed asymptotically at around 80% correct in (a). We found that when performing both tasks concurrently, our subjects’ performance on each was not significantly different from (a) and (b). In a control experiment, where the natural scene was substituted with a stimulus known to require attention, our subjects' performance dropped significantly in the concurrent task. Our findings strongly suggest that ultra-rapid visual categorization of scenes does not require attention.

References

407. Attentional modulation of visual motion perception using novel wavelet stimuli
Naotsugu Tsuchiya1, Geraint Rees2, Jochen Braun3, Christof Koch
We have previously characterized the effects of withdrawing attention on detection and discrimination of static visual stimuli. Here we report attentional modulation of motion perception. A novel motion stimulus comprising spatio-temporally contrast-modulated Gabor wavelets was used to distinguish attentional effects on mechanisms sensitive to component motion from those sensitive to pattern motion.

Methods: Multiple moving wavelets were presented as a composite patch (1° diameter) 4° away from fixation. Each wavelet had a random orientation and phase, and moved orthogonally to its orientation. A local wavelet mask of different orientation and contrast was superimposed on each target wavelet. This composite stimulus is expected to selectively activate component motion mechanisms located in primary visual cortex. A concurrent letter discrimination task presented at fixation was used to withdraw attention from the peripheral motion stimulus. Three observers performed a 2AFC target detection task on the peripheral stimulus, while contrast

analag domain. Spikes serve are made to communicate between maps. Spikes thus serve merely as connectors of far distant analog computing maps. Neurons function as dynamical associators of biophysical signals. In a simplified view, such analog maps can transform a spatial pattern of input spikes almost instantaneously into a different pattern of output spikes.

References

406. Task demand changes the visual category selectivity of single neurons in the human medial temporal lobe
Peter Steinmetz, Leila Reddy1, Christof Koch, Itzhak Fried1
Neurons in medial temporal lobe (MTL) structures, such as the amygdala, hippocampus, and parahippocampal gyrus, respond selectively to categories of visual stimuli (Kreiman et al., 2000). In this report, we investigated how the specific demands of the visual task affected category selective visual responses. We examined the responses of single neurons in the MTL while human subjects (patients undergoing epilepsy monitoring with intra-cranial electrodes prior to epilepsy surgery) alternated between two tasks: the first was to identify pictures in a particular category, such as outdoor scenes, from a set of five categories. The second task was to play a video game, which was projected on top of the pictures. During both tasks, the same visual stimuli were presented, although the subjects needed to focus on different aspects of the stimuli in order to perform the requested task. Of 43 cells recorded in three patients, seven (16%) showed category-selective visual responses, in agreement with a previous report. When the subject alternated between playing the video game and identifying pictures in a category, four of the seven cells (57%) with category-selective responses, changed their selectivity. For three of these four cells, responses to a particular picture category were suppressed when the subject was asked to identify pictures rather than play the video game. These results suggest that the specific cognitive and attentional demands of a visual task change both the overall responsiveness of individual neurons in the medial temporal lobe as well as modifying the extent to which the cells are tuned to categories of visual stimuli. These changes may represent a dynamic reorganization of cortical networks to accomplish different visual tasks.

References

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and local direction of the mask wavelets was systematically manipulated.

**Results:** Novel moving wavelet stimuli can be successfully used to characterize the effects of withdrawing attention on component-motion sensitive mechanisms. 1) Contrast increment thresholds for motion detection showed a ‘dip’ function. 2) Withdrawing attention led to elevation of motion detection thresholds that depended systematically on mask contrast but had little dependence on mask direction. 3) Motion detection thresholds with full attention (or without attention) were surprisingly similar whether the mask wavelets moved locally in either the same or opposite directions as the target.

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### 408. Attention as a result of distributed competition by means of a population code

*Fred H. Hamker*

Recordings in V4, IT, MT, MST, PFC and FEF reveal influences of attention on the average rate activity of neurons. However, it is still missing a global picture of the process of attention, i.e., the origin of spatial attention and the interactions between feature-based and spatial attention. We address this issue by means of a computational approach. We assume that the brain operates in parallel on different variables. These variables are represented by the firing rate of cells and implemented by the connections which determine the tuning curve of a neuron. Cells are often broadly tuned to stimulus aspects and show a preferred stimulus, like a preferred direction, but the activity drops progressively when the similarity between the optimal stimulus and the external stimulus decreases. We account for these effects by using a population code. In such scheme, aggregates of temporal spike trains are described by a spatio-temporal population vector. Thus, an attribute of a stimulus is not encoded by the discharge rate of single neurons, but by the distribution of cell activity at specific locations. We apply this method to achieve new predictions for visual attention by comparing the temporal course of simulated neurons to the average spike rate of real neurons. Our model consists of reciprocally connected functional blocks (V4, IT, PFC, FEF). We adjusted the structure and the parameters of this model to match the temporal dynamics of the firing rate as recorded in several experiments, e.g., effect of spatial attention in, and memory guided visual search. All the attentional effects in these experiments can be described without a map that explicitly represents attention—instead attention is an emergent result of the inherent competition in processing stages. According to our model, memory-guided visual search consists of two phases: parallel feature-based identification in PFC+IT and spatial selection in FEF. We predict that spatial attention is tightly connected to premotor movement neurons in the FEF. Their activity provides a cortical feedback signal to bias, in a top-down manner, spatial attention.

### 409. Analysis of immediate early gene C-FOS mRNA in situ hybridization in the mouse brain during trace and delay fear conditioning

*Chih-Ju Han*, *Laurent van Trigt*, *Raymond Mongeau*, *David Anderson*, *Christof Koch*

Patterns of neuronal activity in the brain of male C57BL/6N mice during fear delay and trace conditioning were compared. Trace conditioning is different from delay conditioning in that there is a temporal gap between the end of the conditioned stimulus (CS) and the start of the unconditioned stimulus (US). It has been shown in humans that trace, but not delay, conditioning requires awareness of the US-CS contingency and is dependent on the integrity of the hippocampus. Mice were housed in the training chambers overnight and fear conditioned on the second day (6 x 16-sec 2K-Hz sine-wave tone followed by a 2-sec 0.5-mA footshocks). The temporal gap between the CS and the US in the trace group was 18 sec, while the CS immediately preceded the US in the delay group. Some mice were sacrificed 30 minutes after training for quantification of c-fos mRNA (an indicator of neuronal activity). In situ hybridization was carried out using a cRNA digoxigenin-labeled probe detected by an alkaline phosphatase conjugated antibody. Other mice were tested with the tone the next day in a different context to confirm the efficacy of the trace and delay conditioning. Preliminary analyses indicated more c-fos mRNA expression in the dorsal lateral septum during trace than delay conditioning. Electrophysiological studies in mice suggest that the septum, which modulates hippocampal activity, might be important in predicting if and when a US occurs.

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### 410. Human visual object categorization is best described by a model with few stored exemplars

*Robert J. Peters*, *Alex Backer*, *Fabrizio Gabbiani*, *Christof Koch*

Despite the success of exemplar-based models of categorization, their full complexity is not captured by their statistical number of degrees of freedom: the generalized context model (GCM) stores all training exemplars in memory, whereas a prototype model (WPSM) stores only one memory trace per category, yet both have the same statistical number of degrees of freedom. Since natural visual categories include many exemplars, the GCM’s outperformance of the WPSM might rely on an implausibly dense neural representation. To make memory use more explicit, we developed a radial basis function "roaming exemplar model" (RXM[n]). The RXM[n] follows the GCM/WPSM in classifying exemplars based on a weighted sum of their attention-weighted similarity to stored exemplars, but departs from the GCM/WPSM in allowing the stored exemplars to vary...
in number per category ([n]) and to be refined through training. Thus, the RXM[n]'s degrees of freedom reflect its memory use. We asked human observers (N=9) to classify sets of Brunswik faces (12 sets, each with 2x10 training exemplars for two categories, and 60 test exemplars). We fitted the RXM[n] (with n=1-10 stored exemplars per category), GCM, WPSM, and linear decision bound model (PBI) with individual subjects' data, and assessed the fits using the Akaike Information Criterion (AIC) to penalize models with higher complexity. The best-fitting model was the RXM[1], followed by the PBI and the GCM. Thus, when models' memory use is made explicit, categorization behavior is best described with few stored exemplars, which are refined through training. Unlike the dense representation predicted by the GCM, this result predicts that a sparse neural representation different from prototype abstraction underlies categorization.

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411. Modeling reverse phi-motion selective neurons in cortex: shunting inhibition revisited
Chunhui Mo1, Christof Koch

Reverse phi-motion is the illusory reversal of perceived movement direction when the stimulus contrast is reversed in each successive frame. Direction-selective cells in striate cortex of alert macaque monkeys showed reversed excitatory and inhibitory region when two different contrast bars were flashed sequentially during a two-bar interaction analyses. While motion energy model predicts reverse phi response, it is unclear how neurons accomplish this. We report detailed compartment simulation of direction selective cells in NEURON implementing a synaptic shunting inhibition scheme. Our results suggest that a simple synapse veto-mechanism that result in strong direction selectivity for normal motion cannot account for the reverse phi-motion effect observed in experiments: showing weak direction selectivity for reverse phi presentation in the same direction as normal motion due to cell's low pass nature. Given the nature of reverse phi-motion, a direct interaction between ON and OFF pathway, missing in the original shunting-inhibition model, is essential to account for the reversal of response. We here propose a double synapse veto mechanism in which an ON excitatory synapse is gated by both a delayed ON inhibition at its null side and by a delayed OFF inhibition at its preferred side and the converse for an OFF excitatory synapse. Mapping this scheme onto the dendrites of a direction-selective neuron show that the model cell responds best to normal motion moving in its preferred direction and reverse phi-motion in its null direction.

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412. The implicit measurement of the neural code
Robert Sneddon

The concept of a “Neural Code” is based on the assumption that the brain performs computations. However, this notion of neural computation is generally stated in a vague manner or not at all. In the present work, this assumption is stated in a formal, quantitative manner. This yields an implicit definition of the neural code. Specifically, any form of encoded information must satisfy basic quantitative conditions. These conditions take the form of functional equations that relate the input of a neural circuit to its output. These functional equations are applied to basic neural circuitry, i.e., an integrate-and-fire neuron as well as a thalamic relay neuron. The predicted behavior of thalamic relay neurons was confirmed, as well as that of an integrate-and-fire neuron. This suggests that this theory is an accurate quantitative formulation of basic neural computation.

413. Human synaptic development is optimal
Robert Sneddon

Information processing in the brain is a complex and sophisticated process that would work poorly if synaptic connections were randomly organized. It would be interesting if a developmental organizing principle exists which describes synaptic development. We postulate that the optimal development of synaptic connectivity is this organizing principle and is a likely product of evolution.

From a Kolmogorov structure measure of synaptic development and an a priori assumption of maximizing connective structure (optimal synaptic development quantified), we derive an equation for the growth and pruning of synapses. We compare the values predicted from this equation to those of 18 sets of human cortical data. The predicted values account for 98% of the variance of the observed values. This finding indicates that optimal synaptic development may be a driving principle for human synaptic development.

414. A visual sensor exploiting mechanical vibrations
Ania Mitros, Oliver Landolt, Thoron Stanford

Most animals use eye movements to enhance visual perception. Continuous movements of the retina cause photoreceptors to scan the image continuously instead of acquiring a discrete number of samples, thereby overcoming the spatial resolution limit imposed by photoreceptor spacing. For example, jumping spiders (Salticidae) rely extensively on scanning for visual prey/mate discrimination and route selection.

The same technique can be used to improve the effective resolution of silicon retinas with focal-plane processing. We are developing a visual sensing microsystem combining an analog VLSI retina chip, a mechanical scanning device and inertial sensors. The purpose of this microsystem is to provide autonomous vehicles or robots with relevant visual data for navigation and obstacle avoidance. The scanning motion is provided
either by existing vibrations of the robot or by an active mechanical device. Thereby, spatial fluctuations of light intensity in the image cause temporal fluctuations in the light received by the photoreceptors. Integrating the photoreceptor signal with an inertial measurement of the scanning path, spatial features such as edges can be extracted from the timing of transitions in this temporal signal. The effective resolution obtained using this approach is much higher than pixel spacing would allow in a fixed retina. A custom analog VLSI chip has been developed, which contains an array 32 by 32 pixels capable of detecting temporal fluctuations and encoding them in the timing of spikes. The spike stream generated by every pixel is merged onto a common output bus using a novel address-event communication scheme.

The first version of the circuit was tested. Although most of the circuitry worked within or better than specifications, a noise source early in the signal processing chain necessitated a redesign of that subcircuit. A second version of the chip was designed and submitted for manufacturing (January, 2001) and is currently under testing. Two mechanical scanning devices have been manufactured. The device for actively inducing vibrations is completed and consists of a spinning mirror driven by an electric motor. The other device exploits parasitic vibrations of the robotic platform without consuming additional power, but requires a redesign. The vibration spectrums available on two robotic platforms were analyzed. The hexapod robot RHEX was found to have insufficient high-frequency vibrations, whereas a small gas-powered helicopter shows promise as a potential test platform for the system.

415. Methods and circuits for focal-plane computation of feature in CMOS visual sensors

Alberto Pesavento¹, Christof Koch

Feature detection and tracking is a fundamental problem in computer vision research. By detecting and tracking features in an image sequence it is possible to recover information about both the motion of the viewer and the structure of the environment. We designed, fabricated and tested a CMOS imager with analog VLSI focal-plane computation for feature detection. The chip implements a feature detection algorithm that is suitable for integration in a compact analog VLSI chip. We review the algorithm, its analog VLSI implementation and experimental results from the chip.

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416. A CMOS imager with focal-plane computation for feature detection

Alberto Pesavento¹, Christof Koch

Feature detection and tracking is a fundamental problem in computer vision research. By detecting and tracking features in an image sequence it is possible to recover information about both the motion of the viewer and the structure of the environment. We designed, fabricated and tested a CMOS imager with analog VLSI focal-plane computation for feature detection. The chip implements a feature detection algorithm that is suitable for integration in a compact analog VLSI chip. We review the algorithm, its analog VLSI implementation and experimental results from the chip.

¹Electrical Engineering graduate student, California Institute of Technology

417. Adaptation of current signals with floating-gate circuits

Alberto Pesavento¹, Timothy Horiuchi², Chris Diorio³, Christof Koch

In this paper we present a new, adaptive spatial-derivative circuit for CMOS image sensors. The circuit removes its offset as a natural part of its operation using a combination of electron tunneling and hot-electron injection to add or remove charge on a floating-gate of an auto-zeroing amplifier. We designed, fabricated and successfully tested a chip with the circuit. Test results show that the circuit reduces the offsets by more than an order of magnitude.

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Publications


under anesthesia. There may be a gate that controls the
neurons to the bird’s own song occurred only in birds
few years ago, we found that the response of song system’s
phenomena that might be linked to the dual function. A
auditory functions? We are beginning to see physiological
How and why do the same neurons serve both motor and
know that all song nuclei respond to auditory stimuli.
contractions of the muscles involved in sound production
within this system a pathway that carries pre-motor vocal
signals from the forebrain to the hindbrain. The pre-motor
signals are the precursors of the signals that cause the
contractions of the muscles involved in sound production
and respiration. Many years ago we found that neurons in
one of the forebrain song nuclei responded to sound, particularly the individual bird’s own song. Today we
know that all song nuclei respond to auditory stimuli.
How and why do the same neurons serve both motor and
auditory functions? We are beginning to see physiological
phenomena that might be linked to the dual function. A
few years ago, we found that the response of song system’s
neurons to the bird’s own song occurred only in birds
under anesthesia. There may be a gate that controls the
entry of auditory signals to the song control system. Our
recent study shows that the gate can be closed or opened
depending on the behavioral and physiological states of the
bird. The gate is open during sleep. How does one know
that birds are asleep? We recorded EEG and auditory
neural responses simultaneously. This study clearly
showed that neural responses to playback of the bird’s own
song occurs only during sleep. When birds drifted from
sleep to wakefulness spontaneously, the gate also shifted
from open to close. Such changes can occur within a
fraction of a second. Birds must hear themselves sing in
order to maintain the integrity of song. The gating may be
a reflection of this process.

418. Auditory spatial receptive fields created by
multiplication
José Luis Peña

Examples of multiplication by neurons or neural circuits
are scarce, although many computational models use this
basic operation. The owl’s auditory system computes
interaural time (ITD) and level (ILD) differences to create
a two-dimensional map of auditory space. Space-specific
neurons in the owl’s auditory map of space represent the
results of all computations involved in the determination of
the ITD and ILD that define, respectively, the horizontal
and vertical dimensions of the owl’s auditory space. The
parallel pathways that process these cues merge in the
external nucleus of the inferior colliculus (ICx) to confer
on the space-specific neurons selectivity for combinations
of ITD and ILD. We studied how postsynaptic potentials
from the two sources interact to produce subthreshold and
suprathreshold responses to ITD-ILD pairs. All the space-
specific neurons studied behaved like analogue AND gates
of ITD and ILD, suggesting that the two inputs are
multiplied instead of being added. A multiplication of
separate postsynaptic potentials tuned to ITD and ILD,
rather than an addition, can account for the subthreshold
responses of these neurons to ITD-ILD pairs. Other non-
linear processes improve the spatial tuning of the spike
output and reduce the fit to the multiplicative model.

419. Cochlear and neural delays for coincidence
detection in owls
José Luis Peña, Svenja Viete, Kazuo Funabiki,
Kourosh Saberi1, Masakazu Konishi

The auditory system uses delay lines and
coincidence detection to measure the interaural time
difference (ITD). Both axons and the cochlea could
provide such delays. The stereoeasis theory assumes that
differences in wave propagation time along the basilar
membrane can provide the necessary delays, if the
coincidence detectors receive input from fibers innervating
different loci on the left and right basilar membranes. The
owl’s nucleus laminaris contains coincidence detector
neurons that receive input from the left and right cochlear
nuclei. If the hypothesis were true, the left and right inputs
to the laminaris coincidence detectors should differ in their
frequency tuning. Monaural frequency tuning curves of
nucleus laminaris neurons showed small interaural
differences. Their preferred ITDs were, however, not correlated with the interaural frequency mismatches. Instead, the neuron’s preferred ITD agreed with that predicted from the distribution of axonal delays. Thus, there is no need to invoke mechanisms other than neural delay to explain the detection of ITDs by the barn owl’s laminar neurons.

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420. Intracellular recording from coincidence detector neurons in owls.

Kazuo Funabiki

The owl’s auditory system measures interaural time differences with a microsecond resolution. The site of this computation is the nucleus laminaris in which neurons respond according to the degree of coincidence of spikes from the two ears. Intracellular recording from these neurons is essential for understanding the biophysical mechanisms of coincidence detection. Since ordinary microelectrodes break en route to the nucleus laminaris, we developed a system of concentric electrodes in which the outer electrode guides and protects the inner electrode until this reaches the target area. Preliminary results showed outward-rectification in response to current injection, indicating that intracellular recording was achieved.

421. Retention of song memory without hearing

Yasuko Funabiki

Learning of song by young birds resembles speech development in human infants. Song learning takes place during a restricted period as in speech acquisition. Hearing one’s own voice or auditory feedback is essential for vocal learning in both songbirds and humans. Song learning consists of two phases; early memorization of a tutor song and later vocal reproduction of the song. In zebra finches, young birds memorize the song of their fathers before 35 days of age and start singing around 40 days of age. The completion of vocal development is called song crystallization, because song does not change for life after this stage. It takes place at about 90 days of age in zebra finches.

The question this work addressed is whether and how long the memory of a tutor song survives, if the bird is made incapable of hearing itself sing. I transferred young zebra finches from their own parents or foster parents of another species to sound proof boxes at 35 days of age. The birds were exposed to loud noise of 110 dB until they were 110 to 200 days of age. This treatment not only prevented the birds from hearing their own voice but also raised their hearing threshold. During this period the birds sang unstable songs that did not resemble the tutor songs. However, when the noise exposure was stopped and as the hearing threshold returned to normal, the birds gradually replaced the unstable songs by song patterns that resembled the tutor models. These results show three important points:

1. The birds could retain the song memory without hearing.
2. The song memory can last long.
3. The plasticity to shape song after the tutor model lasts well beyond the normal time of song crystallization.

422. Rapid modulation of forebrain sensory processing during the sleep-wake transition in songbirds

Teresa A. Nick

The song nucleus HVC (High Vocal Center) sends neural signals for song production and receives auditory input. Using combined measures of electroencephalogram (EEG) and electrooculogram to objectively identify wake/sleep state in zebra finches, we show that HVC activity as well as auditory responses change with physiological states. We used chronically-implanted multi-unit electrodes because neuronal signals can be maintained over long time periods during which spontaneous state changes occur. HVC neurons responded selectively and robustly to playback of the bird’s own song during sleep despite reduced baseline activity. Upon waking, HVC response to song dramatically declined. Similar state-dependent phenomena in downstream nuclei, such as robustus archistriatalis, are likely to be derivatives of those in HVC. Both spontaneous and forced waking during sleep caused HVC auditory responses to cease concomitant with an EEG-measured state change: We found that HVC activity induced by the bird’s own song decreased within 750 msec of light-elicited waking (for three birds, bird woke with light vs. bird did not awaken with light: p < 0.05; for two birds, bird woke with light vs. no light (bird did not awaken): p < 0.05). Such rapid modulation of HVC activity indicates tight temporal control of forebrain processing by brainstem nuclei.

423. Neural dynamics in a feedback control nucleus

Anthony Leonardo

Juvenile zebra finches use auditory feedback to match their own vocalizations to a song template memorized from a tutor. Recent reports have shown that the crystallized song of adult birds can be destabilized by perturbing singing with altered auditory feedback (Leonardo and Konishi, 1999). This song decrystallization demonstrates that auditory feedback is not simply necessary to acquire song but rather is used actively through out life for song maintenance. The neural mechanisms which underly this feedback control process remain poorly understood. However, behavioral and lesion studies indicate that auditory feedback is brought to the motor control system via the projection from nucleus LMAN to RA (Brainard and Doupe, 2000; Scharff and Noltebohm, 1991). Using a newly-developed feedback perturbation system, and a three-channel motorized microdrive (Fee, 2001), we record from up to three simultaneous neurons in nucleus LMAN while adult zebra finches are singing normally and in the presence of auditory feedback triggered to selected aspects of their song. An active sound cancellation system is used to
"denoise" the feedback perturbation from the microphone signal, leaving only the bird's own vocalizations. This permits us to study the timing dynamics in the entire population of recorded neurons from each bird by using the features in the song to time-align the firing patterns of neurons recorded serially. Preliminary results suggest LMAN firing patterns have less sensitivity to feedback perturbations than expected.

Publications
We are interested in information coding in the brain and in the design principles of circuits involved in processing sensory information. We are particularly interested in understanding the role of time, synchronization and oscillations in information coding and in relating the biophysical properties of neurons and synapses to the function of the networks in which they are embedded. We therefore study the cellular, synaptic and network aspects of neural processing, and focused our research this year on four different model systems: the olfactory system of insects (antennal lobes and mushroom bodies, circuits analogous to the vertebrate olfactory bulbs and anterior/posterior piriform cortices); the olfactory bulb of rats; the olfactory bulb of zebrafish; and the motion and anterior/posterior piriform cortices); the olfactory bulb of zebrafish; and the motion bodies, circuits analogous to the vertebrate olfactory bulbs and anterior/posterior piriform cortices); the olfactory bulb of rats; the olfactory bulb of zebrafish; and the motion sensitive part of the visual system of insects. Our work combines experimental (behavioral and electrophysiological) and modeling techniques and aims at understanding functional aspects of brain circuits design and the rules of information coding used by the nervous system.

424. Neuronal priming in olfaction
Alex Bäcker, Gilles Laurent

We have confirmed our previous preliminary report of priming in responses of projection neurons in the antennal lobe of the locust after exposure to an odor at supra-threshold concentrations. In addition, we have found this effect to be odor-specific, restricted to concentrations close to the primer, excitatory or inhibitory, lasting from 10 minutes to an hour and requiring a primer of supra-threshold concentration. The transient nature of the effect suggests it may be used to bias olfactory detection and discrimination toward an odor perceived recently during a previous sampling of the environment.

Reference
Reference

427.  Model of cellular and network mechanisms for odor-evoked temporal patterning in the locust antennal lobe
M. Bazhenov, M. Stopfer, M. Rabinovich, H.D.I. Abarbanel, T.J. Sejnowski, G. Laurent
Locust antennal lobe (AL) projection neurons (PNs) respond to olfactory stimuli with sequences of depolarizing and hyperpolarizing epochs, each lasting hundreds of milliseconds. A computer simulation of an AL network was used to test the hypothesis that slow inhibitory connections between local neurons (LNs) and PNs are responsible for temporal patterning. Activation of slow inhibitory receptors on PNs by the same GABAergic synapses that underlie fast oscillatory synchronization of PNs was sufficient to shape slow response modulations. This slow stimulus- and neuron-specific patterning of AL activity was resistant to blockade of fast inhibition. Fast and slow inhibitory mechanisms at synapses between LNs and PNs can thus form dynamical PN assemblies whose elements synchronize transiently and oscillate collectively, as observed not only in the locust AL, but also in the vertebrate olfactory bulb.

Reference

428.  Model of transient oscillatory synchronization in the locust antennal lobe
M. Bazhenov, M. Stopfer, M. Rabinovich, R. Huerta, H.D.I. Abarbanel, T.J. Sejnowski, G. Laurent
Transient pairwise synchronization of locust antennal lobe (AL) projection neurons (PNs) occurs during odor responses. In a Hodgkin-Huxley-type model of the AL, interactions between excitatory PNs and inhibitory local neurons (LNs) created coherent network oscillations during odor stimulation. GABAergic interconnections between LNs led to competition among them such that different groups of LNs oscillated with periodic Ca$^{2+}$ spikes during different 50-250 ms temporal epochs, similar to those recorded in vivo. During these epochs, LN-evoked IPSPs caused phase-locked, population oscillations in sets of postsynaptic PNs. The model shows how alternations of the inhibitory drive can temporally encode sensory information in networks of neurons without precisely tuned intrinsic oscillatory properties.

Reference

429.  Evolution of odor representations over time and across consecutive brain structures
Stijn Cassenaer
The activity patterns of projection neurons (PNs) in the insect antennal lobe (AL) are modulated in an odor-specific manner that changes over the duration of the stimulus (Laurent, 1996). Hence, the representation of odors, in terms of PN activity, changes over time as well. The dynamic nature of these representations was studied recently in the zebrafish olfactory bulb (OB) (Friedrich and Laurent, 2001). The authors found that chemically-related odors formed clusters in terms of the activity recorded from mitral cells (MCs) in the OB at the onset of stimulus presentation, but were progressively de-correlated during the stimulus interval. That is, the ensemble representations of similar odors were similar during the first few hundred milliseconds of the response, but became subsequently more specific. The implication is that, while the MC responses initially follow receptor neuron activity, they then proceed to occupy the coding space more efficiently as a consequence of the circuit dynamics. Also, this could afford an initial general classification of the odor presented, followed by finer discrimination. In the present work, we want to determine whether the slow temporal patterning of locust PN activity undergoes a similar development. The odors used in the Friedrich and Laurent study were all amino acids. For the locust experiments we are using so-called green odors (Visser and Ave, 1978), namely (alkyl and alkylene) alcohols, ketones, and aldehydes, ranging in carbon chain-length from 5 to 11. Preliminary data suggest that in the locust AL (similar to the zebrafish OB) odors cluster in PN activity space. However, unlike in the zebrafish OB, it appears that different odor-clusters form during the stimulus interval. That is, a given odor appears to excite or inhibit the same collection of PNs as a subset of the other odors at one point, and then share its pattern of PN responses with another subset of odors at a later point. Rather than a de-correlation between related odors during the stimulus interval, we observe changes in the identity of the odors with which a given odor response pattern is correlated.

Experiments currently carried out by Javier Perez-Orive investigate the role of oscillatory synchronization, by recording odor responses from Kenyon cells (KCs), which decode PN activity. A prominent difference between the representation of odors by PNs and KCs, is that the latter is much more sparse. To contrast the KC odor response properties with the PNs' we are making tetrode recordings in the AL, and are characterizing the density of odor representations there. Specifically, we compare the average probability that a given PN or KC will respond to a given odor. In order to assess odor response promiscuity, we are evaluating the average conditional probability that a PN will respond to multiple odors, given that it responds to at least one of the odors tested. Since KCs' odor responses are sparser in the time domain as well, we also compare the response duration in PNs and KCs.

References
430. Odorant information and the alpha lobe
Sarah Farivar
My aim is to study one particular output of the second relay station in the locust olfactory system: the alpha lobe of the mushroom body (MB). The intrinsic neurons of the MB, the Kenyon cells, send their outputs to the two lobes of the MB, the alpha and beta lobes. The alpha lobe cells are extrinsic neurons of the MB - they lie outside of the MB and at least some of their neurites are within in the alpha lobe of the MB. I am specifically using intracellular recording in vivo to characterize the particular responses of these alpha lobe cells to odors, if any exist. This characterization includes determining odor specificity and turning, delineating firing characteristics, and describing the correlation of the responses to that of the populations of cells in the other relay stations. I am also using dye injection and staining to characterize the morphology, locations of cell bodies and outputs of these cells.

431. Firing statistics of simultaneously-recorded olfactory projection neurons in the locust antennal lobe
Ofer Mazor, Christophe Pouzat, Gilles Laurent
We examine the firing properties of projection neurons (PNs) in the antennal lobe of the locust using silicon multi-electrode probes and a new automatic spike-sorting algorithm. Because PNs are the only spiking neurons in this network, cell type identification is unambiguous. We show that, at rest, the firing behavior of PNs is not well described by Poisson statistics and yet, remarkably simple to describe probabilistically. The firing statistics of each PN can be fully described by a "hazard" function and ca. five parameters used to construct it. This characterization applies to all PNs tested across a population of ca. 800 neurons. It describes the continuum of firing properties (from apparent burstiness to tonic firing) observed across PNs and over time.

With this description of single PN behavior at hand, we can detect and test the significance of interactions among simultaneously recorded PNs. We find that even at rest, there are significant positive and negative correlations between most pairs of PNs and that these correlations are present over a wide range of timescales (1ms - 30s). Furthermore, we find clusters of multiple PNs all with positively correlated firing patterns, and with negative correlations across clusters. We are using this method of analysis to gain insight into the internal dynamics of the insect olfactory system, and how these dynamics encode stimulus information. To this end, we are currently examining changes in PN firing behavior and in PN-PN interactions evoked by odor stimuli.

432. Decoding of neural information with oscillatory synchronization
Javier Perez-Orive, Gilles J. Laurent
Although oscillations are observed in the brain areas of many different animals, their functional role is not clear. In the insect olfactory system there are oscillations in the local field potential (LFP) reflecting the existence of odor-specific synchronized dynamic ensembles of neurons (Laurent et al., 1996). Information dependent on oscillatory synchronization of neuronal assemblies has been shown to be functionally relevant (Stopfer et al., 1997). We are interested in looking at the role of oscillatory synchronization in neural coding from a decoding perspective.

The mushroom body (MB) is an insect brain neuropil which receives directly these dynamical inputs and which is involved in olfactory discrimination and memory. We used wire tetrodes to obtain extracellular recordings of the intrinsic cells of the MB (called Kenyon cells, KC). By applying odor stimulation to the locust and recording KC activity, we observe that KCs act as highly specific and precise pattern recognizers: 1) they have very specific olfactory responses, with very few of them responding to more than two odors out of all that were presented; 2) KCs have a very low baseline firing rate, and when they do respond to an odor, it is usually with only one or two spikes; 3) these few spikes of the response are very precisely timed and phase locked to the LFP oscillations.

These results suggest that KCs are acting as transient integrators over a narrow temporal window, which is referenced with respect to the LFP oscillations. Several cellular and circuit properties seem to contribute to this KC behavior. Intracellular recordings of KCs have showed that they have intrinsic biophysical properties which decrease their integration time constant and increase their sensitivity to input coincidences, as their synaptic drive is increased. Glenn Turner, another member of the lab, has found a group of interneurons which seem to be providing non-specific feedforward inhibition to the KCs, counterphase to the excitation they receive. Therefore, it appears like the cellular and circuit properties of the KCs combine with the temporal properties of their input to make them function like an adapting temporal filter, which integrates its input over a very precisely determined temporal window within each cycle of the LFP oscillation. The emerging picture is then that of the MB remapping and sparsening olfactory representations in a manner that is presumably convenient for discrimination and storage of the information. These results provide a specific role for oscillatory synchronization in the encoding and decoding of neural information.
In the locust and other insects, controlled odor presentation in insects and fish, we investigate neural networks whose dynamics in phase space is represented by orbits near the heteroclinic connections between saddle regions (fixed points or limit cycles). These networks encode input information as trajectories along the heteroclinic connections, and these orbits are global attractors under general conditions. We call this behavior winnerless competition (WLC). If there are \( N \) neurons in the network, the capacity is approximately \( e(N-1) \), i.e., much larger than that of most traditional network structures. WLC networks use time as a key element in encoding input information. We show that a small WLC network composed of FitzHugh-Nagumo spiking neurons efficiently transforms input information into a spatiotemporal output. Beyond their biological connection and relevance, these networks offer an interesting strategy for computing with separatrices.

References

433. Dynamical encoding by networks of competing neuron groups: Winnerless competition
M. Rabinovich, A. Volkovskii, P. Lecanda, R. Huerta, H.D.I. Abarbanel, Gilles Laurent
Following studies of olfactory processing in insects and fish, we investigate neural networks whose dynamics in phase space is represented by orbits near the heteroclinic connections between saddle regions (fixed points or limit cycles). These networks encode input information as trajectories along the heteroclinic connections, and these orbits are global attractors under general conditions. We call this behavior winnerless competition (WLC). If there are \( N \) neurons in the network, the capacity is approximately \( e(N-1) \), i.e., much larger than that of most traditional network structures. WLC networks use time as a key element in encoding input information. We show that a small WLC network composed of FitzHugh-Nagumo spiking neurons efficiently transforms input information into a spatiotemporal output. Beyond their biological connection and relevance, these networks offer an interesting strategy for computing with separatrices.

Reference

434. Encoding of odors in turbulent air
Mark Stopfer, Gilles Laurent
In the locust and other insects, controlled odor pulses evoke fast oscillatory synchronization of ensembles of projection neurons (PNs). Superimposed upon these fast (~20 Hz) oscillations are slower, odorant-specific spiking patterns that consist of sequences of excitation, inhibition and quiescence. These features have been shown to contain information about the identities of odorants. Further, the precision of encoding is enhanced when odor pulses are repeated.

To investigate whether these forms of temporal coding occur when odorants are carried in a more natural fashion, we presented odorants within a small wind tunnel (100 x 30 x 20 cm; wind speed: 20-50 cm/sec). Natural odor sources such as fresh flowers or vials of pure or diluted odorants ("green" chemicals such as 1-hexanol, 1-heptanol, and 1-hexen-3-ol or common blends such as cherry and mint) were placed at the upwind end of the tunnel, and a locust with a fixed antenna was placed at the downwind end. To monitor the arrival of windborne odor filaments near the locust's intact antenna, we recorded electroantennograms (EAGs) from a second, isolated antenna wired to a DC amplifier. Simultaneously, we made intracellular recordings from pairs of PNs and local neurons (LNs) and extracellular recordings of the local field potential (LFP) from the mushroom body, a PN projection site. (The LFP provides an indication of synchronized PN firing).

Results from 209, 3 min trials in 20 animals indicate: odor filaments caused distinct, transient deflections in the EAG. In all trials, EAG deflections coincided with distinct bouts of ~20 Hz oscillation in the LFP that were phase-locked to subthreshold oscillations in the LNs and PNs. When PNs responded to the odorant, spikes were generally phase-locked to the LFP. Odorant-specific slow temporal patterns were evident in the firing of PNs. Oscillations and phase-locking increased progressively with filament encounters. Thus, complex and disorderly stimuli can evoke odorant-encoding ensemble activity.

435. The role of lateral horn interneurons in decoding oscillatory activity of projection neurons
Glenn C. Turner, Gilles Laurent
In the locust, odor presentation evokes synchronous oscillatory firing of an assembly of antennal lobe projection neurons (PNs). Both the identities of the neurons in the ensemble and the relative timing of their activity encode information about the stimulus. To better understand how PN ensemble activity is decoded at later processing stages in the locust brain, we have been attempting to identify cells downstream of PNs. PNs extend processes into the lateral horn, a poorly characterized area of the insect brain, in addition to their synapses on Kenyon cell (KC) dendrites in the calyx of the mushroom body. We have identified a group of odor-responsive cells with projections in both the calyx and the lateral horn. It is likely that these cells receive direct input from PNs in the lateral horn, and send their output to the KCs in the calyx. Our analysis suggests that these cells play an important role in decoding PN spike trains by providing delayed feedforward inhibitory input to the KCs that enhances the sensitivity of KCs to coincident PN input.

Intracellular labeling shows that LHI output projections are well suited to be the source of at least some GABAergic inhibitory inputs to KC dendrites (Leitch and Laurent, 1996). Intracellular recordings show that LHIs exhibit strong odor-evoked membrane potential oscillations that are phase-locked to the oscillating local field potential in the calyx, and that LHI spikes occur 180° out of phase with PN spikes. LHIs respond to all odors tested, and show little if any odor-specific temporal patterning. Thus on each odor presentation KCs will receive EPSPs from a specific PN ensemble followed one-half cycle later by a volley of IPSPs from LHIs. This inhibitory input is predicted to reduce the sensitivity of KCs to out of phase PN spikes, thereby narrowing the window of KC excitability. Since the timing of the IPSPs is inherently locked to the timing of PN activity, the KCs automatically adapt their integration window to the period of maximum PN synchrony regardless of the precise frequency of PN oscillations. Thus LHI activity
constitutes a means of adaptively tuning the sensitivity of KCs to coincident oscillatory input, an important component of decoding oscillatory activity of PN ensembles.

Reference

Publications


We found that adaptation to a visual stimulus configuration which gives rise to surface "filling-in" lead to an afterimage of the filled-in surface, even though the area of the surface is beyond the retinal area that had been adapted. This was very counter-intuitive because it was against the traditional notion that the mechanism underlying any kind of afterimage is mainly in the retina. Due to results of some control experiments, we concluded that this at least partly reflects adaptation at the cortical level to an afterimage of the filled-in surface, even though the configuration which gives rise to the "filling-in" lead to an afterimage in the domain of both space and time. To be more specific, we recently found that the way TMS interacts with visual inputs is different between sustained and transient visual inputs, and that the interaction occurs at relatively early cortical levels.

(3) Microscopic psychophysics of visual perception in a very brief time period (1-200 ms) has revealed how the visual system identifies transient visual input in the context of a sustained, or continuously changing, frame of reference. The initial finding of the "flash lag effect" in motion perception has been generalized to other visual attributes such as luminance, color, and spatial frequency. Thus, the feature of the flashed object is judged by subjects as synchronous with a feature of the sustained object that is present at a later time. We have applied the flash lag paradigm to different situations, such as the slit view, and the "elbow illusion" to further understand how the visual system integrate and segregate streams of dynamic visual inputs.

(4) Utilizing our technique combining transcranial magnetic stimulation (TMS) and psychophysics, we have further isolated the exact spatio-temporal details of the visual cortical processes that are directly responsible for pattern and feature filling-in in the domains of both space and time. To be more specific, we recently found that the way TMS interacts with visual inputs is different between sustained and transient visual inputs, and that the interactions are surprisingly non-linear. The results can be compared with our own biophysical model of TMS.

(5) Comparison between infants and adults in cross-modal integration has revealed both qualitative similarities and quantitative differences between them. Although still very preliminary, it is likely that while adults show early and non-linear interaction between visual and auditory inputs, young infants seem to show either more independence or mere summation. Also, we developed a new preferential looking method based on 4-, instead of 2-, alternative forced-choice (AFC) task, which turned out to be more effective and concise than the traditional 1 AFC.

(6) We recently develop a new behavioral paradigm to study the intrinsic and dynamic relationship between bodily, orienting action and emotional judgments such as liking. As the baseline finding, we found that the observer's eye gaze is increasingly more towards the stimulus (a face) which the observer would eventually judge as more attractive in about 500 ms immediately before the preference judgment. Since this type of "cascade" like effect can not be observed in other tasks such as disliking or mere geometric judgment, it seems to be intrinsically related to the affection or attractiveness process. Thus, it is not only that we see an object more because we like it more, but also that we like it more because we see it more. The whole conscious events of emotional experience may rely heavily on this type of interactions below the threshold of consciousness. We are now extensively investigating how robust and generic this "cascade" effect is.

(7) We compared pupilary responses to color flicker in normal and photo-sensitive subjects. The results indicate surprising sensitivity.

(8) Visual coordinate transformations due to eye or visual-frame movements were examined. Also, effects of pop-out by color and orientation were compared and
turned out to be opposite in sign (the former improves, whereas the latter degrades) in the discrimination task performance. These results are informative about dynamic visual processing at intermediate levels.

We will maintain our efforts, with emphasis on a combination of techniques, a wider variety of subjects, the developmental aspects, and the dynamic/adaptive aspects of processing, to further understand visual perception in its relationship to the motor and other perceptual modality systems.

436. A visual illusion induced by sound

L. Shams

Traditionally, vision has been considered the dominant modality in our multi-sensory perception of the world. This view is primarily based on prior observations that in presence of a conflict between the information conveyed by the visual system and other modalities, the overall percept is determined by vision, or the quality of the percepts in the conflicting modality is modified by the visual information, and not vice versa. We report data that counter these views, by showing that auditory information can change the visual percept qualitatively (causing a visual illusion) and this effect occurs even in the absence of ambiguity in the visual domain. We have found a visual illusion which is induced by sound: when a single flash is accompanied by multiple auditory beeps, the single flash is incorrectly perceived as multiple flashes. We refer to this phenomenon as illusory flashing. Our experiments confirmed that the illusory flashing phenomenon is indeed a perceptual illusion. We next investigated the temporal properties of this illusion by varying the relative timing of visual and auditory stimuli. The illusory flashing effect declined from 70 ms separation onwards. However, illusory flashing occurs so long as beep and flash are within approximately 100 ms, consistent with the integration time of polysensory neurons in the brain. Others have shown that the perceived direction of motion of an ambiguous visual stimulus is influenced by auditory stimulation. Our work extends this previous finding by showing that the visual perception can be radically altered by sound even when the visual stimulus is not ambiguous. It is noteworthy that the conditions under which we have found this alteration to occur are by no means peculiar. To the contrary, the stimulus configuration as well as the task are very simple. The illusion is also surprisingly robust to variations in many parameters we manipulated (e.g., flashing disk’s eccentricity and contrast, spatial disparity between sound and flash, shape and texture of the flashing pattern, flash and beep durations). These findings suggest that the illusory flashing phenomenon reflects a fundamental and widespread property of polysensory integration in the brain.


437. Sound modulates visual evoked potentials in humans

L. Shams, Y. Kamitani, S. Shimojo

Purpose: We have previously shown that sound can alter the visual perception radically and qualitatively (1). It is not clear, however, at what level of perceptual processing the interaction between the signals of different modalities occurs. This interaction can occur anywhere from the early sensory processing to late modality-specific to polysensory stages. We aimed to investigate whether the cross-modal influences on visual perception occur at the level of visual pathway, or rather the visual sensory activity is independent of the signals of other modalities as is generally believed. To this end, we compared the visual evoked potentials (VEP) in the presence and absence of sound. Methods: We measured the VEPs of seven participants in three different conditions. Trials, 120 per condition, were randomized. Condition V consisted of visual stimulation (a white disk of 2° size flashed at 5° eccentricity on a black background on a CRT). Control condition A consisted of auditory stimulation (a brief sound lasting for 7 ms played from a speaker adjacent to the screen). Condition AV consisted of audio and visual stimulation; corresponding to the combination of the stimuli in conditions A and V. VEPs were recorded from electrodes MO, O1, and O2. For each electrode, the average of the difference wave AV-(A+V) was calculated across participants. This difference wave was taken as the measure of change in the activity of the visual areas by sound. Results: We found that difference wave had an amplitude which differed from zero significantly between 100 and 200 ms poststimulus. Conclusion: Considering that VEPs prior to 200 ms are believed to be due to modality specific neural activity, these results suggest that the activity in visual cortex is modulated by presence of sound. This implication challenges the general belief that the visual cortical processing is independent of other modalities.


Reference


438. Sound-induced visual "rabbit"

Y. Kamitani, S. Shimojo

Purpose: We previously reported that a single visual flash presented with multiple beeps with short intervals appears to be multiple flashes (1). Do the beeps produce mere impression of brightness fluctuation, or rather independent visual tokens? To demonstrate that the illusory flashes can be perceived independently at different spatial locations, a visual apparent motion display was combined with beeps. Methods: Two vertical bars (13 ms duration each) were flashed 3° horizontally apart with an interval of 106 ms, creating clear apparent motion. Each bar was accompanied by a beep (10 ms duration, simultaneous onset with the bar). Another beep was presented at a various timing between the flashes/beeps.
In a condition without sound, a real additional bar was flashed at an in-between timing at the same position as either of the two bars. Subjects reported the perceived location of the illusory bar associated with the second beep (if any), or that of the additional real bar. The direction of apparent motion was randomized across trials. Results: All subjects (3) reported that an illusory bar was perceived with the second beep at a location between the real bars (difference from the real bars, p < .05). This is analogous to the cutaneous "rabbit" illusion where trains of successive cutaneous pulses delivered at a few widely separated locations produce sensations at many in-between points. The illusory bar appeared closer to the first (second) bar, as the second beep was presented temporally closer to the first (third) beep. The perceived location of the additional real bar was not significantly different from the actual location, thus purely visual "rabbit" was not observed. Conclusion: The sound-induced visual flashing creates visual tokens that can be spatially displaced, indicating visual proper, as opposed to cognitive, alteration, which nonetheless is uniquely induced by sound.


**Reference**


**439. Filling-in induced by high-contrast edge adaptation**

*S. Shimojo, Y. Kamitani*

Purposes: We found that adaptation to an object with high contrast edges delays detection of a subsequent one with low contrast edges at the same location. During this delay the observer perceives only the background which is filled into the test region. We aimed to measure the effect as a function of adapting duration, and to determine if it should be attributed to the afterimage interfering/canceling the test, or rather to failure of edge detection due to adaptation, which leads to filling-in. Methods: A white disk (42.0 cd/m2, 2.8 diameter) was presented at 4.8° eccentricity on a gray background (17.3 cd/m2) for 2, 4, 8, or 16 sec. It was turned off briefly (93 ms) every 500 ms to minimize afterimage formation. Then, a slightly brighter gray disk (18.8 cd/m2) of the same size was presented at the same location. In a 10-sec test period, the subjects pressed a button as long as they saw the test disk, an afterimage, or both. In separate control experiments, they monitored visibility of (a) afterimage on a homogeneous gray background, or (b) the test disk without adaptation. Results: (1) The total invisible time increases as a function of adapting duration, up to 6-7 sec (p < 0.02, 0 vs. 8 or 16 sec. adaptation). (2) Afterimage was visible (up to 4-5 sec with longer adaptation), yet shorter than the total invisible time in the main experiment, at some or all durations in 4 of 5 observers. Discussion: The result (1) can not be explained by visible afterimage since by definition of the task, it would increase (or maintain the same duration of) button pressing. The result (2) is inconsistent with the idea that the test disk and the afterimage cancel each other, which would require the afterimage duration same as, or longer than, the invisible time. The effect may rather be related to failure of edge detection due to peripheral and/or cortical adaptation, which then leads to surface filling-in at a higher-level boundary-based representation.

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**440. A model of magnetic stimulation of neocortical neurons**

*Y. Kamitani, V.M. Bhalodia, Y. Kubota, S. Shimojo*

Neurocomputing Transcranial magnetic stimulation (TMS) has been widely used in studies of human motor and cognitive functions as well as in clinical treatment. Biophysical mechanism underlying its effect is, however, largely unknown. Here, we develop a theory to calculate the effect of magnetic stimulation on arbitrary neuronal structure. Then, we employ a computer simulation which combines a realistic multicompartment model of neocortical neurons and the calculation of the induced electric field. The simulation shows that a single magnetic pulse applied to model cortical neurons can induce brief burst firing followed by a silent period of duration comparable to experimental data of TMS. Our simulation offers a new clue to understand physiology of TMS by demonstrating that magnetic stimulation acts on biophysics of the dendrites in neocortical neurons. *Neurocomputing, 38-40, 697-703, 2001.*

**441. Interaction of TMS-induced phosphenes and visual stimuli**

*D.-A. Wu, Y. Kamitani, F. Maeda, S. Shimojo*

Purpose: Whereas single-pulse transcranial magnetic stimulation (TMS) of the human visual cortex results in scotoma, dual-pulse TMS results in the transient visual percept of an area of brightness, termed a phosphene. In this study, we presented disk-shaped visual stimuli that overlapped the phosphene in the visual field. Subjects evaluated the brightness of the overlapping region when dim or bright disks were either flashed or steadily shown. Procedure: Dual magnetic pulses were delivered by a Neotonus Neopulse stimulator. The coil position over the left occipital lobe, power, and inter-pulse interval were optimized for phosphene induction. Trials began with a dark background (<=0.1 cd/m²), then a large homogeneous disk (15° in diameter) was either: flashed for 93 ms and followed by TMS (40 ms after flash offset), or shown steadily for 6 s, during which (at the 4th second) TMS was triggered. The disks were set either dimmer or brighter than phosphenes perceived on a dark background. The four conditions (flashed/steady x dim/bright) were randomly repeated five times each. After each trial the disk was redisplayed and subjects adjusted the luminance of the region that overlapped with the phosphene to match their percept. Results: 1) For both dim and bright flashed...
disks, the region overlapping the phosphene was reported to be brighter than the rest of the disk (p <.01). However, in steady displays the region was darkened in bright disks, but brightened in dim disks (p <.01). 2) With the 40 ms delay between the flashed disk and TMS, which maximizes the brightening effect, the disappearance of the disk was perceived first, followed by the simultaneous perception of the phosphene and its brighter spatially overlapping region. Discussion: Phosphenes clearly cannot be considered a mere brightening of the visual field. Phosphenes show non-linear brightness interactions with visual stimuli that are both physically and perceptually non-concurrent.

Funding: NIH/ERS and Anonymous donation to Caltech.

442. An enhanced preferential looking method for infant studies
L.B. Shams, S. Shimojo

Purpose: Preferential Looking (PL) method has remained one of the most important psychophysical methods of infant research since its inception in 197X. This method suffers, however, from a serious practical shortcoming. This method relies on a two alternative force choice (AFC) scheme for individual trials. As a result, a large number of trials are required for achieving statistical significance. We have explored a variation of PL which operates upon 4-AFC experimental trials. As the chance level in each trial is .25 (as opposed to .5 in the 2-AFC counterpart), the number of trials to achieve significance is drastically reduced. We used a grating acuity test to verify the feasibility of the carrying out a 4-AFC gaze judgment in infants. Methods: The grating acuity of 10 infants (8-16 weeks of age) was tested using both the conventional PL method, and our 4-AFC variation. The stimuli in the conventional PL test consisted of one circular patch of 100% contrast square wave gratings as the target, and another patch of the same size and shape but containing uniform graylevel value set to the average of the corresponding target brightness. The position of the two patches was five to the left and right of a fixation point in the center of the screen. The stimuli in the 4-AFC condition were identical to those in the 2-AFC condition, except for the number of distractors—four instead of two. The two additional positions were five to the top and bottom of the fixation point. The four patches hence, formed the vertices of a diamond (with the fixation point in the center). In each trial the target appeared at random in one of the four possible positions. Babies were seated in a car seat 57 cm away from the monitor in a dark room, and the judgments were made by the consensus of the two experimenters from a peephole who were not aware of the stimuli on the screen.


443. Do we like what we see more, or do we see more what we like?
C. Simion, C. Scheier, E. Shimojo, S. Shimojo

Goal: To investigate the interplay between orienting eye movements and emotional judgment. Traditionally, emotional judgment is believed to be independent of bodily movements, or to influence them, but not vice versa.

Method: We measured eye movements in subjects who inspected pairs of human faces freely to make a forced-choice decision. They had to decide: (1) which face was more attractive (emotional judgment), (2) which face was less attractive (emotional judgment), or (3) which face was more round (objective decision) and to press one of two keys, in separate sessions. Subjects were allowed to look at the faces for as long as they wanted to.

In a second experiment, we studied the influence of inspection times on preference decisions. We presented faces alternatively in a pair, for 150 and 400 ms, respectively. We wanted to know whether faces shown longer are on average preferred.

Analysis: Inspection times were divided into time bins of 400 ms. The analysis aimed to establish the existence (if any) of a correlation between inspection times of faces and the subjects' emotional or objective judgments. Such correlation would imply a modulation of judgments by eye movements.

Results: Significant correlation was found between the total looking times and choice in the "more attractive" condition, but not in the "less attractive" and the "more round" conditions. Subjects inspected longer the face that they indicated later as more attractive in the majority of trials (P<0.002, total gaze time). Also, the percentage of time that the chosen face was inspected increased constantly from a chance level of 50% to a significant level (average 76%, p<0.009) as subjects approached decision. This indicates a positive feedback mechanism in attractiveness decisions, where eye movements modulate the following emotional judgment. Neither of the other two conditions, i.e., less attractive and more round, rendered a significant correlation.

In the second experiment, we found significant consistency between presentation time and preference (67% 4 % of faces shown longer were preferred, on average).

Discussion: (1) Liking may be a special subjective decision, since disliking does not seem to be its opposite, as it semantically is; (2) Natural emotional decisions may have a strong somatic component, i.e., they may be influenced by orienting bodily movements such as eye movements, as implied in preferential looking phenomenon in infants, rather than relying exclusively on mental "attractiveness criteria." This idea is further supported by the results of the second experiment; (3) This effect seems to be limited to emotional and natural judgments, because an unnatural emotional task (disliking) and a rather objective task (roundness) did not show significant trends.

Supported by Caltech.
Pupillary response to chromatic flicker

B.R. Sheth, S. Shimojo

There is significant evidence for higher-level cortical control of pupillary responses to visual stimuli, suggesting that factors other than luminance changes may induce a pupillary response. In the present study, the pupillary responses to equiluminant flickering stimuli in a range of 3-13 Hz were examined. Flicker stimuli included color-black (luminance-modulated) and color-color (hue-modulated) flicker. Equiluminance was determined both by objective luminance measures as well as by subjective, perceptual equiluminance for each subject. For both objectively and subjectively equiluminant flicker, significant, sustained pupillary constrictions were recorded. The magnitude of these responses was sensitive to both color and frequency parameters; Red-Blue color-paired flicker consistently produced the strongest constrictions. These responses occurred even when the flicker was of a lower luminance, both physically and perceptually, than a preceding nonflickering color, indicating that chromatic rather than luminance-sensitive mechanisms are involved in this response. Interestingly, the color- and frequency-sensitivity of constriction parallels those of flickers which maximally stimulate photosensitive epileptic patients, raising the possibility that chromatic response may be a factor in photosensitivity.

Reference


Coordinate transformations that can help or hurt accuracy

B.R. Sheth, S. Shimojo

In a pointing task, human observers (Os) had to estimate target position (TP), while the reliability of eye position and object cues was varied. Our past experiments showed that the presence of stable objects in the environment enhanced accuracy of estimation. However, we find here that when objects added no extra knowledge, Os still relied on them, only to degrade in accuracy. The O (n=5) had to maintain gaze on a fixation point (FP), while a target (0.4° dia.) was flashed (10 ms) in a 6° x 6° area centered 14.7° left/right of the FP. The area was either a) blank, or b) enclosed by four objects. 500 ms following target offset, the entire screen flashed (45 ms), after which the FP re-appeared at the same location on an otherwise blank screen. The O then had to point a mouse to the TP. Ideally, Os could estimate TP from identical, stable eye position cues in both conditions, and yet, accuracy was significantly worse in b) than in a). In experiment II (n=6), a target was flashed briefly in between the FP and an object. After the screen flash, in separate conditions, either the object and/or FP was displaced, or both FP and object disappeared (eyes can move freely). TP estimates were compared to a baseline in which the FP and object were re-displayed at the same locations. Estimates in all conditions were significantly worse than the baseline, except when the FP and object disappeared after the screen flash, i.e., computation of TP in absolute, world coordinates was nearly as accurate as when reliable eye position and object cues were at hand (baseline). The visual system is, in principle, capable of accurately computing TP in absolute coordinates, yet relies on cue-based coordinate systems even when they are unreliable, reducing accuracy.


Color and orientation pop-outs differentially affect discrimination

B.R. Sheth, S. Shimojo

Visual properties such as orientation, color and luminance are functionally similar in that each of them individually provide a basis for perceptual pop-out. Pop-outs are commonly believed to facilitate information processing in a variety of tasks such as detection and discrimination. Here, we show a special case in which all pop-outs do not facilitate discrimination. A 3 x 3 matrix of (vertically or horizontally) oriented grating patches is shown (followed by a matrix of plaid masks) either in the periphery (10° eccentricity, 1 x 1° area each, 180-300 ms duration) or in the fovea (0.5 x 0.5° area each, 5 ms duration). The observer (n=5 throughout) discriminates the central target’s color or orientation. If the target is a unique color, discrimination improves compared to a control in which all items are of the same color. Conversely, if the target’s orientation is salient, discrimination does not improve but deteriorates compared to a no-orientation pop-out control. If luminance is substituted for color, the effect is similar. There are no attentional differences between conditions. Color does not pop out more than orientation. Phenomenologically, the data show real differences between so-called elementary visual properties. Though counterintuitive, a greater degree of suppression by an orthogonally oriented surround as compared to an iso-oriented one and relatively little suppression of a uniquely colored stimulus by the surround can explain the data. This poses new challenges for physiology and computation.


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Publications


Simion, C., Scheier, C., Shimojo, E. and Shimojo, S. (2001) Do we like what we see more or do we see more what we like? Vision Sciences Society (Abstract), 79.


The Biopolymer Synthesis Center has continued to provide high quality chemical synthesis, purification, and analysis of a wide range of biopolymers at the request of the scientific community at Caltech. We also provide strong technical support to individual research projects through the introduction and the use of the latest technology in both chemistry and biology. Today's emerging new techniques challenge us with their sophistication and complexity as well as their rapid pace of evolution. For a growing number of scientists we have made complex experiments possible by advance design, synthesis, and purification of unique molecules. The intellectual interaction between our staff and the research scientist was essential to the success of each project.

The DNA/RNA Synthesis Laboratory

The total number of DNA molecules synthesized so far in FY'01 (October, 00 – May, 01) showed a slight 5.6% increase compared to that of FY’00. During the first eight months of FY’01, we have synthesized 7,814 deoxyligonucleotides (DNA) for 46 Caltech faculty members and four outside collaborators, including JPL. This represents a total of 23,4268 base additions.

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The three four-column DNA/RNA synthesizers (ABI Model:394-08) operate with full capacity all the time. These synthesizers can be set up as 0.04, 0.2, or 1.0 µM scale synthesis. They are flexible for the use of a variety of modified chemistries, including RNA synthesis. A catalog of DNA reagents for chemical modifications is available in our laboratory. With the expert assistance of our DNA synthesis staff, Caltech scientists can choose from this catalog and design "special oligos" for synthesis. These special oligos are now widely used at Caltech in a large variety of experiments.

Requests for chemically-modified oligonucleotides have been increasing dramatically and continuously and they are more challenging than ever before.

The Peptide Synthesis Laboratory

There are three automated peptide synthesizers in our Peptide Synthesis Laboratory. Our most advanced solid phase automated synthesizer is an ABI Model:433A. It is set up for the application of Fmoc chemistry with the optimized chemical cycles. These cycles are automatically monitored by a conductivity cell. Using Fmoc chemistry, we can selectively introduce phosphorylated sites on any Ser, Thr, or Tyr residues in the peptide sequence. There are two automated peptide synthesizers, ABI Model:430A, set up for the application of t-Boc chemistry. We have synthesized large numbers of short peptides, peptide-protein conjugates to generate heterogeneous antisera or monoclonal antibodies with desired specificity for a large variety of applications. Each peptide made in our laboratory includes CE, HPLC, and MS analysis. MS analysis is usually done at the Protein/Pepptide Microanalytical Laboratory (PPMAL).

The number of peptide molecules synthesized so far in FY’01 remained high with a large increase in their complexity and purity requirements. During the first eight months of FY’01, we synthesized 109 peptides for 18 Caltech faculty members and 12 outside collaborators. This represents a total of 1,489 regular and 115 special amino acid couplings.

A series of technically difficult peptide synthesis were carried out successfully in our laboratory which contributed to the substantial progress made in Professor David Tirrell’s (Chemistry), Peter Dervan’s (Chemistry), Robert Grubb’s (Chemistry), Thomas Meade’s (Biology) and William Dunphy’s (Biology) laboratories.

Angelika Niemz, in Professor Tirrell’s group, is interested to study the effect of trifluoroleucine substitution on the membrane-binding and tetramerization behavior of mellitin. For this study a series of trifluoroleucine-mellitin analogs were synthesized in our laboratory.

Activation of gene expression by small molecule transcription factor is studied by Paramjit Arora and Timothy Best in Professor Peter Dervan’s laboratory. A series of polyproline-based rigid linkers were designed and then successfully synthesized in our laboratory. These polyproline-based rigid linkers are being used to determine the optimum linker size required for transcription activation.

We have synthesized a large number of extremely difficult, very insoluble hydrophobic peptides for Dr. Boris Minev and Professor Maurizio Zanetti at the UCSD Cancer Center. As a news release on the 7th of April, 2000, said: "A team led by Maurizio Zanetti, M.D., UCSD Professor of Medicine and Member of the UCSD Cancer Center, in collaboration with the Institut Pasteur in Paris, has now successfully used a prototype vaccine in cancer cells in vitro to activate a type of lymphocyte called cytotoxic T-lymphocytes (CTL), or killer cells, to destroy cancer cells using telomerase as a target."

For the fourth year in a row in collaboration with Dr. Andrew Carmen and Professor Michael Grunstein at the Molecular Biology Institute, UCLA, we have been synthesizing a large number of histone-peptides which involves the combined application of both t-Boc and Fmoc synthesis. We have found a way to introduce selective acetylation of one or more Lys sidechains in a given peptide sequence.
The David Baltimore group has used flow cytometry to study gene conversion in mammalian cells. Cells with a stably integrated, mutant GFP transgene are transduced with various constructs including wild-type GFP, and the frequency of homologous recombination is scored by correction of the mutant GFP defect. In a separate line of research, members of the Baltimore group have been using the facility for cell cycle analysis, to test the impact of drugs and transduced genes on particular stages of the cell cycle.

The David Anderson laboratory is using the Facility for research on angiogenesis, segregation of endothelial cell subtypes, and neural crest stem cells. Presumptive endothelial precursor cells from rodent embryos at early stages of gestation are preaparatively sorted on the basis of expression of the markers Flk-1 and PECAM. The sorted cells are then tested for their gene expression patterns, developmental potential, and correlation of these properties with expression of transgenic marker genes. Neural crest stem cells are fractionated by a variety of other markers, which may help segregate distinct subsets of stem cells with preferences for giving rise to particular subsets of descendants.

This year over 40 users representing 13 Caltech groups have utilized the facility. The highlights from some of these projects are presented here.

The Facility offers cell sorting and analysis on two full-service, dual-laser cell sorters (Becton-Dickinson FACSVantage SE and Coulter Epics Elite, as well as round-the-clock access to a nonsorting dual-laser flow cytometric analyzer (Becton-Dickinson FACSCalibur). Scheduling is permitted in advance for up to a month, to enable researchers to plan experiments with time-dependent samples. A special aspect of this Facility is the extensive consultation, advice on trouble-shooting, and support for custom applications that is provided to any current or prospective user interested in the use of this technology. More information about the Facility can be obtained at its web site: http://www.its.caltech.edu/~cellsort.

The past year has been one of significant expansion of Facility capabilities by both software and hardware improvements. The FACSVantage was upgraded by the addition of three new detectors onto a new optical bench. This has made it possible to collect data simultaneously on six fluorescence channels along with forward-scatter and side-scatter channels (four channels of detection for the primary Enterprise Argon laser, two channels from the secondary UV beam split off from the Enterprise Argon laser, and two channels of detection from the Spectrum 70 Argon/Krypton mixed gas laser). The three laser beams have a new configuration which aligns the beams serially in the optical path allowing for two delay time circuits, so that similar emissions excited by different wavelengths can be resolved. New omnichrom cross-beam compensation boards have been installed to take advantage of this configuration and allow for much more extensive compensation combinations. The FACSVantage has now performed five-color immunofluorescence using the fluorochromes: FITC, Phycoerythrin (PE), PE-Cy-5 (Cychrome or Tricolor), Allophycocyanin (APC), and PharRed (APC-Cy7). An additional filter set for PE-Cy7 can be ordered which would allow for simultaneous six color immunofluorescence. Enhanced Green Fluorescent Protein (GFP) and Yellow Fluorescent Protein (YFP) can be detected in the FITC and PE channels, respectively.

The facility shared the cost of the purchase of the off-line analysis software package, FlowJo, which has a license that covers ten simultaneous users over the network. The program reads data from all cytometers, generates publication-quality graphics, handles post-sorting compensation through software, does log-to-linear conversions and ratios, generates data movies, exports data directly to web pages, analyzes DNA/cell cycle kinetics, and computes calibration/quantitation for any parameters. The post list mode channel compensation provided by this software is especially helpful for users doing analytical runs alone on the FACS Calibur, for it can save whole experiments by correcting multicolor data that were collected with suboptimal compensation settings.

This year over 40 users representing 13 Caltech groups have utilized the Facility. The highlights from some of these projects are presented here.
The Pamela Bjorkman laboratory is using the Facility to clone stably transfected cell lines that express particular GFP or YFP fusion proteins. These proteins are then used in analyses of the protein domains involved in controlling the trafficking and organization of pairs of proteins, such as the Transferrin Receptor and the Hereditary Hemochromatosis protein (HFE), or the neonatal FcR and its IgG ligand.

The Jose Alberola-Ila group characterizes the impact of signaling intermediates upon T-cell development by flow cytometric analysis of thymocyte subsets from mice with hyperactive or "dominant negative" transgenes. They rely extensively on the definition of stages in the development of these cells by specific combinations of cell-surface markers CD4, CD8, and different variants of the T-cell receptor complex. They also couple this analysis with direct flow cytometric monitoring of apoptotic cells, intracellular staining of Bcl-2 (antiapoptotic protein) expression levels, DNA content analysis, and measurement of population dynamics by intracellular staining of BrdU-labeled cells and counting rounds of cell division by dilution of carboxymethylfluorescein succinimidyl ester label.

The Ellen Rothenberg group similarly uses flow cytometry to characterize developing lymphocyte precursors, focusing on the earliest stages of T-lineage commitment in wild-type or developmentally-arrested mutant mice. Preparative sorting is also used to isolate hematopoietic progenitor populations that are retrovirally transduced to misexpress particular transcription factors, so that the effects of these factors on gene expression and differentiation can be tested. By using bicistronic retroviral vectors, which also drive expression of GFP, it is possible to correlate directly the level of exogenous gene expression with the degree of advancement the cells achieve in any particular developmental system for a simple dose-response analysis. The Rothenberg lab has also been using GFP as a reporter gene to define the sequences of the IL-2 gene that are required for correct expression in transgenic animals. Flow cytometric analysis determines which transgenic constructs reproducibly confer DNA insertion site independence, as well as correct cell-type specificity and inducibility by physiological signals.

**Publications**


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**Monoclonal Antibody Facility**

**Supervisor:** Paul H. Patterson

**Director:** Susan Ker-hwa Ou

**Staff:** Shi-Ying Kau

The Monoclonal Antibody Facility provides assistance to researchers wishing to generate monoclonal antibodies (mAbs), polyclonal ascites Abs, or ascites fluid. In addition to these service functions, the Facility also conducts research on the development of novel immunological techniques.

For example, in collaboration with Li-Ching Lo from Dr. Anderson’s laboratory, we are comparing the footpad immunization protocol with our standard immunization protocol to determine if the former method can yield high affinity IgG mAbs with a very short immunization period.

In its service capacity, the Facility produced mAbs for the following groups during the past year. Li-Ching Lo from Dr. Anderson’s laboratory obtained mAbs against Ngn-2 (expressed in populations of developing neurons). Li-Ching Lo also obtained mAbs against Sox-10 (early glial marker). Nina Sherwood from Dr. Zinn’s
laboratory obtained mAbs against T32-ATPase (T32, a novel gene that can cause misrouting of CNS axons in the Drosophila embryo). Benno Schindelholz from Dr. Zinn’s laboratory obtained mAbs against RPTP52F (receptor-type protein tyrosine phosphatase, which has adhesion molecule-like extracellular domains that may transduce signals in the establishment of proper connections in the nervous system). Benno also obtained mAbs against DK-l (a novel integral membrane protein with high similarity to LIG-1). Caroline Enn from Dr. Bjorkman’s laboratory obtained mAbs against transferrin receptor 2, and against a complex between HFE & TIR. Tara Chapman from Dr. Bjorkman’s laboratory obtained mAbs against LIR-1 (leukocyte immunoglobulin-like receptor 1, a membrane glycoprotein that is expressed on monocytes and B cells and subsets of T, NK and dendritic cells). Joan Jeng from Dr. Kennedy’s laboratory obtained polyclonal ascites against His-LRR (leucine-rich repeats at the N-terminus of Densin-180). Joan also obtained polyclonal ascites against Densin-180.

Dr. K. Wang from BioAgri obtained mAbs against pig beta-interferon protein. Dr. Wang also obtained mAbs against mouse sperm surface protein. Dr. A. Raitano from UCLA obtained mAbs against fusion protein (cDNA from mammalian cell surface protein). Dr. Brian O’Nuallain from the University of Tennessee obtained mAbs that can discriminate between the two structural forms of aggregates of huntingtin.

We are currently working with Qiao Zhou, Li-Ching Lo from Dr. Anderson’s laboratory, Liz Haswell from Dr. Meyerowitz’s laboratory, Anthony West from Dr. Bjorkman’s laboratory, Dr. Brian O’Nuallain from University of Tennessee, Dr. Wen Shi from UCLA, Dr. Beate Sable from Amgen, and Dr. Kyung Han from Penn State University.

Publication

Nucleic Acid and Protein Sequence Analysis
Computing Facility
Supervisor: Stephen L. Mayo
Staff: David R. Mathog

The Sequence Analysis Facility (SAF) provides software, computers, and support for the analysis of nucleic acid and protein sequences. Currently, the SAF hardware consists of a Sun Netra running Solaris (mendel.bio.caltech.edu), a small Beowulf cluster of nine Linux/Alpha nodes, a 32 page per minute duplexing laser printer, and a color laser printer. The Biology Division’s slidemaker, and the SGI and Windows NT workstations that comprise the “structure analysis facility” are also located in our facility. The SAF has over 250 registered users distributed among 50 research groups. In a typical month about 145 of these users make use of at least one machine.

Programs for sequence analysis provided on Mendel include: the GCG and EMBOSS packages (two comprehensive sets of programs); W2H (a web interface to GCG and EMBOSS); OSP and PRIMER (oligonucleotide design); Phred, Phrap, Cross_Match, Consed (sequence assembly), Clustal (multiple sequence alignment); and Phylip (phylogenetic analysis). This software may be used to analyze new sequences or those in the locally maintained databases (Genbank, PIR, SwissProt, etc.). The Beowulf cluster may be used for compute intensive programs such as running BLAST, fasta, and hmmpfam on local databases.

A number of researchers use the SGI workstations for molecular modeling. The programs available for this purpose include InsightII, O, MidasPlus, Setor, Rasmol, VMD, Molscript, XtalView, CCP4, Delphi and GRASP. Users may also analyze models in Swiss PDB Viewer on the Windows NT workstations. All workstations support hardware stereo.

The lecture notes and homework from the introductory course "Fundamentals of Sequence Analysis" are available on the SAF webserver (http://saf.bio.caltech.edu/). In order to make it easier for users to find the topic or program of interest the documentation for all programs available on the SAF computers has been loaded onto this web site and indexed for keyword searching. Nucleic acid sequences from the DNA sequencing facility are also distributed through this site.

Protein Expression Center
Supervisor: Pamela Bjorkman
Director: Peter M. Snow
Staff: Inderjit Nangiana, Cynthia Jones

The Protein Expression Center was established to meet the needs of the Caltech community in all areas relevant to protein expression. The services provided range from the generation of recombinant DNA constructs to expression of recombinant proteins to purification of the expressed proteins.

The tissue culture portion of the facility, which is central to our operation, is equipped with an inverted phase contrast microscope designed for examination of cells, two laminar flow hoods suitable for sterile manipulations, two incubators capable of supporting the growth of mammalian cell lines, and two incubators housing eight spinner plates which are used for the growth of insect cells. The center is currently capable of small-to-medium-scale (100 ml-to 2 liter-scale) production of recombinant proteins in the baculovirus system, which is the most widely used eukaryotic system for the production of recombinant proteins as well as growth of bacterial and yeast cultures up to two liters. The Center is also equipped to express...
proteins, in the methylotrophic yeast *Pichia pastorius*, a potentially powerful expression system. We are currently able to generate more than 10 liters/week of infected cells in the baculovirus system. The Center is equipped with a 15 liter fermenter in which larger-scale bacterial or yeast cultures may be grown. In addition, a 15-liter bioreactor which is suitable for larger-scale production of proteins expressed in baculovirus as well as other eukaryotic systems (such as mammalian cells) is available.

The Protein Expression Center is equipped to perform most common techniques in molecular biology (including PCR, construction and analysis of recombinant DNA vectors, and molecular cloning of DNA molecules). In addition, we are capable of protein purification (FPLC) and analysis using most common biochemical techniques (for example most types of electrophoresis).

During 2000/2001, the Center expressed more than 100 different (requiring the generation of approximately 275 liters of infected insect cells and 20 liters of bacterial cells) from either recombinant baculoviruses or bacteria for a number of investigators both at Caltech and outside of the Institute. Center personnel have made more than 100 new recombinant viruses. The recombinant DNA used for the generation of 25 of these viruses was constructed at the Center. More than 20 viruses were plaque-purified in attempts to improve the expression levels, and more than 40 viruses were titered. In addition, more than 20 of the expressed proteins were concentrated and at least partially purified by Expression Center personnel for the investigators. We have also expressed more than 15 recombinant proteins in bacteria, and in several cases generated the requisite recombinant DNA constructs for the investigators, as well as purified the resultant recombinant proteins. Finally, the Center has assisted investigators not equipped for cell culture in the design and execution of experiments requiring the use of mammalian cells lines, and has served as a source of insect cells for investigators both at Caltech as well as outside the Institute.

Publications


The Protein Microanalytical Laboratory
Director: Gary M. Hathaway, Ph.D.
Associate Biologist: Felicia Rusnak, B.S.
Senior Research Assistant and MPS: Jie Zhou, Ph.D.
Faculty Advisor: William G. Dunphy, Ph.D.
http://www.its.caltech.edu/~ppmal, or http://www.caltech.edu/subpages/analyt.html, or http://www.its.caltech.edu/~bi/bicatalog.html#ppmal

Summary: The Protein Microanalytical Laboratory (PPMAL) provides structure analysis for proteins, peptides and other biopolymers for campus faculty and staff. The laboratory develops new methodologies and techniques and may collaborate on research projects. Partially funded by the Beckman Institute, charges are set to recover residual costs. The laboratory is located in room 204 with offices in rooms 232 and 215 of the Beckman Institute.

Activity Centers
*Biopolymer Mass Spectrometry*
- Tandem mass spectrometry
- Capillary LC/MS and LC/MS/MS
- Matrix-assisted, laser desorption, time-of-flight mass spectrometry
- Post-source decay and prompt fragmentation analysis
- In-gel and on-membrane proteolytic digestion for mass mapping and sequencing by mass spectrometry
- Fragmentation analysis by database search for protein identification
- Post-translational and chemical modification analysis
**Edman Sequencing**
- N-terminal sequence analysis of proteins and peptides by Edman degradation

**High Performance Liquid Chromatography**
- Submillimeter and capillary HPLC for analysis and purification

**Other**
- *De novo* sequence analysis of tandem mass spectrometry derived data
- Chemical modification of peptides for mass spectrometry
- Consultation on sample preparation and error analysis including dissemination of literature materials
- Maintenance of webpage
- Database search by sequence, fragment masses, and composition

**SPECIFICS**

**Mass Spectrometry of Biopolymers** is carried out with our two mass spectrometers: a triple quadruple, tandem mass spectrometer, and time-of-flight mass spectrometer. The liquid chromatography (LC/MS) interface designed by our lab is coupled to a new technique for gradient elution using static minigradients. We construct the reverse phase columns for use within our newly designed sprayer. With this methodology we have obtained good MS/MS fragmentation spectra with as little as 10 femtomoles of peptides (1).

**Protein/Peptide Chemical Sequence Analysis by Edman Degradation**
We use a dual reaction column, chemical microsequencer with subpicomole sensitivity. As demonstrated by the ABRF 2000 (1) sequencing test, the new instrument extends the use of the technology for combined chemical and MS sequencing projects.

**Database Search and Analysis**
The laboratory maintains access to database search via the web with programs such as MASCOT and BLAST using information obtained by both mass and chemical sequencing. These searches often use mass information achieved by collisional fragmentation and predictive software (GPMAW and SHERPA). They are available on our lab computers as independent software, and through the Internet.

**Collaborations and Accomplishments**
During the course of the year the lab interacted with 28 laboratories, their professional staff and associates. We assisted local biotechnology companies associated closely with Caltech such as Xencor, Clinical Microsencors, Insert Therapeutics, Agouron Pharmaceuticals and members of the HHMI at UCLA. Additional faculty are assisted through our efforts on behalf of the Biopolymer Synthesis and Protein Expression Facilities.

Information regarding sample preparation, cost recovery schedules, and other technical aspects of our operation can be accessed through our homepage (http://www.its.caltech.edu/~ppmal). The web site also provides the reader with direct access for comments and questions. We can be reached by email at: ppmal@its.caltech.edu, or by calling 626-395-6388.

**Future Expectations and Directions**
Our laboratory continuously strives to develop new procedures and obtain equipment which will permit both the high throughput mass analysis while maintaining high sensitivity. Our high throughput is reflected in one of the shortest turnaround times of any core facility. The lab’s efforts in enhancing efficiency in biological mass spectrometry has allowed it to encompass the analysis of small organic ions and nucleic acids in addition to proteins and peptides in processing more than 4000 samples annually while still engaging in research project oriented problems.

**References and publications**

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**Genetically Altered Mouse Production Facility**
**Director:** Shirley Pease. FIAT
**Mouse Facility Manager:** Bruce Kennedy, MS, RLATG
**Mouse Facility Supervisor:** Jenny Dancourt, BS, RVT, ALAT
**Embryonic Stem Cell Culture:** Jue Jade Wang, MS
**Cryopreservation and Microinjection:** Juan Silva, BS, LAT
**Staff:** Jennifer Alex; Lilia Anonuevo, ALAT; Cirila Arteaga; Jenny Arvizu; Hector Forero; Hernan Granados; Ana Hernandez; Stephanie Huang, BS; Jorge Mata, ALAT; Jose Mata; Alfonso Meda; Lorena Sandoval

The transgenic technique for gene addition, (Gordon *et al.*, 1980) and targeted gene modification, (Zijlstra *et al.*, 1989) have been established at Caltech since 1984 and 1993, respectively. Gene addition in the mammalian system is accomplished by injecting DNA into the pronucleus of a fertilized egg. Targeted disruption of specific genes, however, requires the manipulation of pluripotent embryonic stem (ES) cells *in vitro* and their subsequent return to the embryonic environment for incorporation into the developing embryo. The resulting chimeric mouse born is useful for two purposes. 1) It is comprised of tissue from two sources, the host embryo and the manipulated stem cells. More importantly, 2) it can be mated, so as to produce descendants that are entirely transgenic, resulting from the ES cell contribution to the germline of the chimeric mouse. The facility in collaboration with Anderson, Simon, Wold and
Varshavsky laboratories has generated multiple transgenic, knockout and knockin mouse strains. Presently, twelve principal investigators and their post-doctoral fellows or graduate students use the facility.

The newly refurbished, pathogen-free barrier-operated mouse facility was opened and re-stocked with pathogen free strains in February 1995. In addition to 76 transgenic, knockout and knockin strains, we also maintain colonies of inbred and outbred animals, which are used to support the development of new lines, by investigators at Caltech. We also have many mouse models on both an inbred and an outbred background, plus intercrosses between two or three different but related mouse models.

In total, we maintain 126 separate strains of mouse. Facility staff provide a complete service to investigators. All colony management operations are carried out by staff at the request of investigators. The work of the staff and Manager of the facility continues to reflect Caltech’s commitment to good laboratory animal practice and our adherence to NIH guidelines.

New transgenic lines have continually been produced and in all, 46 new transgenic lines for gene addition and 35 new knockouts have been produced since February 1995. Facility staff has performed all embryo manipulation involved in the production of these new lines. Microinjection equipment has been set up within the barrier facility, which operates on restricted access as part of the "barrier" itself. A room outside the facility has been allocated by the Division, to be used primarily for teaching grad students, technicians and postdocs, the techniques involved in transgenic mouse production. This room has been operating since July 1996. Investigators have the option of using this room to perform their own microinjection of embryos, rather than using the full technical service available from the Genetically Altered Mouse Facility.

In the generation of mouse models involving the use of embryonic stem cells, we continue to pre-screen candidate clones for injection by the preparation of chromosome spreads. In establishing the chromosome count of each clone prior to injection, we find we are reliable able to predict which clones will generate good chimeras which will transmit through the germline. In effect, we have been able to increase efficiency by 100%, in the production of germline transmitting chimeras.

In cryopreservation, 30 strains are either stored or partially stored. For each strain, between 200 and 500 embryos at 8-cell stage have been preserved in liquid nitrogen. There are currently over 8,000 embryos frozen in total. We shall continue to preserve embryos from every single mouse strain the Core maintains. The advantages of such a resource are many. Unique and valuable mouse strains that are currently not in use may be stored economically. In the event that genetic drift should affect any strain, over time, then the option to return to the original documented genetic material is available. Lastly, in the event of a microbiological or genetic contamination occurring within the mouse facility, we have the resources to set up clean and genetically reliable mouse stocks in an alternative location.

In addition to producing a total of 81 new animal models since February, 1995, we have re-derived 44 pre-existing genetically altered strains of mice. We continue to re-derive unique strains coming in from other institutions, in order that we might maintain a high standard of pathogen-free animals throughout our facilities.

The production of pathogen-free animals enables Caltech to exchange valuable genetically altered mouse models with other academic groups around the world. As more and more mouse models become available and the exchange of animals more frequent, it is essential for Caltech to be in a position to deal effectively with animal lines generated in the facility and in other laboratories. To this end, the Division has funded the refurbishment of two quarantine animal holding areas. This now enables us to safely contain and "clean up" other mouse models coming in from other institutions, without putting our own colony at risk. Since 1995, we have been working towards eliminating MHV from conventional mouse stocks. The conventional holding area in the Alles Building was refurbished during the year 2000. This area has been restocked with pathogen-free animals. Investigators enjoy full access to the facility. Thus, in early 2000 we reached one of our long-term goals in animal management by having cleaned up all our mouse stocks and effectively separated mouse production from animal use and both from the potential threat presented by the introduction of new stocks from other institutions.

Below are the names of the twelve principal investigators and their postdoctoral fellows or graduate students who are presently using the transgenic facility.

Pepe Alberola-Ila
Susannah Barbee, Micheline Laurent, Eric Tse
David Anderson
Xinzhong Dong, Limor Gabay, Emma Drummond, Sebastian Gerety, Amy Greenwood, C.J. Han, Jae Kim, Li Ching Lo, Raymond Mongeau, Yusuke Mukoyama, Donghun Shin, Hai Wang, Mariela Zirlinger, Qiao Zhou
David Baltimore
Eric Brown, Alexander Hoffman, Mollie Meffert, Xiao-Feng Qin
Marianne Bronner-Fraser
Houman D. Hemmati
Scott Fraser
Eric Ahrens, David Crotty, Mary Dickinson, Russ Jacobs, Angelique Louie
Mary Kennedy
Hung Jung Chen, Jenia Khoroseva, Leslie Schenker
Henry Lester
Chi Sung Chiu, Purnima Deshpande, Carlos Fonck, Cesar Labarca, Clemente Neusch, Johannes Schwarz
Paul Patterson
Zhu Min, Rasika Sowmyalaski
Ellen Rothenberg
Gabriela Hernandez-Hoyos, Angela Weiss, Mary Yui

Melvin Simon
Valeria Mancino, Pam Eversole-Cire, Lin Gu,
Wen-shan Hao

Alexander Varshavsky
Yong Tae Kwon, Takafumi Tasaki

Barbara Wold
Libera Berghella, Brian Williams, Jeong Yoon

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Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, J.A.
77:7380-7384.

Liu, X., Wu, H., Loring, J., Hormudzi, S., Disteche, C.M.,

Overbeek, P.A., Aguilar-Cordova, E., Hanten, G.,
Schaffner, D.L., Patel, P., Lebovitz, R.M. and

Zijlstra, M., Li, E., Sajjadi, F., Subramani, kS. and
Bachelor of Science

Orkun Akin*
Timothy Joseph Buschman
Deanna Marie Carrick
Kevin Zi-Jun Chao*
Vivian Fung-Mei Chow*
Jason Han Chua
Jane Garrity*
Iljie Jennifer Kim*
Geoffrey Wilson Meissner*
Nhien Hao Nguyen*
Sean Ariel Pintchovski*
Trisha Arlene Sando
Carrie Shilyansky*
Ryan Matthew Simkovsky*
Ronald K. Siu*
Kayla Elaine Smith
Nancy Chi Wei
Paula Beck Whitten

*Students whose names are followed by an asterisk have graduated with Honor in accordance with a vote of the faculty.

Master of Science

Radu Georgescu
Gilberto Hernandez, Jr.
Natalia Lukina
Kathleen Marie Richter
Doctor of Philosophy 2001

Keith B. Brown — Biology
B.S., Grambling State University, 1992
Thesis: Comparative Studies of Vulva Development in C. briggsae

Chieh Chang — Biology
B.S., Chung Shan Medical and Dental College, 1990
M.S., National Yang-Ming Medical College, 1992
Thesis: Signal Transduction, Regulation, and Developmental Logic of EGFR Signaling in C. elegans

Yong Chi — Biology
B.A., University of California, Berkeley, 1993
Thesis: Negative Regulation of Transcription Factors by Srb10 Cyclin-Dependent Kinase

Anita Gould — Biology
A.A., Simons's Rock College of Bard 1982; B.A., 1985
Thesis: Expression of Eph-family Receptor Tyrosine Kinases and Ephrins in the Tadpole of the Frog Xenopus Laevis, and Possible Roles in the Development of Retinotectal Topography

David Mathew Herman — Biochemistry and Molecular Biophysics
B.S., University of California, San Diego, 1993
Thesis: Stereochemically Modified Polyamides for Recognition in Minor Groove of DNA

Yukiyasu Kamitani — Computational and Neural Systems
B.A., The University of Tokyo, 1993; M.S., 1995
Thesis: Psychobiophysics of Transcranial Magnetic Stimulation

Yang Liu — Biology
B.S., Peking University, 1990
Thesis: Molecular Mechanism of Sulfated Carbohydrate Recognition: Structural and Biochemical Studies of the Cysteine-Rich Domain of Mannose Receptor

Svetlana A. Lyapina — Biology
B.S., Moscow State University, 1994
Thesis: Characterization of the Human SCF Ubiquitin Ligases – Structure, Function and Regulation

Warham Lance Martin — Biochemistry and Molecular Biophysics
B.A., DePaul University, 1989
M.S., California State University, Los Angeles, 1995
Thesis: Protein-Protein Recognition: The Neonatal Fc Receptor and Immunoglobulin G

Wenying Shou — Biology
B.A., Pomona College, 1993
M.S., California Institute of Technology, 1998
Thesis: Diverse Mechanisms of Regulating the Mitotic Cell Cycle

Michael Christopher Vanier — Computation and Neural Systems
B.Sc., McGill University, 1986; M.Sc., 1990
Thesis: Realistic Computer Modeling of the Mammalian Olfactory Cortex

When more than one field of study is listed, the first is the major and the second and others are minors.
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Financial Support

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Arthritis Foundation
Aventis Corporation Science

Beckman Institute
Benjamin Rosen Family Foundation
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Biological Sciences Initiative
Breast Cancer Research Institute
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Cancer Research Institute
CaPCURE
Carl F Braun Trust
Cedars Sinai Medical Center, Los Angeles Center for Consciousness Studies, University of Arizona
Charles B. Corser Fund
Christopher Reeve Paralysis Foundation
Colvin Fund for Research Initiatives in Biomedical Science

Damon Runyon-Walter Winchell Cancer Fund
Damon Runyon-Walter Winchell Foundation
Dana Foundation
DARPA
David & Lucile Packard Foundation
Della Martin Foundation
Department of Energy
Department of the Navy, Office of Naval Research
Deutsche Forschungsgemeinschaft DFG
Deutscher Akademischer Austauschdienst

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Edward H. Schneider Fund
Elizabeth Ross Fellowship
Ellison Medical Research Foundation
Engineering Research Center for Neuromorphic Systems
Ernest D. Zanetti Fund
Ethel and Robert Bowles Professorship
Evelyn Sharp Fellowship

Ferguson Fellowship
Ferguson Fund for Biology
Fletcher Jones Foundation
FORD Foundation
Frank P. Hixon Fund
French Foundation Fellowship

Gates Grubstake Fund
George Beadle Professorship in Biology
German Government Fellowship
Gimbel Discovery Fund
Gordon Ross Medical Foundation
Gosney Fellowship Fund
Grace C. Steele Professorship in Molecular Biology
Grubstake/Colvin Foundation
Grubstakes Award (Caltech)

Helen and Arthur McCallum Foundation
Helen G. and Arthur McCallum Fund
Helen Hay Whitney Foundation
Hereditary Disease Foundation
Hewlett-Packard Company
Hixon Professorship of Psychobiology
Honeywell Technology Center
Howard and Gwen Laurie Smits Professorship in Cell Biology
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National Institute of Arthritis and Musculoskeletal and Skin Disease
National Institute of Child Health and Human Development
National Institute of Mental Health, USPHS
National Institute of Neurological Disorders and Stroke
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National Institute on Deafness and Other Communication Disorders
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National Multiple Sclerosis Society
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FRONT COVER

Elisa Bush, Eloyn Simons, David Dubowitz, Miriam Seading, Marvin Nelson, and John Altmann

Surfaces of the skull and endocranial cavity of *Parapithecus grangeri* based on CT scans performed at Children's Hospital Los Angeles. *Parapithecus* is an early anthropoid from the Fayum deposit in Egypt around 33 million years ago. It is a primitive monkey. This specimen was found by Eloyn Simons and is the most complete early anthropoid skull known. The external surface of the skull has been rendered transparent to reveal the endocranial cavity, which closely matches the external surface of the brain, thus making it possible to reconstruct the outer surface of the brain of this fossil monkey. Dark blue represents the olfactory bulbs and light blue represents the optic nerves. CT scans reveal that *Parapithecus* had a small brain for its body weight, and a small optic nerve compared to living anthropoids. These observations suggest that high acuity vision developed much later than was previously thought, and holds out the possibility that the development of high acuity may have been associated with the development of large brains in the living anthropoids.

BACK COVER

A knock-in mouse carrying a GABA transporter fused with green fluorescent protein (GAT1-GFP) shows plasma membrane GFP expression in GABAergic interneurons of the cerebellum. The brightest structures, pineaux, are aggregates of many axons from basket cells that form inhibitory synapses with the axon hillock of Purkinje cells. Presynaptic terminals also form baskets around the Purkinje cell bodies. Terminals and processes of stellate and basket cells express the transporter-GFP fusion in the molecular layer, above the Purkinje cell layer. The fusion protein is expressed less strongly in the granule cell layer, below the Purkinje cells. Scale bar: 50 μm. See Lester abstracts starting on page 211.

Protein co-factors that regulate aggregation and nuclear localization of huntingtin protein (Htt), the etiological agent in Huntington disease (HD), are of great interest in understanding the pathology of this fatal condition. While Htt is normally found in various locations in the cytoplasm, it aggregates in the nucleus in HD. We have discovered that expression of IKKγ, a regulatory component of IKK signaling, promotes aggregation and nuclear localization of Htt exon-1 in 293 cells. Shown here is a confocal examination of cells co-transfected with wild-type Htt exon-1 and a full-length IKKγ plasmid. Htt is fused to EGFP (upper left panel), IKKγ is stained with anti-HA antibody (upper right), and TOTO-3 is used for nuclear staining (lower left). The lower right panel presents the three colors merged.