FRONT COVER

**HRP-filled axon terminal arbor in the turtle tectum originating from nucleus isthmi**

Nucleus isthmi is a cholinergic nucleus that receives a topographic projection from the tectum and projects topographically back to the tectum. The tectum (= superior colliculus in mammals), which projects to a part of the reticular formation that controls eye and neck muscles, is thought to mediate a "visual grasp" reflex—like the quick turn toward a fly that a frog makes just before snapping it up. Nucleus isthmi may be part of a circuit that triggers such movements, but also helps to arbitrate when several interesting (or edible) stimuli appear simultaneously. The nucleus isthmi terminal illustrated here arises from a myelinated axon (thick trunk entering at the lower right) that turns upward to form a dense cylindrical arbor containing about 3,600 synaptic boutons. Viewed from the surface, the arbor occupies less than 0.5% of the area of the tectum.

BACK COVER

**Correction of a dysmyelinating phenotype in transgenic shiverer mice**

Shiverer mutant mice exhibit a distinct behavioral pattern including tremors, convulsions and early death. Their central nervous system is poorly myelinated and they lack myelin basic protein. The gene encoding myelin basic protein is almost completely deleted. The normal gene encoding myelin basic protein was injected into the pronucleus of fertilized eggs. Transgenic shiverer mice that have integrated the normal myelin basic protein gene into their genome no longer shiver nor die prematurely. The panels are from left to right: A normal mouse with an electron micrograph of a cross section of the optic nerve showing multilayered myelin wrapped around axons (blue). In the center is a shiverer mouse with an electron micrograph of cross sections of the optic nerve showing the complete absence of compact myelin around axons (yellow). A transgenic shiverer mouse with an electron micrograph of the optic nerve from the transgenic shiverer. A greater number of axons are myelinated than in the shiverer mouse (green).


A REPORT FOR THE YEAR 1986-87
ON THE RESEARCH AND OTHER ACTIVITIES
OF THE
DIVISION OF BIOLOGY
AT THE
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA
Constance R. Katz - Annual Report Secretary

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RESEARCH REPORTS

Much of the research work summarized here has not yet been reported in print, in many instances because it is not yet complete. For that reason this report is not intended as a publication and should not be cited as such. Individual projects should be referred to only if specific permission to do so is obtained from the investigator responsible for the material. References are made here to published papers bearing on the projects reported. Publications by members of the Division, covering the period from about June 1986-August 1987, are listed separately at the end of the research reports of each group.
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The Faculty

This year we welcomed three new Assistant Professors to the Division of Biology:

**Christof Koch** was born in Missouri and educated in West Germany, Canada, and Morocco. He obtained his PhD in 1982 from the Max Planck Institute for Biological Cybernetics in Tübingen, and then moved to MIT for postdoctoral work with Tom Poggio. Christof came to Caltech in the fall of 1986 as a joint appointee in Biology and in Engineering and Applied Science, as part of Caltech's new interdisciplinary program in Computation and Neural Systems (CNS). He has moved into laboratories in the basement of the Beckman Behavioral Biology Laboratory and will also have part of his group in the Thomas Engineering Laboratory.

**Howard Lipshitz** came from South Africa in 1978 to do graduate work in Biology at Yale, where he worked with Doug Kankel on the developmental genetics of the nervous system of *Drosophila*. After his Ph.D., he moved to Stanford for postdoctoral work with David Hogness and began work with the bithorax complex of *Drosophila*. He arrived at Caltech in January 1987, and moved into laboratories on the third floor of Kerckhoff, where he will continue work on the developmental genetics of *Drosophila*.

**Paul Sternberg** arrived in June 1987, after completing three years of postdoctoral study with Ira Herskowitz at UCSF. Previously, he was an undergraduate at Hampshire College in Massachusetts, where he majored in both biology and mathematics and then went on to graduate work at MIT. At MIT, he started working with the genetics of the nematode, *Caenorhabditis elegans*, in the laboratory of Bob Horvitz. Although his postdoctoral study at UCSF involved an interlude with yeast genetics, at Caltech he will be concentrating on the genetics and developmental biology of *C. elegans*. He has moved into laboratories on the second floor of Alles and Kerckhoff.

The Division hosted two Wiersma Visiting Professors this year: **Martin Heisenberg**, from Würzburg University, in West Germany, was here for 6 months, working primarily in association with Mark Tanouye. **Terry Sejnowski**, from the Biophysics Department at Johns Hopkins University, visited for 4 months in early 1987.

**Howard Berg** resigned his position as Professor of Biology to move to Harvard where, in addition to his university position, he will join the Land Institute.

We are sorry to report the death on August 14, 1987, of Professor Emeritus **Anthonie Van Harreveld**, at the age of 83. Van Harreveld was one of the original neurobiologists at Caltech. He came in 1934, became full Professor in 1947, and retired as Professor Emeritus in 1974.

**Honors and Awards**

Division Chairman **Leroy Hood** received several awards: The Louis Pasteur Foundation of Los Angeles awarded him its 1987 Louis Pasteur Award for Medical Innovation, and the ARCS Foundation presented him with its 1987 Man of Science award, in recognition of his pioneering research in deciphering the message of DNA. He also received the ISCO Award for Significant Contributions to the Field of Biochemical Instrumentation, and an honorary DSc from the CUNY Mt. Sinai School of Medicine.

**David Anderson** received a Searle Scholars Award from the Chicago Community Trust.

**Norman Horowitz** was awarded the Bicentennial Medallion of Distinction by his alma mater, the University of Pittsburgh, at the 1987 commencement ceremonies at which the University’s 200th anniversary was celebrated.
Honors and Awards (continued)

Christof Koch was awarded an Alfred P. Sloan Research Fellowship and an Office of Naval Research Young Investigator Award.

Mark Konishi presented the 1987 F. O. Schmitt Lecture in Neuroscience at the Rockefeller University.

Ed Lewis was a co-winner of a $20,000 Gairdner Foundation International Award (Canadian) for "identification and analysis of genes that control basic steps in development."

Jean-Paul Revel received an award for excellence in teaching from the Associated Students of the California Institute of Technology.

Mel Simon was elected to membership in the American Academy of Arts and Sciences and was named the Anne P. and Benjamin F. Biaggini Professor of Biological Sciences.

Alan Goldin, a Senior Research Fellow in the laboratory of Henry Lester, was a winner in the competition for a 5-year Lucille P. Markey Scholars Award.

John Ngai, who completed his Ph.D. work with Elias Lazarides this year, won the $2000 Lawrence L. and Audrey Ferguson Prize for the best Ph.D. thesis in Biology.

Developmental Biology

The Institute has been awarded a major 5-year grant from the Lucille P. Markey Charitable Trust for expansion of our research in Developmental Biology. In March, this expansion was signaled by a 3-day symposium on "Cell Lineage and Specification in Development."
Welcome: Marvin Goldberger, President, California Institute of Technology

Opening Remarks: Robert Glaser, Director for Medical Science, Lucille P. Markey Charitable Trust

Lee Hood, Chairman, Division of Biology, California Institute of Technology

SESSION 1: CELL LINEAGE AND CELL SPECIFICATION IN EARLY EMBRYOS

Eric H. Davidson, California Institute of Technology
Cell lineage and spatial regulation of gene activity in sea urchin embryos

J. Richard Whittaker, Marine Biological Laboratory
Determination of cell fate in cell lineages of the ascidian embryo

William B. Wood, University of Colorado
Lineage decisions and sex determination in C. elegans

Charles B. Kimmel, University of Oregon
Embryonic cell lineages of the zebrafish

John B. Gurdon, University of Cambridge
Muscle gene activation in Xenopus development

SESSION 2: STEM CELLS AND SPECIFIC GROWTH FACTORS

Nicole Le Douarin, CNRS Institut de Embryologie
Cell lineage studies in the development of the peripheral nervous system in the avian embryo

Eugene Goldwasser, University of Chicago
The regulation of red cell differentiation

Frederick W. Alt, College of Physicians & Surgeons, Columbia University
Regulation of genomic rearrangement events during lymphocyte differentiation

Irving Weissman, Stanford University School of Medicine
Early differentiation of hematolymphoid cells

SESSION 3: CELL LINEAGE AND DETERMINATION IN THE NERVOUS SYSTEM

Mark Konishi, California Institute of Technology
Cell death in sexual differentiation of the brain

Paul H. Patterson, California Institute of Technology
Factors controlling neuronal phenotypic decisions

Corey S. Goodman, Stanford University
Cell fate and cell recognition during neuronal development in Drosophila

Martin Raff, University College, London
The role of cell-cell interaction in the differentiation of a bipotential glial progenitor cell

Pasko Rakic, Yale University School of Medicine
Principals of neocortical development

SESSION 4: CELL SPECIFICATION IN POSTEMBRYONIC MORPHOGENESIS

Seymour Benzer, California Institute of Technology
A fly's eye view of development

Antonio García-Bellido, Universidad Autónoma de Madrid
The nature of the realizator genes in morphogenesis

Edward B. Lewis, California Institute of Technology
How are the master control genes of the bithorax complex regulated?

Elias Lazarides, California Institute of Technology
Control of early erythroid differentiation and morphogenesis

Jeremy Brockes, MRC Cell Biophysics Unit, London
Cell transitions in amphibian limb regeneration

SESSION 5: CELL HERITABLE STATES OF DIFFERENTIATION

Frederick Meins, Jr., Friedrich Miescher-Institut
Cell-heritable changes in plant development

Davor Solter, The Wistar Institute
Nucleo-cytoplasmic interactions in early mammalian development

Harold Weintraub, The Fred Hutchinson Cancer Research Center
Regulation of DNA replication to once per cell cycle
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Summary: The focus of interest in this laboratory is the developmental expression, the organization, and the evolution of the genome in higher animals. Sea urchin eggs, embryos and larvae serve as experimental systems for much of our work. Other projects concern genes expressed in adult tissues. A number of sea urchin genes active at particular times and in particular cell lineages and cell types have been cloned and characterized. In addition, cloned genes of humans and other primates are being utilized in evolutionary studies. Generally, projects concerned with the molecular biology of sea urchin development are carried out in our Pasadena laboratory, while those at the Kerckhoff Marine Lab are centered on genomic evolution.

Recently developed methods for gene transfer in the sea urchin are being exploited in studies of differential gene expression in the early embryo. The major issue in this work is the molecular basis of the spatial and temporal regulation of structural genes, expression of which underlies the initial appearance of chtodifferentiation. cis-regulatory sequences of the marker genes are being identified, and their specific interaction with trans-acting protein factors that can be extracted from eggs and embryos is being characterized. We regard the developmental origin, activation and spatial distribution of such regulatory factors as a route to a causal interpretation of differential gene activity at the beginning of the life cycle.

1. Occurrence of a Wide Range of Evolutionary Rates within Individual Genomes

Roy J. Britten

Many observations have been made of wide ranges of rates of evolutionary DNA sequence change within genomes, and much of the rate difference appears not to be due to selection but to differences in the underlying mutation rate (see Abstract No. 2). The only example that has been examined in detail by interspecies sequence comparison occurs in a glue gene region of *Drosophila* (see Biology 1986, Abstract No. 133). Two adjacent regions were observed that had a tenfold difference in base substitution but similar amounts of insertions and deletions. Thus, there appears to be a sharp boundary between two different mutation rates (two replicons?).

Some observations of rapidly evolving DNA components depend on the failure of a fraction of single copy DNA to hybridize while the bulk of the DNA has only a modest divergence. We have observed very rapidly evolving fractions of the single copy DNA of Hawaiian picture-wing flies and three other groups have made similar observations among members of the melanogaster and mulleri subgroups of *Drosophila*. In one case studied by Powell at Yale, individuals of the species *D. mercatorium* differed from each other so much that 15% of the DNA failed to hybridize, although the divergence was only about 1% for the sequences that did hybridize. They observed a similar phenomenon among cave crickets. Among sea urchins, there is less "very fast fraction" that does not hybridize in closely-related species. However, we have separated large fractions of *S. purpuratus* DNA that differ by a factor of five in their degree of interindividual variation. The "very fast fraction" is also smaller among mammals but is probably present,
and there is a wide range of rates around the median rate.

2. Evolution of the Mutation Rate of Eukaryotic DNA

Roy J. Britten

As described in last year's annual report (Biology 1986, Abstract No. 59), the rates of evolution are about equal for the pseudogene beta globin, for silent substitutions in coding regions and for total single copy DNA. Recently about 3500 bases of the delta-beta globin intergenic region has been shown to share the same rate. The implication is that all four of these sequence classes are drifting freely at the underlying rate of mutation. Thus, the differences in rate of divergence within the genome discussed in the abstract above may be interpreted as local differences in the mutation rate. There are also large differences among systematic groups in the average rate of evolution of single copy DNA. For example, it is five- or tenfold higher for rodents, Drosophila and sea urchins compared to birds and primates (Biology 1985, Abstract No. 59). Some, but not all of these contrasts, can be attributed to generation time differences.

Apparently the effective rate of mutation may change during evolution, in local regions or throughout the genome (for example, due to changes in replication or repair mechanisms). There might be an effect on variation or fitness and thus the mutation rate is probably under natural selection in the short term. Further, the rate of mutation could affect the evolution of many genes. As a result, a change in the rate of mutation would have a delayed effect on the course of evolution of the species. It is probable that such long-term changes also affect the rate of mutation through natural selection, possibly inter-related with changes in longevity and period required for maturation of juveniles.

3. Interspecies DNA Divergence Assay Suppressing Base Composition Effects

Roy J. Britten, David B. Stout

The relationship of the DNA sequences of organisms is a valuable tool for phylogenetic studies. The bulk of the single copy DNA appears to undergo neutral drift in sequence, and therefore relationships based on sequence divergence simply reflect the branching pattern of the lineages. With appropriate caution, DNA divergence measurements can also be used as an evolutionary clock. Ultimately, a network of single copy DNA divergence measurements will form a basis for understanding speciation and morphological change by detecting, for example, convergent evolution.

The technique utilized most for single copy DNA divergence measurements has been thermal denaturation of duplexes bound to hydroxylapatite, as used by Sibley and Ahlquist for thousands of pairs of birds, primates and rodents. It has the disadvantage that broad melting curves result from the range of base composition. The chaotropic agent tetraethylammonium chloride (TEACL) suppresses most of the width due to base composition. A new medium PT (0.013 M neutral phosphate buffer, 2.0 M TEACL) avoids precipitation while duplexes are efficiently bound and single strands easily eluted from hydroxylapatite. Regardless of composition, long precise duplexes melt at about 70°C and the practical width of the melting curves is about 3°C for eukaryotic DNA. With control of tracer and drive DNA fragment sizes, an accuracy of about 0.1°C should now be possible for relationship studies. The procedure also permits fractionation of duplexes on the basis of precision or length, with much reduced effect of base composition.

4. Recently Copied Human Alu Repeats

Roy J. Britten, Will F. Baron

Alu repeats are a significant source of variation in the human genome. For example, they apparently cause genetically significant effects in the human population such as the LDL receptor gene defects that arise from deletions and duplications terminating in Alu repeats. Recently, sequence comparisons among apes and humans have shown that the sequences of Alus near genes are drifting at the same rate as silent substitutions in coding regions (and single copy DNA), showing that sequence divergence of Alus may be used as a measure of their ages (Sawada et al., 1985).

We have searched for recent additions to the family by isolating all of the precise repeats in human DNA that have less than 5% sequence divergence from each other, using a new technique (see Abstract No. 3). We find that at least 15% of the members of the Alu
family are present in this fraction, due to precise pairing of Alu sequences. Thus, the structure of the Alu family includes many sets of precise relatives which probably result from recent events of duplication or conversion. Many copies appear to have been made in the genome of the human lineage since the divergence from the lineage leading to the Old World monkeys (single copy divergence from human, 7%).

Sequence comparisons (Bains, 1986) among many cloned Alus show an average divergence of about 30% and no pairs closer than 10% in sequence. Thus, it seems likely that copies have been added to the family for a long period of evolution and the process is continuing. One member of the family (located 5' of the epsilon globin gene) that we have studied does not have precise relatives, implying that not all members of the family continue to be copied.

References:

5. The Enhancer Region 5' of the c-fos Gene Includes a Repeating

 Huey Ru Hwu

A 400-500 nucleotide region 5' of the proto-oncogene c-fos has the characteristics of an enhancer and is conserved in sequence between human and mouse genomes (Deschamps et al., 1985). This region is being examined to test the concept that there is a relationship between enhancer elements and interspersed repeated sequences (and possibly mobile genetic elements). Genomic Southern blots of human DNA cut with EcoRI show a broad streak when hybridized with a probe from this region (c-fos 5'). A screen of about a thousand plaques from the Maniatis human genomic library yielded several dozen plaques that hybridized with a range of intensities with this probe, indicating that there does exist an interspersed repeat with homology to a sequence within the conserved 5' region of the c-fos gene. Ten of the most strongly hybridizing have been plaque purified. A 600 nucleotide MboI fragment from one of these shows good hybridization with the c-fos 5' probe and is being subcloned for sequencing.

Reference:

6. Molecular Immunology of the Purple Sea Urchin

L. Courtney Smith

The vertebrate immune system is a finely tuned system of cellular interactions that function to defend the organism from pathogens and other non-self challenges. To accomplish this role, a variety of cell-surface and secreted molecules are employed. Many of these molecules show sequence and topological similarities with each other and have been grouped into the immunoglobulin (lg) gene super family. The evolutionary development of this complex vertebrate system is a topic of general interest and is an underlying impetus for this study.

Because the echinoderms are more closely related to the chordates than are the other invertebrate phyla, they are a good place to begin the search for an invertebrate member of the lg gene family. The coelomocytes, which appear to be the immune effector cells or immunocytes that are involved in responses to foreign cells, are easily obtained in large numbers from the coelomic fluid of the purple sea urchin Stronglylocentrotus purpuratus. This allows the genes (and eventually the proteins) involved in the immune function of these cells to be cloned and analyzed.

We have used several mouse and human T cell-specific genes to probe sea urchin nucleic acids for homologous sequences. RNA gel blot experiments show that mouse Thy 1 cross hybridizes to transcripts from coelomocytes but not from 24-hour embryo transcripts. Genome blots show a set of bands when probed with Thy 1 and a subset of these bands are revealed when human T4 or T8 are used as probes.

A cDNA library was constructed from coelomocytes taken from xenogenetically challenged urchins, and 12 of 40,000 clones rescreened positively for Thy 1. Preliminary restriction analysis of these clones suggests that we have isolated three different cDNAs with shared restriction fragments. Cross hybridizations between clones show that all clones hybridize (to different extents) with several of the others. The DNA sequence will ultimately reveal the exact homology of the cloned urchin sequences to the lg super gene family.
7. Bindin Gene Evolution
Joseph E. Minor Jr.

Bindin is a sperm acrosomal protein that is responsible for the species-specific adhesion of sperm to eggs in sea urchins. The sea urchin species *Stronglylocentrotus purpuratus* and *S. franciscanus* have overlapping ranges on the West Coast and are frequently found in the same tide pools. In addition, they both breed during the same season, releasing their gametes into open water. Since bindin prevents cross-fertilization between these gametes, bindin therefore appears to be the species-isolating mechanism for these species. The aim of this study is to identify what changes in the bindin gene are responsible for the change in fertilization specificity and to ask whether the importance of this gene in species isolation affects its pattern of evolution.

The principle approach is to compare the bindin genes from the sea urchin species *S. purpuratus*, *S. franciscanus*, and *Lytechinus variegatus*. Previous work done by Boning Gao included cloning and sequencing a cDNA that contained 1.9 kb of the 2.5-kb bindin message of *S. purpuratus*. I have isolated cDNA clones that cover at least 2.3 kb of the 3.5-kb message of *S. franciscanus*. I also have prepared a cDNA library from testis poly(A)+ RNA from *L. variegatus*; the bindin message in this species is 3.0 kb. In addition, I have isolated EMBL-3 genomic clones for all three species. With these clones, I should be able to show changes in the bindin protein and to show how these changes were accomplished at the genomic level. I also intend to attempt to change the species specificity of fertilization among these species by transforming sea urchins with foreign sea urchin bindin genes.

8. Analysis of a Gene Expressed in the Sea Urchin Micromere Lineage

Henry M. Sucov

One of the best understood and most easily accessible lineages in the sea urchin is the micromere lineage. Formed as a quartet of particularly small cells at fourth cleavage at the vegetal pole of the embryo, these cells ultimately give rise to a protein-CaCO₃ skeletal structure known as a spicule. As primary cultures of isolated micromeres proceed to develop on schedule, forming spicules often indistinguishable from the in vivo counterpart, it is believed the micromeres are endowed at the time of their formation with the determinants necessary and unique for their ontogeny.

We have isolated and fully characterized a single-copy gene, designated SM50, which is expressed exclusively in the micromere lineage and encodes one of the predominant matrix proteins of the spicule. As a means of addressing which sequences of the SM50 gene might interact with transcription factors, we have cloned the promoter region of the gene so as to direct expression of the reporter enzyme CAT, as described in other reports from this lab. This construct expresses CAT RNA only in the micromere lineage, indicating that proper spatial regulation is maintained with the promoter present on our constructs. Furthermore, we have narrowed the amount of promoter DNA required for expression to the proximal 600 bp 5' of the transcription initiation site. We are in the process of determining by gel retardation assays and DNase footprinting which regions of this promoter interact with protein factors. Ultimately, we hope to determine which of these factors are unique to the micromere lineage. It is conceivable that one or several such factors are localized in the egg cytoplasm at the vegetal pole where the micromeres will form. If so, in addition to being factors required for transcription, these proteins may also have a more general determinative influence in the choice of lineage of the micromere cells that inherit them.

9. Ongoing Studies of Cell Lineage in Sea Urchin Embryos

R. Andrew Cameron, Scott Fraser

Gene expression by developing sea urchins is specific in both time and tissue. The analysis of these patterns requires an understanding of the fate of individual embryonic cells through development into the adult organism.

We constructed a cell lineage diagram that assigns the progeny of cleavage-stage blastomeres to the tissue types of the mesenchyme blastula and pluteus stages. To fill in some of the missing data and test the gratuitous assumptions of this diagram, we have pressure-injected a fluoresceinated lineage tracer into individual blastomeres of eight-cell sea urchin embryos.
and determined the location of the progeny of these cells in the fully developed pluteus. We confirmed the classical assignments of cell fate along the animal-vegetal axis and demonstrated that each blastomere gives rise to a unique portion of the advanced embryo. Clones of cells derived from ectodermal founder cells remain contiguous, while clones of cells descendant from the vegetal plate do not. The locations of ectodermal clones contributed by specific blastomeres require that the larval plane of symmetry lies approximately equidistant from each of the two cleavage planes.

Many later details of the developmental fates of the cleavage-stage blastomeres remain unknown. The lateral blastomeres of the animal hemisphere at the eight-cell stage contribute to both oral and aboral ectoderm, but the point at which these fates diverge is not known. The small micromeres of the vegetal plate region contribute to the coelomic sacs but we do not know if this fate is exclusive. To address these questions, we apply the more delicate iontophoretic method of dye injection to blastomeres at later stages of development. Because less dye is injected, image-enhancing video microscopy is used to detect labeled cells. Preliminary injections of mesomeres at the 16-cell stage indicate that the separation into aboral and oral ectodermal fates occurs at this stage.

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10. P Element-mediated Transformation of the Sea Urchin Genome

Pierre Thiebaud

We are trying to use Drosophila P elements as a vector to transform the sea urchin genome. We constructed the plasmid C1-Cyllla-CAT by inserting the Cyllla-CAT fusion gene into Carnegie 1 plasmid. We injected this construct into Strongylocentrotus purpuratus eggs and are currently studying the fate of exogenous DNA by genome blot analysis of juvenile sea urchins developed from the injected eggs.

11. Sequence Elements Mediating Lineage-specific Expression of Spec1b and Cyllla

Donna L. Livant

Among the genes specifically transcribed in the aboral ectoderm of sea urchin embryos and larvae are the Spec genes encoding calcium-binding proteins and the cytoskeletal actin genes CyIIIa and CyIIIb. Spec mRNAs encode small, acidic proteins related to Troponin C by sequence. The predominant Spec mRNAs, Spec 1 mRNAs, are 1.5 kb in length and begin accumulating in the cells of the aboral ectoderm lineage at approximately 20 hours postfertilization. The Cyllla gene is activated at about the same time as are the Spec 1 genes and in the same cells. Because expression of the Cyllla and Spec 1 genes begins only in the presumptive aboral ectoderm of the blastula stage, and because the kinetics of accumulation of the two types of transcripts are similar, Spec 1 and Cyllla genes are candidates for coordinately regulated genes whose functions are distinct but must be present simultaneously in one cell lineage.

We currently are doing experiments to test the hypothesis that Cyllla and Spec 1b are regulated by the same lineage-specific transcriptional activators. We have demonstrated that at least one trans-activator necessary for Cyllla expression is present in limiting amounts in 24-hour embryos (see Abstract No. 15). If Cyllla and Spec 1b are both regulated by the same limiting trans-activators, it should be possible to use DNA fragments containing the Spec 1b regulatory elements binding these activators in excess to compete for trans-activator binding of the regulatory elements of the Cyllla-CAT construct during embryogenesis. Similar competitive behavior, assayed as decreased CAT activity in 24-hour embryos, by the same molar amounts of Spec 1b and Cyllla regulatory elements would be evidence that these two genes are regulated by the same trans-activators. Recently, we have observed competitive behavior of a particular region 5' of the Spec 1b gene similar to that seen for a fragment 5' to Cyllla containing elements P1-P8 (Abstract No. 20). We are particularly interested in using this system to arrive at an in vivo definition of a lineage-specific regulatory element.

12. In Situ Analysis of DNA Introduced by Microinjection into Sea Urchin Eggs

Barbara R. Hough-Evans

It has been established by in situ hybridization that sea urchin embryos that develop from eggs injected with the fusion gene Cyllla-CAT contain CAT mRNA only in cells of the aboral ectoderm, the tissue to
which the expression of the cytoplasmic actin gene CyIIla is normally confined (Biology 1986, Abstract No. 46). We have applied similar techniques to another group of CAT-containing 72-hour plutei, hybridizing alternate 5 µm sections with a probe for CAT DNA. These slides again show CAT mRNA only in aboral ectoderm, but CAT DNA was present in cells of gut, oral ectoderm, and mesenchyme as well.

In an attempt to quantitate the distribution of injected DNA molecules in the embryo, we have scored serial sections of blastula-stage embryos after hybridization with the CAT DNA probe. At blastula stage, the future aboral ectoderm cells (about half of the blastula cells) cannot be identified morphologically, but can be distinguished only by expression of CyIIla actin and other aboral ectoderm-specific genes. In a preliminary experiment using blastulae that developed from a single batch of CyIIla-CAT-injected eggs, we found that an average of 18 cells per embryo contained CAT DNA while nine cells per embryo were positive for CAT mRNA. Hybridization to whole, squashed blastulae is also being tried. Here the blastulae (essentially hollow balls of about 400 cells) are crushed between slide and coverslip, fixed in 1% glutaraldehyde, and used for in situ hybridizations. Parallel hybridization with histone gene sequences (present 600 times in every nucleus) is used as a positive, quantitative control. We plan to examine the number of nuclei that contain the injected DNA in embryos of very early stages, through cleavage and early blastulae, if this method proves to be feasible.

13. Loss of Spatial Regulation of a Sea Urchin Actin Gene in a Second Species

Robert R. Franks, Barbara R. Hough-Evans

The CyIIla cytoplasmic actin gene of the sea urchin Strongylocentrotus purpuratus is expressed in embryos, beginning at about the blastula stage, and in larvae but not in adult tissues, and its activity is restricted to cells of the aboral ectoderm. When the cloned fusion gene CyIIla-CAT, which consists of about 9.5 kb of 5' flanking sequence and first intron from the CyIIla gene linked to the bacterial CAT gene, is injected into eggs of this species the CyIIla promoter is activated in the embryos that develop at the appropriate stage of embryogenesis, as indicated by the timing of production of CAT mRNA and CAT enzyme. In addition, the gene is expressed only in the aboral ectoderm, based on the results of in situ hybridization experiments with a radioactive probe specific for CAT mRNA. The implication is that the cis-regulatory elements that control the temporal and spatial expression of the CyIIla actin gene are included in the fusion gene and are recognized by trans-activator(s) of the CyIIla gene in embryos. Similarly, when the CyIIla-CAT gene is introduced into the eggs of a different sea urchin species, Lytechinus variegatus, CAT enzyme is produced at the analogous developmental time. Surprisingly, tissue-specific regulation is lost, as in situ hybridization reveals CAT mRNA in all cell types of the developing embryos. These results suggest that the CyIIla DNA elements that confer temporal and spatial regulation are distinct and imply the existence of a negative control element that is not functional, or recognized, in cells of this second species.

14. Interspecies Comparison of Actin CyIIla Regulatory Region

Steven R. Fain, Joseph E. Minor Jr.

Previous work has shown that sea urchin Strongylocentrotus purpuratus (Sp) embryos that develop from eggs injected with a fusion gene comprised of 5' regulatory sequences of the CyIIla actin gene of Sp and the CAT reporter gene express the CAT gene with the same temporal and spatial specificity as the CyIIla gene. When this same fusion gene (with Sp regulatory sequences) is injected into eggs of Lytechinus variegatus (Lv), the CAT gene is expressed at the appropriate time, even though these two species are very distant from each other (Biology 1985, Abstract No. 45). We plan to identify the appropriate actin gene in Lv and compare the 5' regulatory region with that of Sp CyIIla in order to examine the conserved regulatory sequences.

Gel blots of Lv egg and blastula RNAs have shown that actin gene transcripts are not present in eggs and are present in the poly(A)+ RNA of hatching blastula as in Sp. However, in Lv a single transcript size is observed (2.2 kb) compared to the two transcript sizes in Sp (2.2 and 1.8 kb). An oligo(dT)-primed cDNA library has been prepared from Lv hatching blastula poly(A)+ RNA and it contains 500,000 clones of average length about 1.8 kb. Screening with an exon probe indicates that 0.04% of the clones are actin
transcripts, and several have been plaque purified. It has been shown that the 3' untranslated probe used to identify the actin CyIIIa gene of Sp fails to hybridize with Lv DNA but a similar probe for the CyI gene does hybridize well with Lv DNA. Therefore, gel blots of these cDNA clones have been hybridized with the CyI 3' untranslated probe and an exon probe as a first step in identification. Surprisingly, 5 out of 5 of these clones reacted with the CyI probe, indicating that CyI transcripts are more prevalent in Lv embryos, whereas at this stage in Sp, CyIIIa transcripts predominate.

15. Titration of CyIIIa-CAT Gene Expression in Sea Urchin Embryos

Donna L. Livant, Barbara R. Hough-Evans, Ann E. Cutting

CyIIIa gene transcription begins in presumptive aboral ectoderm cells of the early blastula stage sea urchin embryos. The expression construct CyIIIa-CAT contains approximately 8 kb of sequence upstream of the CyIIIa gene joined to the bacterial CAT gene. After injection into unfertilized sea urchin eggs, CyIIIa-CAT is expressed in the same cells at the same time as the endogenous CyIIIa gene.

We have injected varying numbers of CyIIIa-CAT molecules into eggs and observed the variation in molecules of CAT enzyme per embryo at 24 hours with respect to the number of molecules of CyIIIa-CAT recovered in 24-hour embryos. The titration curves generated from these data show nearly linear increase in CAT enzyme per embryo as the number of CyIIIa-CAT DNA molecules per embryo increases until a point is reached beyond which further increases in CyIIIa-CAT DNA result in no increase in detected CAT enzyme per embryo.

These results demonstrate that at least one trans-activator necessary for the expression of the CyIIIa gene is present in limiting or near-limiting amounts in the 24-hour embryo. We have also observed at least a fivefold variation in the number of CyIIIa-CAT DNA molecules per embryo required to reach saturation in CAT expression. This result suggests that the concentration of the trans-activator necessary for CAT expression varies in the embryos developing from the eggs of different females. We are currently measuring the number of nuclei in 24-hour embryos containing CyIIIa-CAT DNA in order to estimate the concentration of this activator in the nucleus of the developing embryo. We are very interested in the possibility that this limiting trans-activator corresponds to one of the factors defined in vitro by Calzone and co-workers (see Abstract No. 20).

16. Identification of Gene Regulatory Elements by In Vivo Competition Analysis

Robert R. Franks

DNA transformation studies have shown that the fusion gene CyIIIa-CAT, which contains the CyIIIa actin gene promoter of the sea urchin Strongylocentrotus purpuratus linked to the bacterial CAT gene, contains DNA elements that interact with trans-activators of the CyIIIa gene in developing embryos, and that these elements are sufficient for the ontogenic regulation of this promoter. It also has been shown that CyIIIa trans-activators are present in limiting amounts since they can be titrated by transforming embryos with increasing amounts of CyIIIa-CAT DNA. In order to identify those CyIIIa DNA elements that compete for binding of these factors, embryos are transformed by coinjecting CyIIIa-CAT and a given upstream CyIIIa DNA fragment in excess. We have thus far identified several DNA fragments extending from the CAT site to about 2.5 kb upstream that act as competitors for the binding of CyIIIa transcription factors, based on a decrease in CyIIIa-CAT gene activity in transformed embryos. These DNA regions coincide with CyIIIa gene fragments that have been shown in DNase I and exonuclease III protection assays to interact with DNA binding proteins obtained from embryo nuclear extracts (see Abstract No. 20). Fragments extending further upstream, between -2.5 kb and -9.5 kb, do not compete, nor does a 1.3 kb fragment containing the 3' half of the first intron of the CyIIIa gene, which is also present on the CyIIIa-CAT construct. Progressively smaller DNA fragments are being used in these competition assays to delineate the relevant control elements.

17. Coregulation of the Sea Urchin Myosin Heavy Chain Gene with Muscle Actin

Samuel J. Rose III

A fragment of a Strongylocentrotus purpuratus (Sp) myosin heavy chain (MHC) gene was isolated from a genomic recombinant DNA library by cross reaction
with a cloned Drosophila melanogaster (Dm) MHC probe. A portion of a 227 nucleotide Sp coding sequence that is included in this fragment predicts a peptide very closely homologous with a region of the Dm sequence. The MHC gene sequence is present in a single copy per haploid Sp genome, and the gene is utilized in adult as well as embryonic muscle. The quantity of MHC transcript was measured in embryos of various stages by single-strand RNA probe excess titration. Transcripts are not observed until post-gastrular stages, after which they accumulate rapidly. The time course of accumulation closely parallels that measured earlier for muscle actin message.

18. Transcriptional Regulation of the Sea Urchin Muscle Actin Gene
Donna L. Livant, Jeffrey D. Tekanic

We are interested in the transcriptional regulation of the muscle (M) actin gene of sea urchin as an example of a zygotic gene activated late in embryogenesis in only a few cells. Unlike many other transcripts, no M actin transcripts are found in the egg. Muscle actin transcripts are found about 60 hours post-fertilization only in 20-40 cells associated with the coelomic sacs of gastrulating embryos. In these cells, the M actin transcripts accumulate to about 1000 molecules per cell by late pluteus.

We have isolated the M actin gene from S. purpuratus, including approximately 8 kb of upstream sequence and 2 kb of downstream sequence. We expect the M actin regulatory elements to be present in these sequences. By analogy with previous results for the cytoskeletal actins Cyllla and Cyl, we are searching for these elements 5' to the translational start site of the gene. We have subcloned the 8 kb of 5' flanking sequence and have made an expression construct with the bacterial CAT gene in order to assay this sequence for M actin regulatory elements. This construct expresses CAT in four-day embryos. We are currently examining the time course of CAT expression more closely to see if it corresponds to that of the endogenous M actin gene. We also are determining the location of the M actin transcriptional start site by S1 analysis and primer extension. By analogy with the Cyllla and Cyl actin genes, we expect it to be 1.5-2 kb upstream of the translational start site.

19. Larval Gene Expression
R. Andrew Cameron

The larva stage intervenes between the embryo and the adult in the sea urchin life cycle. The larva, which is adapted to swim and feed in the water column, furnishes a way to disperse the genotype and precociously develop structures needed to form the adult. Metamorphosis marks the transition from the larva to the adult and, once competent, the larva may delay metamorphosis until a suitable substrate is encountered.

The sea urchin larvae probably demonstrates unique patterns of gene expression. For example, several genes, including a member of the actin gene family (Cyllla), are expressed exclusively in the aboral ectoderm, a tissue that is completely re-absorbed at the end of the larval existence. Some of these genes have been proposed to function by causing the collapse of the larval tissue at metamorphosis.

We have measured amounts of total RNA and the number of specific actin transcripts per larva at several stages of larval development. Before feeding, the sea urchin embryo contains 2.8 ng of total RNA; at two weeks postfertilization, this has increased to 18 ng, at four weeks to 40 ng, and at five weeks, after the larvae have become competent to metamorphose, to 104 ng. The number of transcripts of an actin gene expressed during both larval and adult existence (Cyllla) increases from 9.1 x 10^4 at two weeks to 41 x 10^4 at four weeks and 52 x 10^4 at five weeks. In contrast, the larval-specific actin transcripts (Cyllla) increase from 1.8 x 10^5 at two weeks to 3.8 x 10^5 at four weeks, but decrease at five weeks to 3.0 x 10^5.

The decrease in Cyllla expression probably reflects the differentiated state of this tissue just before the re-absorption that occurs at metamorphosis.

20. Sea Urchin Actin Transcription Factors
Frank J. Calzone, Nadine J. Thézé, Pierre Thiebaud, Ronald L. Hill

Transcription of the Cyllla genes is activated synchronously in a relatively large number of founder cells at the early blastula stage (14-18 hr post-fertilization). We have identified eight different genomic sequence elements 5' to the start of transcription of the Cyllla gene which react in vitro with a high degree of specificity with proteins extracted from
blastula stage nuclei. A quantitative study has shown that the synthesis and appearance in nuclei of each of the eight DNA binding proteins (called P1-P8) are regulated differently during development. Three factors (P3, P4, P5) are present in the cytoplasm of the unfertilized egg. De novo synthesis is not necessary to explain the level of the maternal factors in nuclei of the blastula stage embryo. Factors P1, P2, P3, P4, P5, and P8 have been detected in embryo nuclei prior to blastula stage (7 hr postfertilization); however, the maternal factor, P5, appears to be absent from nuclei prior to activation of the Cylla gene. Factors P4 and P7 have not yet been examined in the 7-hour embryo. Interestingly, gel retardation data suggest that the structures of P3, P4, and P5 extracted from embryo nuclei differ slightly from the proteins extracted from the egg cytoplasm, although proteins from both sources have the same sequence specificity. It is possible that structural modifications are required to activate the maternal proteins. We are very interested to know if any maternal factor is inherited specifically by the progenitors of aboral ectoderm.

21. DNA-Protein Interactions in the 5' Regulatory Sequences of the Cylla Gene

Nadine J. Thézé, Pierre Thiebaud, Frank J. Calzone, Ronald L. Hill

We have used DNase I footprinting assays to identify sequence-specific DNA-binding proteins in crude nuclear extracts of sea urchin embryos. These proteins interact selectively with regulatory sequences in the 5' flanking region of the Cylla actin gene (see Abstract No. 20). We are looking at a 2.5 kb 5' sequence which is known to be important from in vivo competition experiments (Abstract No. 16) for the activation of the Cylla gene. This 2.5 kb DNA has been sequenced and subcloned in shorter fragments ready to use for this study. This sequence also will be used for 5' flanking sequence comparison studies with other sea urchin genes (Abstract No. 14).

22. Identity between Trans-Activators of Cylla Gene and Other Sea Urchin Genes

Pierre Thiebaud, Nadine J. Thézé, Frank J. Calzone

In order to know whether the DNA binding proteins interacting with the 5' regulatory regions of the Cylla gene (Abstract No. 20) interact with other sea urchin genes, we will compare by gel retardation competition experiments the 5' sequences of the muscle actin gene, Cyl actin gene, spicule matrix protein gene, and Spec gene with the 5' sequences of the Cylla gene.

23. Expression of Mitochondrial Genes in Early Mouse Embryos

Lajos Piko, Kent D. Taylor

The mouse egg contains about 90,000 mitochondria which undergo morphological and functional differentiation beginning with the 4- to 8-cell stage. In order to gain insight into mitochondrial genetic activity in early embryos, we determined the contents of mitochondrial DNA (mtDNA) and the steady-state amounts of 12S and 16S mitochondrial rRNAs and the mRNAs for cytochrome c oxidase subunits I and II (COI and COII) in dot hybridization experiments with cloned mtDNA probes during development from the one-cell to the blastocyst stage. The mtDNA content remained constant during this period at about 2.13 pg or 119,000 mtDNA molecules per embryo, suggesting an absence of mtDNA replication. The amounts of mitochondrial rRNA and the mRNAs for COI and COII varied markedly depending on developmental stage. They remained low between the end of oocyte growth and the late two-cell stage but increased 25-50X during cleavage from two-cell to early blastocyst (32 cells), which takes about two days. In the early blastocyst, the number of mitochondrial mRNA molecules was estimated at 7.9 x 10^6 or about 23% of the total embryo poly(A)^+ RNA. These results suggest that the mitochondrial genome is largely inactive in the egg and two-cell embryo but that a high rate of mitochondrial transcription is initiated during cleavage. The evidence indicates that the steady-state concentration of mitochondrial gene transcripts during this period is regulated primarily at the level of transcription.

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Summary: Our laboratory is interested in the structure, organization, regulation, evolution and function of receptors of the immune response. We have focused on the study of two of these classes of receptors, the class I molecules encoded by the major histocompatibility complex and the T-cell receptors. In the abstracts that follow, both of these systems have been analyzed in a variety of different ways.

The receptors of the immune system all share immunoglobulin homology units and, accordingly, are members of the immunoglobulin gene superfamily. Members now include a wide variety of different molecules, as shown in Table 1 (opposite), and we are interested in designing general strategies for identifying new members of the immunoglobulin gene superfamily.

We have studied the structure, organization and expression of myelin basic protein (MBP), a major component of the myelin sheath. This past year we succeeded in curing the shiverer mouse, which has a deletion in its MBP gene, by constructing a transgenic shiverer mouse with a functional MBP gene. In addition, we are beginning to study the evolution of MBP and P0, another component of myelin, by isolating these genes from the shark, *Heterodontus*.

We have established two types of microchemical facilities—one, the developmental, is dedicated to the task of developing new chemistries and instrumentation for synthesizing, mapping and sequencing genes and proteins. The second category—the applied microchemical facilities—handles the protein sequencing (directed by David Teplow) and DNA and protein syntheses (directed by Suzanna Horvath) requirements for the Division.

Dr. Kent has employed our protein synthesis capacities to carry out a series of fascinating structure-function studies on interesting hormones. He is also analyzing the proteins involved in the diseases AIDS and hepatitis B.

Table 1

<table>
<thead>
<tr>
<th>IMMUNOGLOBULIN GENE SUPERFAMILY</th>
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<tr>
<td>Antigen Recognition</td>
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<td>T-cell receptors</td>
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<td>Immunoglobulins</td>
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<tr>
<td>MHC molecules (class I, class II, ( \beta_2 )-microglobulin)</td>
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<tr>
<td>Nonantigen-Presenting Class I-like Receptors</td>
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<td>Qa</td>
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<td>Tla</td>
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<tr>
<td>CD1</td>
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<tr>
<td>T Cell-Associated Molecules</td>
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<td>CD3 ((\gamma, \delta, \epsilon))</td>
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<tr>
<td>CD4</td>
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<td>CD8</td>
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<td>Ig-Binding Molecules</td>
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<tr>
<td>Poly Ig receptors</td>
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<tr>
<td>( \alpha/\beta ) glycoprotein</td>
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24. Genomic Organization of the Mouse T-Cell Receptor \( \beta \) Gene Family

*Éric H. C. Lai, Richard K. Barth*

The antigen-binding T-cell receptors are encoded in mice by two families of genes—the \( \alpha \) genes located on
chromosome 14 and the β genes on chromosome 6. We have focused our study on the structure and organization of the β genes. The β genes are composed of a constant gene (Cβ) and variable (Vβ), diversity (Dβ) and joining (Jβ) gene segments, which rearrange and are joined together at the DNA level during T-cell differentiation to generate an assembled Vβ gene. The structure of the Dβ-Jβ-Cβ region has been well characterized. However, the relative order and location of the Vβ gene segments are not known. The murine β gene family contains just 20-30 Vβ gene segments by statistical estimates, 19 of which have been identified from cDNA or genomic clones.

We have employed three distinct but complementary methods to map the genomic organization of the β gene family. First, deletional mapping has permitted us to determine the relative order of the Vβ gene segments. On the chromosome that has experienced a Vβ-Dβ-Jβ deletional arrangement, the Vβ gene segments located between the rearranging Vβ and Dβ gene segments are lost and hence the presence or absence of Vβ gene segments can be determined by Southern blot analysis. An examination of many T-cell lines with different rearranged Vβ gene segments allows the generation of a deletion map of the β gene family. Second, restriction enzyme mapping of germline genomic DNA was carried out by field inversion gel electrophoresis, which has the capacity to separate DNA fragments ranging in size from 20 to 1600 kb. Finally, the fine structure of a portion of the β gene family was examined by analysis of a cosmid clone. Together, the three approaches have allowed us to map 19 Vβ gene segments and the Dβ-Jβ-Cβ region as shown below:

\[ V_β^{2.4-16-10-1-5-2-8.3-5.1-8.2-8.1-{[9,11,12,13]-6-15-3-7-D_β-J_β-C_β-14} \]

The Vβ gene segments of the β gene family span approximately 450 kb of DNA, excluding one potential gap in the DNA fragment alignments.


Eric H. C. Lai, Patrick J. Concannon

The generation of an immune response depends upon the interaction of various hematopoietic cell types among which T cells and their receptors play a central role. The human T-cell receptor, like their murine counterparts, is a heterodimer with its α and β chains each having a variable (V) region, which recognizes antigens, and a constant (C) region, which anchors the T-cell receptor to the T-cell membrane. During the past year, we have focused our work on analyzing the β gene family. The β chain is encoded by three gene segments, variable (Vβ), diversity (Dβ) and joining (Jβ), which rearrange and recombine 5' to a Cβ gene during T-cell differentiation. There are two Cβ genes, each associated with a Dβ and either six or seven Jβ gene segments to their 5' sides. However, the number and locations of the human Vβ segments are unknown. A statistical analysis has suggested that there are about 60 functional Vβ gene segments and at present, there are 37 distinct Vβ gene segments published in the literature. We are interested in mapping the location of all the human Vβ gene segments.

We have used the same three approaches: 1) deletional analysis of Vβ gene segments in T-cell lines, 2) Southern blot analyses of field inversion gels with Vβ probes, and 3) restriction enzyme analyses of cosmid clones (see Abstract No. 24). We have mapped 40 Vβ gene segments as well as both constant gene clusters to within 600 kb of DNA. Human Vβ gene segments with varying degrees of nucleotide homology were highly interspersed and we estimate that the size of the β gene family is ~1000 kb. Extensive gene duplication has occurred in the human β gene family and this accounts for the increase of the number of human Vβ gene segments as compared to the mouse. Comparisons of the human and murine Vβ gene segments revealed more than 12 homologues (>65% homology). A striking observation is that the relative order of the human and murine Vβ homologues appears to be conserved along the chromosome, suggesting that there may be selective pressure operating to maintain the order of these gene segments along the chromosome.
peptide antigen in the context of the MHC-encoded I-A<sup>K</sup> molecule, whereas the other five T-cell hybridomas are I-E<sup>K</sup> restricted. The I-A<sup>K</sup>-restricted cells recognize determinants contained within the amino-terminal residues 74-86, while the I-E<sup>K</sup>-restricted cells recognize determinants of the C-terminal residues 85-96. These observations suggest that specific interactions between certain residues of the antigen and the restricting MHC molecule are important for determinant selection.

We isolated eight β gene rearrangements from these cells and subjected them to DNA sequence analysis. We identified five distinct V<sub>β</sub> gene segments (V<sub>β</sub>2, V<sub>β</sub>6, V<sub>β</sub>8.3, V<sub>β</sub>10, V<sub>β</sub>12) and six distinct J<sub>β</sub> gene segments (J<sub>β</sub>1-1, J<sub>β</sub>1-2, J<sub>β</sub>1-3, J<sub>β</sub>1-5, J<sub>β</sub>2-2, J<sub>β</sub>2-5) used by these T cells. Hence, the T cells we analyzed are not derived from a single set of rearranged receptor genes that have, for example, undergone somatic hypermutation events. Our results indicate that T-cell responses to single determinants such as small peptide antigens are made up of many independent clonotypes.

We have isolated several genomic rearranged a clones using specific J<sub>a</sub> probes. In the cells in which we cannot identify a rearrangement, we have constructed cDNA libraries. One of these cell lines is using V<sub>a</sub>8.3. Using hybridization probes for V<sub>a</sub>2, V<sub>a</sub>4, V<sub>a</sub>5, V<sub>a</sub>6, V<sub>a</sub>7, V<sub>a</sub>9 and V<sub>a</sub>11, we do not detect expression on Northern blots. Our sequence analysis is in progress on α-genomic and α-cDNA clones.

Both the β and α gene analyses have been extended to lysozyme-specific T-cell hybridomas derived from C57BL/6 and B6-C-H-2<sup>bm12</sup> mice. Some of these T-cell hybridomas recognize the peptide region 74-90. There are only three amino acid differences between the I-A<sup>b</sup> molecule and the I-A<sup>bm12</sup> molecule that these cells recognize with peptide. Our current data indicate several different cell lines use V<sub>β</sub>3 and V<sub>a</sub>12 (V<sub>a</sub>12 is a previously undescribed mouse V<sub>a</sub> gene segment that we have identified by sequence analysis) based on Northern blots. Sequence analysis will reveal the detailed structure of the α and β genes.

27. Human T-cell Receptor V<sub>β</sub> Gene Polymorphism

Patrick J. Concannon, Richard A. Gatti

Southern blot hybridizations with human T-cell receptor V<sub>β</sub> gene probes were used to determine the sizes of the various V<sub>β</sub> gene subfamilies. An analysis of DNA samples from 100 unrelated individuals identified a single individual who lacked one V<sub>β</sub> gene segment. A second individual had an apparently different repertoire of V<sub>β</sub> gene segments in one subfamily, as assayed by hybridization, possibly due to a gene conversion event. An analysis with four restriction enzymes of DNA from 30 consanguineous donors detected restriction fragment length polymorphisms associated with 12 of 14 V<sub>β</sub> gene segment subfamilies examined. In an analysis of DNAs from a large panel of unrelated individuals, some alleles at these loci were found to be in linkage disequilibrium, indicating a potentially close physical linkage. The segregation of three polymorphisms, two associated with V<sub>β</sub> gene segment loci and one associated with the C<sub>β</sub> genes, was compatible with Mendelian inheritance, and demonstrated that highly informative haplotypes could be generated. The high degree of polymorphism observed in the human T-cell receptor β chain complex should allow exploration of possible associations between T-cell receptor genes and inherited diseases involving the immune system.

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28. Analysis of the 5′ End Control Element for T-cell Receptor Gene Expression

Nancy C. Lan, Kai Wang

In contrast to T cells, mouse Tk<sup>−</sup> L cells which were transiently or stably transfected with complete T-cell receptor V<sub>β</sub> genes do not express detectable transcripts, suggesting that the gene is transcriptionally silent in fibroblast cells. This project focused on studying the role of 5′ end regions of T-cell receptor genes in their tissue-specific expression.

Several approaches are taken in this study: 1) Constructs containing 5′ end regions of T-cell receptor V<sub>a</sub> and V<sub>β</sub> genes linked to a tissue-nonspecific transcription unit containing the bacterial gpt gene, SV40-derived splice and polyadenylate sites were transfected into L and T cells. A modified mouse H<sub>4</sub> histone gene is also present in these constructs to serve as an internal control for transfection efficiency. Dot blot analysis of RNAs prepared from L or T cells transfected with constructs containing V<sub>a</sub> promoter but not β promoter indicates severalfold higher expression of gpt gene in T cells than in L cells.
The sizes and the levels of gpt and H4 gene transcripts in these transfected cells are currently being analyzed by S1 analysis. 2) DNase I hypersensitive sites at 5' end region of a pair of Va and Vb genes were studied in T cells which express these genes. We found a DNase I hypersensitive site at 0.3 kb and 2.3 kb upstream from the coding region of the Va and Vb genes, respectively. These regions of Va but not Vb are included in our transfection constructs; however, whether these sites correlate to the specific expression of these genes in T cells will await deletion (Va) and extension (Vb) studies in the future. 3) The 5' end regions of several Va and Vb genes have been sequenced. We are currently analyzing these sequences and examining conserved sequences among these genes. 4) Footprinting and gel mobility shift experiments involve 5' end regions of Va and Vb genes using nuclear extracts from T cells or non-lymphoid cells are in progress. The results of these studies should provide us with some information on the promoter regions (or sequences) of T-cell receptor genes which are, in part, responsible for their tissue-specific expression, and on nuclear factors which might play a role in governing such a specificity.

29. Antigen/MHC-specific Activation of Murine T Cells Transfected with Functionally Rearranged T-Cell Receptor Genes

Chia-lam Kuo

The genes encoding the α and β chains of the T-cell receptor isolated from an antigen-specific, MHC-restricted murine T-cell hybridoma were introduced into a mouse T-cell line of helper lineage by electroporation. In order to examine the contributions of those gene products to antigen and/or MHC specificity, the resultant transfectants were tested for functional antigen and/or MHC recognition. Only those transfectants that express both the introduced α and β genes contributed by the normal T cell can respond specifically to the appropriate antigen-MHC pair. None of the transfectants that expressed the introduced single α or β gene, or a paired hybrid gene, i.e., one gene from the normal T cell and the other from the fusion partner, can respond to the same combination of antigen and MHC product recognized by the donor T cell. However, one clone expressing the transfected genes that encode the α and β chains of the fusion partner shows reactivity to the antigen-presenting cells even in the absence of the antigens. These data suggest that the αβ heterodimer of the T-cell receptor is required to define the fine specificity of a T cell.

30. Chimeric Receptor Genes as a Probe of T-cell Antigen Recognition and Function

Joan Goverman

B cells bind soluble antigen through immunoglobulin (Ig) receptors, while T cells recognize antigen only when it is presented on a cell surface in association with polypeptides encoded in the major histocompatibility complex (MHC). Characterization of the T-cell receptor has revealed that it is similar in many respects to Ig: both are heterodimeric structures in which each individual chain is composed of a variable (V) and a constant (C) region. The Ig chains are designated light (L) and heavy (H) and the T-cell receptor chains are designated α and β. The V regions are involved in binding antigen while the C regions are involved in membrane attachment and effector function. The V regions in both receptors have remarkably similar tertiary structures and are encoded by genetic elements distinct from those that encode the C regions. These characteristics have enabled us to design a strategy to investigate mechanisms of T-cell antigen recognition and activation. We have constructed chimeric receptor genes in which the V regions of the T-cell receptor are replaced with those of an Ig, thus generating T cells specific for a soluble, well characterized antigen instead of cell-surface antigen associated with MHC molecules. T cells expressing the chimeric receptors will be used to study receptor-protein interactions and T-cell activation requirements. The effects of soluble versus cell surface-bound antigen, antigen multivalency and association of antigen with MHC molecules of different classes will be studied under conditions in which the T-cell receptor is triggered in a physiological fashion, but separated from the context of a MHC molecule.

We have generated constructs containing two hybrid genes: one containing a VH gene segment joined to a Cβ gene segment and the other containing a VL gene segment joined to a Ca gene segment. We have introduced these constructs into a T-cell
thymoma, EL4, and demonstrated that we obtain good levels of expression of both of the hybrid genes. Using FACS analysis and anti-idiotpe antibodies specific for the V_H gene segment, we observed significant expression of the hybrid V_H-C_B chain on the cell surface. We are carrying out immunoprecipitation experiments to determine whether this hybrid chain is expressed on the surface associated with the endogenous a chain. Cells expressing the V_L-C_a hybrid gene are currently being investigated for expression of hybrid protein. T cells expressing both hybrid genes will then be analyzed for the ability to respond to antigen under various conditions.

31. Molecular Characterization of T-cell Receptors Directed Against Myelin Basic Protein

Dwight H. Kono, James L. Urban, Vipin Kumar, Suzanna J. Horvath, Raúl A. Saavedra

Experimental allergic encephalomyelitis (EAE) is an autoimmune disease of the central nervous system induced in genetically susceptible animals by immunization with myelin basic protein (MBP). Aspects of the disease have similarities to human post-rabies immunization, encephalitis and multiple sclerosis. EAE is also a prototypic model for T cell-mediated autoimmune disease since T-helper cell depletion can prevent and cure the disease and MBP-specific T-helper clones can transfer the disease to naive animals. In addition, the ability to develop disease is MHC restricted and different peptide fragments of MBP cause disease in different strains of mice. Our project involves molecular characterization of the immune response to MBP necessary for the development of EAE in SJL mice.

We have identified a minor epitope in the C-terminal of small mouse MBP, residues 86-97, which can induce severe EAE in SJL mice. This epitope appears to be the "EAE-causing" epitope previously described in the C terminal of small rat MBP. We are also screening for other "EAE-causing" MBP epitopes. We have generated cell lines and clones to the peptide determinants of both SJL and B10.PL mice. Currently, clones are being sequenced to determine if the T helper response is oligoclonal, with usage of only a limited number of variable gene segments as has been found with pigeon cytochrome c.

The next aspect of the project involves various methods to treat the disease using the data we have obtained from the molecular characterization. As such, we have begun to study the effects on disease of tolerizing mice with peptides and the use of T-cell receptor-specific monoclonal cytotoxic T cells and antibodies generated against peptide-specific T-cell clones. We will also be using probes generated from the sequencing data to study the immune response on a molecular level to contrast this response to an autoantigen (MBP) with the "normal" immune response.

32. The Molecular Specificity of Experimental Autoimmune Thyroiditis

Richard K. Wilson, James L. Urban

Experimental autoimmune thyroiditis (EAT) is a T cell-mediated autoimmune disease induced in genetically susceptible animals upon immunization with autologous thyroglobulin (TG) or adoptive transfer of syngeneic thyroglobulin-responsive T cells. EAT and analogous human autoimmune thyroid diseases are characterized by infiltration of the thyroid by mononuclear cells, hyperstimulation of hormone secretion and tissue destruction usually resulting in the inhibition of thyroid function. We have produced and cloned several mouse T-cell lines that are reactive to bovine TG and cause varying degrees of autoimmune thyroiditis in mice. Additionally, we have produced several anti-TG T-cell hybridomas. Using the T-cell lines and hybridomas, we have localized the disease-causing epitope to a small fragment of TG, which will be partially sequenced and fine-mapped using synthetic peptides. Furthermore, T-cell receptor-specific cDNAs from the disease-causing cell lines have been prepared and will be sequenced and compared to determine if predominant usage of specific V_d and/or V_b gene segments occurs in autoimmune thyroiditis. The knowledge gained from these experiments will eventually allow us to engineer cytotoxic anti-receptor antibodies capable of specifically deleting the autoreactive anti-TG T-cell subset from diseased animals.

33. Experimental Allergic Encephalomyelitis: T-cell Receptor V-Region Gene Expression

Bernhard Arden, Hartmut Wekerle

Experimental allergic encephalomyelitis (EAE), an animal model for autoimmune disease, can be induced in Lewis rats by injection of myelin basic protein
(MBP). T-cell lines specific for MBP have been isolated from Lewis rats. The MBP-specific T-cell lines can transfer EAE to naive syngeneic recipients. They recognize the encephalitogenic peptide sequence from amino acid residues 68-88 of MBP. These cells carry the W3/25\(^-\), OX8\(^-\) surface marker, a phenotype defining rat T helper/inducer cells. These T-cell lines recognize MBP with class II-MHC antigens on astrocytes and subsequently kill the antigen-presenting cells. The aim of this study is to determine whether certain variable (V) gene segments are predominantly used in this T-cell response. A cDNA library has been constructed from messenger RNA of one of these T-cell lines. The expressed Va and V\(\beta\) genes are isolated using mouse constant region probes, and their nucleotide sequences will be determined. The clonal heterogeneity in DNA genomic rearrangements of these T-cell lines is investigated using mouse \(\beta\)-chain probes.

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34. Large-Scale Production of Tcr in a Baculovirus Expression System

James L. Urban, William K. Funkhouser, Nancy A. Costlow

To produce relatively large amounts of T-cell receptor molecules for further study, we have developed a baculovirus expression system for the Tcr similar to that used to generate high levels of secreted human interferon-B (Smith et al., 1983) and interleukin-2 (Smith et al., 1985). Genes encoding the \(\alpha\) and \(\beta\) chains of a murine alloreactive Tcr recognizing the Kb class I MHC molecule were isolated from a cytotoxic T-cell cDNA library and separately introduced into the polyhedron gene of the Autographa californica nuclear polyhedrosis virus. This gene normally encodes a 29,000 MW protein that is the major structural component of viral occlusions and accumulates to very high levels, accounting for 50-70% or more of the total protein of infected cells. Sf9 insect cells infected with either the \(\alpha\) or \(\beta\) chain recombinant virus produce high amounts of a novel 3.3 kb RNA transcript consisting of the \(\alpha\) or \(\beta\) chain coding sequences and polyhedron 5' and 3' untranslated regions. The level of message expression is 1,000-10,000 times that found in murine T cells on a per-cell basis. Furthermore, this high level of expression does not decrease when Sf9 cells are co-infected with both the \(\alpha\) and \(\beta\) chain recombinant viruses. We are currently analyzing the nature of the proteins produced in these cells using two-dimensional gel electrophoresis, and we are exploring the possibility of producing large amounts of secreted Tcr by introducing stop codons in the \(\alpha\) and \(\beta\) chain transmembrane regions.

References

35. Diversity of the Chicken T-cell Receptor

Bernard Vernooy, Chia-lam Kuo

In order to survive, vertebrates must be able to mount an immune response to many foreign antigens, and thus they must have a very diverse repertoire of immunoglobulins and T-cell receptors. Mammals generate this diversity by a number of mechanisms. Two or three gene segments (V-J or V-D-J) are rearranged and juxtaposed to one another to create a V gene. Junctional diversity is created since the rearrangement points are variable and additional nucleotides can be inserted at the rearrangement junctions. In immunoglobulins, somatic mutation generates additional diversity. Lastly, two different chains make up one receptor and the chains can be joined together in all pairwise combinations. Thus, a very large number of different receptors can be made, starting from a relatively small pool of gene segments.

The diversity in the immunoglobulin light chain in chickens is created in a very different way. Here, there is only one functional V gene segment that re-arranges with the single J segment in all B cells. Junctional diversity is limited, but additional diversity is generated through several gene conversion events between the rearranged V gene and any of 25 distinct pseudogenes. These somatic gene conversion events are restricted to the V gene and do not occur in the J or leader segments. They involve stretches of 10 to 120 bases and result in 4 to 6 converted segments at the end of the bursal maturation. The chicken heavy chain may have a similar mechanism for generating diversity. It is of interest to find out how the T-cell receptor diversity is generated in chicken. Will there
be one mechanism for generating diversity in antigen receptors in the chicken, or are there two different mechanisms?

To address this question, we are constructing a chicken thymus cDNA library from which we will try to isolate chicken T-cell receptor cDNAs by cross hybridization with mammalian probes. If this does not work, we will use a subtracted probe (T cell-B cell) to isolate the T cell-specific cDNAs. The T-cell receptor clones among these cDNAs should show rearrangements on Southern blots of the thymus and cloned T cells.

36. Genes Encoding the \( V_H \) Region of Anti-\( \alpha(1+3) \) Dextran Antibodies

Carol Readhead, Joan L. Klotz\(^1\), Teresa Jeffroy, Roger Perlmutter\(^2\)

The response of mice to the polysaccharide \( \alpha(1+3) \) dextran is dominated by a particular idiotype. This dominant idiotype is defined in terms of the dextran-binding myeloma protein J558. Striking evidence of the predominance of this idiotype was given by Schilling \textit{et al.} (1980) when they sequenced 21 dextran-binding myeloma and hybridoma proteins and found that 17 out of the 21 proteins were identical to J558 in the \( V_H \) segment. This implies that the \( V_H \) gene segment encoding J558 plays a major role in the \( \alpha(1+3) \) anti-dextran response.

Southern blot analysis of mouse DNA showed that there are over 50 bands that hybridize to a J558 \( V_H \) gene segment probe. (The probe was given to us by Roy Riblet who had isolated a rearranged genomic clone from a J558 myeloma library.) By screening a band-limited library (4-6 kb) of genomic BALB/c DNA, we were able to determine the minimum number of \( V_H \) gene segments in each hybridizing band. Over a hundred clones homologous to J558 were present in the library. These clones were analyzed by restriction map techniques to determine which were identical. Eighty clones were still distinct. Many of these \( V_H \) gene segments were on EcoRI fragments of the same size. Thus, the individual hybridizing bands apparently represented ten or more homologous \( V_H \) gene segments. If so, then many more than 50 J558-like \( V_H \) gene segments exist. A previous study, using Cot curve analysis, has shown that there are 500-1000 \( V_H \) gene segments homologous to J558 (Livant \textit{et al.}, 1986).

Within the group of 110 clones isolated from the limited library, we have identified 13 \( V_H \) gene segments that share homology with the rearranged J558 \( V_H \) segment, both in the coding region and in regions more than 1 kb 5' to the coding region. These \( V_H \) gene segments have been sequenced and are closely homologous to J558 although they are more closely related to one another than they are to J558. These studies and those of Donna Livant show that a very large number of \( V_H \) gene segments belong to the dextran antibody family and that within this large family, subsets of genes exist that are very closely related to each other.

References:


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37. Molecular Mapping of the Major Histocompatibility Complex in the BALB/c Mouse

Erich C. Strauss, Stephen W. Hunt III

The murine major histocompatibility complex (MHC) consists of six genetically-defined regions: K, I, S, D, Qa, and Tla. Molecular cloning and chromosome walking experiments have resolved a 600 kb gene cluster that contains the K and I regions, a 300 kb gene cluster that spans the S region, a 500 kb gene cluster that contains the D and Qa regions, and two clusters of 160 kb and 90 kb that constitute the Tla region. The relative orientation and distances between the gene clusters of the K, I and S regions have been determined. The K region defines the proximal end of the MHC. The I region is 75 kb distal to the K region and 170 kb proximal to the S region.

We are interested in determining the physical map position and orientation of the D, Qa and Tla regions in the MHC of the BALB/c mouse. To establish a large-scale genomic restriction map that encompasses the S, D, Qa, and Tla regions, field-inversion and pulse-field gradient gel electrophoresis, in conjunction with Southern blot hybridization, are being employed. Genomic DNA is digested with restriction enzymes.
that have six or eight base pair recognition sites and
that contain one or two CpG dinucleotide sequences.
The CpG dinucleotide is known to be underrepresented
in mammalian DNA. As a result, digestion of genomic
DNA with these enzymes generates restriction frag­
ments in the range of 50 kb to greater than one mega-
base. Using this approach, a physical map has been
constructed that links the two Tla clusters and orients
them with respect to each other. Our data indicate
that the 160 kb Tla cluster is separated from the 90 kb
Tla cluster by a distance of 150 kb.

Currently, we are using single-copy probes derived
from the S and D regions to investigate the distance
separating these gene clusters. Moreover, we have
isolated low-copy probes from the Qa region and each
end of the Tla region to determine the relative orient­
tion and physical distance separating the 400 kb Tla
gene cluster from the 500 kb D-Qa gene cluster.

38. Biology of the Major Histocompatibility
Antigens: A Model for Studying Cell-Cell
Interactions in the Immune System

Iwona Strzyowski

Cell-mediated response to pathogens involves a
concerted interaction of many components of the
immune system. The identitities of some of these
components and their relative contributions to the
immune responses are known; many others remain to
be discovered. We study the mechanisms by which
murine transplantation antigens (or major histo­
compatibility complex class I molecules) function in
the recognition and elimination of the virally-infected
and neoplastically-transformed cells. The class I
molecules can be subdivided into two
categories: classically-defined transplantation anti­
gens, which are encoded by the H-2 subregion of the
major histocompatibility complex; and molecules re­
lated to them by amino acid sequence homology, which
are encoded by the Qa/Tla subregion. Mice encode 2-3
highly polymorphic H-2 class I genes and 20-40
non-polymorphic Qa and Tla genes. The Qa/Tla molecules
differ from H-2 antigens in molecular weight, more
restricted tissue distribution, and requirement for cell
membrane attachment. In most cases the proteins en­
coded by the individual Qa/Tla genes, their functions,
and the mechanisms leading to their specific tissue
distribution have not been identified.

We have undertaken a systematic study of the
Qa/Tla genes and their products. The Qa genes from
BALB/c and C57BL/10 mice were transfected into
different cell lines and their expression was studied.
One of the expressed products was characterized in
detail. We have shown that the Q7 gene encodes a
membrane-bound as well as a secreted form of the
serologically-defined Qa-2 antigen. The Q7 gene
introduced into liver-derived cells is expressed as a
membrane-bound antigen and as a secreted molecule.
In transfected L cells it is expressed only as a soluble
protein. Both these products are biochemically
indistinguishable from the Qa-2 molecules synthesized
by the activated T cells. Biochemical analysis
revealed that the Qa-2 antigen encoded by the Q7
genome is anchored to the liver cell membranes by a
phospholipid tail. We are now trying to determine if
this feature is responsible for tissue-specific Q7
expression as secreted or membrane-bound molecule.
Studies are also under way to identify molecular
mechanisms involved in the phospholipid tail attach­
ment, the role of this tail in altering the mobility of
the Qa molecules in the membranes, and its functional
significance for the Qa class I antigens.
2. Cytotoxic T Lymphocytes from Mice with Soluble Class I Q10 Molecules in Their Serum Are Not Tolerant to Membrane-bound Q10

Donald W. Mann, Iwona Stroynowski, Kathleen N. Blackburn, James Forman

One of the unanswered questions of the immunology of the major histocompatibility antigens relates to the mechanisms by which organisms become tolerant to their own self-H-2 molecules and mechanisms by which they "learn" to use self-H-2 molecules for presenting foreign antigens to cytotoxic T cells. It is believed that early developmental onset and the sites of the transplantation antigen expression are important in the induction of the tolerance and H-2 restriction.

To test if the soluble forms of the class I molecules are sufficient to induce the tolerance, we have studied cytotoxic T-cell responses in mice carrying high concentrations of Q10 molecules in their serum. The Q10 molecules are class I Qa region-encoded molecules which are secreted from liver during the prenatal life as well as during the adulthood. The amino-terminal portion of this molecule can also be expressed as an integral membrane protein by splicing the 5' end of the Q10 gene to the 3' end of the H-2d gene and transfecting the hybrid gene into the murine L cells. Using these transfecants as target cells, we have determined that mice with circulating Q10 molecules can generate cytotoxic T cells reactive with hybrid Q10/Ld molecules. Thus, the presence of the soluble Q10 molecules in the serum is not sufficient to eliminate anti-Q10 alloreactive cytotoxic T cells from the host's T-cell repertoire. The generality of this observation is now being tested for other soluble class I H-2 molecules.

3. MHC Class I Antigen Expression and Growth of Tumors

Michael Nishimura, Iwona Stroynowski
Kathleen N. Blackburn, Suzanne Ostrand-Rosenberg

We have examined the role of MHC class I antigens in the rejection of murine hepatoma BW7756. An in vitro isolate of BW7756, Hepa Ova, lacks the H-2Kb antigen expression and does not form tumors in syngeneic mice, raising a possibility that tumorigenicity may be related to the H-2Kb antigen expression. To test this hypothesis, Hepa Ova cells have been transfected with the H-2Kb gene and clones expressing high levels of the H-2Kb antigen were isolated. None of the H-2Kb-transfected Hepa Ova clones are tumorigenic in syngeneic mice. However, tumors are formed in sublethally irradiated or athymic nude mice. Hepa Ova and the H-2Kb transfectants are equally lysed by natural killer effectors, indicating that expression of the H-2Kb antigen has no significant effect on natural killer-mediated lysis. These results indicate that: 1) rejection of Hepa Ova is T cell-mediated; 2) expression of the H-2Kb antigen has no effect on the tumorigenicity of Hepa Ova; and 3) expression of the H-2Kb antigen has no effect on the susceptibility of Hepa Ova to lysis in vitro or in vivo natural killer cells.

References:

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39. Molecular Analysis of Interferon Responsive Regions in Murine Class I Genes

Bette Korber, Iwona Stroynowski

Interferons (IFNs) regulate the expression of a wide variety of molecules including several components of the immune system. They are important modulators of anti-viral and anti-tumor responses, and they influence cell growth and differentiation. We have studied IFN-induced expression of murine H-2 genes. These genes encode class I transplantation antigens which play a critical role in the destruction of virally-infected and
malignantly-transformed cells. Interferon-induced enhancement of H-2 antigen expression is thought to increase the ability to perform their physiological functions.

In order to dissect the molecular events involved in the IFN regulation of these genes, the 5' flanking region and the DNA downstream from the transcription initiation site of the class I genes were analyzed separately. The promoter regions of the H-2D<sup>d</sup> and H-2L<sup>d</sup> genes were linked to the structural gene encoding chloramphenicol acetyl transferase (CAT). Conversely, the structural gene of the H-2L<sup>d</sup> molecule was linked to the non-IFN-regulated promoters. These constructs were transfected into L cells and their ability to respond to IFN was measured. We found that both the promoter and the region of the gene downstream from the transcriptional start site contribute to the overall regulation of the class I genes. The downstream region was shown to control the major component of the response in L cells. Accordingly, the regulation of class I genes by interferons appears to be complex and involves at least two different mechanisms.

Reference:

40. Regulatory Elements in the Promoter Region of the Class I Genes

Bette Korber, Iwona Stroynowski

The sequencing of the 5' flanking region of the murine class I genes led to the identification of multiple DNA motifs that are highly homologous to the transcriptional regulatory elements found in other genes. The H-2D<sup>d</sup> promoter contains a TATA box, two CCAAT elements, two class I enhancer regions and the interferon consensus sequence, IRS. We have carried out deletion analysis of the H-2D<sup>d</sup> 5' flanking region to identify the sequences that are involved in the interferon-mediated regulation of class I gene transcription. The IRS region was found to be required for type I (α + β) and type II (γ) interferon responses in all cell types tested. In some cells the response to type I interferon required additional DNA sequence, which is located adjacent to the IRS. In other cells this element was not necessary. The magnitude of the induction varied between different cell lines. These results suggested that cell-specific factors are involved in interferon-mediated regulation of class I antigen expression. To define these factors, footprinting experiments were performed. The pattern of DNase I protections indicates that nuclear factors bind to motifs in the IRS, both enhancer regions and the CCAAT and TATA regions. Tissue specificity of these factors and their presence in interferon treated/untreated cells are now being studied.

41. Class I Genes Contain an Interferon Responsive Sequence Downstream of the Transcription Initiation Site

Sherry A. Kempin, Bette Korber, Iwona Stroynowski

Class I antigen expression undergoes modulation during the ontogeny and during the adult life. It is maintained at different basal levels depending upon the tissue or cell type; in addition, it can be regulated by various agents. Both type I (α and β) and type II (γ) interferons have been proposed to play a physiological role in the developmental onset of the class I antigen expression and in the enhancement of their expression on the adult cells.

Studies by Korber et al. (see Abstract No. 39) indicate that the sequences 5' as well as 3' of the transcription initiation site are involved in the interferon-mediated regulation of class I gene expression. We have focused on the molecular basis of the mechanism that is dependent on the 3' sequences. Initial experiments were designed to test whether these sequences, when transposed to a location upstream of the promoter, would enhance the rate of the transcription initiation. Class I gene fragments were inserted in front of a deleted version of the H-2D<sup>d</sup> promoter followed by the chloramphenicol acetyl transferase (CAT) gene. These constructs were transfected into L cells and the expression of the CAT protein was measured. Preliminary results suggest that the constructs are not inducible by γ-interferon. Therefore, it is unlikely that the 3' sequences act to potentiate transcription in a position-independent manner. Studies in progress will measure steady state levels of the class I mRNA in interferon-treated and untreated cells to determine if the induction occurs at the transcriptional or posttranscriptional level. With further analyses, we hope to identify and characterize the 3' interferon responsive sequences.
42. Construction and Expression of the Genes Encoding Soluble Transplantation Antigens

Laura M. Hoopes, Keith D. Lewis, Iwona Stroynowski

Classically-defined transplantation antigens are membrane-bound molecules that can present antigens to specialized T cells. To study parameters involved in the T cell-class I antigen interactions, we have constructed genes encoding secreted transplantation antigens. The rationale for this approach was twofold: to test the ability of the soluble class I antigens to induce tolerance in transgenic mice; and to purify large quantities of the class I proteins for crystallography and for in vitro studies of solution interactions between class I and T-cell receptor molecules.

Since present evidence suggests that the great majority of the epitopes recognized by the cytotoxic T cells are confined to \(\alpha_1\) and \(\alpha_2\) regions of the class I proteins, we have designed and constructed hybrid genes encoding the \(\alpha_1\) and \(\alpha_2\) domains of the classical H-2 L\(d\) antigen fused to the \(\alpha_3\) domain of the secreted Qa molecules, Q7 or Q10 (see Abstract No. 38).

Both constructs were transfected into the cultured cell lines. The L\(d/Q10\) gene expressed at least tenfold higher levels of the secreted hybrid protein than L\(d/Q7\) gene and was characterized further. The synthesis and secretion of the L\(d/Q10\) molecules were compared in fibroblastic L cells, Hepa-I cells (hepatoma cells secreting liver proteins) and GC cells (pituitary tumor cells secreting growth hormone). Quantitative immunoprecipitations of the L\(d/Q10\) molecules from the cell lysates and the concentrated supernatants indicated that the Hepa-I transfectants synthesized twice as much hybrid protein and secreted -5 times as much as the L-cell transfectants; they synthesized -10 times as much and secreted -30 times as much as the GC cell transfectants. The two-dimensional gel analysis and Endo F enzyme digestions established that the soluble L\(d/Q10\) products are glycosylated proteins of the predicted molecular weight and the predicted isoelectric point. Studies of the kinetics of the secretion, the subcellular location of the soluble molecules and the biochemical purification of the L\(d/Q10\) molecules are in progress.

43. The Expression and Function of L\(d/Q10\) Chimeric Molecules in Transgenic Mice

Laura L. Hoopes, Keith D. Lewis, Carol Readhead, Jessica A. Dausman, Iwona Stroynowski

We are using transgenic mice with the L\(d/Q10\) chimeric molecules, which we have shown are secreted efficiently by transfected cells, to ask whether self-tolerance education can be accomplished by means of secreted rather than the usual membrane-bound transplantation antigen molecules. The basic design of our experiment is to obtain one or more transgenic mouse lines without any cell membrane-bound L\(d\) molecules, but with high levels of the chimeric L\(d/Q10\) molecules in their serum, and to challenge these mice with transplants having membrane-bound L\(d\) molecules. If membrane binding is required for the H-2 molecules to be recognized as self-MHC, these transplants will be rejected, but if the primary recognition signals in the \(\alpha_1\) and \(\alpha_2\) exons of the secreted molecules have acted as educators for self tolerance, then the transplants will be accepted. Other measures of CTL function will also be used to evaluate how efficiently, if at all, the secreted L\(d/Q10\) molecules have been perceived as authentic L\(d\) molecules by the transgenic mouse immune system.

We have at least 15 progeny from injection experiments using the L\(d/Q10\) construct that are ready to be analyzed for presence and expression of the gene. First, using dot blotting of the tail DNA, we will test for the presence of the vector sequences. Then, blood samples will be tested using Western dot blots reacted with monoclonal antibody 30-5-7. Animals that appear positive will be bred, and when positive progeny are available, the spleens, livers, thymuses, and lymph nodes will be disaggregated and biosynthetically labeled. The lysates and the supernatants of the biosynthetically-labeled disaggregated tissues will be analyzed by immunoprecipitation and two-dimensional gels. We are using monoclonal antibody 30-5-7 and are preparing a polyclonal rabbit antiserum with specificity to the transmembrane region of Q10\(d\), for use in these experiments.

In addition to analyzing the tolerance of these animals, the RNA present in different tissues will be examined to see whether the molecules are synthesized predominantly in the liver, like Q10, or in all tissues except nerves and early embryos, like L\(d\), or...
some other pattern of expression is observed. In addition, lines that have the gene but do not express it will be examined for methylation, DNase I sensitivity, and structural integrity of the transgene, in order to find out how it is regulated in non-expressing cells with intact genes.

44. Expression of Class I Genes in Normal Murine Cells

Stephen W. Hunt III, Kurt A. Brorson, Hilde Cheroutre, Fung-Win Shen

The class I antigens are a family of highly-related proteins which include the transplantation antigens H-2-K, H-2-D and H-2-L, the class I-like antigens Qa-1, Qa-2,3, and thymus leukemia (TL). The transplantation antigens are involved in T-cell recognition of virus-infected and transformed cells. The function of the class I-like antigens is unknown.

Genes encoding the class I antigens have been cloned from several different strains of mice, including BALB/c, which was done in our laboratory. There are at least 35 class I genes in the BALB/c mouse. Until our study, only seven of the 35 class I genes were known to be expressed. To study the expression of individual members of the class I gene family, we generated a set of gene-specific oligonucleotide probes that hybridized to a single, or at most, a few class I genes. These probes have been used to characterize class I gene expression in cDNA libraries made from mouse tissues.

A thymus cDNA library was screened with a class I gene-specific probe derived from the highly-conserved, fourth exon of the H-2D\textsuperscript{d} gene. A total of 72 class I clones were isolated and were subsequently identified by a combination of hybridization to the gene-specific probes and DNA sequencing. We have determined that at least 15 different class I genes are transcriptionally active in the thymus. One cDNA represents a new class I gene that was absent from a BALB/c cosmid library characterized previously in this laboratory. Similarly, we have characterized class I gene expression in a T-helper cell cDNA library. At least eight class I genes are expressed in this cell line. These genes represent, as expected, a subset of the class I genes expressed in the thymus cDNA library. It will be interesting to determine whether all T-helper cells express this same group of class I genes and whether T cells with different effector functions (i.e., cytotoxic, suppressor) express a different subset of class I gene transcripts.

We have begun to study the tissue distribution of several of the genes that were expressed in the thymus. The "37" gene is expressed in the thymus, spleen and lymph nodes and to a much lower level in the liver. Additionally, the "37" gene is transcribed in three suppressor T-cell hybridomas and a B-cell hybridoma. The "37" gene is not expressed in the thymoma BW517, the fusion partner of the suppressor hybridomas, suggesting that the transcriptionally active "37" gene was donated by the suppressor parent. The T9 cDNA probe, which recognizes both the T9 and T17 genes, shows the same pattern of hybridization as the "37" probe. These results suggest that some of the nonclassical class I antigens may be important in the development or functioning of various T- and B-cell populations. We are in the process of determining the tissue distribution of the other class I genes that are expressed in the thymus.

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45. Identification and Characterization of Class I Genes Expressed in Bone Marrow of the Adult BALB/c Mouse

Hilde Cheroutre, Stephen W. Hunt III, Kathleen Segesman

In the BALB/c mouse, more than 30 class I genes have been defined as members of the Qa/Tla group, but little or nothing is known about the expression, the protein product, or function of these class I-like molecules. As described in the previous abstract, Dr. Hunt has looked at the expression of Qa/Tla genes in adult thymus tissue and using the same strategy, we describe here the analysis of expression of those genes in adult bone marrow.

We were able to identify eight different class I gene transcripts in the bone marrow by screening a cDNA library with the specific transmembrane probes, as previously described by Dr. Hunt. Some other class I clones could not be further characterized with these probes and sequencing will be necessary in order to determine their nature. Several observations are striking in comparing the class I gene expression in the bone marrow and thymus. The three transplantation antigens H-2K, L and D are expressed in both tissues,
but the level of H-2K expression in bone marrow is markedly reduced when compared to the thymus. This suggests that certain cells in the bone marrow have greatly reduced levels of H-2K expression as contrasted with the H-2D and H-2L, or that there are cell subpopulations in the bone marrow lacking H-2K expression. On the other hand, the expression of the class I gene "37" in these tissues is the inverse of H-2K. In both tissues the level of expression of each individual class I gene ranges from 1% to 30% of the total class I gene expression, possibly suggesting that some class I genes will be found on almost all cells in these tissues, whereas others will be found only on small subpopulations. The bone marrow expresses two class I genes (K1 and T4) not seen in the thymus, and the thymus expresses eight class I genes (H-2D2, H-2D4, Q7, Q8/9, T3, T13, T18, Thy15.1 and Thy19.4) not found in the bone marrow. This could suggest that the distinct lineages developing in these organs express distinct class I genes. The intriguing question is whether these molecules play a role in the growth and differentiation of specific cell lineages.

46. Characterization of Four Unusual Murine Class I Major Histocompatibility Complex Genes

Kurt A. Brorson, Hilde Cheroutre, Stephen W. Hunt III

We are interested in characterizing class I gene expression in various tissues of the BALB/c mouse. In the thymus, we have determined that at least 15 different class I genes are expressed. We are studying four of these genes, T9, T17, T18 and Thy19.4. We have sequenced cDNAs for each of these genes from the thymus library. Using the sequence data, we have created gene-specific cDNA probes for the T9/T17 gene pair and the T18 and Thy19.4 genes. The Thy19.4 probe was used to isolate a genomic 7.2 kb HindIII fragment which contains the entire Thy19.4 gene. In order to characterize the protein products of these genes, constructs have been made that contain each of these genes, along with an SV40 enhancer. The constructs were then transfected into BW5147 cells by electroporation. So far, we have identified transfectants that express both the T9 and T18 genes at the mRNA level. These transfectants are being used to produce full-length, properly spliced cDNA clones for these genes. In addition, rats, neonatally tolerized to irradiated transfecteds in order to produce antiserum and monoclonal antibodies to the gene products of the T9 and T18 genes. In the future, we plan to use the monoclonal antibodies to characterize which tissues express these molecules and to determine their function.

47. Expression of a Myelin Basic Protein Gene in Transgenic Shiverer Mice: Correlation of the Dysmyelinating Phenotype

Carol Readhead, Brian J. Popko, Naoki Takahashi1, H. David Shine2, Raúl A. Saavedra, Richard L. Sidman2

Myelin of the central nervous system serves to facilitate the conduction of nerve impulses along axons. The myelin sheath consists of multiple wrappings of the oligodendroglial cell-surface membranes around segments of axons. During maturation, the cell-surface membrane compacts and eliminates the cytoplasm. The inner surfaces then become apposed to form the major dense line. Myelin basic protein, one of the most abundant proteins of the myelin sheath, is associated with the major dense line. Mice homozygous for the autosomal recessive mutation, shiverer (shi), lack myelin basic protein (MBP) and exhibit a distinct behavioral pattern including tremors (shivering), convulsions and early death at 90 to 150 days. We have previously demonstrated that shiverer mice have a partial deletion in the gene encoding MBP (Roach et al., 1985). We now have introduced the wild-type MBP gene into the germline of shiverer mice by microinjection into fertilized eggs. Transgenic mice homozygous for the introduced gene have MBP mRNA and protein levels that are approximately 25% of normal and produce compacted myelin with major dense lines. Corrected temporal and spatial expression of the MBP gene is achieved with a genomic MBP cosmid clone containing 4 kb of 5' flanking sequence and 1 kb of 3' flanking sequence. These homozygous transgenic shiverer mice no longer shiver and have lived for over a year (Readhead et al., 1987).

In collaboration with Richard Sidman and David Shine, we are initiating a more detailed morphometric analysis of the central nervous system of the transgenic mouse.
References:

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48. Myelin Basic Protein Gene Structure and Expression in Myelin-deficient Mice

Brian J. Popko, Carmie Puckett, Carol Readhead

Mice homozygous for the autosomal recessive mutation myelin deficient (mld) exhibit decreased central nervous system (CNS) myelination, tremors, and convulsions of progressively increasing severity that lead to an early death. The myelin of mld mice is characterized by a deficiency in the myelin basic protein (MBP), an important structural component of CNS myelin. Southern blotting analyses and the characterization of MBP cosmid clones indicate that the mld MBP locus is organized as a tandem duplication that spans approximately 100 kb. The upstream mld MBP gene contains an inversion of its 3' half, whereas the downstream gene appears normal. This gene organization results in the reduction and abnormal developmental regulation of MBP transcription in mld mice. We have introduced into the germline of mld mice a wild-type MBP gene using standard microinjection techniques. Mld mice transgenic for the MBP gene form compact myelin, no longer tremor or convulse, and live longer than the mutant animals, indicating that the mld mutation is a defect in the MBP gene. We are currently conducting experiments to determine why the mld MBP gene organization results in abnormal MBP gene expression.

49. The Myelin Proteins and Genes in Primitive Vertebrates

Lance Fors, Ruedi Aebersold, Raúl A. Saavedra

Myelin of the vertebrate nervous system is a dynamic structure that provides electric insulation and thus facilitates the rapid conduction of nerve impulses along axons. The major myelin proteins in the central nervous system (CNS) of mammals are proteolipid protein (PLP) and myelin basic protein (MBP), whereas in the peripheral nervous system (PNS) the major proteins are P0 and MBP or another basic protein, P2, depending on the species. Myelin as a concentric multilamellar structure appears first during vertebrate evolution in sharks; and therefore, shark represents the system of choice to study the essential proteins that form the myelin structure. We have isolated the major CNS myelin proteins of the shark Heterodontus, separated them in polyacrylamide gels and obtained extensive N-terminal and internal fragment sequence. The sequence data unequivocally show that the major proteins of shark CNS myelin are homologous to mammalian P0 and MBP. Both shark P0 and shark MBP appear in our preparations as multiple bands, and together account for 95% of total CNS myelin proteins. These shark proteins show a provocative homology to their mammalian counterparts, 60% and 53%, respectively. Thus our results support the notion that myelin has evolved as an organelle where two protein components, a hydrophobic one, like PLP or P0, and a hydrophilic one, like MBP or P2, play the major structural roles. Furthermore, we have used this protein sequence data to help generate oligonucleotide probes corresponding to both shark proteins and screened a shark brain cDNA library. We have isolated cDNA clones for both P0 and MBP, and we are now in the process of characterizing those clones.

Our future plans include: 1) use the shark cDNA probes to clone and analyze the genomic structure of both shark genes; 2) compare the genomic structure of the shark genes to that of their mammalian counterparts; and 3) use the transgenic system available in our laboratory to determine whether the shark genes can functionally substitute and thus re-establish normal neural function in the mutant mice "shiverer" and "jimpy."

50. Structural Analysis of Scrapie-Associated Proteins

David B. Teplow, Brian T. Chait 1, Stanley B. Prusiner 2

Scrapie is a fatal, progressive, neurodegenerative disease of sheep. Human counterparts of scrapie include Kuru, Creutzfeldt-Jakob disease and Gerstmann-Sträussler syndrome. Scrapie and its related disorders were thought to be caused by a "slow virus." However, fractionation of scrapie infectivity produces an isolate composed almost entirely of a single protein species, with no associated nucleic acids. The term "prion" has
been used to describe this class of infectious proteinaceous pathogens. To better understand the pathobiology of scrapie, we have studied the structures of a number of related prion proteins isolated from hamsters infected with the scrapie agent. We have also been involved in developing methods to isolate a homologous protein from normal animals. Prior work has demonstrated that the "normal" protein is structurally distinct from the scrapie protein. We are now defining the structures of both proteins and identifying the differences between them, using a combination of classical protein chemistry and plasma desorption mass spectrometry. These methods allow us to define the primary structures of proteins and peptides as well as characterize posttranslational modifications.

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51. Identification of the Binding Domain of the AIDS Virus on the CD-4 Protein (Helper T-Cell Antigen Receptor)

Bradford A. Jameson, Stephen B. H. Kent

The CD-4 protein present on T lymphocytes is the phenotypic marker for helper T cells (also referred to as T4 cells). This protein, which is involved in the recognition of MHC class II bearing antigen-presenting cells, is commonly known as the T4 antigen receptor and has been shown to act as the cellular receptor for the human immunodeficiency virus (HIV) and related T4-tropic viruses. A panel of functionally distinct monoclonal antibodies has been produced by Ortho Diagnostics (kindly provided by Dr. Gideon Goldstein). Several of these antibodies are capable of blocking the binding of HIV as well as inhibiting the MHC class II-restricted presentation of foreign antigen by antigen-presenting cells. Using a nested array of synthetic peptides (28-35 amino acids in length) derived from the primary amino acid sequence of the CD-4 protein, we have mapped the binding domains of several of the CD-4-specific monoclonal antibodies. The antibodies that block HIV binding and MHC class II restricted antigen presentation appear to cluster in and around residues 137-167 on the CD-4 protein. We are currently attempting to map the remaining monoclonal antibodies from this panel as well as attempting to competitively block the HIV binding to T4 cells using synthetic peptide analogues of this binding domain.

52. Studies of the Outer Envelope Protein (gp120) of the AIDS Virus

Bradford A. Jameson, George M. Shaw1, Linda K. Martin, Stephen B. H. Kent

The human immunodeficiency virus (HIV) has been shown to be the causative agent of the acquired immunodeficiency syndrome (AIDS). It has also been shown that the envelope protein (gp120) of this virus contains the structures recognized by neutralizing antibodies and by the host cell receptor. We have designed experiments intended to test two independent, but not mutually exclusive hypotheses. The first is that the clusters of sequence hypervariability within the gp120 protein among various virus isolates correspond to immunodominant epitopes which are capable of binding anti-viral neutralizing antibodies. The second is that the surface of the infectious virus, in part, mimics the surface of antigen-presenting cells.

Two regions of the gp120 protein are particularly good candidates for being involved in this viral binding process and, consequently, effective targets for neutralizing antibodies. These regions reside in the carboxy-terminal half of the gp120. The first region has a remarkable resemblance to the disulfide loop of the HLA class II a chain. The second region displays an array of amino acids that are reminiscent of the heavy chain variable region of the immunoglobulin protein. Both of these regions contain clusters of conserved and hypervariable amino acids.

One of the major obstacles to be overcome in the development of an efficacious vaccine against HIV is that each virus isolate appears to exhibit unique, highly divergent envelope proteins. Although each virus bears a subset of immunodominant epitopes which are common to all HIVs, antibodies generated in response to these group-specific epitopes do not neutralize the infectivity of the virus. The immunodominant epitopes present on the viral surface that do give rise to neutralizing antibodies appear to be type-specific. Because the number of viral serotypes within the HIV family is unknown, the exploitation of type-specific antibodies is only of limited value. Future vaccines against HIV must rely on either complete information regarding the serotypic diversity of the virus family or one must find and exploit group-specific determinants on the viral surface that are capable of giving rise to virus neutralizing antibodies.
Even though the immunodominant neutralization epitopes of the HIV appear to be type-specific, it is entirely possible that there exist immunorecessive epitopes (i.e., epitopes that are rarely, if ever, recognized by the immune system in the context of the native protein) that are group-specific and can confer immunity against all serotypes of the virus. We have synthesized a series of overlapping peptides (varying between 30-65 amino acids in length) covering the entire carboxy-terminal half of the gp120 protein. We are currently using these peptides to determine both type-specific and group-specific epitopes capable of binding neutralizing antibodies as well as testing these peptides for their ability to competitively block the interaction between helper T cells and the HIV.

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53. Characterization of the Hepatitis B Virus X-gene Product
Bradford A. Jameson, Dorothee Wemicke-Jameson, Stephen B. H. Kent

The hepatitis B virus (HBV) genome has only four major open reading frames encoding: the core protein, the viral polymerase, the envelope protein and the "X" protein. As its name implies, the HBV-X gene product has no known associated function. The experiments that we are conducting are designed to yield primary information concerning the structure and function of this protein and its link, if any, to the production of hepatocellular carcinomas. Using the amino acid sequence of the HBV-X protein, peptides corresponding to both the amino and carboxy termini were synthesized. We also intend to synthesize a panel of nested peptides (~30 amino acids in length) which cover the entire sequence of the protein in overlapping fashion. The synthetic peptides are being used to screen sera samples from patients with medically defined histories for X-reactive antibodies.

The peptides have been used also to create monospecific antisera. These antisera, in turn, are being used as probes to isolate and identify (via protein sequence analysis) HBV-X and X-related proteins as well as to define the cellular location of such proteins. In collaboration with Dr. Myron Tong (Huntington Memorial Hospital, Pasadena, CA), we have obtained a continuous adherent cell line, which was established from a hepatocellular carcinoma of a hepatitis surface antigen-positive patient. Although no free HBV-DNA has been found in this cell line, Southern blots indicate the presence of integrated HBV-DNA in at least five different sites. Metabolically-labeled cells were probed for the presence of an HBV-X protein or an immunologically-related protein with both the amino- and carboxy-terminal anti-peptide antibodies. In this manner, we have found specific recognition of a 17 kDa protein band corresponding to the predicted molecular weight of the HBV-X protein. We are currently attempting to sequence and characterize this protein.

54. T-cell Epitopes of Hepatitis B Virus Pre-S-coded Envelope Proteins and the Creation of Hybrid Antigens

Although there is a great deal of information concerning the nature of antibody-binding sites (B-cell epitopes), much less is known about the protein regions involved in the major histocompatibility complex (MHC)-restricted helper T-cell response (T-cell epitopes). We are using hepatitis B virus (HBV) as a model system to identify and fine-map T-cell epitopes located on the HBV envelope protein. The identification and fine mapping of these epitopes are being performed via standard T-cell proliferation (thymidine uptake) assays using either synthetic peptides selected from a nested panel of peptides or whole protein as challenge antigens.

In collaboration with Dr. Marc Girard (Pasteur Institute, Paris, France) and Dr. Elizabeth Muchmore (New York University Medical School), we are conducting a series of experiments involving a panel of chimpanzees inoculated with an experimental hepatitis B virus vaccine and subsequently challenged with live virus, or control animals which received only a virus challenge. Blood samples have been drawn at two-
week intervals and the peripheral blood lymphocytes tested for T-cell reactivities against our panel of Pre-S-coded test antigens. Preliminary results indicate that the T-cell responses among the primates appear to be qualitatively different than those obtained in comparable experiments in congenic mouse strains. Although the major T-cell epitopes found in the murine studies (Milich et al., 1986) seem to be utilized in the primate system, the fine-specificities seem to vary between individual animals.

Another major goal of this project is to explore the possibility of creating hybrid antigens comprised of T-cell epitopes from one protein which are juxtaposed with a B-cell epitope from a different protein. Such experiments are intended to yield vital information concerning the autonomy of T-cell epitope function and the fundamental attributes of an effective immunogen. This information should provide a foundation for the ultimate development of rationally designed synthetic vaccines.

References:

55. Synthetic Analogs of Hepatitis B Virus Proteins as Potential Diagnostic Reagents
A. Robert Neurath¹, Nathan Strick¹, Stephen B. H. Kent, Karen F. Parker

Several of the hepatitis B virus (HBV) DNA-coded proteins and antibodies (Ab) to them are important serological markers for HBV infection. These include the structural components of the virus: the HBV envelope (env) protein and the nucleocapsid core. Proteins that are not components of virions and Ab to them are also markers for HB. These include HBe antigen (HBcAg) and anti-HBe, X-protein and the corresponding Ab. HBcAg is closely related to the protein of the nucleocapsid. The nature of X-protein is being investigated. The minor components of the virus, i.e., DNA polymerase, protein kinase and a protein linked to the 5' end of HBV DNA, do not appear to be antigens suitable for serological tests.

The HBV env proteins consist of several related species sharing a 226 amino acid sequence (S-protein) and differ from each other by absence or presence of N-terminal extensions 55 [Pre-S(2)] or 163 (174) [Pre-S(1)+Pre-S(2)] amino acids long. Epitopes on Pre-S(1) and Pre-S(2) sequences are more immunogenic than epitopes on the S-protein. Ab to the distinct segments of the HBV env protein appear at different times during HBV infection and have distinct diagnostic significance. An Ab response to the internal nucleocapsid appears early during infection and is a marker for infection rather than for recovery. The antigen serving as a major diagnostic marker for HBV infection is S-protein. The detection of other antigenic markers (HBeAg) and of Pre-S(1) and Pre-S(2) sequences usually correlates with active HBV replication and the detection of HBV DNA by nucleic acid hybridization.

Synthetic analogs of several HBV proteins have been prepared. The most promising with respect to diagnostic applications are the peptides derived from the HBV env protein. Studies with these peptides lead to the identification of epitopes which are essential for protection against disease. Studies with analogs of the S-protein permitted the evaluation of binding affinities of Ab elicited by HBsAg and demonstrated the relevance of binding affinity for protection. Peptides derived from the Pre-S(1) and Pre-S(2) sequence are highly immunogenic and elicit Ab with binding affinities similar to those elicited by the HBV env protein. These Ab's are protective.

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56. Antibodies Recognizing Human Serum Albumin Are Not Elicited by Immunization with Pre-S(2) Sequences of the Hepatitis B Virus Envelope Protein
A. Robert Neurath¹, Nathan Strick¹, Karen F. Parker, Stephen B. H. Kent

Antibodies to the Pre-S(2) region of the hepatitis B virus (HBV) envelope protein and to human serum albumin (HSA) were allegedly detected at about the same level in sera of humans with acute or chronic hepatitis B (Hellström et al., 1986). It was claimed that anti-HSA antibodies arise as a result of an immune response to the Pre-S(2) sequence and that they were involved in hepatocellular damage during hepatitis B. In order to determine whether or not an immune response to the Pre-S(2) sequence indeed results in elicitation of anti-HSA, over 100 sera from animals and humans immunized with HBsAg containing Pre-S(2) sequences, or with synthetic peptides derived from the Pre-S(1), Pre-S(2) and S regions of the HBV
env protein (in either free form or linked to distinct carriers) were assayed for anti-HSA. The results revealed the following. 1) Immunization with the native Pre-S(2) sequence or with unconjugated synthetic peptides derived from that sequence does not result in elicitation of anti-HSA. Therefore, the alleged appearance of anti-HSA during hepatitis B cannot be directly related to an anti-Pre-S(2)-specific immune response. 2) Some synthetic peptides, whether or not they were derived from the Pre-S(2) sequence, when linked to certain carriers but not to others, elicited in rabbits an anti-HSA response, which was markedly lower than the response to the homologous peptide. These anti-HSA antibodies could be separated from anti-Pre-S(2)-specific antibodies by affinity chromatography and did not recognize the synthetic peptide (in free form) used for immunization. The elicitation of anti-HSA was restricted to certain synthetic peptide-linker-carrier combinations and was not restricted to Pre-S(2) regions of the HBV env protein reported to be involved in the recognition of glutaraldehyde-polymerized serum albumin. The use in active immunoprophylaxis of hepatitis B of unconjugated peptides from the Pre-S(2) sequence with proven high immunogenicity will avoid carrier/linker-mediated induction of antibodies not relevant to protection against HBV.

References:

1The Lindsley F. Kimball Research Institute of the New York Blood Center, New York, NY.

57. Antibodies to Synthetic Peptides from the Pre-S(1) and Pre-S(2) Regions of One Subtype of the Hepatitis B Virus Envelope Protein Recognize All HBV Subtypes

A. Robert Neurath1, Stephen B. H. Kent, Nathan Strick1, Karen F. Parker, A.-M. Courouge2, M. M. Riottot3, M. A. Petit4; A. Budkowska5; Marc Girard2, J. Pillot4

Immunodominant B- and T-cell epitopes have been demonstrated recently on the Pre-S(1) and Pre-S(2) regions of the hepatitis B virus (HBV) envelope protein. Synthetic peptide analogs corresponding to the Pre-S(2) region elicit virus-neutralizing antibodies and protect chimpanzees against HBV infection. Antibodies raised by immunization with peptides derived from the Pre-S(1) sequence block the site involved in HBV attachment to cell receptors, and are expected to be virus-neutralizing. There is a high amino acid variability in Pre-S sequences coded for by genomes of distinct HBV subtypes which have already been sequenced. Additional amino acid variability within Pre-S sequences of HBV subtypes which have not yet been sequenced is possible. To further assess the potential of Pre-S sequences as components of improved hepatitis B vaccines, it was necessary to determine whether or not Pre-S sequences corresponding to all known hepatitis B virus subtypes cross-react immunologically. Antisera raised against synthetic peptide analogs carrying the immunodominant epitope of the Pre-S(1) and Pre-S(2) sequence, respectively, and corresponding to two HBV subtypes, adw2 and ayw, each recognized Pre-S(1)- and Pre-S(2)-specific epitopes on all serological subtypes of the HBV envelope protein. Thus, the sequence variability within the Pre-S(1) and Pre-S(2) regions does not represent an impediment to the development of synthetic peptide or genetically engineered hepatitis B Pre-S immunogens for worldwide immunization.

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58. Systematic Location of Immunodominant Epitopes in Pre-S-coded Domains of the HBV Envelope Proteins

Stephen B. H. Kent, Karen F. Parker, A. Robert Neurath1, Nathan Strick1

There is great current interest in the "mapping" of epitopes on the protein constituents of pathogenic microorganisms. Recently, this has been approached by the parallel synthesis of hundreds of small (6-8 residue) peptides, followed by assay of their cross reactivity with, for example, immune sera from post-infection individuals or neutralizing monoclonal antibodies. Here we present an alternative, more generally effective strategy for the systematic location of epitopes, which has resulted in a number of interesting observations including the discovery of a novel class of biologically active peptides. Our approach is based on the synthesis of large (~30 amino acid residue) peptide fragments of the protein under study. This has several
advantages. A total of only 2 x n residues of synthesis completely spans in overlapping fashion a protein of "n" residues; furthermore, initial screening of the relatively few large fragments is more convenient and allows subsequent more detailed studies to be focused on the few immunochemical hotspots of the protein under study, rather than needlessly duplicate these efforts over the whole molecule. In addition, large peptides are more likely to contain complete epitopes, and have a pronounced tendency to adopt conformations similar to those in the native protein. This allows the location of epitopes dependent on secondary or even tertiary structure, as long as significant contributions arise from continuous segments of the peptide chain. Application of these concepts to the Pre-S-coded domains of the M- and L-proteins of the HBV envelope has resulted in location of two immunodominant epitopes of the virus and the location of the host-cell receptor binding site on the L-protein. All three epitopes have been successfully mimicked by approximately 30-residue synthetic peptides. The peptides mimicking the immunodominant epitopes are inherently strong immunogens, invoking powerful immune responses in experimental animals without coupling to carrier molecules. The molecular basis of this immunogenicity, i.e., the presence of both T-cell and B-cell epitopes in these peptides, has been further dissected. These observations have general implications for the development of synthetic vaccines.

Characterization of Prion Proteins with Monospecific Antisera to Synthetic Peptides

Ronald A. Barry¹, Marion T. Vincent¹, Stephen B. H. Kent, Stanley B. Prusiner²

The sialoglycoprotein PrP 27-30 is the major macromolecular component in highly purified preparations of prions derived from scrapie-infected hamster brain. Immunoblotting studies demonstrated that this protein is generated by partial protease digestion of a larger precursor (PrPSc) with an apparent molecular weight of 33-35 kDa, and that a protease-sensitive cellular PrP isoform, designated PrPC, is present in normal hamster brain. To characterize the relationships among these proteins, enzyme-linked immunosorbent assay and immuno-

blotting studies were undertaken with rabbit antisera raised against three synthetic PrP peptides from different regions of the prion protein. All three antisera were found to specifically react with the prion proteins, and failed to identify other lower or higher molecular weight proteins. Our results provide evidence that the primary structures of PrP 27-30, PrPSc and PrPC are related; this conclusion supports molecular cloning studies indicating that these proteins are encoded by the same chromosomal gene.

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60. E-Domain Peptide of Rat Pro-Insulin-like Growth Factor II: Validation of a Radioimmunoassay and Measurement in Culture Media and Rat Serum

Vincent W. Hylka¹, Stephen B. H. Kent, Daniel S. Straus¹

We recently discovered a peptide derived from the carboxy-terminal portion of the E-domain of rat pro-insulin-like growth factor II (proIGF-II) in medium conditioned by BRL-3L rat liver cells. This peptide begins at residue 117 in the proIGF-II sequence. To measure physiological concentrations of this peptide in serum, we established a radioimmunoassay for a synthetic peptide [rat proIGF-II(117-156) = E-domain peptide], corresponding to the carboxy-terminal 40 amino acids of rat proIGF-II. The 41-residue peptide, [Tyr¹¹⁶]-proIGF-II(117-156), was also synthesized and iodinated for use as tracer. Using polyclonal antibodies, we established a standard curve that measured as little as 25 pg/tube. Tracer was not displaced by insulin, rGH, mEGF, bPTH, oFSH, TRH, or LHRH under our assay conditions. However, a synthetic analogue of the E-domain peptide, [Phe¹¹⁷]-proIGF-II(118-156), showed displacement similar to the synthetic E-domain peptide. Serial dilutions of either culture media or rat serum exhibited displacement parallel to the standard curve. Measurement of E-domain peptide in serum-free media conditioned by BFL-3A rat liver cells showed a time-related increase in E-peptide concentration over a 72-hour period. Analysis of E-peptide immunoreactivity from conditioned media after gel filtration chromatography in 1 M acetic acid revealed a single peak which had a molecular weight (determined by Western blot) iden-
tical to the synthetic E-peptide standard. The concentration of immunoreactive E-domain peptide levels in serum of 5-day-old rat pups was 30-40 times higher than concentrations found in the serum of adult rats. Gel filtration chromatography of adult rat serum in 1 M acetic acid revealed a single major peak of immunoreactivity eluting at a position similar to the elution position of the E-domain peptide from BRL-3A rat liver cell conditioned medium. The radioimmunoassay described here should prove useful for measurement of somatic output of E-domain peptide under different physiological conditions.

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61. Structure-Function Studies of Lymphokines by Total Chemical Synthesis
Ian Clark-Lewis, A. Lopez 1, Matthew Vadas 1, John W. Schrader 1, Stephen B. H. Kent

Two hemopoietic growth factors, murine interleukin-3 (IL-3) (160 residues) and human granulocyte-macrophage colony-stimulating factor (GM-CSF) (127 residues), were synthesized using automated solid-phase peptide synthesis methods. The optimized chemical methods used gave synthetic proteins in high yields with the expected physiochemical properties and correct primary structures. The synthetic molecules had the range of biological activities attributed to materials generated by recombinant DNA technology. The high activity of the unpurified product allowed structure-function studies to be performed. Synthetic analogs of IL-3 in which alanines have been substituted for cysteines have allowed determination of the disulfide bridge that is essential for IL-3 activity. In the case of GM-CSF, experiments with synthetic fragments have defined a region essential for activity. The results suggest that the automated peptide synthesis approach is particularly useful for determining the minimal structure required for activity, and which amino acids are essential for activity. Furthermore, the availability of large quantities of these molecules will be useful for further structure determinations and in vivo studies of native and engineered lymphokines.

Reference:

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2Marine Biological Laboratory, Woods Hole, MA.

62. Structural Requirements in the Egg Laying Hormone (ELH) of Aplysia californica
Dorothea L. Schiller, Brian T. Chait 1, Felix Strumwasser 2, Stephen B. H. Kent

Egg laying in Aplysia californica is associated with a stereotyped behavioral array. It has been postulated that this is caused by the coordinate release of a number of different peptides from a polyprotein precursor which also contains ELH (Scheller et al., 1983). Our goal was to determine the structural requirements for this activity. ELH, a 36-residue peptide amide, was synthesized by improved manual solid-phase methods. Injection of synthetic ELH (100 μg) induced normal egg laying in 7 of 7 animals, proving that ELH alone is sufficient to induce the complete behavioral array associated with egg laying. The fragments 8-36 and 1-29 amide (1500 μg) were inactive while 1-35 amide (200 μg) retained full activity. A series of analogs was designed and synthesized in which the N-terminal 20 residues of the molecule were replaced with three different amphiphilic helices and the C-terminal 16 residues preserved. None of these analogs were active in the egg laying assay. Further investigations are under way to more precisely define the actual requirements for bioactivity in the ELH molecule and to investigate the interaction of ELH with the various target-tissue receptors.

Reference:

1The Rockefeller University, New York, NY.
2Marine Biological Laboratory, Woods Hole, MA.

63. The Total Chemical Synthesis of Proteins
Dorothea L. Schiller, Ian Clark-Lewis, David Woo 1, Stephen B. H. Kent

The goal of total chemical synthesis of proteins is the preparation of homogeneous compounds made up of a single molecular species of known covalent structure and, for proteins, of defined tertiary structure. Classically, synthetic organic chemistry has relied on unambiguous synthetic routes to establish the covalent structure of the molecule under study. Peptides, once they contain more than a few amino acids, and proteins are not directly susceptible to this approach. Thus, one of the critical aspects of chemical synthesis
of large peptides and of proteins is the preparation of products with a high degree of purity and verified covalent structure. The key to the preparation of high purity synthetic products is the synthetic procedure used: a crude product containing a high proportion of the target molecule is a prerequisite to effective purification. Over the past two years, this synthetic methodology has been applied in our laboratory to structure/function studies of the protein mitogen interleukin-3, where we have located an essential disulfide bond by total chemical synthesis of a series of mutant proteins. An optimized total chemical synthesis of the protein mitogen human TGF-α in a form suitable for two-dimensional NMR studies has also been achieved. Methods used to establish the degree of purity of the synthetic products include: analytical HPLC, gel electrophoresis, and isoelectric focusing. The covalent structure of the products is determined by Edman degradation, mass spectrometry, and peptide mapping. Finally, the three-dimensional structure of synthetic proteins can be established by NMR methods. Using highly refined synthetic chemistry and modern purification and characterization techniques, the science of proteins is now being brought within the realm of organic chemistry.

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64. Total Chemical Synthesis of Peptides for Structure/Function Studies

Karen F. Parker, Linda K. Martin, Dorothea L. Schiller, Paolo Mascagni, Stephen B. H. Kent

Methods for the chemical synthesis of peptides have been refined to the point where the total synthesis of polypeptide chains of 50 to 150 amino acids is routine. This brings to fruition the original goal of peptide chemists: the total chemical synthesis of proteins for investigation of the structural origins of their activities. We have developed a generally applicable chemistry for the automated assembly of protected peptide chains at rates as fast as 20 minutes per residue and with yields of 99.4–99.8% per amino acid residue together with methods for the precise characterization of the protected peptides. Complete deprotection is achieved with strong acids (HF, sulfonic acids) without chain cleavage artifacts and with minimal side reactions due to the protecting groups. Purification of the synthetic products was carried out by reverse-phase HPLC and their characterization by isoelectric focusing on immobilized pH gradient gels, quantitative Edman degradation, and mass spectrometry. In the past two years these highly optimized synthetic methods have been used for the total synthesis of a variety of peptides including HIV-1 and HBV envelope protein-derived sequences, and the egg laying hormone of Aplysia californica. Current studies include T4-cell receptor-derived peptides and cytochrome c-derived peptides.

65. Application of Microsequencing Techniques to Proteins of Biological Interest


The goal of our work involving microchemical instrumentation/microchemical methods has been to provide the technical basis to study the primary structure of proteins available in small amounts.

We achieved major improvements in methods of sample preparation for N-terminal and/or internal sequence analysis, in the Edman chemistry, sequenator design and data acquisition/data analysis. Our progress in these areas is discussed in specific abstracts in this volume.

We have applied the newly developed techniques in collaborative efforts to a variety of proteins of biological interest (see Table 1 on following page) in order to define structural relationships between proteins and posttranslational modification, to determine the molecular nature of proteins in conjunction with electronic amino acid sequence databases or to provide the basis for the molecular cloning of the coding gene.

For N-terminal sequence analysis, samples were prepared by electroblotting onto chemically modified glass fiber filters, followed by covalent attachment to the glass. For internal sequence analysis, proteins were generally isolated by one- or two-dimensional electrophoresis and electroblotting onto nitrocellulose. Isolated proteins were enzymatically cleaved on the support and fragments were separated by narrow-bore (2 mm) HPLC. Sequence analysis was generally performed at the 2–20 pmole level.
### Table 1
Summary of Sequenced Proteins

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<tr>
<th>Sample</th>
<th>MW (kDa)</th>
<th>Isolation method</th>
<th>Sequence obtained</th>
<th>Use</th>
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<td>SDS-PAGE/HPLC</td>
<td>Multiple internal</td>
<td>Cloning, homology</td>
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<td>Shark myelin protein P₀</td>
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<td>SDS-PAGE/HPLC</td>
<td>Multiple internal</td>
<td>Cloning, homology</td>
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<td></td>
<td></td>
<td>Glass blot</td>
<td>N-terminal</td>
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</tr>
<tr>
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<td>27</td>
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<td>Multiple internal</td>
<td>Cloning, homology</td>
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<td>Rh(D) human red cell antigen</td>
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<td>SDS-PAGE/HPLC</td>
<td>Multiple internal</td>
<td>Cloning</td>
</tr>
<tr>
<td>Human mitochondrial F₁-ATPase subunit</td>
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<td>Multiple internal</td>
<td>Confirm identity</td>
</tr>
<tr>
<td>Human mitochondrial protein ARP</td>
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<td>Multiple internal</td>
<td>Cloning, homology</td>
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<td>Human CSF protein #5</td>
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<td>Transformation modulated protein plastin</td>
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<td>2-D gel/HPLC</td>
<td>Multiple internal</td>
<td>Cloning</td>
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<td>Various hepatitis B virus surface proteins</td>
<td>33,36</td>
<td>Glass blot</td>
<td>N-terminal</td>
<td>Confirm identity</td>
</tr>
<tr>
<td>Heat shock protein</td>
<td>70</td>
<td>2-D gel/HPLC</td>
<td>Multiple internal</td>
<td>Confirm identity</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor-Ⅰ</td>
<td>55</td>
<td>SDS-PAGE/HPLC</td>
<td>Internal</td>
<td>Confirm identity</td>
</tr>
<tr>
<td>Human recombinant interleukin-2</td>
<td>14</td>
<td>Glass blot</td>
<td>N-terminal</td>
<td>Identify post-translational modification</td>
</tr>
<tr>
<td>Human α tubulin</td>
<td>53</td>
<td>2-D gel/HPLC</td>
<td>Multiple internal</td>
<td>Confirm identity</td>
</tr>
<tr>
<td>Human α tubulin</td>
<td>51</td>
<td>2-D gel/HPLC</td>
<td>Multiple internal</td>
<td>Confirm identity</td>
</tr>
</tbody>
</table>

**66. N-Terminal and Internal Sequence Analysis of Shark Myelin Proteins Isolated from Polyacrylamide Gels**

_Ruedi Aebersold, Raúl A. Saavedra, Stephen B. H. Kent_

We have developed a general approach to obtain N-terminal and/or multiple stretches of internal sequence information of low microgram amounts of proteins separated by polyacrylamide gel electrophoresis. The techniques were applied to determine partial amino acid sequences of the major myelin proteins in the shark (Heterodontus) central nervous system (CNS). For N-terminal sequence analysis (P₀; N-terminal of MBP turned out to be blocked), proteins were electrophoretically transferred to modified glass fiber sheets where they were retained by covalent attachment for direct sequence analysis. For internal sequencing, proteins were electroblotted onto nitrocellulose and cleaved by enzymatic digestion on the support. Cleavage fragments were eluted off the matrix and separated by reverse-phase HPLC prior to sequence analysis in a gas-phase sequenator.

The sequence data identified two major proteins of shark CNS myelin, MBP and P₀. For both proteins, which together account for 95% of total CNS myelin, several forms of different molecular weight were identified by sequence analysis. Shark MBP and P₀ showed a strong homology to their mammalian counterparts. The comparative analysis of the main structural proteins of shark CNS myelin and mammalian peripheral nervous system myelin showed strong similarities between the two structures, whereas the composition of structural proteins in mammalian CNS myelin was different.
67. Internal Amino Acid Sequence Analysis of Proteins Separated by One- or Two-Dimensional Gel Electrophoresis by In Situ Protease Digestion on Nitrocellulose

Ruedi Aebersold, John Leavitt, Raúl A. Saavedra, Stephen B. H. Kent

We have developed a general two-step method for obtaining peptide fragments for sequence analysis from picomole quantities of proteins separated by gel electrophoresis. After separation by one- or two-dimensional polyacrylamide gel electrophoresis, proteins are electrophoretically transferred (electroblotted) onto nitrocellulose, the protein-containing regions are detected by reversible staining, cut out and each protein digested in situ by proteolytic enzymes such as trypsin or V-8 protease. The resulting peptide fragments are separated by narrow-bore (2 mm) reverse-phase HPLC, collected and sequenced in a gas-phase sequenator. The peptide maps after in situ digestion are reproducible but different from maps of the same proteins digested in solution. Excellent peptide recoveries and the absence of extraneous contaminants in the separation of the peptide fragment mixture allow the generation of extensive internal sequence information from picomole amounts of protein. The method thus overcomes the problem of obtaining amino acid sequence data from N-terminally blocked proteins and provides multiple, independent stretches of sequence which can be used to generate oligonucleotide probes for molecular cloning, and/or used to search sequence data bases for related proteins. This method has been successfully applied to the routine amino acid sequence analysis of a wide range of proteins isolated from one- and two-dimensional polyacrylamide gels. The simultaneous preparation of multiple samples in a single experiment has obviated sample preparation as the rate-limiting step in protein sequence analysis.

68. Sequence Analysis of Proteins from Whole-Cell Lysates After Separation in Analytical Two-Dimensional Gels

Ruedi Aebersold, John Leavitt, Stephen B. H. Kent

We have developed a general approach that, for the first time, allows the sequence analysis of proteins detected in two-dimensional gels, using the two-dimensional gel itself as an isolation method in combining with electrophoretic transfer (electroblotting) of proteins from the gels to sheets of filter paper (Aebersold et al., 1986). The blotted proteins are sequenced on the filter paper supports, or are subjected to enzymatic analysis (see Aebersold et al., 1987; Abstract No. 67, this volume). The peptide- or protein-containing spot or band is cut out and placed directly in the cartridge of the gas phase protein sequenator (Hewick et al., 1981) for determination of the N-terminal amino acid sequence.

One of the chief features of this approach is that it is a parallel sample preparation technique in which all components of a mixture of proteins are simultaneously prepared for sequence analysis. This has caused a fundamental change in our approach to research problems involving protein sequence analysis. Rather than the laborious preparation of a protein in highly purified form, it is now more efficient to work directly from mixtures of proteins. The preparation of proteins from two-dimensional gels by electroblotting is generally applicable, but the amounts of proteins are at the lower limit of current sequence analysis methods.

The amounts of proteins present in standard analytical two-dimensional gels are quite low. For proteins in the middle range of abundance, the amounts are typically 10-100 nanograms of each species, corresponding to 0.2-2 picomoles for a 50 kDa protein. With currently available sequencing technology, it is possible to determine the N-terminal 5-15 amino acid residues using 5 picomoles of sequenceable protein.

Sensitivity is thus the factor limiting routine sequence analysis of proteins separated by two-dimensional gels. A further 100-fold increase in the sensitivity of protein sequence analysis is urgently needed to take advantage of the powerful analytical separation and electroblotting techniques described here. Our research program to achieve this increased sensitivity has been described (see Abstract No. 69).

The application of these techniques has been illustrated by the partial sequence analysis of a number of unknown proteins from whole-cell lysates. This is an enormously powerful technology that will have profound implications in the systematic development of therapeutics and diagnostics. The groundwork for this
has already been laid in the past decade with the identification of marker proteins for tumorogenicity and for degenerative nervous and other human diseases. Furthermore, the ability to routinely sequence proteins from whole-cell lysates after separation by two-dimensional gel electrophoresis will change the way many biological research problems are approached. As a result, protein molecular biology will be an equal partner with genetic engineering techniques in the research laboratory.

References:

69. High Sensitivity Solid-Phase Microsequencing in a Modified Gas-Phase Sequenator

Heinz Nika, Ruedi Aebersold, Stephen B. H. Kent

We have used the chromophoric reagent dimethylamino-azobenzene isothiocyanate (DABITC) (Chang et al., 1978) for automated solid-phase microsequencing in a modified gas-phase sequenator using 100- to 500-fold less protein than analyzed previously in conventional solid-phase instruments (Salnikow et al., 1981). The approach we have taken includes: 1) a high-yield covalent attachment of protein to a chemically-activated glass fiber support; and 2) the development of solid-phase Edman degradation protocols using DABITC/PITC double coupling (Chang, 1983) and vigorous solvent conditions for the effective removal of excess reagent and by-products. Results were obtained with picomole amounts of protein (typically 10-50 picomoles) spotted on chemically-activated glass fiber supports or electrophoretically transferred (electroblotted) from SDS-polyacrylamide gels.

References:

70. Novel Strategies for Isolation of Proteins and Peptides for Sequence Analysis


We have developed a general method for the extensive internal amino acid sequence analysis of picomole amounts of proteins separated by one- or two-dimensional gel electrophoresis. The proteins are electroblotted onto nitrocellulose, stained, the protein-containing regions cut out and each protein digested in situ by proteolytic enzymes (e.g., trypsin, V-8). Peptide fragments are released from the blot into the supernatant and are separated by narrow-bore reverse-phase HPLC and sequenced in a gas-phase sequenator. This method has been routinely applied to a wide range of unknown proteins isolated from one- and two-dimensional polyacrylamide gels.

In order to use our novel solid-phase Edman degradation techniques, we have developed methods for the routine covalent attachment of picomole quantities of peptides and proteins in high yield. After separation by polyacrylamide gel electrophoresis, proteins are electroblotted onto DITC-glass fiber paper, where they are immobilized by stochastic reaction of the α- and ε-amino groups. After reverse-phase HPLC separation, peptides are collected on aminophenyl-glass fiber discs and selectively attached through the α-carboxyl groups by buffered water-soluble carbodiimide. The covalently attached samples have been sequenced with PITC or DABITC at the low picomole level.

71. Folding and Oxidation Studies of Chemically-synthesized TGF-α

Dorothea L. Schiller, Brian T. Chait1, Stephen B. H. Kent

Human transforming growth factor type α (TGF-α) is secreted into the culture medium of several retrovirally transformed fibroblasts as well as synthesized by tumor cell lines and during early fetal development. TGF-α induces a mitogenic response and interacts with the receptor for epidermal growth factor (EGF).

This 50 amino acid protein was chemically synthesized using an automated peptide synthesizer (Applied Biosystems, Inc., Foster City, CA) and solid phase protocols developed in our laboratory. After cleavage of the peptide from the resin and removal of the side-chain protecting groups, various methods were
investigated to fold and oxidize the chemically synthesized protein to obtain active protein with the correct tertiary structure. Among these were the classical air oxidation, and a combination of different "shuffling reagents," i.e., a mixture of the reduced and an oxidized thiol compound which allows the incorrectly formed protein disulfides to shuffle to the correct ones (Creighton, 1974). These experiments showed that air oxidation gives the poorest results. Furthermore, it is crucial to keep the protein in the fully reduced state until disulfide formation is initiated by adding the thiol compounds under an inert argon atmosphere and under buffer conditions where correct folding of the peptide chain occurs. From all the tested shuffling reagents, oxidized/reduced dithiothreitol gave the best results. TGF-a prepared in this way was characterized by mass spectrometry and isoelectric focusing and showed full biological activity.

Reference:

72. Data Analysis for Protein Sequencing
Wade M. Hines, Christopher T. Dodd1, Steven M. Clark, Stephen B. H. Kent

We have developed software for the numerical graphical analysis of chromatographic data generated in the protein sequencing experiment. Chromatograms are digitized with 12-bit resolution and are saved on hard disk and floppy. Off-line digital data analysis consists of high and low frequency filtering, peak detection, peak identification, and peak quantization by non-linear least squares fitting and integration. Color graphics display the processing as it occurs. The routines are highly interactive, allowing the user to optimally tailor analysis conditions by adjusting constants and limits used in the various algorithms. Graphics also serve the user in the display of processed and unprocessed data. In the identification of PTH-amino acid peaks, chromatograms are displayed both in full and with an inset window which shows an expanded view of each peak overlayed on the corresponding region of the previous sequencing cycle. This representation proves extremely useful in analyzing the changes from chromatogram to chromatogram that define the amino acid sequence of a protein. "Synthetic chromatograms" are a useful graphic representation of analyzed data in which the integrated PTH signals are represented as idealized Gaussians and plotted on a flat baseline. This system has enhanced our ability to read sequence at and below the picomole level of signal. It has been instrumental in our ability to sequence proteins blotted from two-dimensional gels and internal peptides derived from electrobotted proteins.

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73. Design of Synthetic Peptides Mimicking the Immunologic and Biologic Functions of the Pre-S Sequence of the HBV Envelope (ENV) Protein
A. Robert Neurath1, Nathan Strick1, Karen F. Parker, Stephen B. H. Kent

Earlier studies from our laboratory and others have established that synthetic peptides derived from the Pre-S(2) [Pre-S(120-145) and Pre-S(133-151)] and the Pre-S(1) regions [Pre-S(21-47)] of the HBV env protein elicit virus-neutralizing (protective) antibodies and antibodies inhibiting the attachment of HBV to hepatocytes, respectively. These results suggested that peptides from the Pre-S region are potential components of synthetic hepatitis B vaccines. In order to realize this potential, peptides optimally mimicking Pre-S sequences within the native HBV env protein have to be identified. To accomplish this goal, peptides of different lengths derived from the Pre-S(1) and Pre-S(2) sequences were synthesized and their antigenicity and immunogenicity were determined. The effect of amino acid substitutions within the peptide sequences on their reactivity with polyclonal and monoclonal antibodies was investigated. These studies established the location of residues essential for antigenicity and immunogenicity of Pre-S(1) and Pre-S(2) sequences. The importance of additional residues located at the C and N termini, corresponding either to the natural Pre-S sequence or to amino acid residues absent in the natural sequence, was investigated. Essential residues for recognition of hepatocyte receptors within the Pre-S(21-47) sequence were identified.

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A Synthetic Peptide Vaccine for Hepatitis B. Conjugation of Cysteine-linked and Disulfide-containing Peptides to a Single Carrier

Chin Sook Kim, Karen F. Parker, A. Robert Neurath, Stephen B. H. Kent

In order to obtain a third generation vaccine for HBV, we need a new adjuvant/carrier and epitopes from both the Pre-S-gene- and S-gene-coded envelope proteins. Toward this goal, we have been working on a convenient method to hook the epitopes onto the carrier.

As previously described (Neurath et al., 1984, 1986), peptides containing the immunodominant Pre-S-coded epitopes were linked to the carrier by reaction of a free sulfhydryl group in the peptides with the maleimide-modified carrier. The S-protein-derived peptide (Howard et al., 1984; Zheng et al., 1986) contains a disulfide loop essential for its activity so that sulfhydryl-linking is not possible. As an alternative strategy, a C-terminal homoserine residue was added to the S-protein-derived peptide epitope with the aim of reacting amino groups in the carrier with the homoserine lactone peptide. Suitable Nα-acetyl peptides have been synthesized, deprotected, cyclized to the disulfide-containing homoserine lactone form, and purified. Reaction of these peptides with a variety of amines was studied under a range of reaction conditions to optimize conjugation to the carrier. Candidate synthetic vaccines containing peptide epitopes specific for each of the L-, M-, and S-proteins of the HBV envelope are being evaluated for immunogenicity with adjuvants suitable for vaccine formulation.

References:

The Lindsley F. Kimball Research Institute of the New York Blood Center, New York, NY.

Caltech Protein Sequenators

Steven M. Clark, Wade M. Hines, Charles F. Spence, Petros Arakelian, Stephen B. H. Kent

We have designed and built an improved protein sequenator which is ideally suited to protein sequencing chemistry development. The instrument has a programmable controller, based on the 6502 microprocessor, which allows independent control of all valves. Any desired chemistry can be entered into the menu-driven operating system by using a light pen. The controller also allows ramping of the cartridge compartment temperature for exploration of optimal conditions for coupling and cleavage reactions. Reliability and serviceability were considered in the controller and plumbing design. The cartridge sub-assembly can be easily removed from the instrument for sample loading. Other hardware features include the continued use of the Wittman-Liebold/Caltech zero dead volume valves, the use of narrow range, low pressure regulators with flow restrictors for accurate reagent and solvent delivery, and the incorporation of threaded bottle caps in the reagent and solvent manifolds for easy reservoir changes. These instruments offer very substantial advantages over commercially available protein sequenators because of the completely flexible hardware configurations and their program versatility. Thus, we have been able to adapt the design to do routine solid-phase sequencing at the picomole level using liquid reagents and powerful solvents which reduce chemical background (see Abstract No. 69).

The Optimization of Automated DNA Sequence Analysis

Jane Z. Sanders, Barbara J. Otto, Lloyd M. Smith

A commercial version (Applied Biosystems, Inc.) of the prototype DNA sequencer recently constructed here is currently being tested in our laboratory. This instrument uses laser excitation of fluorescent dyes to detect bands of DNA present in polyacrylamide gels. The fluorescent dyes are covalently attached to the universal M13 oligonucleotide primer used in the enzymatic generation of DNA fragments in the (Sanger) A, C, G and T dideoxy sequencing reactions. A different colored fluorophore is used in each of the four reactions, which are then combined and co-electrophoresed in a slab gel. A sensitive laser-based detector scans the gel near its bottom and measures fluorescence at each of four wavelengths. The DNA sequence is inferred from the temporal order of bands of the four colors migrating through the detector.
Data from a maximum of 16 lanes are stored in a microcomputer during a 12-hour run for subsequent automated analysis and base-calling. At present this instrument is capable of sequencing 300 bases per lane at 95-98% accuracy for a theoretical full-use output of about 10,000 bases per day.

We are interested in improving the performance of this instrument with regard to accuracy, resolution, and amount of data obtained per run. The issue of accuracy is of particular importance. The enzymatic method of sequence analysis is well known to be less accurate than the chemical method. This is due to the properties of the enzyme used, the Klenow fragment of DNA polymerase I. This enzyme does not terminate with equal frequency at all bases of a given type during the sequencing reactions. This causes substantial variability in the amplitude of the different peaks obtained on the instrument, which in turn leads to errors in the sequence, when small but real peaks are lost in adjacent larger peaks or in baseline noise.

We are investigating the use of an alternative enzyme, T7 DNA polymerase (available commercially as Sequenase). Our preliminary results indicate that this enzyme greatly reduces amplitude variability in automated sequencing, leading to significantly reduced error rates. A thorough statistical analysis of data obtained with both enzymes is under way.

77. The Chemical Synthesis of Fluorescent Dye-Oligonucleotide Conjugates Containing Multiple Dye Molecules

Robert J. Kaiser, Lloyd M. Smith

In order to enhance the sensitivity of the fluorescence-based DNA sequencing approach (see Abstract No. 76), we have developed a novel chemistry for efficiently incorporating multiple aliphatic amino groups into internal positions of a synthetic oligonucleotide. These amino groups then serve as points of attachment for dye molecules, thus allowing for the introduction of multiple fluorescent moieties into an oligonucleotide. Under appropriate conditions, a linear relationship between the number of dyes per oligonucleotide and the resulting fluorescence should be observed.

Our strategy is based on the standard solid-phase phosphoramidite DNA chain assembly chemistry, utilizing the cyanoethyl phosphoramidite derivative of 5'-O-(4,4'-dimethoxytrityl)-2'-N-(N-trifluoroacetyl-glycyl)-2'-amino-2'-deoxyuridine. This molecule can be incorporated into internal positions of the synthetic oligonucleotide, since the position of the amino group does not interfere with formation of the 3'-5' phosphodiester backbone. Utilizing a modification of the standard reaction cycle, coupling yields of 96+% are routinely achieved. After normal cleavage and deprotection, the resultant product is an oligonucleotide containing the desired number of amino groups ready for conjugation with dye.

We have initially focused on oligonucleotides containing two amino groups contained in a non-hybridizing "tail" 5' to the priming portion of the sequence and separated from one another by a single nucleotide. These oligonucleotides give good yields of dye conjugates, with the two-dye oligonucleotide derivatives being readily separable from incompletely reacted material by reversed-phase HPLC. We have synthesized four such two-dye primers to be compatible with the fluorescence-based sequencing technique, containing the dyes fluorescein, dichlorodimethoxyflourescein, tetramethylrhodamine, and Texas Red, another rhodamine derivative. All four oligonucleotides are effective primers in Sanger (dideoxy) sequencing using conventional radioisotopic detection. The two fluorescein-based two-dye oligonucleotides have approximately twice the fluorescence yield as the corresponding one-dye oligonucleotides. In contrast, the two rhodamine-based two-dye oligonucleotides exhibit significant fluorescence quenching relative to the one-dye oligonucleotides. We are continuing to investigate alternate arrangements of dye molecules in these oligonucleotides to eliminate this problem.

78. Automated Fluorescence-based Chemical DNA Sequencing

Robert J. Kaiser, Randolph B. Lipscher, Jane Z. Sanders, Lloyd M. Smith

The commercial automated fluorescence-based DNA sequencer (see Abstract No. 76) currently utilizes the Sanger (dideoxy) method of DNA sequence analysis. The fluorescent label is incorporated into the nested set of DNA fragments obtained in this method via a fluorescent dye-conjugated primer. An alternative method of DNA sequence analysis, the chemical
method of Maxam and Gilbert, is also amenable to adaptation for use in fluorescence-based DNA sequencing.

We are investigating two approaches to fluorescence-based chemical sequencing. The first approach involves primer-directed enzymatic strand synthesis on a single-stranded template to produce the desired fragment of DNA for sequencing. The chemical sequencing reactions are then carried out on this enzymatically synthesized strand to generate a nested set of fragments each ending in a known base or bases. A small, fluorescently-labeled piece of synthetic DNA is then enzymatically ligated to the fragments and the sequence determined by electrophoresis and fluorescence detection using the commercial instrument.

The second approach is designed to eliminate much of the time-consuming ethanol precipitation and lyophillization involved in the current chemical sequencing methodology. The approach is made possible by the chemistry we have developed to introduce reactive amino groups into synthetic oligonucleotides, either at the 5' terminus or into internal positions (see Abstract No. 77). By utilizing different amino-protecting groups—an acid labile group for introducing a 5'-terminal amino functionality and a base labile group for introducing an internal amino functionality—we can discriminate between these two reactive sites in a single oligonucleotide. We have used this to advantage by preparing an oligonucleotide containing a protected amino group at its 5' end and a free amino group internally. This oligonucleotide is then covalently attached to a solid glass support via a long, hydrophilic tether ending in an amino-reactive functionality. We are able to achieve a loading of several tens of picomoles of oligonucleotide per milligram of glass support using this chemistry. This support-bound oligonucleotide will then be used in a strategy similar to that described in the first approach to produce a nested set of fragments covalently attached to the support. The advantage of this method is that leftover reagents used in both the strand synthesis and the chemical sequencing reactions can be removed simply by thoroughly washing the support. The fragments can then be fluorescently labeled by deprotecting the 5'-amino group of the primer and reacting with dye, once again removing excess reagents by washing. The dye-labeled fragments are then cleaved from the support and analyzed on the automated fluorescence-based sequencer.

79. A Gene Detection Assay Capable of Distinguishing Single Nucleotide Differences

Ulf Landegren, Robert J. Kaiser

Mutated genes frequently differ from wild type by single nucleotide changes. Currently existing techniques for detecting genes are cumbersome or fail to distinguish all possible mutations. A technique has been developed that permits a rapid and general identification of known allelic variants of a gene. The principle that is exploited in the assay is to allow two oligonucleotides to anneal in solution to immediately adjacent regions on the gene under investigation. By the action of a ligase, these two oligonucleotides can then become joined covalently and this event is scored as indicative of the presence of the gene sequence. The ligation event is sensitive to mismatches in the apposed ends of the oligonucleotides and thus permits single nucleotide distinction.

Initial experiments to prove the general feasibility of the approach were performed by analyzing the result of the ligation as an increase in size of radiolabeled oligonucleotides after separation by polyacrylamide gel electrophoresis. A more convenient assay was developed by introducing a biotin group on one of the two oligonucleotides and labeling the other with $^{32}$P. After hybridization and ligation, biotinylated oligonucleotides are permitted to bind to streptavidin agarose beads. These are subsequently washed by filtering over a filter paper and any radioactivity captured by the ligation of the two oligonucleotides can be detected by autoradiography. Sets of oligonucleotides representing allelic variants of the human $\gamma$-globin gene were prepared and used to demonstrate the capacity of the technique to identify single nucleotide changes in cloned and genomic DNA. The technique is rapid, specific and sensitive, does not require high molecular weight DNA and should be amenable to automation and nonradioactive detection.

80. A Continuously Variable Field Gel Electrophoresis Apparatus

Steven M. Clark, Eric H. C. Lai, Petros Arakelian

The recent availability of restriction enzymes such
as NcoI and SfiI, as well as methods for preparation of intact high molecular weight DNA, strongly motivate improvements in methods for the separation of large DNA fragments. Methods that have previously been used for separation of large DNA fragments in the 50 kb to 9000 kb range include pulsed field gel electrophoresis (G.E.), field inversion G.E. and voltage-clamped homogeneous electric field G.E. In all of the previous techniques there have been drawbacks: each apparatus is suited to only one kind of separation technique and field optimization for a repetitive separation has been laborious. We have designed and built a prototype instrument to do continuously variable field gel electrophoresis in which 24 electrodes arranged in a hexagonal array are independently controlled by a computer. This design allows for both temporal and spatial control of the electric field throughout the separation process giving equal or superior resolution as compared to previous apparatuses and eliminating DNA mobility differences between lanes. The electrode control unit was designed for two modes of operation, stand-alone and PC driven. This feature will help in the optimization and reproducibility of any particular type of separation.

81. Development of Novel Computer Tools for the Analysis of Nucleic Acid and Protein Sequence Data

Tim Hunkapiller

The latest releases of the national nucleic acid database, Genbank and Protein Information Resource (PIR) have close to 18,000 entries and exceeds 50 million characters of data and annotation. This total has been nearly doubling in size every year. With recent advances in sequencing technologies pioneered in this lab and probable major new initiatives to characterize entire genomic sequences, the amount of available data may soon be increasing exponentially. These data have become a critical resource for molecular biologists as both an archive and research tool. As a consequence, molecular biologists must have the computer tools that allow timely and convenient access to these data in coordination with the handling and analysis of their own extensive data as well as the algorithmic methods necessary for extracting meaningful information from this wealth of data. We have developed a comprehensive sequence database and analysis system that currently resides on a HP9000-840 (VAX 8650-class) superminicomputer employing a state-of-the-art RISC-based architecture. This resource is maintained as both a facility for day-to-day data handling for members of both our laboratory as well as the Division and as a facility for the development of novel algorithmic and database tools.

Many of these utilities are concerned with the time-consuming search for relationships between different protein or DNA sequences. The computation effort of many of the applied algorithms is exponentially related to the length of the data being considered. The projected growth in data promises to far outstrip the ability of any algorithmic scheme and all but the most powerful and expensive computers to analyze it in a reasonably timely fashion. We are currently evaluating two particular schemes to insure our continued ability to analyze this information. The first concerns the application of novel computer architectures. Primarily, this means the application of new parallel computer systems such as the Hypercube series developed here at Caltech. These, along with variations on this architecture such as that of the massively parallel Connection Machine, offer interesting possibilities for supercomputer power applied to molecular biology data analysis. It will be most important to assertain which analytical problems lend themselves to parallel definition.

The second possibility involves the development of specialized processors. We are currently collaborating with TRW Inc. in assessing the applicability of a new pattern-matching engine developed for text databases, the Fast Data Finder (FDF). The FDF is a systolic array of specialized character-processing chips. Patterns are loaded into an array of the chips and the data to be searched is piped through in a continuous stream. The convolutions and conditional (i.e., "spelling" stringencies, list options, etc.) of the pattern are mapped in with the actual characters and considered as the data streams through. No iterations are required regardless of the complexity of the search. The FDF has already shown the potential of speeding up certain types of problem solving by orders of magnitude. However, there are important sequence comparison algorithms and questions that probably will
not be tractable with text processor functions. Primary among these are mathematically rigorous operations that define the minimum distance between sequences based on user-defined penalty parameters. We are currently collaborating with members of the Jet Propulsion Laboratory on the design of such a chip.

**PUBLICATIONS**


=======================================================================

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Senior Research Fellows: John V. Cox, Catherine M. Woods
Research Fellows: Alexander Artisheky, Peter M. Burger, Nigel R. Burns, Marius A. Carioi, Horst Hinssen, Michael Rodriguez, Frank Sangiorgi
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Special Graduate Student: Chantal M. Champagne
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Summary: Differentiation, leading to cellular diversity, constitutes a major aspect of the regulation of the development of metazoa. Genetic material permits the orderly expression of certain genes, together with the suppression of others, in such a way as to specify the morphogenetic pathway of lineages. We wish to know what patterns of expression are inherent to the differentiation program of a given lineage and what genetic events are affected by influences arising from the environment into which the stem cells of these lineages migrate to differentiate. For such an analysis to be experimentally feasible, we need well-characterized cellular systems in which the mechanisms of determination and subsequent differentiation are accessible to study. The erythroid lineage...
of the hemopoietic system represents one of the best characterized cell populations suitable for studies of determination and cellular morphogenesis during differentiation. Not only can the cells at various stages of differentiation be purified from embryos with relative ease, but also early progenitor cells can be immortalized, at least for a few generations, through the use of retroviruses. Our ability to develop in vitro techniques where cells of different lineages can be propagated in tissue culture has allowed us to investigate the contribution of the cell cycle to differentiation and maturation in a fashion that has not been possible previously with any other lineage. To analyze the molecular mechanisms that underlie the determination of the morphogenetic pathway of erythroid cells, we have concentrated on the expression and assembly of proteins responsible for the maintenance of the cell's shape. Red blood cells exist in nature either nucleated or enucleated. With the exception of mammals, in most other vertebrate species red blood cells are nucleated. In mammals (e.g., mice), the primitive lineage of red blood cells that develops as a cohort in the bloodstream from the yolk sac in the first few days of embryonic development remain nucleated. The definitive lineage of the red blood cells is initiated a few days later, first in fetal liver and after birth initially in the spleen and then in the bone marrow. These cells enucleate in all three organs giving rise to enucleated reticulocytes. In vertebrates where the cells remain nucleated (e.g., chickens), both the primitive and definitive lineage cells remain nucleated irrespective of the site of development. We wish to know what genes are involved in the retention of the nucleus in nucleated cells and how their expression has changed in mammals. By comparing the expression of these genes in the primitive vs. the definitive lineage of mammals, we can define what patterns of expression are inherent to the developmental program of these cells vs. what patterns are dictated by the microenvironment in which the definitive cells differentiate. Having established the patterns of expression of these genes and the control of assembly of their protein products in two representative species, chicken and mouse, we should be in a position both to define biochemical factors responsible for facilitating enucleation in mammals and not in chickens. Furthermore, by manipulating the expression of these genes in mice to mimic the expression patterns observed in chicken red blood cells, we should be able to begin manipulating enucleation in vivo in transgenic animals. This approach should provide us with the means experimentally to manipulate in general the morphogenetic pathway of the red blood cell lineage.

The discoidal shape of the nucleated red blood cell is defined by three structural domains: a submembranous network of proteins principally based on spectrin, actin, protein 4.1, ankyrin and the anion transporter; a marginal band of microtubules disposed at the equator of the cell; and a system of intermediate filaments based on the protein vimentin disposed perpendicular to the marginal band and interconnecting the two opposing sites of the plasma membrane through the spectrin network to the nucleus. Enucleate red blood cells lack a marginal band and intermediate filaments. We wish to know not only the pattern of expression of the genes that make up the skeleton of the red blood cell but also the mechanism(s) of assembly of their gene products which gives rise to the asymmetric distribution of these three cytoskeletal domains. We also wish to know how the expression of these genes has changed in mammals to allow these cells to ultimately lose microtubules and intermediate filaments, enucleate and assume a biconcave shape. Finally, by manipulating the expression of these genes in transgenic animals, we should be in a position to begin manipulating the normal course of morphogenesis of the mammalian erythroid lineage. The studies presented below describe various aspects of our experimental efforts to elucidate the pathway of structural differentiation of the red blood cell.

82. Expression of Transfected and Endogenous Vimentin Genes during Erythropoiesis In Vitro

John J. Ngai

The expression of vimentin during erythropoiesis serves as an attractive model system in which to study the regulated expression of intermediate filament proteins during cellular differentiation and morphogenesis. Murine erythroleukemia (MEL) cells normally repress the levels of endogenous mouse vimentin mRNA during inducer-mediated differentiation in vitro, resulting in a subsequent loss of vimentin
filaments. The expression of vimentin in differentiating MEL cells reflects the disappearance of vimentin filaments during mammalian erythropoiesis in vivo. In contrast, chicken erythroid cells express high levels of vimentin mRNA and vimentin filaments during terminal differentiation. The striking difference in the changes of vimentin mRNA abundances (and ultimately, of vimentin filaments) in terminally differentiating mammalian and avian erythroid cells presents an opportunity to study not only the factors responsible for the dynamic positive and negative regulation of the vimentin gene, but also the basis for the evolutionary divergence of the two erythropoietic programs with regard to vimentin expression. We have examined the behavior of transfected chicken and hamster vimentin genes in MEL cells and have found that chicken vimentin mRNA levels increase significantly in differentiating transfected MEL cells, whereas similarly transfected hamster vimentin genes are negatively regulated. In vitro nuclear run-on transcription experiments reveal that endogenous mouse vimentin gene transcription rates decrease only about twofold in differentiating MEL cells, as compared to the extensive (~25-fold) decline in mouse vimentin mRNA levels. This apparent disparity in gene transcription rates and steady state levels suggests that the decline in mouse vimentin mRNA levels in differentiating MEL cells is due in part to posttranscriptional mechanisms. In contrast, the increase in chicken vimentin mRNA levels in differentiating transfected MEL cells roughly correlates with an increase in transcription of the chicken gene. Together these results suggest that the difference in vimentin expression in avian and mammalian erythropoiesis is due to a divergence of cis-linked vimentin sequences that are responsible for transcriptional and posttranscriptional regulation of vimentin gene expression. Our results further suggest the existence of general erythroid-specific trans-acting factors that have been functionally conserved from birds to mammals and interact with chicken vimentin regulatory sequences in murine erythroid cells in a manner appropriate for chicken vimentin sequences in a chicken erythroid environment. Lastly, transfected chicken vimentin genes produce functional vimentin protein and stable vimentin filaments during MEL cell differentiation, demonstrating that the accumulation of vimentin filaments is determined primarily by the abundance of newly synthesized vimentin.

83. Vimentin Expression in Murine Primitive Erythrocytes

Frank Sangiorgi, Catherine M. Woods

A sequential change in the site of erythropoiesis and in the appearance of two morphologically distinct erythroid cell lineages occurs during the embryonic and fetal development of the mouse (Kovach et al., 1967). Primitive erythroid cells develop from yolk sac blood islands from the 8th to 12th day of gestation (dg) and persist in the fetal circulation until the 16th day. Unlike the erythrocytes of the adult, these primitive cells remain nucleated throughout their development and synthesize predominantly embryonic hemoglobins. Large numbers of enucleated erythrocytes of the definitive lineage containing adult hemoglobins are subsequently formed in the fetal liver from 12 to 14 dg.

Our early investigations of these primitive cells have focused on vimentin, a constituent of the intermediate filament cytoskeleton. Western blot and immunofluorescence analyses have demonstrated a decrease in the amount of this protein from 11 to 15 dg. Two-dimensional gel electrophoresis of metabolically-labeled cells depicts a decrease in protein synthesis in general and in vimentin synthesis in particular by day 11. Analysis of the mitotic state of these cells by acridine orange staining-flow cytometry presents a profile of proliferating cells from 8 to 11 dg. By the 12th day the cells have ceased dividing and are found in the G0 compartment of the cell cycle for the remainder of their lifespan.

These studies suggest that a vimentin filament network in murine primitive erythroid cells is present early in development, and that the synthesis of this protein decreases rapidly after day 11. The cells continue to proliferate until day 12, at which time cell division ceases. Thus, during development, the decrease in vimentin synthesis is a programmed event, regardless of the nucleated state of the cell. Studies on the level of vimentin mRNA and of vimentin gene transcription during development of these cells is currently under way.

Reference:
84. Targeting the Expression of an Exogenous Vimentin Gene to the Erythroid Lineage of Transgenic Mice

Frank Sangiorgi, John J. Ngai

The hematopoietic system provides a useful model to examine the change in intermediate filament organization during cellular differentiation. Vimentin, one type of intermediate filament protein, is expressed variably throughout the differentiation of the hematopoietic cell lineages.

A structural role for vimentin filaments in chicken erythrocytes has been inferred from several studies (Granger and Lazarides, 1982; Capetanaki et al., 1983). These filaments form a network that attaches to the cell membrane and appears to anchor the centrally located nucleus. In contrast, vimentin filaments are not observed in the mature, enucleate mammalian erythrocyte. In our studies utilizing murine erythro-leukemia cells, the decrease in vimentin mRNA and disappearance of vimentin filaments is a rapidly inducible event during erythropoiesis in vitro (Ngai et al., 1984). This evidence leads to the hypothesis that enucleation during mammalian erythropoiesis is facilitated by the loss of vimentin filaments.

In order to examine the role of vimentin filaments in erythroid morphogenesis in vivo, we have attempted to direct the expression of chicken vimentin to murine erythroid cells. A chimeric gene incorporating a full-length vimentin cDNA under the control of the mouse ß-major hemoglobin promoter has been injected into fertilized mouse oocytes. This arrangement of sequences will also serve to target the expression of vimentin in a temporal-specific manner (Russell and Bernstein, 1966). The resultant mice from the microinjection and transplantation procedures will be analyzed for integration of the foreign DNA into the genome. Following identification of several transgenic mice, these animals and their offspring will be examined for the assembly and cytoplasmic localization of chicken vimentin in erythroid tissues.

References:
86. Erythroid Anion Transporter Assembly is Mediated by Recruitment onto a Preassembled Membrane Cytoskeleton

John V. Cox, Jeffrey H. Stack

The mechanisms regulating the association of the anion transporter (band 3) with the peripheral membrane cytoskeleton of chicken erythroid cells were investigated by pulse-chase and steady state RNA and protein analyses. These studies have revealed that the extent of association of band 3 with the membrane cytoskeleton varies during chicken embryonic development. Pulse-chase studies have indicated that band 3 polypeptides do not associate with the membrane cytoskeleton until they have been transported to the plasma membrane. At this time, band 3 polypeptides are slowly recruited, over a period of hours, onto a preassembled membrane cytoskeletal network and the extent of this cytoskeletal assembly is developmentally regulated.

Only 3% of the band 3 polypeptides are cytoskeletal-associated in 4-day erythroid cells versus 93% in 10-day erythroid cells and 36% in 15-day erythroid cells. This observed variation appears to be regulated primarily at the level of recruitment onto the membrane cytoskeleton rather than by different transport kinetics to the membrane or differential turnover of the soluble and insoluble polypeptides and is not dependent upon the lineage or stage of differentiation of the erythroid cells. Steady state protein and RNA analyses indicate that the low levels of cytoskeletal band 3 early in development most likely result from limiting amounts of ankyrin and protein 4.1, the membrane cytoskeletal binding sites for band 3. As embryonic development proceeds, ankyrin and protein 4.1 levels increase with a concurrent rise in the level of cytoskeletal band 3 until, on day 10 of development, virtually all of the band 3 polypeptides are cytoskeletal bound. After day 10, the levels of total and cytoskeletal band 3 decline, while ankyrin and protein 4.1 continue to accumulate until day 18, indicating that the cytoskeletal-association of band 3 is not regulated solely by the availability of membrane cytoskeletal binding sites at later stages of development. Thus, multiple mechanisms appear to regulate the recruitment of band 3 onto the erythroid membrane cytoskeleton during chicken embryonic development.

87. Sequence Analysis of the Ankyrin Gene

Marios A. Cariolou

Ankyrin is a major polypeptide component of the cytoskeleton of erythroid cells and functions to link spectrin to the transmembrane anion transporter. It is also present in a variety of other cells such as neurons and muscle (Nelson and Lazarides, 1984a,b). The exact nature by which ankyrin interacts with other cytoskeletal components in cells, and also, whether ankyrin exists as a single molecule or as a family of closely related molecules, is the major objective of this investigation.

Preliminary results indicate the presence of two polyadenylated ankyrin mRNA species that may give rise to different ankyrin-like polypeptides. Recently, a new λZap cDNA library was constructed in order to obtain clones with large inserts to complete the sequence analysis of erythroid ankyrin. From the primary amino acid structure of ankyrin, we will propose a model for its possible spatial arrangement and functional properties in cells. Furthermore, by using sensitive molecular probes we will study the mechanisms involved in regulating the expression of ankyrin and ankyrin-like polypeptides in erythroid and non-erythroid cells, as well as define structural domains involved in its binding to spectrin and the anion transporter and sites of fatty acid acylation (Staufenbiel and Lazarides, 1986).

References:

88. Expression of Erythroid Membrane-Skeleton Polypeptides in AEV- and S13-Transformed Erythroid Progenitor Cells

Catherine M. Woods, Peter K. Vogt

The avian erythroblastosis virus (AEV) ES4 strain and the S13 virus both transform early erythroid progenitor cells and block their differentiation at the CFU-e stage of development, i.e., well before the stage of terminal differentiation (reviewed in Graf and Berg, 1978). Progenitor cells transformed with S13 can differentiate spontaneously or, by use of a temperature-sensitive mutant of the virus, can be induced by temperature shift to differentiate along a
normal pathway. Hence, we have initiated studies to investigate whether these systems would enable us to analyze the initial steps involved in the expression and assembly of the erythroid membrane skeleton. We have found that these cells do express major peripheral components of the membrane skeleton, which have hitherto been regarded as being unique to terminally-differentiating cells. Thus, in addition to the more universal non-erythroid αγ-form of spectrin, these cells contain αS-spectrin, ankyrin and protein 4.1 at steady state, albeit at a much reduced level on a per-cell basis than found in mature erythroid cells. These proteins assemble under the plasma membrane to form a rudimentary membrane skeletal array but do not appear to be as cross-linked as in the mature red cell. Surprisingly, however, these cells do not express or accumulate anion transporter, the protein that is considered to be the ultimate membrane receptor for the erythroid membrane skeleton. Northern analysis has confirmed that the anion transporter cytoplasmic mRNA is uniquely lacking in fully-transformed cells (Woods et al., 1986).

The much lower steady state levels of the peripheral membrane-skeleton constituent polypeptides found in these transformed erythroid progenitor cells compared to even mitotic erythroblasts appear to be entirely due to the instability of the assembled polypeptides to catabolism at this stage. This instability appears to correlate with the lack of the anion transporter, since upon induction to differentiate the cells begin to express the anion transporter and concomitantly the assembled peripheral components begin to accumulate. In order to directly assay the role of the anion transporter in stabilizing the peripheral skeletal components from catabolism separately from the other multitude of changes occurring in parallel with the expression of the anion transporter during the terminal differentiation steps, we have initiated a series of experiments to try and introduce plasmid constructs carrying the cDNA for the whole coding region of the anion transporter under an inducible promoter into these transformed cells. The relative stability of the assembled spectrin network in the presence or absence of the anion transporter can then be assayed.

Another series of experiments has been aimed at determining whether this regulation of expression of the anion transporter is operating at the level of transcription or of transcript processing and transport from the nucleus. Nuclear run-off experiments indicate the gene is being expressed. Furthermore, whereas no anion transporter transcripts can be detected in cytoplasmic RNA, high molecular weight transcripts can be detected in nuclear RNA isolated from uninduced transformed cells. Together, this suggests that synthesis of this protein is regulated at the level of processing and transport from the nucleus. This will be investigated in more detail. We are presently attempting to analyze the changes that are occurring in this process as the cells are induced to differentiate.

References:

89. The Role of Fatty Acid Acylation in Assembly of Ankyrin onto the Plasma Membrane

Catherine M. Woods

From in vitro biochemical studies, it has been concluded that the protein ankyrin, which serves as the major membrane-linking protein for the spectrin-actin network through its interaction with the transmembranous anion transporter, assembles onto pre-assembled anion transporter during the biogenesis of the membrane skeleton (see Abstract No. 86). However, from our studies with AEV and SIJ cells, it is clear that ankyrin and hence spectrin can assemble onto the plasma membrane, albeit unstably in the absence of the anion transporter. The question then arises as to whether an alternative membrane-binding site exists for ankyrin in these cells. Biochemical analysis of native ankyrin-spectrin-actin complexes from the membrane has failed to reveal the presence of any potential polypeptide candidate for an alternative binding site. An alternative possibility is that ankyrin itself interacts directly with the lipid bilayer. Previous studies from this laboratory had shown that ankyrin is actively fatty acid acylated with palmitic acid in both immature and mature red cells (Staufenbiel and Lazarides, 1986). We have shown that ankyrin in transformed erythroid progenitor cells is likewise actively acylated with palmitate once associated with
the membrane. Inhibitors of fatty acid biosynthesis appear to inhibit assembly of newly-synthesized ankyrin onto the membrane in these cells. This in turn is associated with inhibition of the assembly of newly-synthesized spectrin. These studies are being pursued further to determine the exact role that fatty acid acylation plays in the initial assembly of ankyrin onto the plasma membrane and its mechanism.

Reference:

90. Mechanisms of Generation of Multiple Protein 4.1 Variants in Erythroid Development

Jeffrey H. Stack

Protein 4.1, an extrinsic membrane protein that plays an important role in stabilizing the membrane cytoskeleton, is found in chickens as a multiplet of isoforms ranging in molecular weight from 77 kDa to 175 kDa (Granger and Lazarides, 1984). The relative ratios of the different variants changes in a developmentally-regulated manner. Protein 4.1 cDNAs have been isolated and RNA blot analysis showed a single 6.5 kb RNA. S1 nuclease protection experiments have shown that this single size class RNA is composed of at least four distinct species and the relative ratios of these RNAs are also developmentally regulated. In addition, genomic DNA blots demonstrated that the gene encoding protein 4.1 exists as a single copy in the haploid chicken genome (Ngai et al., 1987). Possible mechanisms for generating multiple RNAs include different sites of transcription and/or translation initiation and termination and different polyadenylation sites. In order to determine the actual mechanism, we have concentrated on optimizing the conditions in which full-length 4.1 cDNAs are synthesized. Sequence analysis of cDNA clones in conjunction with in vitro transcription/translation systems should reveal the splicing pattern(s) responsible for generating this complex set of proteins.

It is possible that different protein 4.1 variants contain different functional domains (see Abstract No. 91) and the regulated expression of these molecules could have profound effects on the assembly and stability of the membrane cytoskeleton. Once full-length cDNAs are isolated and analyzed to determine the polypeptide encoded by a particular cDNA, manipulation (deletion), in vitro biochemical, and transfection studies can begin to address the functional significance of the different protein 4.1 variants.

References:


Nigel R. Burns

Following extensive studies on the erythrocyte membrane cytoskeleton, it has become apparent that the stability of this polyhedral network is conferred, at least in part, by the formation at junction points of a high affinity complex between spectrin, actin and protein 4.1. In the chicken erythrocyte, protein 4.1 exists as a heterogenous set of polypeptides, which in the adult mature cell consists of at least seven polypeptides ranging in molecular weight from 77-170 kDa. The expression of these polypeptides is developmentally regulated to the extent that in immature, proliferative erythroblasts variants of 77 kDa and 87 kDa predominate. As the cells undergo terminal differentiation, the pattern of expression changes so that higher molecular weight forms become more abundant (Granger et al. 1984). It has been shown that the chicken haploid genome contains a single gene and that this gene gives rise to multiple 6.6 kb mRNAs in a developmentally-regulated manner (Ngai et al., 1987). It has been concluded that both posttranscriptional and translation regulation are utilized in the generation of the diversity of chicken erythrocyte protein 4.1.

This complicated pattern of expression of protein 4.1 has led us to initiate investigation on the functional diversity, which we consider probably underlies the structural diversity, of this class of protein. In particular we wish to delineate the influence of the changing pattern of protein 4.1 expression upon the morphology of the cytoskeleton.

We have developed a method for purifying chicken erythrocyte protein 4.1 to homogeneity. Using this material in in vitro assay systems, we have been able to demonstrate that the different routes of mRNA processing, which the diversity of protein 4.1 variants
represents, is indeed reflected in differential function. All the protein 4.1 variants in the adult mature cell, with the exception of the 77 kDa variant are capable of binding to the membrane cytoskeletal protein, spectrin. The inability of the adult mature 77 kDa polypeptide to bind to spectrin precludes it from forming high affinity complexes in conjunction with actin, in the membrane cytoskeleton. Consequently, this particular variant cannot act to stabilize the membrane cytoskeleton in the manner in which the other variants can. Further in vitro assays are being developed to elucidate a role for the 77 kDa variant and to further examine the extent to which a single gene can encode for multiple polypeptides of different functionalities.

As an aid for these studies, and to enable us to localize individual protein 4.1 variants in situ, we have raised a panel of monoclonal antibodies which show restricted cross reactivity between variants. So far we have been successful in isolating two hybridomas whose antibodies recognize only a single variant as well as cell lines that recognize only two and three variants. We anticipate that this repertoire will be increased on further subcloning. These antibodies will be used to indicate the distribution of the various protein 4.1 variants, at both the light and electron microscope levels, during erythroid differentiation as well as during the cell cycle.

References:

92. 8-Amyloid Expression in Transgenic Mice.
A Model for Alzheimer's Disease

Michael Rodriguez, Frank Sangiorgi

Senile dementia of the Alzheimer's type (SDAT) is characterized in humans by the accelerated accumulation of abnormal fibrillar structures within neuronal cell bodies, in areas of degenerating neurons in the perineuronal space and in the walls of blood vessels. These structures, known as neurofibrillary tangles, amyloid plaque cores and vascular amyloid, respectively, are composed of a common polypeptide termed Aβ, which is believed to be derived from a normal protein known as β-amyloid. Of fundamental importance in tracing the etiology and pathogenesis of Alzheimer's disease is whether the pathological and behavioral changes in this disease are a direct consequence of Aβ deposition in the brain or the result of some other event, with Aβ deposition being an interesting secondary, albeit diagnostic, epiphenomenon. Since in man the pathological changes of SDAT are qualitatively similar to those described in the normal elderly population, it is not possible to completely dissociate SDAT from the process of aging. Furthermore, although several hypotheses have been proposed to account for the pathogenesis of Alzheimer's disease, most of these are not testable, since humans are not amenable to direct experimental investigation and there is no satisfactory animal model for this disease.

We propose to address some of these issues by constructing an animal model system. Transgenic mice harboring the β-amyloid cDNA under the control of the inducible mouse metallothionine I promoter will be used to evaluate whether overproduction of β-amyloid is necessary and/or sufficient to generate the diagnostic pathological features of Alzheimer's disease, including the production of Aβ. Initially we will raise antibodies to the β-amyloid protein by cloning the β-amyloid cDNA into the high expression vector pHSe6. This vector can be used to obtain milligram quantities of protein that can be used as an immuno- gen. Using antibodies and cDNA probes to β-amyloid and Aβ, we plan to establish the pattern of expression, synthesis and turnover of these polypeptides in normal mouse development and in several cell lines grown in tissue culture including PC12 and mouse embryonal carcinoma cells, which can be induced to differentiate towards a neuronal phenotype. Subsequently, we plan to induce overproduction of β-amyloid in neuronal cell lines and transgenic mice and assess the effects on the normal pattern of expression, synthesis and turnover of this protein. These studies will allow us to evaluate under what conditions Aβ is produced in vivo, how this process is controlled as a function of aging, how amyloid fibril formation is initiated and propagated during this process, and whether these fibrils act as aggregation sites for the other proteins normally found in NFTs. Overall, this experimental approach will allow us to establish whether the manipulation of only one genetic parameter in a model system is sufficient to reproduce the structural, functional and behavioral abnormalities of SDAT.
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Summary: In multicellular organisms, the determination of anterior-posterior (A-P) embryonic polarity is fundamental to subsequent development, since the entire body plan is constructed relative to this axis. Little is known about the molecular mechanisms by which embryonic polarity is established and how axial information is transduced into cell fate. The major focus of research in our laboratory is on the molecular genetic analysis of A-P axis formation in Drosophila. We have commenced a molecular analysis of four maternal effect loci required for A-P axis establishment and are also carrying out a search for cDNAs representing transcripts localized along the A-P axis in the unfertilized egg. After the A-P axis is established, a variety of genes act to subdivide the developing embryo into periodic repeats and, ultimately, into segments. The homeotic genes combine axial and periodic information in order to specify segmental identity. We are studying a gene located in the bithorax homeotic gene complex of Drosophila; this late bxd gene might be involved in specifying the identity of one of the adult segments.

93. Molecular Analysis of Embryonic Polarity Genes

Howard D. Lipshitz, Susan R. Halsell, Anne Frey, William W. Fisher

We have begun the molecular analysis of four loci that are required for the establishment of A-P embryonic polarity. These loci were identified by Schupbach and Wieschaus (1986a,b) as genes whose expression is required in the Drosophila germline for the correct formation of the A-P embryonic axis. Two of the genes, torso and trunk, are required for the formation of the poles of the embryo; mutants lack the extreme anterior and posterior structures. A third mutation, spliced, causes the deletion of more central structures—only the poles remain. In exuperantia mutants, anterior structures are absent and are replaced by posterior structures resulting in a bipolar (double-posterior) embryo. Drs. Schupbach and Wieschaus have kindly provided us with the mutants and we are proceeding with attempts to molecularly clone these loci. Our initial approach is to generate P element-induced female sterile mutations and then to test these for allelism with the genes of interest. These attempts are in their initial stages; we have established 4,500 individual lines that we are testing for female sterility. We also are isolating X-ray-induced mutations in order to map the loci cytogenetically. Once we have cloned these genes, we will proceed to analyze their structure as well as the spatial and temporal expression of their RNA and protein products during oogenesis and early embryogenesis.

References:
94. cDNAs Representing RNAs Localized in the Unfertilized Egg

Da-Li Ding

We are particularly interested in the possibility that certain of the RNAs that encode proteins involved in specifying the A-P embryonic axis are non-uniformly distributed in the egg, as well as in the mechanisms by which RNAs become asymmetrically localized in cells. The *Drosophila* egg is a giant, ellipsoidal cell whose major axis is 540 µm and whose minor axis is 200 µm. Most of the contents of the oocyte are synthesized in a set of 15 interconnected nurse cells and then transported into the oocyte during oogenesis. These features make the egg uniquely suitable for a search for cDNAs representing RNAs localized along the A-P axis. We have designed a miniature guillotine that we are using to bisect frozen eggs along this axis. Our strategy is to then purify anterior (A) and posterior (P) RNA and to construct A and P cDNA libraries in the JSW A1 vectors designed in the Meyerowitz laboratory (Palazzolo and Meyerowitz, 1987). We then proceed to construct subtracted (A minus P) and (P minus A) libraries and screen these for cDNAs representing RNAs localized to the anterior or posterior regions of the egg. Confirmation of localization, as well as information regarding the spatial and temporal expression of these RNAs, will be obtained by tissue *in situ* hybridization to ovaries. Subsequently, we will carry out molecular and genetic analyses on the loci encoding these RNAs.

Reference:

95. Molecular Analysis of the Late *bxd* Gene of the Bithorax Complex (BX-C)

Howard D. Lipshitz, William W. Fisher

We have previously carried out a detailed analysis of the transcriptional products of the *bithoraxoid* (*bxd*) region of the *Ultrabithorax* (*Ubx*) domain of the BX-C (Lipshitz *et al*., 1987). These studies revealed that the only mRNA encoded by the 40 kb *bxd* region is a 0.8 kb mRNA that probably encodes a 101 amino acid protein. This "late *bxd* gene" is expressed from the mid-third larval instar through the adult stages of development. We have presented arguments in favor of the possibility that the late *bxd* protein acts together with the homeo domain-containing *Ubx* protein family to specify the identity of a specific adult segment (parasegment 6) (Lipshitz *et al*., 1987). We are commencing a detailed analysis of the spatial expression of the late *bxd* mRNA and protein in late larvae, pupae and adults. Subsequently, we plan to carry out a functional analysis of the late *bxd* gene by analyzing the effects of BX-C mutations on its expression, as well as by means of P element-mediated insertion of a fully functional late gene back into the *Drosophila* germline. These studies could provide the first direct link between the expression of a specific BX-C protein and a spatially and temporally localized homeotic phenotype.

Reference:

PUBLICATIONS


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The research in our laboratory involves two different organisms, both used because of the ease with which they lend themselves to experiments in classical and molecular genetics. They are the fruit fly *Drosophila* and the flowering plant *Arabidopsis*.

The *Drosophila* experiments fall into two classes, those studying the hormonal, tissue- and time-specific regulation of a cluster of genes transcribed only in the larval salivary gland, and those involving development of the nervous system and eye. The salivary gland-expressed genes are glue genes; that is, they code for messenger RNAs which are translated to proteins that the larva secretes at the end of the larval stage, and which serve to attach the puparial case to its substrate for the prepupal and pupal periods. This year we report continued progress in delineating the cis-acting regulatory sequences of these genes, in understanding the relation of the activity of these genes to the chromatin state change called polytene chromosome puffing, in determining the trans-acting regulators of the genes, and in studying their evolution. The other fly experiments include the molecular cloning and characterization of genes expressed specifically in the eye and other head regions of the fly. One gene characterized in detail has been the ops in gene coding for the photoreceptor pigment of one specific type of receptor cell, the R7 cell. In addition, a large collection of head-specific genes has been cloned as a beginning of a complete analysis of the gene products that make eye and nervous system different from other tissues.

The *Arabidopsis* work has included further progress in establishment of methods that should make it possible to clone any gene for which a mutant phenotype is known. The isolation starts with mapped DNA fragments near the gene of interest; the gene is then cloned by successive isolation of overlapping cloned segments of the genome. A map of random DNA fragments has been established and is rapidly expanding, and transformation methods for detecting the cloned gene of interest have also been worked out. In addition, detailed characterization of genes expressed in developing seeds, and of methods for their study, is reported, as is further progress in characterization of the repetitive DNA elements of the *Arabidopsis* genome.

Regulation of Puffing and Gene Expression at the 68C Locus

Peter H. Mathers

The *Drosophila* 68C chromosome location contains three glue genes, *Sgs-3*, *Sgs-7* and *Sgs-8*, which are developmentally expressed in a coordinate stage- and tissue-specific manner. Regulation of these three genes is under the control of two known factors: the steroid hormone, ecdysterone, and the product of the X-chromosomal locus, 2B5. Mutations that affect production of these factors have been used to show that proper glue RNA accumulation from 68C requires both of these products. While puffing is usually thought to be associated with high transcriptional activity, these mutations, which have a strong effect on RNA accumulation, have no effect on chromosome decondensation, or puffing. We are using these mutations and glue gene fragments reintroduced into the fly using germine transformation to analyze the requirements for correctly regulated puffing and gene expression.

As we reported last year (see Biology 1986, Abstract No. 126), a 2B5 mutant, *l(1)npr-1*, fails to express a transformed copy of *Sgs-3* which has only 130 base pairs (bp) upstream of the RNA start site and one kilobase of coding region. This 130 bp region has been shown by others in the lab to be sufficient for correct tissue and time regulation but does not provide for properly abundant transcript levels. In an attempt to determine whether the 2B5 product was required upstream or downstream of the *Sgs-3* start site to give proper RNA accumulation, reciprocal promoter-transcript fusions were constructed between *Sgs-3* and *Sgs-4*. *Sgs-4*, located at position 3C, is expressed (but at reduced levels) in the *l(1)npr-1* mutant and therefore acts as a positive control. After a site-directed mutagenesis of the *Sgs-3* start site to allow these fusions, fusion constructs were introduced back into the fly via P-factor transformations. The two reciprocal fusions and the mutagenized *Sgs-3* control construct were all found to accumulate RNAs of the expected size and specificity but at reduced levels, presumably resulting from inappropriate initiation and/or stability of the novel transcripts. A careful analysis is now under way to determine which, if any, of these constructs will express in a *l(1)npr-1* background. This analysis will provide us with...
information on the process of 2B5 activation of 68C gene expression.

Finally, we have begun to analyze the requirements for chromosomal puffing. We are using germline transformants with varying 68C transcription lengths and varying promoter strengths to determine how length of transcribed DNA and mass of transcribed RNA affect the amount of chromosomal decondensation. Already, we have a construct that produces near normal levels of the 1.1 kb Sgs-3 RNA but fails to puff. In addition, mutations at 2B5 and in ecdysterone synthesis that allow puffing while failing to accumulate RNA will be studied to determine how this fits into an overall picture of chromosome decondensation and gene activity.

97. Evolution and Expression of the Sgs-3 Glue Gene

Christopher H. Martin, Elliot M. Meyerowitz

The Drosophila 68C glue genes are strictly regulated by time, tissue, and steroid hormone. As an approach to locating cis-acting sequences necessary for this regulation, one can look at how the gene and its flanking regions change over the course of evolution. Essential sequences should remain relatively conserved amidst a background of relatively rapidly diverging, non-essential sequences. Another goal was to learn more about the processes of evolution acting on the Sgs-3 gene. This gene differs from the other two located at 68C by the presence of a central, threonine-rich region that contains 37 repeats of a 5 amino acid sequence. The sequences of the Sgs-3-like glue gene from three Drosophila species were determined and compared to the D. melanogaster sequence. Islands of conserved sequence are present in the upstream sequences, which are known to be involved in regulation of the Sgs-3 gene; these results are being applied to various site-directed mutagenesis experiments in the lab. Additionally, much was learned about the evolution of the Sgs-3 glue gene. For example, while all of the Sgs-3-like glue genes contain a central threonine-rich region, the length and consensus amino acid sequence of the repeat region varies considerably.

We wanted to determine if the regulatory regions are sufficiently conserved among the species to interact with the regulatory factors of D. melanogaster. This was done by introducing Sgs-3 glue genes from two other Drosophila species into the D. melanogaster genome utilizing P factor-mediated transformation. The constructs used all expressed at near normal levels in the D. melanogaster background. This demonstrates that the trans-acting factors of D. melanogaster can interact with these foreign genes so as to yield proper, regulated expression.

98. Regulatory Elements of Sgs-3

Margaret Roark, K. Vijay Raghavan, Carol A. Mayeda

We have successfully adapted a transient expression assay for the rapid analysis of the regulation of Sgs-3. Gene fusions of Sgs-3 with the Drosophila Alcohol dehydrogenase gene or with E. coli β-galactosidase sequences allow the histochemical assay of the function of regulatory sequences. Plasmids containing the Sgs-3-lacZ or Sgs-3-Adh fusion constructs are injected into pre-blastoderm embryos at the anterior end and the salivary glands from the larva into which the embryo develops are dissected and assayed for enzyme activity.

The transient expression assay has allowed us to further delimit two regulatory elements defined earlier. The first region (130 bp of sequence 5′ of the mRNA start site of Sgs-3) lies close to the gene and by itself is sufficient for low levels of expression. The second region lies between -130 bp and -2300 bp 5′ of the mRNA start site: the presence of this region together with the 130 bp region results in high levels of expression. Using the transient assay and by the synthesis of appropriate deletions, we have shown that the 130 bp element is not only sufficient but is also necessary for high level expression of an Sgs-3-Adh fusion gene. (The transient assay may not be sensitive to expression at levels 10% or less than normal; sequences that express at these low levels in germline transformants are negative in the transient assay.)

The region that confers high level of expression has been narrowed down to between -650 and -220 bp 5′ of the mRNA start site. This element does not have some of the characteristics attributed to enhancers: it does not act in an inverted orientation and it does not activate a heterologous promoter.

Constructs that are negative in the transient assay are being examined in germline transformants to see if they express at low levels.
Saturation Mutagenesis of an Upstream Regulatory Element

K. Vijay Raghavan, Takeshi Toda, Margaret Roark

We have shown that sequences that lie 130 bp 5' of the mRNA start site of Sgs-3 are both necessary and sufficient for gene expression. A saturation mutagenesis of this region has been undertaken: the purpose is to identify point mutants that fail to express an Sgs-3-Adh fusion protein in the transient expression assay. Such mutations will not only define the regulatory element at the level of single nucleotides but will allow the selection of mutants in other genes that restore function. Such extragenic revertants could define loci that encode trans-acting regulatory factors.

We have devised a mutagenesis scheme that allows for the rapid identification of point mutants in specific regions (Figure 1). This, combined with the rapidity of the transient expression assay (see the previous abstract), allows a detailed analysis of tissue and stage specificity of gene expression. The scheme relies on the use of the chemical tetramethylammonium chloride (TMA). In its presence, at appropriate concentrations, the melting temperature of DNA-DNA hybrid molecules can be made independent of base composition. Chemically mutagenized single-stranded DNA is made double stranded by primer extension, the mutagenized region of interest (the 130 bp region in this case) is digested with appropriate restriction enzymes and cloned into the transient expression vector (a plasmid containing the Sgs-3-Adh gene fusion). Each colony is amplified in a microtiter well and a fixed volume spotted onto a nitrocellulose filter. Ten replica filters are made in this manner and each filter probed with a different 20 nt oligonucleotide probe that spans part of one strand of the 130 bp region. The probes together cover both strands of the region where mutants are desired. The filters are washed in the presence of TMA under conditions where even a single base mismatch will result in the probe melting off. Absence of hybridization of a particular colony to a particular probe identifies the region of the mutations. Such colonies are picked from the master plate and plasmid DNA from them sequenced to verify the change introduced.

This scheme has worked efficiently in a pilot run and many point mutants have been isolated and are being tested in the transient assay.

Figure 1. Production and identification of point mutations in the proximal regulatory element.
100. Regulation of the Sgs-7 and Sgs-8 Glue Protein Genes

Mark D. Garfinkel, Carol A. Mayeda

We have continued studies on two histochemically-assayable fusion genes derived from the divergently-transcribed Sgs-7—Sgs-8 gene pair (Biology 1986, Abstract No. 129). In the normal arrangement of these genes, Sgs-8 is transcribed leftward, Sgs-7 is transcribed rightward, and 475 base pairs separate their 5' termini. In our gene fusions, Sgs-8 was joined to the E. coli β-galactosidase gene so as to make an enzymatically-active hybrid protein, and the 5' untranslated region of Sgs-7 was joined to the 5' untranslated region of D. melanogaster Alcohol dehydrogenase such that alcohol dehydrogenase would be translated from a hybrid mRNA. The entire 475-base-pair intergenic region was reassembled. P factor-mediated gene transfer was used to create six independent germline transformants carrying the fusion gene pair. All synthesize both alcohol dehydrogenase and β-galactosidase enzyme activities and chimeric RNA molecules with the expected tissue and temporal specificity. When adult flies homozygous for three different autosomal transformation events are mated to virgin females heterozygous for the mutation l(1)mpy-1 (known from previous work to define a trans-acting regulator of the 68C glue protein genes) and progeny male third-instar larvae are processed for histochemical reactions, only males that carry a l(1)mpy+ allele manufacture alcohol dehydrogenase and β-galactosidase enzymes in their salivary glands. That Sgs-7—Adh fusion gene expression is dependent upon the l(1)mpy+ gene product rules out several mechanisms for this requirement in 68C glue protein gene expression, including those that involve sequence-specific interactions with regions of the Sgs-7 transcript (i.e., the intervening sequence) or transcribed DNA that was replaced by the alcohol dehydrogenase segment.

The embryo-microinjection transient expression procedure has been used to test in vitro-synthesized deletions of the intergenic region for expression of the histochemically-marked fusion genes. When constructs containing the entire 475-base-pair intergenic region or 445 base pairs upstream of the Sgs-8—lacZ fusion gene were microinjected into syncytial cleavage embryos, abundant β-galactosidase enzyme activity was seen in the salivary glands of one-third of the surviving third instar larvae. When constructs containing 425 base pairs or less upstream of the Sgs-8—lacZ fusion gene were tested, no third instar survivors showed any β-galactosidase activity. Sequences required for Sgs-8 gene expression in this assay mapped with unexpected proximity to the Sgs-7 gene. In the case of the Sgs-7—Adh fusion gene, constructs retaining 340 base pairs and 240 base pairs of upstream sequence supported essentially wild-type gene expression; those retaining 215 base pairs and 145 base pairs gave reduced levels of gene activity, and a construct retaining 95 base pairs of 5' flanking sequence was inert in the transient expression assay. These results are consistent with the presence of an enhancer-like element required for the expression of both Sgs-7 and Sgs-8 which is asymmetrically positioned within the intergenic region. Experiments to test this hypothesis, and to verify the transient expression results using germline gene transfer, are in progress.

101. Developmentally-regulated Transcription in the Larval and Prepupal Salivary Gland

Bruce A. Hamilton

The larval salivary gland of Drosophila persists through prepupal development, with rapid changes in chromosomal puffing pattern and the variety of newly synthesized proteins (suggesting that the gland serves some as yet undetermined secretory function after extrusion of the larval glue: Tissières et al., 1974; Mitchell et al., 1977; Ashburner and Berendes, 1978). We are now cloning and characterizing some of these prepupal genes, which are abundantly expressed only in the salivary gland. As an extension of the transcriptional regulation studies of the glue genes Sgs-3, Sgs-7, and Sgs-8, we will use these prepupal genes as heterologous acceptors for promoter fusion constructs with glue gene regulatory elements. Using a histochemically-assayable reporter gene, we can then assess the ability of acceptor promoter to complement functions deleted from the donor (Sgs) sequences. We hope this will give us an understanding of the developmental field of some transcription factors required by these developmentally regulated promoters and of any constraints on spatial arrangement or compatibility in their interaction with other factors.
We are also currently evaluating an Sgs-3 distal 5′ flanking sequence implicated by deletion analysis (see Abstract No. 98) cross-species sequence conservation (Abstract No. 97), homology to other genes, and nuclease sensitivity (Ramain et al., 1986) in regulating level of expression of the gene. The sequence is a direct repeat of the decanucleotide AAGCTAAATT, which occurs between bases -596 and -615 of Sgs-3 in D. melanogaster. A single, perfect copy of this sequence is present 5′ of the Sgs-3-like gene of D. simulans (at -442) and single copies matching 9 out of 10 bases lie upstream of the corresponding genes of D. erecta (-364) and D. yakuba (-370). A 9 out of 10 base pair copy is also found in the short mutual upstream region between Sgs-7 and Sgs-8 (whose regulatory sequences overlap; see Abstract No. 100) and a 7 of 10 base pair homology is reported upstream of Sgs-4 (-405). The sites in D. melanogaster all correspond to reported DNase I hypersensitive sites (Shermoen and Beckendorf, 1982; Ramain et al., 1986). More convincingly, recent work in our lab has shown that these sequences are within Sgs-3 and Sgs-7 upstream regions whose deletion reduces the level of gene expression (Abstract Nos. 98 and 103). We have made and are making constructs that attach the direct repeat sequence to the proximal 130 bp 5′ of Sgs-3, which by themselves give a very low level of expression, or delete it from a longer 5′ region, which expresses at wild-type levels; we will test these constructs in a transient assay.

References:

102. Expression and Evolution of Eye-specific Genes
Karl J. Fryxell
I have identified genes in D. melanogaster whose expression is limited to eyes, photoreceptor cells, or R7 photoreceptor cells, by differential screening with [32P]cDNAs derived from the heads of mutant flies that have reduced amounts of these tissues and cells (Fryxell and Meyerowitz, 1987). I identified two opsin genes within this group by hybridization with synthetic [32P] oligonucleotides and determined the complete DNA sequence of one opsin gene, which is specifically expressed in R7 photoreceptor cells (some aspects of the partial sequence were discussed in last year’s Annual Report). Another R7 opsin gene has been isolated in the laboratory of G. Rubin (University of California, Berkeley); these two genes are apparently expressed in different subsets of R7 photoreceptor cells (Montell et al., 1987). The extreme specificity of the expression of these genes will provide a toehold from which to begin an analysis of the development and physiology of R7y and R7p photoreceptors (Hardie, 1986).

I am also interested in the process by which a precursor cell decides whether or not to become a neuron, and have therefore begun to study (by in situ hybridization and DNA sequencing) the genes that I previously found to be expressed in non-neuronal cells in the fly eye. I have recently found that the proportion of neuronal to non-neuronal cells in the fly eye may be either increased or decreased by appropriate mutations at the Notch locus. This observation is of interest in itself, but it also allows us to produce ommatidia with any desired number of photoreceptor cells. I plan to show which opsin genes are expressed by these photoreceptor cells, using a combination of in situ hybridization and multiple-label immunofluorescence experiments.

The increasing number of reports in the past year of the sequences of opsins and other, possibly homologous membrane proteins suggested to us that these data may be sufficient to provide some general information about the evolution of the nervous system. In order to show this, I have devised a new method of sequence alignment that can produce an accurate overall alignment of 15 sequences of 400 amino acids each in about one day on a personal computer. The major conclusion of this work is that color vision arose independently in insects and vertebrates. I have also been able to show that vertebrate β-adrenergic receptors and muscarinic acetylcholine receptors belong to the opsin gene family and constitute a single branch within this gene family.
References:

103. Max Headclones

Michael J. Palazzolo, David R. Hyde, K. Vijay Raghavan, Kirk MacKlenburg, Seymour Benzer, Elliot M. Meyerowitz

To identify molecules that are essential to the development and function of the Drosophila nervous system, we have begun to isolate cDNA clones that correspond to genes that are expressed at low levels in the adult head and not detectably expressed in the 0-1 hour embryo. Differential hybridization and subtractive hybridization are two methods that have been used to identify genes that are expressed more abundantly in one tissue than another. These procedures are based on screening nitrocellulose filter replicas of cDNA and genomic libraries with cDNA probes. Control experiments show that it is difficult to identify clones that are homologous to RNA molecules which are present in the probe at less than 1 part in 200. RNA reassociation kinetics suggest that most of the tissue-specific mRNA sequences are present at a much lower concentration than 0.5%. Thus, it is unlikely that these standard procedures can be used to identify cDNA clones that relate to most of the genes that are expressed in a nervous system-specific fashion. We have developed procedures that are several orders of magnitude more sensitive than differential and subtractive hybridization. Our strategy is based on sorting a subtracted head-minus-embryo cDNA library constructed in λSWAJ3 (Palazzolo and Meyerowitz, 1987). Our methodology is so sensitive because it takes advantage of the ability to rapidly identify tissue-specific mRNAs by RNA-RNA (Northern) hybridization analysis using single-stranded, high specific activity, cRNA probes synthesized directly from DNA prepared from individual λ cDNA clones. Using these methods, we have isolated almost 500 non-cross-hybridizing clones that correspond to genes that are expressed in the head but not the embryo. RNA reassociation kinetics also suggest that there are a limited number of such sequences, perhaps as few as 1600 (Levy and Manning, 1981). If so, cDNA molecules that we have already cloned should identify a substantial fraction of these genes. We have begun to characterize the cDNA clones that have been isolated. These initial experiments will determine: 1) the chromosomal locus that corresponds to each of the cDNA clones already isolated, 2) the particular tissue and cell types in which each corresponding gene is expressed, 3) the localization of the corresponding encoded protein products, and 4) the effects on the developing embryonic nervous system caused by a deletion of the corresponding chromosomal locus.

References:

104. A Restriction Fragment Length Polymorphism Map of the Arabidopsis Genome

Caren Chang, Arthur W. DeJohn

Continuation of the work previously described (Biology 1986, Abstract No. 124) has resulted in a restriction fragment length polymorphism (RFLP) map of the Arabidopsis genome (see Figure 2). The motivation for obtaining this map is to make available an alternative method for cloning Arabidopsis genes by using RFLP markers as starting points for chromosome walking. Arabidopsis is the only higher plant in which chromosome walking is feasible because, unlike in other plants, dispersed repetitive elements that would disrupt the walk are virtually absent in Arabidopsis. To construct the map, we examined meiotic segregation of RFLP markers in the progeny of a cross between wild-type Neiderzenz and a Columbia strain that is homozygous for three visible markers: angustifolia, glabra-1, and thiazole requiring (mapped on chromosomes 1, 3 and 5, respectively). The F1 plants were allowed to self-cross, as were the resulting F2 individuals. The visible markers were scored in the F3 generation to reveal the genotype of each F2 parent. To observe segregation of RFLPs, genomic DNA blots for 106 different F3 pools were probed with genomic DNA clones capable of detecting RFLPs between the two parental lines. From this segregation data, we calculated map distances for pairwise combinations of clones. The RFLP markers were obtained from cloned Arabidopsis genes and from random genomic lambda clones that had been screened for low copy number. Approximately 48% of the random clones...
were polymorphic for at least one of the enzymes EcoRI, XbaI, or BglII. Chromosome walking should become generally applicable when the map density reaches roughly 250 RFLP markers so that the average distance between adjacent markers is 300 kb. The map so far contains 41 markers in five linkage groups; three of these linkage groups are assigned to chromosomes 1, 3, and 5 based on their linkage to the visible markers that were included in the cross. We are continuing to add markers to the map, and with a second cross we are presently identifying chromosomes 2 and 4.

![Figure 2. RFLP map of the Arabidopsis genome. Markers are designated by the number (or name) of the genomic DNA clone that detects the RFLP at the indicated position. an, gl-1, and tz are the visible markers. Distance units are in centiMorgans. Of the five linkage groups, chromosomes 1, 3 and 5 have been identified; their lengths are consistent with those in the established genetic map of visible markers.](image)

105. A Walking Plant Transformation Cosmid Vector

**Martin F. Yanofsky, John L. Bowman**

The ability to clone any locus for which there is a visible phenotype would be an advance in the study of plant development at the molecular level. This soon may be the case in *Arabidopsis* by using a RFLP clone as a starting point and then walking to the gene of interest. Currently, a RFLP map of the *Arabidopsis* genome is being constructed (see Abstract No. 104) which will be dense enough to make walking a feasible proposition. To facilitate the walking and transformation processes, a single vector has been constructed to perform both these functions.

This cosmid vector contains synthetic bacteriophage promoters from SP6 and T7 flanking a unique BglII site. Small end-specific RNA probes can be generated by in vitro transcription of recombinant cosmids digested with restriction enzymes that do not interrupt the promoter region. Flanking the promoters are NotI and SfiI sites which facilitate mapping and allow the excision of full-length inserts. An *Arabidopsis* library constructed in this vector (-10 kb) should
contain inserts with an average length of 30-35 kb. The vector can be conjugatively transferred from *E. coli* to *Agrobacterium tumefaciens* where it will be incorporated into a disarmed Ti plasmid by homologous recombination (due to a region of the vector that has homology to the T-DNA region of the Ti plasmid). The resulting chimeric plasmid will contain a right T-DNA border, the inserted fragments, a hygromycin phosphotransferase gene for selection of transformed plant cells, a left T-DNA border sequence, and the vir genes (required for transfer of the T-DNA from *Agrobacterium* to plants). Transfer of insert DNA into the plant genome will be mediated by *A. tumefaciens* and stable incorporation will be selected for in the presence of hygromycin.

Initial experiments will involve complementation of *Arabidopsis thaliana* mutations that have specific effects on floral development (Biology 1986, Abstract No. 123). Beginning with a RFLP clone that maps near such a mutation, walking will proceed in the direction of the desired locus. As walking proceeds, isolated clones will be transformed into the mutant background and assayed for complementation. Since this method of complementation was recently shown to work for the *Adh* gene (Biology 1986, Abstract No. 125), it is likely that the technique will be applicable to the isolation of nearly any gene for which there is a mutant phenotype.

### 106. Complementation of an Arabidopsis ADH Null Mutant by Genetic Transformation

**Caren Chang**

Using *Agrobacterium*-mediated transformation, we have demonstrated that the cloned *Arabidopsis Adh* gene can restore tissue- and stage-specific alcohol dehydrogenase (ADH) activity to an *Arabidopsis* ADH null mutant. The interest of this work, in addition to establishing a transformation protocol within our lab, is the analysis of ADH as a biochemical tag for future promoter fusion experiments. Also of importance is characterization of the insertions in the transformed genome including frequencies, sites, configurations, and stability. The transformation procedure we followed was that of Lloyd et al. (1986) in which transgenic plants are regenerated from leaf-derived callus tissue. Leaf explants of an ADH null mutant were infected with *Agrobacterium* carrying a binary transformation vector into which we had cloned the genomic *Adh* gene (Chang and Meyerowitz, 1986). The vector allows selection of transformants on hygromycin B. Shoots that were produced by callus under hygromycin selection were regenerated into mature plants. We have nine independent transformed lines based on genetic criteria and genomic DNA blots. Progeny resulting from self-fertilization in the regenerated plants were tested for resistance to hygromycin B, as well as stained for root-specific ADH activity. In another assay for ADH activity, the progeny were tested for sensitivity to allyl alcohol, which is toxic to the wild type but non-toxic to mutant plants. Six of the lines display a 3:1 ratio of both hygromycin B resistance and ADH activity (including allyl alcohol sensitivity), supporting the presence of a single active transformed copy. Preliminary genome blot hybridizations for these six lines are consistent with a single integration site per line for the binary plasmid, although the number of copies per site is not known. Third and fourth generation plants continue to show inheritance predicted for a single active locus. Progeny from the remaining three lines give different segregation patterns; one line has more than one locus of ADH activity with a single locus of hygromycin resistance, another line lacks ADH activity while having resistance to hygromycin, and the last line has ADH activity while lacking hygromycin resistance. These three lines have yet to be analyzed by genome blot hybridization.

**References:**


### 107. Seed-specific Gene Expression in Arabidopsis thaliana

**Patty P.-Y. Pang**

Four classes of genes expressed specifically in seeds have been isolated from an *Arabidopsis* genomic library using cDNA from 7-8-day-old embryos. Three of the classes hybridize to abundant RNA species of approximately 1700 nucleotides long, and one to an RNA species approximately 650 nucleotides. One of the three large seed-specific genes cross-hybridizes to and is 86% homologous with the *Brassica napus* 12S
storage protein gene (R. E. Pruitt thesis, 1985). DNA sequence of a second large seed-specific gene shows a 75% homology to the 12S storage protein genes. Partial DNA sequence of the third large gene has been determined and also shows a high degree of homology to the 12S protein genes. Our results thus suggest that there are at least three distantly-related 12S seed storage protein genes in *Arabidopsis*. Each of the three 12S protein genes are single copy in most *A. thaliana* ecotypes, while seed storage protein genes are present in multiple copies in other plants: thus, the organization of the storage protein gene family in *Arabidopsis* is the simplest known.

The developmental profiles of the seed protein gene messages are very similar. They are expressed in the later stages of embryogenesis. The smaller message, however, appears earlier during development. In *B. napus*, the transcription of the 12S cruciferin gene is increased in excised embryos when the plant hormone abscisic axic (ABA) is present (DeLisle and Crouch, 1987). I have analyzed seed protein messages from *A. thaliana* ABA-deficient and -insensitive mutants. Results show no significant changes in the level of expression except that in the ABA mutants, the onset of expression of the genes may be 1-2 days later in embryogenesis than in wild type, suggesting that there are other factors involved in the regulation of the expression of the storage protein genes.

In order to analyze the 5' region required for the expression of the 12S genes, gene fusions of the *A. thaliana* *Adh* gene to the promoters of the 12S genes have been reintroduced into *A. thaliana* using a binary vector *Agrobacterium* Ti-mediated transformation system. An *Adh* protein fusion construction to one of the 12S promoters will also be introduced into *A. thaliana* so that selections for trans-acting mutations that do not allow expression of the seed storage proteins can be performed.

**Reference:**

108. Factors Controlling Seed-specific Gene Expression in *Arabidopsis*

**Martin F. Yanofsky**

During dicotyledonous plant embryogenesis, a number of mRNAs are abundantly expressed that code for seed storage proteins. These proteins most likely serve as a store of nitrogen for the developing seedling. This study has focused on one such seed-specific gene that encodes an uncharacterized 148 amino acid protein product and is present in three copies in the Landsberg *Arabidopsis thaliana* genome. Experiments are in progress to identify and characterize the cis- and trans-acting sequences required for seed-specific expression.

Cis-acting regulatory sequences will be identified by transcriptionally fusing the presumed promoter region of this gene to an easily assayable marker. Experiments are currently in progress to determine the relative sensitivity of several markers including plant alcohol dehydrogenase, bacterial β-glucuronidase, and firefly luciferase. Transgenic plants will be generated by *Agrobacterium*-mediated transformation, and expression of the chimeric marker gene will be assayed and compared with a closely linked constitutively expressed gene. In addition, the nucleotide sequence will be determined for all three copies of this gene, and this may identify conserved sequences involved in regulating expression.

Trans-acting factors required for regulated expression of this gene will be identified by isolating mutants defective in its expression. Selection of EMS-induced mutants will be applied on transgenic plants that contain the *Adh* gene under transcriptional control of the seed-specific promoter. Since *Adh* activity is lethal to seeds germinated in the presence of allyl alcohol, mutants should be readily obtained. Mutants will be further characterized and the wild-type gene isolated by chromosome walking and complementation.

109. Using Transposable Elements as Genetic Tags in *Arabidopsis*

**Kevin G. Mossie**

We have taken two approaches to determine if it is possible to use transposable elements as genetic tags in *Arabidopsis*.

The first approach involves the direct screen of genomes from different *Arabidopsis* races for the presence of putative endogenous transposable elements. Toward this goal, two different approaches have been used: 1) screening, isolation and characterization of dispersed middle repetitive DNA elements in
Arabidopsis and examination of these elements for structural properties characteristic of known transposable elements, including the presence of terminal repeats at the ends of these elements, target site duplications, and the presence of "empty" and "filled sites" and 2) screening the genomes of Arabidopsis races for retroviral-like sequences using mixed oligomers derived from sequences best conserved in reverse transcriptase genes of both retroviral and transposable element origin (Yuki et al., 1986).

The second approach involves the use of exogenous transposable elements in Arabidopsis and screening them for the ability to transpose in Arabidopsis. We have chosen as element of choice a characterized copia retrotransposon from Drosophila melanogaster (Mount and Rubin, 1986) for the reason that the element has been sequenced and appears to be structurally functional (Mount and Rubin, 1986), and that the mechanism of transposition of this class of elements has been determined (Boeke et al., 1985). A selection scheme to determine the transposition of this element in Arabidopsis has been devised that takes advantage of the observation that newly-transposed elements lose exogenously-introduced intron fragments (Boeke et al., 1985). Briefly, a selectable marker encoding the hygromycin phosphotransferase (hph) gene will be introduced within the copia element at an orientation opposite to that of the major copia transcript which serves as template for element-encoded reverse transcriptase enzyme. The hph will itself be interrupted by an intron fragment in the same orientation as the copia transcript, but opposite to the hph transcript. In this orientation, the intron can only be spliced out of the copia transcript, and the selection scheme employed therefore demands that a cDNA copy of this spliced transcript re-integrate into the genome to produce a functional hph gene which can be selected for. The transposition of the exogenous copia element in Arabidopsis should therefore correspond to the activation of the hph marker.

References:

110. In Situ Hybridization in Arabidopsis thaliana
John L. Bowman, Elliot M. Meyerowitz
A valuable technique to help elucidate spatial and temporal gene expression is in situ hybridization to RNA. To optimize the conditions, a series of experiments was performed using a 12S seed storage protein gene. The spatial and temporal expression of this gene is known and thus it provided a reasonable experimental system.

Tissues were fixed in a buffered glutaraldehyde solution and probed with 3H-labeled RNA. The probes were synthesized from plasmids containing SP6 promoters placed in front of the cloned 12S seed storage protein gene in either orientation. Thus, both the anti-sense and sense strands were synthesized with the sense strand, providing a convenient control for non-specific hybridization. Good signal-to-noise ratios were obtained with exposure times of less than a week for this abundant transcript. In situ hybridizations of a less prevalent transcript, the Adh gene, are in progress.

Abundant mRNA for the 12S seed storage protein was found to be localized in the embryo; neither the seed pod nor seed coat was found to contain the mRNA. Thus, in situ hybridization confirmed the result that the 12S seed storage protein gene is expressed exclusively in the embryo and provided a more detailed picture of its localization within the embryo itself as the procambium did not appear to express the gene.

It is hoped that this procedure will provide critical information on the spatial and temporal expression of genes involved in floral morphogenesis.

PUBLICATIONS


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**Summary:** T-cell effector function presents a number of fascinating developmental questions. T cells are functionally specialized into helper cells, which secrete lymphokine hormones in response to antigen stimulation, and killer cells, which bind and directly lyse target cells. Both helper and killer T cells utilize the same pool of T-cell receptor gene segments and develop from common precursors in the thymus. It is completely unknown when their developmental pathways diverge, or under what microenvironmental influences. Also, unlike many other differentiated cells, mature T cells retain the ability to proliferate, but under very tight regulation. Their characteristic responses to particular growth signals play an important role in their function as immune effectors. During their differentiation, on the other hand, immature T-lineage cells in the thymus go through multiple rounds of accelerated proliferation in response to a different set of signals. This explosive growth process culminates in the maturation of a few percent of the progeny and the death of all the rest.

Our laboratory is interested in several aspects of T-cell development: in the molecular basis of the determination of T cells to become helpers or killers; in the relationship between growth control signals for immature versus mature T cells; and in the commitment of pre-T cells to mature or to die.

We discovered recently that the ability to secrete the T-cell growth factor interleukin-2 (IL2), previously thought to be restricted to mature helper T cells, could already be found in the immature thymocyte population provided that the cells were stimulated appropriately. IL2 secretion is controlled at the transcriptional level, and we found that a select fraction of the immature cells can accumulate IL2 transcripts upon stimulation. This expression is clearly distinct from that in mature cells since the peak number of transcripts in immature cells is severely limited relative to that in mature helper cells. We are now trying to define the precise maturation status of the earliest inducible cells. In parallel, we are attempting to develop a system to assay the molecular basis of IL2 inducibility in lymphoid cells of different maturation states, using transient expression following gene transfer.

We are also interested in determining whether the earliest IL2-inducible cells have precursor activity, and if so, how many functional classes they can give rise to. We do not yet know whether inducibility itself, high-level inducibility, or some other cell property is the sign of irreversible commitment to a helper cell fate. For example, immature IL2-inducible cells may be equally inducible for response products associated with totally different functions, and commitment may be a loss of bipotentiality. Thus, we
are beginning to study the expression of serine esterase genes which are reported to be killer cell specific.

Finally, a lead on the timing of commitment to maturation versus death has recently come from our studies of growth control mechanisms in the thymus. We have examined subpopulations of cells that can be distinguished by their expression of various phenotypic markers into a "mature" class, a "precursor" class, and a third, enigmatic but numerous class of "nonmature" cells that fills the thymic cortex. While there is general agreement that most cortical thymocytes die, the developmental status of these cells has remained controversial. The question is whether they are by-products of differentiation which are already committed to die, or bona fide precursors about to undergo an unusually hazardous maturation event. Since regulated IL2 receptor expression is universally associated with mature T-cell immune competence, we have assayed different populations of thymocytes for their ability to make this response. We have found that expression of receptors for IL2 is inducible in mature and early immature thymocytes alike, but not in the major cortical population of thymocytes. This seems to indicate that the cortical cells have suffered a loss of function with respect to their own precursors. Such an event might be expected to occur at a developmental branch point where these cortical cells left the maturation pathway. We are therefore anxious to define precisely the stage at which this putative loss of function is first detectable, especially with respect to proliferation and T-cell receptor surface expression.

111. IL2 mRNA Accumulation by Subpopulations of Splenic T Cells

Kathleen L. McGuire, Ellen Rothenberg

We have been studying the regulation of interleukin 2 (IL2) gene expression in normal mouse T cells as a molecular criterion of one type of functional competence. Briefly, using the techniques of quantitative RNase probe protection analysis and in situ hybridization, we have attempted to estimate the amount of mRNA for the T-cell growth hormone, IL2, accumulated by individual cells in various populations of T cells. In previous work, we found that splenic T cells accumulate high levels of IL2 RNA (i.e., greater than 150-200 copies per positive cell) at 24 hours of stimulation with the calcium ionophore, A23187, and the phorbol ester, 12-0-tetradecanoylphorbol-13-acetate (TPA). We were interested in determining the phenotype of the cells responsible for the IL2 production seen in the spleen. We used a monoclonal antibody (MAb), which specifically recognizes the CD8 and CD4 cell-surface glycoproteins, to mediate complement (C') elimination of the two classes of splenic T cells bearing one or the other marker. Such treatments result in pools of splenic cells specifically depleted of CD8+ or CD4+ T cells. CD4+ cells are usually helper T cells, including most physiological IL2 producers, and CD8+ cells include killers that do not usually produce IL2 in response to antigen. Following stimulation with the ionophore and phorbol ester, in situ hybridization and quantitative RNase probe protection analysis suggested that CD4+ T cells accumulated -500 copies per average producer, but CD8+ T cells also accumulated IL2 mRNA to an average of 370 copies per positive cell. C' elimination with both Abs abrogated IL2 mRNA accumulation, confirming that only T cells were activating the IL2 gene under these conditions. FACS analysis using fluorescent staining with an Ab specific for T cells suggested that about 90% of all T cells can activate the IL2 gene when stimulated with A23187 and TPA. We can conclude from these studies, then, that both CD4+ and CD8+ cells are contributing to the IL2 production seen. However, while Ab and C' elimination of CD4+ cells decreases total IL2 mRNA accumulation by ~70% under these stimulation conditions, this treatment decreases IL2 mRNA by >90% when the cells are stimulated with Con A and TPA. These results suggest that there is a population of CD8+ cells that have the ability in principle to accumulate IL2 mRNA but do not do so when triggered through their cell-surface receptor for Ag by Con A. Therefore, it would appear that A23187 and TPA may give a qualitatively different type of activating signal from that of receptor ligands, such as Con A and Ag. Further studies are required to define the basis for the differences in IL2 mRNA accumulation between these two activation regimes.
112. Activation of the IL2 Gene by Single Positive Thymocytes

**Kathleen L. McGuire, Ellen Rothenberg**

We had previously shown that approximately 15% of total thymocytes accumulate IL2 mRNA when stimulated for 24 hours with calcium ionophore and TPA. However, only 2-3% of total thymus accumulated the high levels of IL2 mRNA characteristic of splenic T cells. The rest of the producing cells in the thymus (~13% of total) transcribed the IL2 gene in response to stimulation but could only accumulate very low levels of IL2 mRNA, to an average of 10-20 copies per positive cell. This low-producing subset was found to include approximately 15% of the immature, precursor population of thymocytes known as the double negative cells (CD4⁻, 8⁻) but was not limited to cells of this phenotype. To determine the contribution by cells of the "single positive" (CD4⁺ or CD8⁺) mature phenotypes to IL2 production in the thymus, we used monoclonal Abs specific for the CD4 (L3T4) and CD8 (Lyt2) cell surface markers to isolate complement elimination of specific populations. C' elimination with anti-CD4 results in a population of thymocytes that is ~50% CD8⁺ and 50% double negative. The remaining cells are 100% viable after stimulation, as are the double negative cells when cultured alone, and 15% of the total population accumulates IL2 RNA to average levels of 130 copies per positive cell. From these data we calculate that 15% of CD8⁺ cells in the thymus can activate the IL2 gene and that many of these cells accumulate high levels of IL2 mRNA per positive cell—about 250 copies/cell when the double negative cell contribution is subtracted out. The majority of the cells of this phenotype do not, however, produce detectable IL2 RNA in this assay system.

When a monoclonal Ab specific for CD8 is used to mediate complement elimination, the cells isolated are initially ~50% CD4⁻ (CD8⁻) and 50% double negative (CD4⁻, 8⁻). Unlike CD4⁻-eliminated cells, this population as a whole is only 70% viable after stimulation with calcium ionophore and TPA, suggesting that 50% of the CD4⁺ cells in the sample die in culture overnight. When in situ hybridization analysis is used to determine the number of IL2-produced cells after stimulation, 44% of the remaining population is found to be positive for accumulated IL2 RNA. Taking into account the behavior of the double negative cells, this implies that 90-100% of surviving CD4⁺ cells activate the IL2 gene. RNase probe protection analysis reveals that these cells accumulate an average of 125 copies/positive cell. This is the same average value as for the double negative and CD8 single positive population described above, but is surprising in this case because here the double negative cells represent 20%, not 50%, of the IL2 producers. Thus, at least 50% of the CD4⁺ cells seem to accumulate only the low levels of IL2 mRNA (10-20 copies/cell) characteristic of the low-producing cells in total thymus (see above) and not the high levels expected for mature CD4⁺ cells. This suggests that all CD4⁺ cells are not equivalent and may imply a maturation heterogeneity in this population previously undescribed.

113. Separation of Mature and Nonmature IL2-producing Cells Using the Lectin PNA

**Kathleen L. McGuire, Ellen Rothenberg**

The lectin peanut agglutinin (PNA) preferentially binds to "nonmature" cortical thymocytes and not to mature medullary thymocytes. Separation of thymocytes by panning on PNA-coated petri dishes, therefore, results in two populations of cells. The PNA⁺ population is enriched in cells with the CD4⁺CD8⁻ phenotype typical of cortical cells (~90% of the total), although some CD4⁺ (6%) and CD8⁺ and double negative thymocytes (5%) consistently fractionate as PNA binding cells. The PNA⁻ or medullary population is significantly enriched in single positive (40%) and double negative (20%) cells. Fractionation of thymocytes in this way allowed for an 80% recovery of viable cells with 16% of the total in the PNA⁻ fraction. 36% of surviving PNA⁻ cells accumulated IL2 RNA after stimulation to an average of 250 copies per positive cell. After correction for cell death, this is equivalent to 2% of the total thymus input, which we previously estimated as the percent being mature IL2-producing cells (see previous abstract).

The PNA⁺ population was found to be 20% positive for IL2 mRNA after stimulation. In contrast to the PNA⁻ cells, however, these cells accumulated an average of only 20 copies per positive cell. These data allow for two conclusions. The first is that the IL2-producing cells are not mature contaminants. If they were, the producing cells would be less numerous but accumulate the much higher levels of IL2 mRNA per
positive cell that are characteristic of the PNA\textsuperscript{−} population. Second, we can conclude that the low-producing population in the thymus (see previous abstract) can be defined by the PNA\textsuperscript{+} subset. The number of positive cells is approximately twofold higher than can be accounted for by the single positive and double negative cells in the PNA\textsuperscript{+} population. This confirms that at least some of the double positive cells in the thymus must contribute to the IL2-producing subset of the thymus. Additionally, our calculations suggest that about 10\% of the double positive cells in the thymus activate the IL2 gene and that these cells account for about 60\% of the IL2-producing cells in the thymus.

We can conclude from these studies, then, that the small percentage of thymocytes that accumulate high levels of IL2 mRNA bear the single positive cell-surface phenotype characteristic of mature T cells. The majority of the IL2-inducible thymocytes, however, accumulate low levels of IL2 RNA and include cells of the double positive, CD4 single positive, and double negative phenotypes. The demonstration of IL2 gene activation in the immature double positive cells proves that not all of these cells are functionally inert. At least some of the cells can respond to these stimulation conditions. Whether this response is indicative of a maturation pathway which will result in mature T cells or merely of a remnant activity which will soon be lost remains to be determined.

114. Induction of Interleukin-2 Gene Expression by Various Modes of Activation

Julia A. Yang, Ellen Rothenberg

We are interested in understanding how interleukin-2 (IL2) gene expression is regulated in T lymphocytes. Expression of IL2 mRNA is restricted to T lymphocytes and is dependent upon activation, i.e., resting T lymphocytes do not express IL2 mRNA. Antigen-specific, MHC-restricted recognition by mature T lymphocytes involves interaction of foreign antigen in conjunction with MHC determinants on the surface of an antigen-presenting cell and the T-cell receptor (TCR). The TCR is composed of two polymorphic, integral membrane glycoproteins (\(\alpha\) and \(\beta\), or \(\gamma\) and \(\delta\)) which are associated with a complex of invariant CD3 (or T3) polypeptides, \(\gamma\), \(\delta\), \(\epsilon\), \(\zeta\) and \(\pi 1\delta\), on the cell surface. The CD3 complex is believed to be involved in transmembrane signal transduction.

Our lab has recently reported that different populations of T lymphocytes are able to express IL2 mRNA when stimulated using the phorbol ester, TPA and calcium ionophore, A23187 (McGuire and Rothenberg, 1987) and induction of IL2 gene expression in different T lymphocyte populations is heterogeneous. We are currently using antibody directed against the \(\epsilon\) subunit of the CD3 complex, in the presence and absence of TPA, to activate T lymphocytes and induce IL2 gene expression. Activation using antibodies directed against the CD3 complex is expected to mimic in vivo activation more closely than using TPA and calcium ionophore. We are comparing induction of IL2 gene expression using anti-CD3 antibody with that using TPA and calcium ionophore, and with that using the lectin Concavalin A in the presence of TPA, in different T lymphocyte populations. Further studies with the addition of the lymphokine IL1 will establish whether certain populations of cells require additional signals. These studies will help us understand which populations of T lymphocytes are able to respond like "mature" T lymphocytes by expressing IL2 mRNA upon activation via the T-cell receptor.

We are also examining the ability of different sub-populations of double negative thymocytes to express IL2 mRNA in response to various modes of stimulation. Expression of IL2 mRNA and production of IL2 protein can be regarded as a developmental "marker" for thymocytes with the capacity to develop into mature helper T cells. It is known that the double negative population is phenotypically heterogeneous. Some of the cells express cell-surface T-cell receptor-CD3 complexes and may represent mature cells of a minority T-cell lineage. We are now investigating whether IL2 inducibility in the double negative population is confined to these cells or whether it includes cells with verifiable precursor function.

Reference:

115. Differential Regulation of T-cell Receptor/T3 Genes in Immature Thymocytes

Christine Kinnon, Kathleen L. McGuire, Ellen Rothenberg

Immature thymocytes that lack both Lyt-2 (CD8) and L3T4 (CD4) expression can respond rapidly to
stimulation with phorbol ester and calcium ionophore by expressing some gene products characteristic of mature, activated T cells. We studied the effect of such stimulation on the number of copies per cell of RNAs that encode components of the T-cell receptor/T3 complex. Although upon stimulation, mRNAs for T-cell receptor \( \beta \)-chain and T3-6 accumulated to higher levels, the cells did not express \( \alpha \)-chain transcripts from rearranged or germline genes. The lack of \( \alpha \)-chain mRNA expression shows that cells can make certain responses to stimulation without first progressing to a conventionally mature state. Transcripts from the Cy1 (13.4) and Cy2 (10.5) genes were differentially regulated. The rarer Cy1 transcripts were strongly induced, while the initially abundant Cy2 transcripts showed a modest decrease in transcripts per cell within 24 hr. These results define a repertoire of highly selective biosynthetic responses to stimulation that characterize the developmental state(s) of the double negative thymocyte population.

116. Analysis of the Mouse Interleukin-2 Gene Promoter

Thomas J. Novak, Ellen Rothenberg

We have constructed a number of recombinant plasmids in which the bacterial chloramphenicol acetyltransferase (CAT) gene is under the control of the mouse IL2 gene promoter. Each construct contains the IL2 proximal promoter and mRNA cap site but only differ in the amount of IL2 upstream sequence they possess. Relative to the start site of transcription, the four constructs contain either 0.1, 0.32, 1.8 or 2.8 kb of 5' flanking DNA. These plasmids, along with pRSV-CAT as a positive control, were introduced into a variety of hematopoietic and non-hematopoietic cell lines by protoplast fusion. Transient CAT activity was measured and the number of plasmid copies per cell was determined two days later. In most instances, transfected cells were stimulated for the last 12 hours of culture with phorbol ester (TPA) and \( \text{Ca}^{2+} \) ionophore (A23187) and compared with identically transfected cells that were not stimulated. Treatment with TPA/A23187 can induce transcription of the endogenous IL2 gene in helper T cells, as described in the previous abstracts.

Mouse L-cell fibroblasts do not express IL2-driven CAT activity either constitutively or inducibly when transfected by protoplast fusion or calcium phosphate coprecipitation, despite the presence of many plasmid copies per cell (1-20 copies/cell by protoplast fusion; >150 copies/cell by calcium phosphate).

All four constructs are expressed constitutively in mouse and human T-cell lines with the human helper T-cell line JURKAT yielding the highest activity per gene copy. Two mouse helper T-cell lines, EL4.E1 and BW5147, and a mouse cytotoxic T-cell line, MTL2.8.2, express comparable but lower amounts of CAT activity two days posttransfection. Treatment with TPA/A23187 in the 12 hours preceding the assay generally results in only a five- to tenfold increase in amount of CAT expressed per gene. However, as expression of pRSV-CAT is also increased by the same fraction, this induction does not appear to be IL2 gene specific but more likely due to general cellular activation.

The constructs containing 0.1 and 0.32 kb of IL2 upstream sequence are also expressed in the B-cell line NS-1; this activity is comparable to that seen in T cells. However, constructs containing 1.8 and 2.8 kb of upstream DNA are not able to direct CAT synthesis in this B-cell line. Qualitatively similar results are found for the macrophage line P388D1 and the IL3-dependent pre-mast cell line 32D.

These results are reproducible but do not fully correlate with data derived from the human IL2 gene, which shares 85% homology in the nucleotide level with the mouse IL2 gene up to position -500 (relative to cap site). Our experiments with the shorter constructs show less cell-type specificity of expression than would be predicted from the more limited studies done in other labs. In most of the cell lines we have examined, moreover, specific inducibility is poor. The relatively high expression we obtain with our smaller constructs, even those missing putative enhancer sequences, may be due to promiscuous transcript initiation in the vector sequences upstream. We are currently analyzing the 5' terminus of CAT mRNA by probe protection to be certain that it is all authentically initiated at the IL2 start site. On the other hand, it is striking that we see no difference between the abilities of helper and killer T-cell lines to express any of the constructs.

We have begun the process of isolating stable transformants by including dominant selectable markers on our plasmids. Perhaps more rigorous cell-
type specificity will be found when the IL2-CAT gene is integrated into chromatin rather than present as a supercoiled, extrachromosomal plasmid. Finally, we are actively exploring the possibility that cell-type specificity and/or inducibility are controlled by intragenic or downstream sequences.

117. Constitutive Coexpression of IL2 and IL2 Receptor Genes in a Murine Cytotoxic T-cell Line

Thomas J. Novak, Ellen Rothenberg

In its simplest form, antigen-driven T-cell proliferation requires the production of T-cell growth factor (IL2) by helper T cells and the interaction of this newly synthesized IL2 with IL2 receptors (IL2R) expressed on antigen-activated helper and/or cytotoxic T cells. Although expression of IL2 and IL2R gene products in response to antigen is transient, it is relatively easy to isolate cell lines that constitutively express IL2 receptors on their surface. These cell lines absolutely require exogenously-added IL2 to proliferate in culture, and when deprived of it they rapidly die. To assess the possibility that the genes encoding IL2 and IL2R can function as co-oncogenes, we have transfected a cDNA encoding murine IL2 under the control of the Rous sarcoma virus LTR (RSV LTR) into an IL2-dependent, cytotoxic T-cell line, MTL2.2.2, which expresses IL2R constitutively. The recombinant plasmid used, pRSV-IL2 (Neo), carried as a selectable marker the neomycin phosphotransferase gene from Tn5 under the control of the SV40 early promoter, which allowed selection of stable transformants by growth in the presence of the cytotoxic drug, G418. Five clones were isolated and their DNA examined by DNA gel blot analysis for the presence of an intact RSV IL2 gene; four were clearly positive while the fifth appeared negative. None of the clones grows in the absence of exogenously-added IL2. However, at least four of the five constitutively expressed IL2 mRNA, as determined by cytoplasmic RNA dot blot analysis. The steady state level of IL2 mRNA in three of the clones was only about three times lower than that seen in an equal number of induced EL4-E1 cells, a thymoma programmed to make IL2. The two remaining clones expressed much lower though easily detectable levels of IL2 mRNA. RNA gel blots and RNA probe protection assays are now being used to determine the length and start site, respectively, of this IL2 mRNA. We also have an IL2-specific monoclonal antibody from Dr. Tim Mosmann (DNAX Corp.) that should allow us to determine unambiguously if any of these transformants are synthesizing IL2 protein.

118. Serine Esterase Inducibility as a Lineage Marker of Thymocytes

Russell J. Hill, Ellen Rothenberg

We are currently comparing the ability of thymocyte subpopulations to produce serine esterase mRNA in response to TPA and A21387 with our previous data on the inducibility of the IL2 gene. These serine esterase genes are believed to help mediate cytotoxicity because they are present in cDNA libraries made from cytotoxic cell lines (CTLs) but are absent from helper T cell (Th) libraries (Gershenfeld and Weissman, 1986; Lobe et al., 1986). They also have homology to proteins that function in the complement lytic cascade. Since these genes have putatively lineage-specific expression, with IL2 and other lymphokines being produced by mature Th cells, whereas serine esterases are made by mature CTLs, the inducibility of these genes in an immature thymocyte may serve as a marker for its developmental potential.

We wish to address the following issues. First, we want to know how exclusive mature CTL and Th cells are in their expression of these function-related genes. For example, are only those cells that will later exhibit cytotoxicity capable of transcribing serine esterase genes, or are other cells in principle capable of transcribing these genes, though they do not exhibit a cytotoxic phenotype because of other differences? Secondly, if the expression of serine esterase genes and IL2 is mutually exclusive to any degree in mature T cells, how far back in T-cell development does this exclusion exist? Is there a common progenitor thymocyte inducible for both IL2 and serine esterase genes, or is there lineage exclusion as early in development as when these genes first become inducible? Finally, because CTL activation is a slow, multistep process in which serine esterase mRNA does not accumulate until two to three days after activation, we want to determine the requirements for initiating serine esterase transcription. Is the delay in transcription an intrinsic
property of the cascade of regulatory proteins controlling gene activation in CTL? Alternatively, is the gene competent to be transcribed earlier but awaiting an additional signal, which is normally made some time after stimulation? We are testing the latter possibility by adding lymphokines at the beginning of stimulation to look for accelerated serine esterase induction.

References:

119. Retroviral Perturbation of Thymocyte Subpopulations
Russell J. Hill, Ellen Rothenberg

Since the purification of thymocyte subpopulations by such methods as complement elimination, panning and sorting by FACS is often a laborious procedure, which is also limiting in the number of thymocytes that can be prepared, we have sought to produce cell lines that can be used as models for different thymocyte subpopulations.

It has been well demonstrated that different retroviruses and different oncogenes have tropisms for cells of specific types and developmental stages, such that they are able to immortalize particular cell types selectively. We have therefore started injecting various retroviral constructs into mice intrathymically in an attempt to induce neoplasms of different developmental stages of T cells. Although so far no permanent transformation has been observed, we have detected an interesting phenomenon. The viruses induce perturbations in the normal ratios of thymocyte subpopulations as detected by two color FACS analysis for CD4 and CD8. This perturbation passes through the thymus as a wave, first detectable at 3 to 4 weeks after infection, that peaks at around 5 or 6 weeks after infection and then subsides so that it is no longer detectable at 7 to 8 weeks. One viral construct containing the abl oncogene caused a temporary increase in double negative and CD8 single positive thymocytes, whereas a ras virus construct caused an increase in double negative and CD4 single positive thymocytes. We believe that these viruses may be tropic for different thymocyte lineages and cause increased proliferative ability in their target cell, although they appear to be incapable of transforming any cell type stably under the infection conditions used. Further work using higher titer virus stocks will be necessary both to demonstrate the reproducibility of these observations and also to obtain cell lines.

120. A Specific Block in Thymocyte IL2R Inducibility
Paul D. Boyer, Ellen Rothenberg

Activation of mature T cells results in the induction of surface receptors for the T-cell growth factor, IL2. Since T-cell development takes place in the thymus, we were interested in the association between IL2R inducibility and thymocyte maturation. Thymocytes were fractionated using several techniques and IL2R inducibility was assayed in these fractions by immunofluorescent staining of cells that either had or had not been stimulated for 20 hours with the phorbol ester TPA and the calcium ionophore A23187. Although not physiological, these conditions permit the examination of gene expression potential in cells that may not yet be capable of physiological activation through the T-cell receptor.

Unlike mature T cells, which are all inducible for IL2R expression, fewer than 25% of all thymocytes can be induced to express IL2 receptors. Stimulation of mature or medullary-type thymocytes (15% of total) and immature or cortical-type thymocytes (85% of total) separated with the lectin peanut agglutinin results in IL2R induction in 75% and only 14% of the cells, respectively. Similarly, fewer than 15% of cortical-type cells isolated by anti-H-2K elimination are inducible for IL2R expression. Clearly, the bulk of thymocytes are incapable of this typical mature T-cell response. This lack of inducibility results from a specific molecular block in the majority of cortical cells and not from a complete inability to respond to stimulation, since IL2R non-inducible cells do respond to stimulation by increasing their H-2Kb surface expression.

We have also shown that at least 90% of CD4-CD8- thymocytes, a population that includes precursors of all other thymocytes, are able to express IL2 receptors. The developmental significance of the IL2R inducibility in the vast majority of these most-immature thymocytes, in mature, medullary-type thymocytes, and in all mature T cells emphasizes the specific block in IL2R inducibility found in most
cortical-type thymocytes and suggests a linkage with their imminent intrathymic death.

121. Molecular Analysis of the Specific Block in IL2R Inducibility in Most Thymocytes

Paul D. Boyer, Ellen Rothenberg

We have shown that approximately 75% of stimulated thymocytes are specifically blocked in their ability to induce surface IL2R expression. The vast majority of these cells are small, cortical-type thymocytes that have a 95-99% probability of dying in situ. Understanding the mechanism responsible for this block may provide insight into developmental processes occurring in the thymus, perhaps even into the little understood process of programmed cell death.

Our approach to analyze the molecular basis for this block is to ask in what regards cortical-type thymocytes, of which 85-90% demonstrate this block, differ significantly from medullary-type thymocytes, of which less than 25% demonstrate this block. Although surface H-2Kb expression is not blocked in IL2R non-inducible cells, this particular expression could result from posttranslational modifications and not de novo protein synthesis. We therefore compared the accumulation of metabolically-labeled proteins in these populations with or without stimulation with the phorbol ester, TPA, and the calcium ionophore, A23187. There is a three- to fourfold increase in the rate of protein synthesis per viable cell over 18 hours of stimulation in medullary-type cells, while there is no demonstrable increase at all in cortical-type cells. There is evidence, however, that even this stable level of protein synthesis in cortical-type thymocytes is in fact a low response to stimulation, since these cells show a decrease in protein synthesis when cultured in the absence of stimulation. We are investigating this difference in protein synthesis further by immune precipitation of IL2Rs from these lysates.

To determine whether the lack of increase in protein synthesis was due to reduced RNA accumulation, total cellular RNA was isolated from these populations cultured in the presence or absence of TPA and A23187. Medullary-type thymocytes accumulate tenfold more RNA per viable cell than cortical-type thymocytes. In addition, cortical-type cells accumulate only fivefold more RNA with stimulation than without, while in medullary-type cells this difference is tenfold. Hybridization of an IL2R cDNA probe to the isolated RNA demonstrates 80- to 90-fold more IL2R RNA in medullary-type cells than in cortical-type cells. Taking into account differences in accumulated RNA and in the amount of surface IL2R expressed in the two populations, it is likely that cells that are blocked in their ability to induce surface IL2R2 are also blocked from accumulating IL2R RNA. We are now investigating the molecular basis for the block in IL2R RNA accumulation in cortical-type thymocytes.

PUBLICATIONS

Boyer, P. D. and Rothenberg, E. V. (1987) Interleukin-2 receptor inducibility is specifically blocked in nonmature thymocytes. Submitted for publication.


Summary: The work in our laboratory is focused on two basic questions. (1) What are the mechanisms that cells use to measure changes in concentration of specific chemicals, e.g., neurotransmitters, hormones or growth factors, and how are these "signals" converted (transduced) into changes in intracellular metabolism? (2) How do cells initiate and maintain specific heritable patterns of gene expression? We have been exploring these questions in a variety of biological systems. In prokaryotic organisms, our attention has been concentrated on flagellar phase variation and on bacterial chemotaxis.

Phase variation results from a gene rearrangement that switches the expression of flagellar genes in a heritable fashion. Thus, starting with a homogeneous population of cells, the rearrangement leads to clonal dimorphism. Our efforts over the past three years have provided a clear picture of the requirements for gene rearrangement. We demonstrated the requirement for three proteins, a recombinase, an enhancer binding protein and the DNA binding protein HU. In the past year we have begun a detailed analysis of the mechanism involved in recombinase and enhancer protein binding. In a collaborative program we have generated a peptide fragment of Hinc recombinase which can act as a specific DNA cleaving reagent and we are trying to modify its specificity.

Bacterial chemotaxis provides an important model system for studying signal transduction. Bacteria show complex behavioral responses to a variety of ligands. There are clear analogies between the structure of the receptors and the properties of the signal integration and transduction systems in E. coli and in a variety of other cells. A relatively complete genetic and biochemical picture of the components of the chemotaxis system is available. We have been able to purify most of the soluble proteins required for signal formation and we have prepared dominant mutants in the C-terminal portion of the transmembrane receptor. Soluble fragments of the receptor mutants are still capable of signaling in intact cells. In the past year we have had considerable success in reconstituting bacterial chemotaxis in vitro. This provides an opportunity to compare the biochemical properties of the system with the behavior of the organism.

In eukaryotic systems we have been studying signal transduction mediated by G proteins. These complexes of three polypeptides are essential in coupling changes at a variety of cell surface receptors to the activation or inactivation of systems that generate intracellular "second messengers." Our efforts have been directed at gaining a clear picture of the diversity of G-protein systems that exist in individual cells and that are specifically expressed in highly differentiated cells. We would like to understand the mechanisms that these systems use to integrate information and the parameters that allow them to be effective in specialized systems such as in the visual system. We are reconstituting the signaling systems by introducing genes corresponding to modified G proteins into cells in tissue culture and into mice to form transgenic animals. We are studying the distribution of specific G protein expression in complex tissues, in brain and in retina.

We have, for a number of years, explored the pattern of changes in gene expression in mammalian spermiogenesis. There is evidence that suggests that heritable changes in the pattern of gene expression in...
male alleles of specific genes are determined during gametogenesis and we believe that such changes may occur during meiosis. In order to understand the mechanisms involved, we must first have a better understanding of gene expression and of the molecular events involved in meiosis and gametogenesis. Our work this year has been directed at characterizing the pattern of mRNA expressed at different stages in spermatogenesis.

122. Interactions Between Fis and the Recombinational Enhancer of the 
Hin Inversion System

Anna C. Glasgow, Reid C. Johnson, Michael F. Bruist, Melvin I. Simon

Hin-mediated inversion of a 996 bp DNA segment controls expression of the flagellin genes in Salmonella typhimurium. We have shown previously that the recombinational enhancer of the Hin inversion system stimulates this site-specific recombination reaction in vitro 150-fold in the presence of the Escherichia coli host factor, Fis. To gain an understanding of the roles of the enhancer and Fis in stimulating the Hin-mediated inversion reaction, we have used nuclease and chemical protection/interference studies and gel retardation assays to examine the interactions between Fis and the recombinational enhancer. These studies combined with mutational analysis defined the enhancer sequences required for Fis binding and function. We have found that Fis binds with different affinities to two domains within the enhancer sequence; the apparent binding constants for the hirL-distant and -proximal domains are approximately 24 nM and 120 nM, respectively. Binding studies with the wild-type enhancer and with isolated proximal and distal domains demonstrated that the interaction of Fis with each domain is independent of binding to the other domain. The disposition of purine contacts made by Fis in each domain, determined by methylation interference/protection studies, indicates that Fis binding at each domain is primarily to one side of the DNA helix. Based on the position within the enhancer sequence of these purine contacts and of a conserved sequence between the two domains, CAPu..TCA.C, the two binding sites of Fis are 48 bp apart and therefore separated by approximately four and a half turns of the DNA helix. The importance of the relative disposition of the Fis binding sites on the DNA helix was examined by assaying the activity of mutant enhancers containing various sized insertions between the two enhancer domains. It was found that the correct helical positioning of the two binding sites on the DNA helix was critical for enhancer function, while the binding affinity of Fis for each domain was unaltered in these mutants. Therefore, it appears that the two Fis molecules must sit on opposite faces of the DNA to function in stimulating Hin-mediated recombination. These results support a model for the role of the recombinational enhancer in Hin-mediated inversion in which the interaction between the recombination sites bound by Hin and the Fis-enhancer complex results in the proper topology to stabilize the synaptic complex and allow strand exchange to occur.

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123. Binding Properties of Hin Recombinase to its hix Recombination Sites In Vivo

Kelly T. Hughes, Melvin I. Simon

Salmonella phase variation results from the specific alternating inversion of 995 base pairs of chromosomal DNA. When the invertible segment is in one orientation, the H1 flagella antigen is expressed. When this segment is inverted to the other orientation, the H2 flagella is expressed. This reversible switching event is mediated by the Hin recombinase acting on two 26-base-pair recombination sites, hixL and hixR. The switching occurs in the middle of these hix sites. We have undertaken a study to characterize the direct DNA-protein interactions of Hin with the hix sites in vivo. We used the bacteriophage P22 challenge phage system developed in Phil Youderian's lab for this study. In this system, the Hin recombinase is treated as a repressor protein binding to hix operator sites. The P22 ant gene when overexpressed locks the phage into a lytic growth cycle. When the normal operator for the ant promoter is replaced by a hix sequence, the normal repressor of P ant does not recognize the hix operator and does not bind. Consequently, the ant gene is overexpressed and the phage cannot lysogenize. Frequency of lysogeny is measured by selecting for kanamycin resistance cloned into the challenge phage. When the cells being infected carry a low copy number plasmid that has Hin under the control of the tac-
promoter, the hixL and hixR challenge phages are able to lysogenize at frequencies dependent on the concentration of the Hin-inducing agent IPTG, which induces the tac-promoter and thereby results in Hin expression. The lysogenization frequency as a function of increasing levels of IPTG results in a curve consistent with Hin binding to hixL and hixR in a cooperative manner. When hix 14-base-pair half-sites are used as operators, only one can bind Hin enough to allow lysogeny. However, the frequency for lysogeny using the half-site is very low compared to the full-site and shows a binding curve consistent with Hin binding the half-site in a noncooperative manner. When a single base pair is introduced into the middle of the hixL operator, binding is still cooperative, but the overall frequency of lysogenization is down tenfold. When four bases are inserted in the middle of hixL, binding is eliminated; the frequency of lysogenization is the same as a poor half-site.

By using the challenge phage system, we have been able to show direct binding of Hin recombinase to hix recombination sites in vivo. Hin binds to hixL and hixR acting as a repressor protein for the ant gene of P22. The binding of Hin to hixR is cooperative. This cooperative binding is not observed for hix half-sites or when two hix half-sites are rotated to opposite sides of the DNA helix by inserting four base pairs in between.

124. Isolation and Characterization of hix Recombination-site Mutants Defective in Binding Hin Recombinase

Kelly T. Hughes, Carol C. Lee, Melvin I. Simon

We have successfully used the bacteriophage P22 challenge phage system developed by Phil Youderian's lab to study Hin recombinase binding to the hix recombination sites. In this system, Hin acts as a repressor binding to hix sites, which have been cloned into the operon sites of the P22 ant gene promoter. When Hin is present, the ant gene is repressed and the phage is temperate in its growth on Salmonella. When Hin is absent, the ant gene product, Antirepressor, is overexpressed and the phage is locked into a lytic growth cycle. Antirepressor binds the P22 C-2 repressor (equivalent to the CI repressor of bacteriophage λ) and inactivates it. This results in the inability to lysogenize.

We have used this system to select for hix operator mutants that can no longer bind Hin. A cell that carries a P22 C-2 ant lysogen is not immune to infection by a hix challenge phage and inhibits the C-2 repressor expressed by itself and the lysogen. When a cell carries both a P22 C-2 ant lysogen and a plasmid expressing Hin, it is immune to infection by the hix challenge phages. This is because Hin binds to the hix operator preventing ant gene expression and the C-2 repressor from the lysogen represses the P_L and P_R promoters on the challenge phage preventing growth. At a low frequency (10⁻⁵), plaques arise that are able to express the ant gene. Essentially all of these phage-producing plaques on a C-2 ant + Hin + host are mutated at the hix site (operator constitutives) which bind Hin at reduced levels or not at all.

We have sequenced hundreds of hix mutants defective in binding Hin. Among the mutants sequenced are plus and minus frameshifts of one or more bases within the central 16 base pairs of the hix sites. More importantly, we have identified seven single bases when substituted by other bases greatly reduce binding to Hin. The bases are identical to those shown by in vitro methylation-inhibition studies to be essential for Hin binding (see Abstract No. 122). We conclude that we have identified DNA bases which interact directly with Hin. We are currently isolating Hin suppressors which bind the mutant sites and identify which amino acids in Hin are directly interacting with specific bases in the hix sites.

125. Biochemical Characterization of the Cytoplasmic, C-terminal Domain of the Tar Protein

Nachum Kaplan, Melvin I. Simon

Tar is one of the four chemotaxis receptor proteins of E. coli. Previous studies from this and other laboratories have indicated that these proteins can be divided into several functional domains: 1) a periplasmic N-terminal region responsible for ligand binding, 2) two transmembrane regions, and 3) a cytoplasmic C-terminal region possibly involved in signal transmission and adaptation. In order to further study the functional properties of the C-terminal fragment, it was cloned into a multiple copy plasmid under the control of a tac promoter. In addition, two mutations that were made previously in the whole Tar protein
were separately introduced onto the C-terminal overproducing plasmids. When present on the whole protein, these mutations, termed 436V and 461L, resulted in a dominant tumbling only and smooth swimming only phenotype, respectively. Upon induction with IPTG, cells carrying the plasmids produced about 10% of total cellular protein as the fragments. A scheme utilizing ion-exchange and Cibacron Blue liquid chromatography was developed to purify the C-terminal fragments to homogeneity. Typical yields of pure fragment were 5 mg, 30 mg and 20 mg per liter of induced cell culture for the wild type, 436V and 461L mutants, respectively. The production of the fragments was followed by performing Western blots of whole cells using anti-Tar antibodies. It was found that the wild-type fragment was heavily degraded in vivo, whereas the 436V and 461L mutant fragments were degraded to a much lesser extent. This might explain the much lower yield of purified wild-type fragment (see above). In addition, only the 436V fragment but not the wild-type or 461L fragments was modified in vivo by the chemotaxis methylating and demethylating enzymes, CheR and CheB. Sizing experiments using a FPLC gel filtration column showed that in a physiological buffer, all pure fragments existed as a 100,000 dalton (Da) aggregate (monomer size = 32,000 Da). The 461L fragment showed an additional aggregation species of 240,000 Da that was in equilibrium with the 100,000 Da form. The 240,000 Da form was abolished in the presence of octyl glucoside. Under the latter condition, all three fragments existed as 135,000 Da aggregates. Taken together, all the above data indicate that there might exist conformational differences between the wild-type and mutant fragments that reflect their phenotypic differences. Finally, the three purified fragments are being examined by circular dichroism spectroscopy and are being crystallized in preparation for x-ray diffraction analysis (in collaboration with Dr. J. S. Remington, University of Oregon, Eugene, OR).

126. Functional Analysis of the Cytoplasmic Domain of the Receptor-Transducer Protein Tar
Kenji Oosawa, Norihiro Mutoh1, Melvin I. Simon

The four receptor-transducer proteins that are involved in chemotaxis in E. coli have been identified as transmembrane proteins. It has been proposed that they have four functional domains that are responsible for ligand-binding, transmembrane signaling, intracellular signaling, and adaptation. Because receptor-transducers are transmembrane proteins, reconstitution of the chemotaxis system and biochemical analysis was relatively difficult. The cytoplasmic portion of the receptor-transducer protein has been purified following digestion by endogenous protease. But this fragment did not show activity in vitro. To examine the activity of the cytoplasmic domain of the receptor-transducer protein in vivo, we attempted to construct a gene encoding only the carboxy-terminal fragment of Tar. This fragment started from methionine 43 amino acids downstream of the carboxy terminus of the second transmembrane region. The C-terminal fragment protein was identified in the cytoplasmic fraction by immunoblotting of SDS-polyacrylamide gels with anti-Tar antibody. Deamidation and methylation of wild-type fragment protein and of fragment with a variety of tar mutations were examined. Some mutant fragment proteins were deamidated and methylated, whereas wild-type and other mutant fragments were not modified. Also, effects on the C-terminal fragment (when it was the sole receptor-transducer molecule in the cell) of changes in cytoplasmic pH were examined. Cells with a mutant C-terminal fragment that was able to be modified showed a transient smooth swimming response to a decrease in cytoplasmic pH in the same manner as cells with the whole Tar protein. These results showed that carboxy-terminal fragment protein retained partial function of the cytoplasmic domain of the receptor-transducer protein.

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127. The Expression of G Protein Genes in Human Myeloid Cells
Thomas T. Amatruda, Melvin I. Simon

We plan to characterize the G protein genes that are expressed in human myeloid cells to determine the role of G proteins in growth, differentiation and function of these cells. The physiological functions of myeloid cells are modulated by a variety of membrane-acting stimuli, several of which act by means of G proteins. G proteins also may play a role in terminal differentiation of myeloid cells. This
phenomenon is induced in vitro by pharmacological agents such as phorbol esters and dibutylryl cAMP which interact with G protein-regulated systems.

We constructed a cDNA library with RNA extracted from HL-60 myeloid leukemia cells and are now screening this library with oligonucleotide and cDNA probes for G protein subunit genes. Probing with the cDNA for the β subunit of bovine transducin, we isolated HL-60 cDNAs which encoded two distinct β subunits. One of these is identical in predicted amino acid sequence with bovine transducin, while the other is a novel β subunit (β2). In collaboration with B. Birren and H. Fong, we determined that the β2 subunits expressed in bovine and human tissues are identical in predicted amino acid sequence, and that both β subunit genes are expressed in a number of bovine tissues and human cell lines. We are now working with N. Gautam to express the β2 subunit in bacterial and cell-free translation systems.

We are examining the pattern of expression of the genes encoding α and β subunits of G proteins in HL-60 cells induced to differentiate to granulocytes by exposure to DMSO or retinoic acid. As probes for the α subunits, we are using Ga cDNAs isolated from rat olfactory epithelium and probing under conditions of reduced stringency. These studies will be extended to other myeloid cell lines and to cells of other hematopoietic lineages which can be studied in vitro and in vivo.

128. Diverse Structures and Expression of βγ Subunits of Guanine Nucleotide Regulatory Proteins

Henry K. W. Fong, Thomas T. Amatruda, Bruce W. Birren, Melvin I. Simon

Retinal transducin and other well-characterized G proteins are composed of three subunits. We have identified and analyzed cDNA clones of the bovine transducin β subunit which may be highly conserved or identical to those in other G proteins. The encoded β subunit has a molecular weight of 37,375 and is comprised of repetitive homologous segments arranged in tandem. There is also significant homology in primary structure and segmental sequence between the β subunit and the yeast CDC4 gene product. The transducin β subunit polypeptide is encoded by a 2.9-kilobase (kb) mRNA. However, there exists in retina other β-related mRNAs that are divergent from the 2.9-kb mRNA on the basis of oligonucleotide and primer-extended probe hybridizations. All mammalian tissues and clonal cell lines that have been examined contain at least two β-related mRNAs, usually 1.8 and 2.9 kb in length.

These results indicated that there is a diversity of β subunit-related mRNAs that could encode different proteins. We have now isolated and analyzed homologous cDNA clones that encode a novel β subunit from bovine adrenal, bovine brain and a human myeloid leukemia cell line, HL-60. We refer to the transducin β subunit as β1 and the novel protein as the β2 subunit. The β1 and β2 proteins are about 90% identical, but there are divergent as well as conserved amino acid substitutions between them. The divergent substitutions do not occur in the residues of the identifiable repeat motif and are clustered to a small extent only in two regions, between amino acid residues 26 to 37 and residues 178 to 184. It also appears that divergent substitutions are found more often in the NH2-terminal half of the homologous repeats. The major mRNA that encodes the bovine β2 subunit is 1.7 kb in length. It is expressed at lower levels than β1 subunit mRNA in all tissues examined. Both the β1 and β2 messages are expressed in cloned human cell lines. Hybridization of cDNA probes to bovine DNA showed that β1 and β2 are encoded by separate genes. The amino acid sequences for the bovine and human β2 subunit are identical, as are the amino acid sequences for the bovine and human β1 subunit. This evolutionary conservation suggests that the two β subunits have different roles in the signal transduction process.

Together the β and γ subunits of G proteins act as a tightly bound entity that reversibly associates with the α subunit. The expression of the transducin γ subunit is retina specific. It is synthesized as a discrete 73-amino-acid polypeptide and is very hydrophilic and acidic; it has 19 acidic and 11 basic amino acids as well as three cysteine residues. Furthermore, there is significant similarity between the carboxyl terminus of the γ transducin peptide and the carboxyl termini of ras oncogene products. Preliminary results suggest that homologous cDNAs can also be isolated using the Ty cDNA probe.
129. G Protein Function in PC12 Cells

Ryn Miike-Lye, Melvin I. Simon

We are examining the function of G proteins and regulation of their synthesis in a cultured cell line in which signal transduction can be induced to change in a controlled manner. The differentiation of PC12 cells into sympathetic neuron-like cells, induced by nerve growth factor (NGF), provides a powerful model system to ask what sites in a G protein are important for its function in vivo. By oligonucleotide-directed mutagenesis, I have made a mutation in the α subunit of Gs that is analogous to one of the activating mutations in ras p21. The site of this mutation lies in the major region of homology between ras p21 and G proteins. By transfecting the mutant Gs into PC12 cells, we will be able to determine whether this mutation is functionally analogous, i.e., whether the α subunit of Gs will also become constitutively activated. The increased activity of adenylate cyclase predicted by such an activation will be detectable in PC12 cells transiently expressing the mutant Gs by either of two assays. First, elevated cAMP acts synergistically with NGF to greatly accelerate neurite outgrowth. Second, a variant of the PC12 cell line, which contains a stably integrated copy of the CAT gene under the control of a cAMP-dependent enhancer, will be transfected with the mutant Gs. In this case, elevated cAMP will be detected by an increase in CAT activity.

A current model proposes that free αs and α1 subunits available to interact with receptors and cyclase are inactivated by a common pool of βγ subunits, leading to the prediction that the amount of α, β and γ subunits within a cell may be tightly controlled. Thus, we are asking if G-protein subunit synthesis is regulated in a situation where there are large changes in the amount of receptors coupled to G proteins. To date, we have not observed any large changes in the amount of α-subunit mRNA as a function of differentiation.

130. Analysis of the Transducin β and γ Subunits by Expression In Vitro and in E. coli

Narasimhan Gautam, Melvin I. Simon

In the rod outer segments, the cGMP phosphodiesterase is activated when rhodopsin is photolysed. Transducin, which comprises three subunits, α, β and γ, mediates this response. The α subunit activates the phosphodiesterase while the βγ complex facilitates the binding of α to rhodopsin. The β subunit is conserved in other signal-transducing G proteins. Recent evidence indicates that the βγ complex might act independently as an activator.

Our aim has been to correlate the structure of β and γ with their function by expressing the normal and altered forms of these proteins in E. coli or yeast, purifying and assaying them after reconstitution. So far we have been able to express β in E. coli. The level of soluble and presumably functionally active β has been increased by using protease-deficient strains and changing induction conditions. It has not been possible to express the γ subunit either in E. coli or yeast.

Isoelectric focussing showed that both β and γ are highly acidic proteins although transducin is associated with the membrane in vivo. The γ subunit possesses a cysteine at the carboxyl tail which is conserved in the ras oncogene product. Posttranslational processing events lead to the attachment of palmitic acid to this amino acid in the ras gene product and an increase in its mobility in SDS gels. Incubating rabbit reticulocyte lysate with palmitic acid showed that the lysate possesses the capacity to acylate proteins. To examine whether γ is processed analogous to the ras gene product thus making it hydrophobic, RNA was synthesized in vitro from the γ cDNA and translated in vitro in the reticulocyte lysate. The resultant protein had the same mobility as native γ. The protein synthesized from a γ cDNA in which the cysteine had been mutated to leucine had the same mobility, indicating that γ is probably not processed in the same manner as the ras protein.

Using synthetic peptides as antigens, we have now generated antibodies to the β and γ proteins. These antibodies cross react with both the native and denatured forms of these proteins. Cross reaction with a proteolytic digest of β shows that these antibodies are specific to different regions of β.

In collaboration with T. Amatruda, we have begun characterizing the protein product of the β2 cDNA by synthesizing it in vitro and in E. coli. This cDNA is very homologous to transducin β but is distinct in its expression. We plan to determine the distribution of
the $\beta_2$ product in different tissues and in various G proteins.

131. Reconstitution of Signal Transducing Systems In Vitro

Michael A. Lochrie

I have isolated bovine cDNAs encoding two retinal-specific G protein (transducin) $\alpha$ subunits, $T_R\alpha$ and $T_C\alpha$, which are 81% identical in amino acid sequence. $T_R\alpha$ is a rod photoreceptor-specific protein that has been exhaustively studied. It activates a cGMP phosphodiesterase after excitation by photoactivated rhodopsin. Hydrolysis of cGMP results in closure of a cation channel and membrane hyperpolarization. $T_C\alpha$ is a cone photoreceptor-specific protein. Its function in cones is not known but is likely to be similar to that of $T_R\alpha$ in rods. My goal is to compare and explore the molecular mechanisms of transducin $\alpha$ subunit function by expressing cDNAs, purifying the resulting proteins, reconstituting signal transducing systems in vitro, and then comparing the activities of natural and mutant proteins. These studies may provide a biochemical basis for interpreting the results of experiments in which the same mutant gene will be introduced into an in vivo system such as a transgenic mouse of a cultured cell system that exhibits G protein-dependent behavior. Concurrently, we hope to obtain enough protein to initiate X-ray crystallographic studies. The photoreceptor system is ideal for reconstitution studies because more is known about it than any other G protein system, the components can be purified to homogeneity in large quantities, and cDNAs have been isolated for all of the components of the basic receptor-G protein-effector cascade.

I have constructed E. coli plasmids and strains for the expression of the cone $\alpha$ subunit and the rod $\alpha$, $\beta$, and $\gamma$ subunits individually or in combinations. The expression is regulated, results in unfused proteins, and the levels of production are reasonable. However, it appears that these bacterially-produced proteins do not fold properly since they precipitate, are rapidly degraded, and have little activity. Attempts to either secrete $T_R\alpha$ or produce it cytoplasmically in yeast have been unsuccessful, possibly because of similar reasons. Currently, I am investigating the expression of $T_R\alpha$ in a baculovirus/insect cell system, which has potential advantages over bacteria or yeast, and the plausibility of refolding the bacterial proteins to gain activity.

Collaborative projects are continuing or being initiated in the following areas: 1) G protein/photopigment gene expression in retinoblastoma cell lines, 2) expression of G proteins during mouse retinal development, and 3) analysis of mouse mutants with retinal degenerative diseases.

Figure 1. Excision of plasmid with cDNA insert from lambda cloning vector.

132. A New Cloning Vector

Michael Strathmann, Melvin I. Simon

In order to identify novel G-protein subunits, we are developing new methods for cloning cDNA corresponding to rare members of protein families. These methods will allow us to characterize unique G proteins that may represent only a small fraction of the G protein pool in a particular cell population.

To facilitate this analysis, we have constructed a cloning vector that combines the advantages of plasmid and phage vectors. A plasmid, flanked by direct repeats of a 600 bp internal fragment of the $\beta$-lactamase gene, was inserted into lambda (see Figure 1 above) and a polylinker into which cDNA can be cloned was inserted into the plasmid. Recombination between the repeats rescues the plasmid and confers ampicillin resistance on the host. Consequently, cDNA can be
rapidity "subcloned" by infecting a lysogenic host and selecting for ampicillin resistance. The surviving cells harbor the cDNA in plasmids which can be sequenced and further manipulated.

Any plasmid with the \( \beta \)-lactamase gene can be easily incorporated into this vector. A phage that contains only the 600 bp \( \beta \)-lactamase fragment is grown on a host carrying the plasmid of interest. An Amp\(^{5} \), lambda\(^{-}\) strain is infected with the lysate and spread onto Amp plates. One-half of the phage purified from the resulting colonies contain the plasmid. Thus, one can simply generate new lambda vectors tailored to a specific cloning strategy.

133. Chromosomal Localization of G Protein-encoding Genes in Mouse

Vivian H. Cohn, Pamela Eversole-Cire, Melvin I. Simon

The family of signal-transducing proteins known as G proteins (guanine nucleotide-binding proteins) are responsible for coupling extra- and intracellular signals in a variety of systems. Each G protein is composed of three subunits—\( \alpha \), \( \beta \), and \( \gamma \). Multiple genes have been identified coding for each subunit. In order to study the organization of this multi-gene family and the relationships between the various subunit genes, we are investigating their chromosomal locations in mouse.

Chromosomal assignments were determined using restriction fragment length polymorphisms (RFLPs) to analyze recombinant inbred (RI) strains of mice. Positions have been determined for two retinal transducin \( \alpha \) genes (\( T_{\alpha r} \) and \( T_{\alpha c} \)), a \( \beta \) gene (\( G_{\beta 1} \)), a \( G_{i} \) gene (\( G_{i 2} \)), and a \( G_{s} \) gene (\( G_{s 1} \)). \( T_{\alpha r} \) and \( T_{\alpha c} \) map to chromosomes 9 and 17, respectively. These two genes are structurally related, yet they are each expressed in distinct photoreceptor cell types. Thus, it is perhaps not surprising to find that they are localized on different chromosomes. \( G_{s 1} \) and \( G_{i 2} \) are responsible for a stimulation or inhibition of intracellular adenylate cyclase levels, respectively. \( G_{s 1} \) was shown to have an RFLP distribution pattern among RI strains that is quite different from the other \( \alpha \) genes studied. Weak linkage to several markers suggests a position on chromosome 1. \( G_{i 2} \), on the other hand, shows a pattern identical to \( T_{\alpha r} \) in 42 out of 42 RI strains. Thus, it appears that these two genes both lie on chromosome 9, probably less than one million base pairs apart, and are linked to Ltw-3 (a liver protein 2-D variant) and Fv-2 (Friend virus-2). \( G_{s 1} \), one of two \( \beta \) genes so far identified, was found to be weakly linked to markers on chromosome 19.

Although mapping genes provides information about their organization on only a gross level, it clearly is a useful starting point for examining the relationships among members of this gene family. Further, it is a way of investigating positional relationships with other genes with which they may interact. One example of this is the linkage determined between \( T_{\alpha c} \) and \( rds \) (retinal degeneration slow) on chromosome 17. Other possible G protein-disease relationships have been implicated, such as the association between \( G_{s} \) and pseudohypoparathyroidism (which has not been localized).

134. Tissue-specific Expression of an Embryonic \( U_{1} \) Small Nuclear RNA Gene in Transgenic Mice

Elsebet Lund, James E. Dahlberg, Melvin I. Simon

Mammalian cells contain 25-30 \( U_{1} \) snRNA genes, each of which is transcribed once every 3-4 seconds. In mice, one class of these genes (the so-called adult or \( mU_{1a} \) genes) is expressed constitutively; the other class (the embryonic \( mU_{1b} \) genes) is expressed only in embryos and in adult tissues, such as testis, that contain a high proportion of non-terminally differentiated cells. The strength of snRNA promoters makes them attractive for expression of cloned genes.

The feasibility of using the \( mU_{1} \) promoters to direct gene expression in transgenic mice was tested by making a chimeric template containing the \( mU_{1b} \) promoter and a human \( U_{1} \) (h\( U_{1} \)) RNA coding region; human \( U_{1} \) RNA is very similar to, but clearly distinguishable from, mouse \( U_{1} \) RNAs. Transgenic mice containing the chimeric \( mU_{1b}/hU_{1} \) gene were obtained by microinjection of linear DNA fragments into fertilized mouse eggs.

Analysis of one of these mice showed that the gene was incorporated into the germline and expressed in progeny mice. Moreover, this expression was subject to the same control as the endogenous embryonic \( mU_{1b} \) genes. Specifically, the h\( U_{1} \) RNA accumulated in adult testis but not adult brain or liver.

These experiments show that transgenic mice can be used to study the control of snRNA synthesis. For
example, they show that accumulation of mU1b RNA is likely to be controlled during transcription rather than by degradation of specific forms of the RNA.

Moreover, these results show that it should be possible to exploit the mU1 promoters to study other genes. In particular, they can be used to drive synthesis of anti-sense RNAs at specific times during development.

135. Antibodies Specific for the Periplasmic, N-terminal Region of the Tar Protein

Nachum Kaplan, Suzanna J. Horvath, Melvin I. Simon

Tar is one of the four chemotaxis receptor proteins of E. coli. These proteins are located in the cytoplasmic membrane and are distinguished by their specificities for different ligands; each protein binds or responds to a specific set of attractants and repellents. Ligand binding is via the periplasmic N-terminal portion of the protein. In order to further probe the ligand binding site(s) and the role of the N-terminal domain in signal transduction across the membrane, we set out to prepare synthetic peptide antibodies specific for this region. Amino acid sequences for raising the antibodies were chosen according to the following criteria: 1) areas of least amino acid homology between Tar and the other receptor proteins, and 2) hydrophilic-rich stretches for ease of synthesis and antibody elicitation. The sequences chosen were amino acids 106-126 and 162-182 of the Tar protein. Solid phase synthesis was performed manually. Midway through synthesis, aliquots were removed to produce the 11-mers 11-6-126 or responds to a specific set of attractants and repellents. Ligand binding is via the periplasmic N-terminal portion of the protein. In order to further probe the ligand binding site(s) and the role of the N-terminal domain in signal transduction across the membrane, we set out to prepare synthetic peptide antibodies specific for this region. Amino acid sequences for raising the antibodies were chosen according to the following criteria: 1) areas of least amino acid homology between Tar and the other receptor proteins, and 2) hydrophilic-rich stretches for ease of synthesis and antibody elicitation. The sequences chosen were amino acids 106-126 and 162-182 of the Tar protein. Solid phase synthesis was performed manually. Midway through synthesis, aliquots were removed to produce the 11-mers 11-6-126 and 172-182. All four peptides were conjugated to keyhole limpet hemocyanin and injected into rabbits. The rabbits were boosted and bled at two-week alternating intervals. Approximately five weeks after the initial injection, high levels of anti-Tar antibodies were elicited by all peptides; these high titers stayed constant for several months thereafter. The 21-mer peptides produced an approximately twofold higher titer than the 11-mer peptides. In addition, the two 21-mer unconjugated free peptides were also injected into rabbits; they elicited sera of approximately the same titer as the corresponding conjugated peptides. All the sera were found to be specific for: 1) whole Tar protein but not Tsr (a second chemotaxis receptor protein), 2) the N-terminal half of Tar (an amber fragment) but not the C-terminal half (a cloned fragment). The antibodies have been used in identifying the cellular location of several Tar amber fragments. In addition, they will be used in immunoaffinity purification of Tar, and possibly in behavioral studies using cells that have been semipermeabilized to allow the antibodies to reach the cytoplasmic membrane and to bind to the chemotaxis receptors, thus either blocking or enhancing chemotactic responses.

PUBLICATIONS


Summary: Our work on the mechanism of tRNA and mRNA splicing in yeast (Saccharomyces cerevisiae) has made good progress during the last year. If a theme has emerged, it is that these reactions take place on large complexes or structures and that our ability to dissect the components of these complexes is greatly enhanced by the power of the genetic approaches that are possible in the yeast system.

In tRNA splicing we have continued our work on the two principal enzymes that catalyze this reaction: the tRNA ligase and the endonuclease. The endonuclease, an integral membrane protein, has been purified ~100,000 fold but we have not as yet identified the polypeptide constituent(s). During the next year we will intensify our efforts to finish this purification and will in addition take a new genetic approach to identification of the gene for endonuclease. We have completed the nucleotide sequence of the gene for tRNA ligase and also of a closely-linked gene that is essential for yeast growth, is very likely co-regulated with ligase but is as yet of unknown function. tRNA ligase is a protein with three independent activities. We have made some progress in mapping these activities to domains of the protein. We can now purify tRNA ligase in 50-100 mg quantities and during the next year will seek to obtain crystals of the protein which are suitable for X-ray crystallographic structure determination.

There are some nine different tRNA precursors in yeast that contain intervening sequences. Each is recognized by the endonuclease which cleaves the precursor at 5' and 3' splice sites to release the linear intron. We now understand much more about the mechanism of recognition of the splice sites by endonuclease through the construction of altered tRNA-Phe precursors. The altered genes are transcribed in vitro by bacteriophage T7 RNA polymerase.

The endonuclease must recognize some feature of the mature tRNA domain common to all of the precursors. It then measures the distance to the splice junction. This distance is the same position relative to the mature domain in all precursors. If the distance is changed, by virtue of insertion of a base pair into the anticodon stem, the intron becomes larger by two bases. This result proves that it is the position of the intron relative to the mature domain that is recognized and not the sequences at the splice junction. Dr. C. Greer at UC Irvine has discovered that tRNA ligase also forms a complex with tRNA precursors. By using tRNA precursors that contain the photosensitive nucleoside analogue 5-bromouridine, we have shown that a specific crosslink can be made between the precursor and tRNA ligase. This crosslink is to a single uridine in the intervening sequence. This approach will be used in the future to explore the details of RNA-protein interactions in tRNA splicing and in mRNA splicing as well.

In mRNA splicing the precursor must be folded so that the 5' and 3' splice junctions come into close proximity. A large number of components, including five small nuclear ribonucleoprotein particles (snRNPs) are required to assemble the precursor into a splicing substrate. The resulting complex is called the spliceosome. We now understand in broad outline the pathway of assembly of the spliceosome. It is a complicated process in which snRNPs recognize sequences in the precursor and form initial complexes. Interactions between snRNPs, followed by addition of more
snRNPs, are probably required to fold the complex into its active form. In order to understand this process in greater detail, we must enumerate all of the components and obtain them in pure form. Our approach to dissecting this complicated structure is a genetic one. A set of nine genes, RNA2-11, had been isolated more than 15 years by Lee Hartwell and Calvin McLaughlin. We have now shown these to be directly involved in mRNA splicing. These genes were identified by screening a bank of temperature-sensitive mutants of yeast. It is interesting that 59.6% of the mutants in a random collection of temperature-sensitive mutants have this phenotype. The reason for this high proportion is not known, but it has allowed us to expand the original collection of RNA mutants and we shall continue to characterize these genes. During the last year we have cloned and sequenced the RNAll gene and we have shown that the RNAs gene is part of a 30S complex, quite likely a snRNP and is also part of the spliceosome. New mutants that we have isolated show different phenotypes than any of the original RNA mutants. We have isolated a mutant that blocks the second stage of the splicing reaction causing the accumulation of the lariat-exon 2 intermediate. We also have a mutant that accumulates large quantities of the lariat intron product, apparently retained in a large complex with some of the snRNPs.

We have continued our collaboration with Jeffrey Miller at UCLA to create a complete set of amber suppressors in E. coli. We have encountered an interesting problem. Five of the suppressors, thr, arg, asp, ileu and val, insert lysine instead of the cognate amino acid. This result suggests that all five of these amino acyl tRNA synthetases have the anticodon as a primary recognition site and that the lysyl tRNA synthetase is relatively promiscuous in recognizing tRNAs with the amber anticodon. It does not, however, recognize all such tRNAs and this may be because tRNA<sup>LYS</sup> is a member of a set of tRNAs which are similar in structure and which includes all five of the mischarged suppressors.

136. Yeast tRNA Ligase Sequence: Studying the Domains

Shawn Westaway

tRNA ligase is one of two enzymes required for tRNA splicing in Saccharomyces cerevisiae. The enzyme is likely a single polypeptide of 95 kDa and possesses three activities responsible for the second stage of the tRNA splicing mechanism: a cyclic phosphodiesterase, a polynucleotide kinase, and a ligase activity. The tRNA ligase protein has been purified and its gene has been cloned (Phizicky et al., 1986). We have sequenced the gene and analyzed the transcripts from this region (Westaway, Phizicky and Abelson, manuscript in preparation). The inferred amino acid sequence of the tRNA ligase reading frame reveals a basic protein of 827 amino acids, which at present is not homologous to other known proteins of similar function. We have found two other open reading frames, ORF1 and ORF2, near the 5' end of the tRNA ligase gene (see Abstract No. 137).

We are pursuing the study of the tRNA ligase protein by attempting to dissect the domains responsible for the four splicing activities listed above. Fragments of the tRNA ligase protein have been isolated, each of which contains only some of the different activities of the intact polypeptide, but all of which are recognized by anti-tRNA ligase antibody (see Abstract No. 138). The amino-terminal amino acid sequence for three of these purified fragments (65 kDa, 40 kDa, and 25 kDa) has been obtained. We are cloning these fragments using the DNA sequence from the ligase gene, by inserting appropriate stop and start codons using oligonucleotide-directed mutagenesis and removing all other DNA sequences of the mature tRNA ligase protein. By overproducing these peptide fragments in E. coli, we remove any possibility of contamination by other portions of the tRNA ligase protein or other yeast proteins. Since the fragments resulting from intact tRNA ligase are recognized by anti-tRNA ligase antibody, we should be able to detect the cloned fragments of the protein as well. Purification of these cloned peptide fragments, followed by assays for each separate enzymatic activity, should verify the results obtained using the peptide fragments of intact tRNA ligase. In this manner we hope to effect the localization of the adenylation site of tRNA ligase, as well as a systematic elucidation of the distinct domains of tRNA ligase necessary for each function during the entire splicing reaction.

References
137. Open Reading Frames Near the tRNA Ligase Gene

Shawn Westaway, George Komatsoulis

Sequencing of the clone containing the gene for tRNA ligase revealed two other open reading frames, ORF1 and ORF2, near the 5' end of the tRNA ligase gene (Westaway, Phizicky and Abelson, manuscript in preparation). ORF1 precedes the tRNA ligase reading frame and overlaps the ligase coding region by eight nucleotides but is out-of-frame with respect to ligase. The overlapping nature of ORF1 led us to determine if this ORF was translated in vivo, either independently or as a frameshift fusion polypeptide to the tRNA ligase reading frame. Using site-directed oligonucleotide mutagenesis in M13, we have constructed an ochre stop codon within ORF1 before the region of overlap. By employing site-specific homologous recombination, we have replaced the wild-type ORF1 with this ochre mutation in a diploid strain of yeast. Genetic analysis of this mutant yeast strain reveals no deleterious phenotypic effects. Our results indicate that translation of ORF1 is not essential for normal mitotic or meiotic cell viability.

However, this is not the case for the second open reading frame. ORF2 begins 342 nucleotides upstream from tRNA ligase and proceeds in the opposite direction. This ORF produces a transcript of approximately 2.1 kb that begins only 125 nucleotides away from the tRNA ligase transcription initiation site. In order to study this open reading frame, we have constructed a mutation in ORF2 by filling in a unique restriction site which should throw the coding region out of frame. Replacement of the wild-type sequence with this mutation, followed by genetic analysis, indicates that ORF2 is a single-copy essential gene. Results of our genetic complementation experiments suggest that there are sequences within the amino-terminal coding region of tRNA ligase necessary for the production of the ORF2 gene product. Is this gene coregulated with tRNA ligase, or is its product related to tRNA metabolism in general? To study this question, we have fused ORF2 and part of its 5'-untranslated region to the GAL1-GAL10 (galactose) upstream activating sequence in order to conditionally control the production of its product. The ORF2 mutant strain (described above), when transformed with this construct, is healthy when grown on galactose, which induces the production of intact ORF2 product from the plasmid. The cell dies when switched to glucose media (which represses the galactose-controlled gene). We are using this conditionally lethal strain to study the in vivo role of ORF2 in the yeast cell. Sequencing of the ORF2 gene is in progress, and may yield further clues as to the function of this gene in yeast.

138. Activity Domains of Yeast tRNA Ligase

Qi Xu

The splicing reaction of yeast tRNA proceeds in two distinct stages. In the first stage, there are two precise endonucleolytic cleavages at the mature sequence boundaries to produce the tRNA halves and linear intervening sequence. In the second stage of the splicing reaction, the tRNA halves are joined in an ATP-dependent step. All four of the enzymatic activities required in the second stage of splicing reaction—an adenylated enzyme intermediate, a tRNA ligase, a polynucleotide kinase and a cyclic phosphodiesterase—coassemble on a glycerol gradient with a single 90 kDa polypeptide.

The S. cerevisiae tRNA ligase has been cloned and expressed in E. coli on a plasmid containing the ligase gene under the control of the tac inducible promoter. This ligase expressed in E. coli is a fully active, 90 kDa polypeptide. Along with the 90 kDa polypeptide there are a number of discrete lower molecular weight fragments that are antigenically related to tRNA ligase.

These major fragments can be produced by trypsin digestion of the 90 kDa ligase protein. We have purified the three major fragments (65, 40 and 25 kDa molecular weight) to homogeneity. Amino acid sequences of the 65 kDa and 40 kDa fragments reveal these two fragments to have the identical amino-terminal sequence as the 90 kDa ligase protein, but the 25 kDa protein is from the C-terminus of the protein. The 90 kDa ligase as well as the 65 kDa and 40 kDa fragments can be adenylated while the 25 kDa fragment cannot. The 65 kDa fragment possesses the majority of the polynucleotide kinase activity, whereas the 25 kDa fragment has less than 5% of the original kinase ability, and the 40 kDa fragment has no kinase activity. The 65, 40 and 25 kDa fragments do not have the ability to catalyze the ligation of tRNA half-molecules. Addition of the 25 kDa fragment with the
65 kDa fragment restores tRNA ligase activity. These data indicate that yeast tRNA ligase has distinct activity domains that must act in concert to achieve the complete tRNA ligation reaction.

139. Substrate Recognition and Splice Site Selection in Yeast tRNA Splicing

Vicente M. Reyes

We have developed a new method for the synthesis of pre-tRNA as substrate for in vitro splicing and for other studies. In this system, the S. cerevisiae pre-tRNA Phe gene containing a 19-base IVS has been fused downstream to a bacteriophage T7 promoter and a BstNI cleavage site placed at its 3' end. To synthesize the pre-tRNA, the plasmid is first cleaved with BstNI and then incubated with T7 RNA polymerase. We have proven that this synthetic pre-tRNA has the correct sequence and has mature 5' and 3' termini (approximately half of the molecules end with 3'CCA, and the other half with 3'CCAG); we have also shown that it is spliced efficiently and accurately (both cleavage and ligation are accurate) by the purified yeast splicing endonuclease and ligase. To take advantage of this system, we have constructed precise base alterations in this pre-tRNA with the goal of understanding how the tRNA splicing enzymes recognize their substrates. Our results show, among others, that alteration of residues C56 and U8 abolishes cleavage (but that of G19 does not), and restoration of the tertiary base pairing between residues 19 and 56 by constructing the double mutant C19-G56 does not rescue cleavage. These results, together with the fact that residues C56 and U8 are (a) totally conserved among all tRNAs, and (b) both located on the surface of the pre-tRNA molecule, lead us to think that these two residues are probably among the points of contact with the endonuclease (and G19 is not) during the splicing event.

We have also elongated the anticodon stem of the pre-tRNA by inserting one base pair. This variant precursor is spliced, but the IVS is two bases longer than normal; we have shown that the splice sites have been shifted one base upstream and one base downstream of the wild-type 5' and 3' splice sites, respectively. Additionally, deletion of the (highly conserved) U residue preceding the anticodon gives rise to a spliced precursor, but the IVS is one base shorter, and is generated by cleavage at the normal 3' splice site but the 5' splice site has been shifted one base downstream. These results suggest a conceptual model for the mechanism of splice site selection by the endonuclease (Figure 1): the enzyme seems to locate the splice sites by "measuring" fixed distances from a reference point within the mature domain of the pre-tRNA (presumably in the thoracic region where most of the highly conserved residues are concentrated), and then appropriate cleavages are made. We currently are testing this model by inserting additional base pairs into the anticodon stem of the pre-tRNA to see whether the IVS excised would be progressively longer (by two bases per bp insertion).

Another question we would like to address is whether the unspliced variant precursors we have constructed are defective at the level of binding to the endonuclease, or at the actual cleavage event.

Figure 1. Metric model for splice site selection by the yeast tRNA splicing endonuclease. In this model, the enzyme is envisioned to locate the splice sites by "measuring" fixed distances from some fixed point(s) in the domain where it binds the pre-tRNA, as opposed to recognition of short, unique sequences at the splice junctions, as in mRNA splicing.

140. Crosslinking Uridine-Analog Incorporated Precursor RNAs to RNA Processing Proteins

N. Kyle Tanner

Eukaryotic tRNA genes are sometimes interrupted by an intron within the anticodon loop. These introns
are removed during RNA processing through a two-step mechanism. In the first step, the 5' and 3' splice sites are cleaved by an endonuclease and the intron is removed. In the second step, the splice junctions are ligated together by a tRNA ligase. Yeast tRNA ligase has been purified in our laboratory to near homogeneity and the endonuclease has been highly purified. Our laboratory has also synthesized an unmodified yeast tRNA precursor that acts as a substrate for the processing enzymes. I have shown that pre-tRNA transcripts with all of the uridines replaced by bromouridine are recognized and processed by endonuclease and by ligase. Irradiation of the BrU-pre-tRNA and ligase with long wavelength UV light results in a single major covalent crosslink between the RNA and the protein. The site of this crosslink has been determined for the pre-tRNA and work is being done to localize the crosslink on the protein. Specific proteins associated with the endonuclease activity are also crosslinked when irradiated in the presence of BrU-pre-tRNA.

I am currently using other photoactivated uridine analogs in RNA transcripts in association with Michelle Hanna at the University of California, Irvine. These analogs will be used to determine the protein-nucleic acid interactions that occur in pre-tRNA splicing. In addition, I am testing these analogs for their use in studying the protein-nucleic acid interactions involved in pre-mRNA splicing.

141. Testing for Intron Function in a Unique and Essential Yeast tRNA Gene

Calvin K. Ho

A subset of the tRNA genes in S. cerevisiae contain introns that are transcribed as part of a precursor tRNA and subsequently excised in a multi-step splicing process. While the enzymes and mechanisms involved in tRNA splicing have been the focus of intensive study, less is known about the significance of introns in tRNA biosynthesis, apart from their participation in splicing. Nonetheless, a clear role for tRNA introns has emerged from studies on two intron-containing suppressor tRNA genes. Deleting the intron in SUP6 (Tyr+ochre) and SUP53 (Leu3+amber) produces tRNAs that 1) lack base modifications in the anticodon, and 2) are less efficient suppressors in vivo. These examples point to the importance of the intron in ensuring proper modification of the primary transcript and in producing mature tRNAs of optimal efficiency. However, not all tRNAs associated with introns contain anticodon base modifications, so this function may not be a general one.

To assess whether tRNA introns have other positive roles in tRNA gene expression, we are testing the consequences of deleting the intron from tRNA\textsubscript{Ser}\textsubscript{UGG}, which lacks nucleoside modifications in its anticodon. In contrast to the many tRNA genes that are multicy copy, the gene coding for tRNA\textsubscript{Ser}\textsubscript{UGG} (sup61+) is unique and indispensable: nonsense suppressors of this gene cause recessive lethality. It is therefore possible to investigate the role of the intron on wild-type tRNA function as well as on suppressor function. The intron\textsuperscript{−} gene (SUP61\textsubscript{ΔIVS}) was constructed by oligonucleotide-directed deletion mutagenesis and introduced into diploid yeast by gene replacement techniques. Sporulation yielded four viable spores per tetrad, indicating that the intron is not absolutely essential for functional expression of sup61\textsuperscript{+}. Haploids carrying the intron deletion appear to be indistinguishable from isogenic intron\textsuperscript{+} strains as judged by growth at various temperatures and in media supplemented with a variety of sole carbon and nitrogen sources. Moreover, intron-deficient strains of opposite mating type are able to produce diploids, and homozygous intron\textsuperscript{−} diploids sporulate to produce germinating progeny spores. There also appear to be no phenotypic effects associated with over-expressing SUP61\textsubscript{ΔIVS} from 2 \mu containing plasmids.

In an effort to detect subtle effects, SUP61\textsubscript{ΔIVS} and isogenic sup61\textsuperscript{+} strains will be co-cultured in a chemostat in a growth competition experiment. Finally, we plan to examine the effects of excising the intron from suppressor genes derived from sup61\textsuperscript{+}. Intron deletions have been incorporated into SUP61-a amber and SUP61-o ochre suppressors and the intron\textsuperscript{+} and intron\textsuperscript{−} suppressor genes have been cloned into CEN plasmids. Transformation of strains carrying suppressible alleles with these plasmids will establish whether the intron is important for maintaining suppressor efficiency.
142. Assembly of the Spliceosome in Yeast

Soo-Chen Cheng

The assembly of the spliceosome was studied by electrophoresis on native polyacrylamide gels. Four splicing complexes, A1, A2-1, A2-2 and B, were identified during the time course of the splicing reaction. The order of the assembly process appeared to be B+A2-1+A1+A2-2. Complexes B, A2-1 and A1 contained predominantly precursor RNA, whereas complex A2-2 contained precursor RNA, the splicing intermediates and the excised intron but no spliced message. Titration of the splicing reaction with EDTA in the presence of 3 mM MgCl₂ showed accumulation of increasing amounts of complex A1 but decreasing amounts of complex A2-2 with increasing concentrations of EDTA. This indicates that some components required for converting A1 to A2-2 were inhibited by EDTA. At 5 mM EDTA, complex A1 accumulated with only small amounts of complex A2-2, suggesting the assembly steps B+A2-1+A1 required very low levels of Mg²⁺, perhaps in order of nanomolar. Step A1+A2-2, on the other hand, required higher concentrations of Mg²⁺. Heat inactivated ma2 mutant extract is also incapable of forming complex A2-2, resulting in accumulation of A1.

Examination of the involvement of small nuclear RNAs, snR6, snR7, snR14 and snR20, in these complexes by Northern hybridization revealed that snR14 appeared only in A2-1, snR6 and snR7 are in complex A1, A2-1 and A2-2, whereas snR20 is in all four complexes.

When these small nuclear RNAs in the splicing extract were examined on the native gels independent of the splicing reaction by Northern hybridization, we found that snR7, snR14 and a portion of snR6 were associated with each other, forming large complexes, which underwent ATP-dependent dissociation to release smaller complexes of snR6, snR7 and snR14. SnR20 was not involved in these complexes. Evidence suggests that the dissociation of the snR6-snR7-snR14 complexes by ATP is not the sole role of ATP for the early step of the complex assembly. These results can be incorporated into a model of the assembly of the yeast spliceosome (Figure 2).

143. Purification and Visualization of the Yeast Spliceosome

Susan E. Goeltz, Michael W. Clark

To understand the mechanism by which introns are precisely and efficiently excised from pre-mRNA, one must first identify the molecules that combine to form "spliceosomes." We are approaching this problem in two ways. First, we have been attempting to purify intact spliceosomes. Second, we are beginning to purify the particles (the snRNPs) that combine to form a spliceosome.

In our attempts to obtain pure spliceosomes, we have used two methods. One is based on unusual
sedimentation properties that spliceosomes have on glycerol gradients. Their mobility decreases at high salt (400 mM) relative to that seen at low salt (25 mM). Thus, by running sequential glycerol gradients at different salt concentrations, spliceosomes pure enough to examine with electron microscopy have been obtained. These spliceosomes appear to be spherical with dimensions of 200 Å x 230 Å. The other method for purifying spliceosomes is based on affinity chromatography of spliceosomes containing a modified pre-mRNA. This RNA contains some uridine-S-S-biotin residues. Such spliceosomes can be bound to a solid matrix to which avidin has been coupled. The spliceosomes can then be released from the avidin matrix gently and specifically with a reducing agent that cleaves the disulfide bond that has been placed between the mRNA and the biotin group.

We have also started to purify the snRNPs involved in mRNA splicing. Initially, we are focusing on the complex that contains snRNA 20. Thus far, individual steps for the purification include ammonium sulfate precipitation, chromatography on a sizing column (FPLC superose 6) and on hydroxylapatite, and differential sedimentation. We are hoping that a combination of these steps will result in an active and relatively pure snRNP.

144. Temperature-sensitive Splicing Mutants in Yeast
Usha Vijayraghavan

A screen for RNA processing mutants in yeast was undertaken. Ethyl-methanesulphonate mutagenesis was used to create a bank of strains that were temperature sensitive (ts) for growth. Individual isolates from the bank at log phase of growth were shifted to nonpermissive temperature for 2 hours, followed by total RNA extraction. The RNA from these strains was screened on Northern blots, using a probe specific to the yeast actin intron sequences. Several candidate mutants have been isolated and rescreened at both permissive and nonpermissive temperature. Of a screen of 600 ts lethals, 25 positives were isolated after the rescreen.

Complementation analysis with the mna1-11 strains (previously shown to accumulate pre-mRNA) shows that three strains failed to complement mna2, three mna3, four mna6 and one mna9. The mutants that complement the mna1-11 defects fall in three classes: nine that accumulate precursor, one the lariat intermediate and one released intron. The ts defect in all of these classes is recessive, and in all cases tested segregates 2 ts+ : 2 ts− upon sporulation of the appropriate heterozygous diploid. Cosegregation of the ts and RNA defect has been established in a few cases. Two isolates of the class accumulating lariat intron-exon 2 intermediate fall into the same complementation group and cosegregate with the ts defect. Accumulation of intron was seen in three strains. In one, the intron accumulation cosegregates with the ts defect, but this is not so in another with a similar RNA defect. Evidence for the presence of the lariat intermediate or intron in these strains was obtained by a combination of Northern blot and primer extension analysis. Completion of the genetic analysis for all of the new groups is in progress. Further characterization of these mutant strains including tests of whether these RNA processing defects are general and affect most intron containing precursors is in progress.

145. RNA11 Gene Product and the Spliceosome
Tien-Hsien Chang

The splicing of mRNA in yeast takes place on a high molecular weight complex with a sedimentation coefficient of 40S, termed the "spliceosome." In order to thoroughly understand the mechanism of mRNA splicing, it is necessary to enumerate the individual components involved in the spliceosome assembly. The mna mutants in yeast accumulate intron-containing pre-mRNAs at the nonpermissive temperature. We have previously shown that the RNA gene products are essential for mRNA splicing in vitro (Lustig et al., 1986; Lin et al., 1987). It is possible that some of the RNA gene products are intrinsic components of the spliceosome. If verified, the RNA gene products would be potential candidates for spliceosome-specific markers and therefore would greatly facilitate the mRNA splicing research in the future. We first explored this possibility on RNA11 gene product.

We employed a combination of in vitro transcription and translation techniques to synthesize full-length 35S-labeled RNA11 protein. The in vitro synthesized RNA11 protein is capable of rescuing the defects of mna11 mutant extract at the nonpermissive
temperature. In this process, most of the input $^{35}S$-labeled RNA11 protein turned out to be in a 30S complex of uncertain function and a minor fraction of the RNA11 protein was incorporated into the 40S spliceosome. Unlike the formation of the 40S spliceosome complex, the assembly of the 30S complex apparently requires neither exogeneously-added ATP nor pre-mRNA. We speculate that the 30S-complex is likely to be one of the snRNPs in yeast and may serve as a structural intermediate for the assembly of the functional 40S spliceosome.

To further clarify the complicated picture, we overproduced the RNA11 protein in *E. coli* and obtained a partially purified fraction consisting of the RNA11 protein which can complement the heat-inactivated *ma5* extract. We have also raised antibodies against RNA11 protein using gel-purified RNA11 protein from an *E. coli* overproducer. By immunological methods, we will determine if the RNA11 protein is in the spliceosome assembly pathway or is an intrinsic component of the spliceosome.

References:

146. Characterization of the RNA5 Gene of *S. cerevisiae*
Gloria Dalbadie-McFarland

The gene products of the *S. cerevisiae* genes RNA2 through RNA11 have been previously shown by Lin and Lustig to be directly involved in splicing the transcripts of intron-containing, nuclear, protein-coding genes; splicing of these mRNA precursors occurs in a large ribonucleoprotein complex known as the spliceosome.

The RNA5 gene product is required at an early step in the splicing process. Previous *in vitro* studies of splicing extracts prepared from a temperature-sensitive *ma5* mutant imply that the RNA5 gene product is either an integral component of the spliceosome or is required for its assembly.

In order to obtain the RNA5 gene product in sufficient quantities to study its properties and its role in splicing in detail, I have cloned and characterized the RNA5 gene and will attempt to overexpress it in *E. coli*.

A DNA fragment was cloned from a yeast genomic library by complementation of a temperature-sensitive growth defect of an *ma5* mutant strain. The cloned DNA specifically complements the *ma5* defect and does not complement temperature-sensitive defects in other RNA genes. I have shown by tetrad analysis that a single copy of this DNA fragment integrated in the yeast genome is tightly linked to the RNA5 gene, strongly suggesting that this is indeed the RNA5 gene.

Using probes derived from the cloned DNA, I have identified a single, low abundance, polyadenylated transcript. DNA sequence analysis and precise mapping of the 5'end of the transcript have revealed an open reading frame that could encode a 43 kDa protein. Further complementation analyses are in progress to confirm that the predicted protein is the RNA5 gene product. The gene will subsequently be overexpressed in *E. coli* and the protein will be used to obtain antisera. This will be a valuable tool in the study of the RNA5 protein, its role in the splicing pathway, and its association with other components of the spliceosome.

147. Characterization of the RNA4 Gene of *S. cerevisiae*
Josette Banroques

A temperature-sensitive mutation in the RNA4 gene of *S. cerevisiae* causes accumulation of intron-containing mRNA precursors. It has been previously shown by *in vitro* studies that the product of the RNA4 gene is implicated in mRNA splicing at an early step of the process.

The RNA4 gene has been isolated by genetic complementation and cloned on a multicopy plasmid (Last et al., 1984). It has been shown that cloned extra copies of RNA4 are able to restore the splicing activity at restrictive temperature. The gene has been characterized by restriction mapping and subcloning. DNA sequencing is in progress now. Upstream of the coding sequence, a conserved sequence TAGTAAAG has been found in several RNA clones, suggesting that it is involved in the expression and/or the regulation of these genes.

Immunological studies will allow us to investigate the role of the protein in the mRNA splicing and to search for its interactions with the other components of the spliceosome.
148. The mal Mutation Disrupts the Location of Proteins Found in the Yeast Nucleolus

Michael W. Clark, Judith L. Campbell

By immune fluorescent and nucleolar-specific silver staining techniques, we have determined that the single-strand DNA binding protein, SSB-1, is located in the yeast nucleolus. We have also identified another single-strand DNA binding protein, SSB-36Kd, that binds silver under the conditions for nucleolar-specific staining and is presumably another nucleolar protein.

We have used these nucleolar proteins as markers to examine the morphology of the nucleolus in one of the yeast RNA processing mutants, mal. At non-permissive temperature, by SSB-1 antibody fluorescent and nucleolar specific silver staining, the mal nucleolus appears disrupted. The staining patterns are no longer confined to a cap-like nucleolus filling one-third of the nucleus, as they are in the wild-type cells, but appear to be located over the entire nucleus. At the same temperature, wild-type and RNA splicing mutant, ma2, cells do not show this nucleolar disruption.

The mal mutation causes the accumulation of precursor rRNAs, precursor tRNAs and some precursor mRNAs. From these biochemical data, two models for the function of the RNAl gene product have been proposed. In one model, the RNAl protein is directly involved in the transport of mature RNAs out of the nucleus into the cytoplasm. In the other model, the RNAl protein is somehow involved in a nuclear organization mechanism. The mal mutation then prevents proper alignment of the RNA processing enzymes and prevents them from functioning. Our data of nucleolar disruption caused by the mal mutation supports the latter model of a nuclear organization mechanism involvement for the RNAl protein.

We are presently screening human autoimmune sera that reacts with mammalian nuclear and nucleolar proteins and antisera made to specific mammalian nucleolar proteins for cross-reactivity with yeast nuclear and nucleolar components. We will use any cross-reacting sera and antisera made against known yeast RNA splicing components to further examine, at an optical and electron microscopic level, the disruptive effects on nuclear organization of mal mutation.

149. Construction of Amber Suppressor tRNA Genes in E. coli: Determination of Specificity

Jennifer Normanly, Lynn G. Kleina1, Jeffrey Miller1

The aim of this project (a collaboration with Jeffrey Miller and coworkers at UCLA) is to expand the current collection of amber suppressor tRNA genes in E. coli as a means of facilitating amino acid substitution studies and protein engineering. Previously, we described the construction of amber suppressor alleles corresponding to E. coli Phe, Cys, Pro, Gly, His, Asp, Lys, Arg, Thr, Glu, Ala, Val, Met(m) and Ile tRNAs (Biology 1986, Abstract No. 17). These tRNA genes, constructed from synthetic oligonucleotides, are expressed constitutively from a strong promoter on multicopy plasmids. All of the synthetic genes have been shown to produce functional amber suppressor tRNAs, which vary in efficiency.

Our present efforts are directed at determining the exact specificity of these suppressor tRNAs. We are utilizing an assay described previously (Biology 1985, Abstract No. 15), whereby the tRNAs are used to suppress an amber mutation in a protein-coding sequence. Isolation and sequencing of the resultant protein reveal which amino acid(s) the suppressor tRNA inserts in response to the amber codon. So far by this method, we have determined that the suppressor tRNAs for His, Ala, Phe, Pro, Lys and Cys insert the predicted amino acid. The Glu suppressor inserts glutamine (20%) as well as glutamic acid (80%). Interestingly, the Gly suppressor (derived from the tRNA86t) inserts a mixture of glycine and glutamine, while a Gly suppressor obtained through genetic manipulations of tRNA86t (provided by E. Murgola) inserts only glycine. The Met(m) suppressor inserts predominantly glutamine. This result was not unexpected, as the importance of the anticodon in methionine tRNA synthetase recognition of Met(t) tRNA has already been established. Altering the anticodon of Met(t) tRNA to CUA, as we have done with Met(m), results in mischarging by the glutamine tRNA synthetase in vitro (Schulman and Pelka, 1985).

The most surprising finding was that suppressor
alleles of tRNAs Ile, Arg, Val, Thr and a mutant Asp tRNA all inserted lysine. Only the Thr suppressor inserted some of the predicted amino acid (16% threonine, 84% lysine). This result raises some interesting questions regarding the role of the anticodon in aminoacyl tRNA synthetase recognition. For some tRNAs, Met(t), Met(m), Gwy, Gly, UCC, and Trp (Yaniv et al., 1974), alteration of the anticodon results in partial or complete mischarging by the glutamine tRNA synthetase. The anticodon of tRNAs Ile, Arg, Val, Thr, and Asp would also appear to play a significant role in aminoacyl tRNA synthetase recognition. Why is one group of tRNAs mischarged by lysine and another by glutamine?

It turns out that there are two lysine tRNA synthetase genes in E. coli, one of which is expressed in response to heat shock (Hirshfield et al., 1984). All other species of tRNA in E. coli have only one corresponding aminoacyl tRNA synthetase. Perhaps one of the lysine tRNA synthetases is primarily responsible for the mischarging. If so, we are hopeful that mutation or deletion of the gene encoding the indiscriminate synthetase will diminish the mischarging that we observe.

To date, we have been able to increase the available collection of useful amber suppressor tRNAs from five to 11. To obtain a complete set of amber suppressor tRNAs, it may ultimately be necessary to construct specialized strains in which the gene for an aminoacyl tRNA synthetase corresponding to a given suppressor tRNA is overexpressed in conjunction with the presence of either a mutant glutamine or lysine tRNA synthetase.

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PUBLICATIONS

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Summary: The elucidation of the informational content of the human mitochondrial genome has recently been completed (Biology 1986, Abstract No. 24). The emphasis of our research has now shifted to the nuclear role in mitochondrialogenesis and to nucleo-mitochondrial interactions. At the same time, the information that we have acquired concerning the
general mechanisms of mitochondrial DNA (mtDNA) transcription and RNA processing in mammalian mitochondria and the development of in vitro assay systems have opened the way for a dissection of the enzymatic machineries involved. Furthermore, the identification of the mitochondrial translation products corresponding to all the reading frames of human mtDNA and the availability of polypeptide-specific antibodies have made it possible to investigate whether there is regulation of gene expression at the translational level in mammalian mitochondria.

In the past year, we have made significant progress in the areas mentioned above. Thus, an important breakthrough, with potential far-reaching implications for studies on nucleo-mitochondrial interactions and on mitochondrial gene expression, has been the development of a new approach for mitochondria-mediated transformation involving injection of drug-resistant organelles into sensitive cells. This development may open the way for the much sought after but still elusive mtDNA-mediated transformation of eukaryotic cells. In the area of nuclear gene control of the oxidative-phosphorylation apparatus, cDNAs encoding the human ADP-ATP carrier have been cloned, and evidence has been obtained for the existence in HeLa cells of two forms of specific mRNA for this protein; furthermore, the cloning of the nuclear genes for the six major catalytic subunits of the respiratory chain NADH dehydrogenase is under way.

In the area of mtDNA transcription, an in vitro submitochondrial system capable of supporting transcription of an exogenous template with high specificity is being used to study the mechanisms involved in the control of initiation of transcription on the heavy (H) strand and light (L) strand and termination of rDNA transcription at the 3' end of the 16S rRNA gene on the H strand. A significant finding in this area has been the identification of a requirement for ATP hydrolysis at an early step of mtDNA L-strand transcription. At the same time, we have begun a systematic effort by several approaches to isolate specific proteins that play a role in the regulation of initiation or termination of mtDNA transcription or in mtDNA replication. In another line of investigation, an in vitro tRNA-processing assay system using natural and artificial tRNA-containing transcripts of bacterial or mitochondrial origin is being used both for the purification of the RNase P and the identification of its RNA component and for defining the steps involved in the processing of the polycistronic transcripts of mtDNA.

In the area of mitochondrial protein synthesis, evidence from in vivo pulse-labeling experiments in HeLa cells has shown large variations in the efficiency of translation of the various mitochondrial mRNAs, suggesting that translational regulation plays a significant role in mitochondrial gene expression in mammalian cells, as is the case in yeast. Two complementary in vitro approaches are being utilized for the study of such regulation. One involves the use of HeLa cells permeabilized with digitonin, which provide a means of studying mitochondrial protein synthesis in situ in the absence of soluble cytoplasm, and are thus suitable for the analysis of the role of cytosolic components in controlling translational events. The other approach involves a submitochondrial protein synthesizing system, which is being developed from HeLa cells, utilizing appropriately tailored mitochondrial genes cloned in SP6 and T7 promoter-containing vectors. Along another line of investigation, the regulation of mitochondrial protein synthesis is being investigated in the brain, an organ with a high rate of O2 consumption and high functional demands on the oxidative-phosphorylation system. In particular, changes in mitochondrial gene expression in relationship to development and senescence are being analyzed in rat brain presynaptic endings; this neuronal compartment is particularly interesting in view of the crucial role that mitochondria play in synaptic function.

In the other area of interest in our laboratory, the novel submicroscopic extrachromosomal elements detected by pulsed field gradient or inversion field gel electrophoresis in some human cell lines with dihydrofolate reductase gene amplification (Biology 1986, Abstract No. 29) have been further characterized by restriction enzyme digestion, which has confirmed a size of -650 kb for these elements. Furthermore, electron microscopy of metaphase spreads has revealed the presence of chromatin fibers with a similar DNA content and with an apparently circular configuration, which are probably related to the above elements. Investigations on the structure of these elements, their expression, their stability in the
absence of selective pressure, their capacity to replicate and their role in gene amplification are presently being actively pursued.

150. Control of Transcription of Human Mitochondrial DNA In Vitro

Nalini Narasimhan

We are using a soluble in vitro transcription system to study the mechanisms involved in the control of initiation of transcription on the heavy strand and light strand of human mitochondrial DNA (mtDNA) and the potential termination of rDNA transcription at the 3' end of the 16S rRNA gene on the heavy strand.

A characteristic feature of the human mitochondrial RNA polymerase is that it requires a high concentration of ATP (0.5-1 mM), which is ~15-20-fold higher than that required of the other NTPs. In the crude mitochondrial lysate, the ATP concentrations for optimum transcription of both heavy and light strand mtDNA cover a broad range, with a maximum around 2-5 mM. However, the shapes of the ATP response curves for the two transcription events are different. Non-specific ATP degradation during the assay cannot account for the high ATP requirement. The ATP analog, AMP-PNP, which contains a non-hydrolyzable 8-\gamma bond, cannot substitute for ATP in the transcription reaction, although it can stimulate transcription in the presence of a low concentration of exogenous ATP. We have obtained evidence that AMP-PNP cannot support an early event in human mtDNA transcription (formation of preinitiation complex or initiation), but can substitute effectively for ATP in the subsequent elongation steps. These results indicate that ATP hydrolysis is probably involved in an early event in transcription.

At present, we are investigating the exact nature of this ATP requirement in order to learn whether the RNA polymerase itself or one or more other specific proteins are involved in the ATP-dependent step.

151. Identification and Characterization of Proteins Involved in the Control of Human Mitochondrial Transcription and Replication

Brigitte Kruse, Nalini Narasimhan

The location of all the genes and most of the transcription control elements on the human mitochondrial genome has been determined. There are several lines of evidence indicating that, aside from the RNA polymerase, some specific proteins which interact with the human mitochondrial DNA play a role in the regulation of transcription and/or replication.

At present, we are trying to enrich mitochondrial DNA binding proteins from a crude mitochondrial lysate using DNA-cellulose chromatography. We have prepared small DNA fragments containing transcription or replication control elements in order to analyze the protein fractions by gel retardation experiments. The precise determination of the protein binding sites will be done by DNase I footprinting. Finally, affinity chromatography using chemically-synthesized oligonucleotides corresponding to the protein binding sites should result in an efficient purification of these factors. Functional tests on the purified proteins will be carried out with an in vitro transcription system which has been developed in this laboratory.

152. Characterization of RNase P from HeLa Cell Mitochondria

Ram S. Puranam

Regions of the heavy strand of mitochondrial DNA coding for the rRNAs and poly(A)-containing RNAs are in nearly every case contiguous at both ends to a tRNA sequence. This genetic arrangement supports a model in which the heavy strand of mtDNA is transcribed from either one of two promoters as a single polycistronic RNA. This transcript, punctuated by the tRNA sequences, is processed by precise endonucleolytic cleavages to eventually yield the mature rRNAs, poly(A)-containing RNAs and tRNAs. According to the tRNA punctuation model of mitochondrial RNA processing, the structure formed by the tRNA sequences provides the required recognition signal for the processing enzyme(s) to cleave the primary transcripts. Substrate recognition by one of the mitochondrial RNA processing enzymes appears to be similar to that of Escherichia coli RNase P, an enzyme that generates the 5' termini of mature tRNAs by endonucleolytic cleavage at the appropriate sites in the tRNA gene transcripts.

Earlier work in this laboratory (Doersen et al., 1985) has shown the presence of a ribonuclease P-like activity in HeLa cell mitochondria. This enzyme has been shown to process a precursor to E. coli suppressor tRNA^{TY} at the same site as the E. coli RNase P,
producing the 5' end of mature tRNA\textsuperscript{TYR}. This enzyme has RNA and protein components, which are necessary for its function, as judged by the sensitivity of its activity to micrococcal nuclease and pronase treatment, respectively.

I have standardized the conditions for purifying the RNase P from HeLa cell mitochondria and HeLa cell cytoplasm, where a similar activity resides. For this purpose, I used DEAE-cellulose, phosphocellulose and heparin-agarose chromatography and sedimentation velocity fractionation in glycerol gradients. Work on identifying the RNA and protein components and their coding sequences is in progress.

Reference:

153. RNA Processing in Human Mitochondria
Cesare Rossi

This project is aimed at characterizing the enzymatic activities involved in the processing of the polycistronic mitochondrial DNA (mtDNA) transcripts in HeLa cells. In principle, this phenomenon can be either cotranscriptional or posttranscriptional, and the enzymes could act independently or be organized in a multifunctional complex.

A good working model for approaching this question is offered by the studies on RNA splicing in the nucleus: the first requisite for such studies was the availability of a reaction's precursor, namely, an intron-containing RNA species. Since this molecule can be processed correctly by nuclear extracts, it is now possible to go on to identify the factors responsible for the activity.

Our data from experiments of S1 nuclease digestion of hybrids between ssDNA probes and mitochondrial RNA (mtRNA) enriched in high molecular weight species show that processing intermediates are present in vivo. They contain a tRNA sequence still joined to the 3' end of the RNA coded by the adjacent gene, while a cut has already occurred at the 3' end of the tRNA sequence (see the discussion of the "tRNA punctuation" model, Attardi, 1985).

To satisfy the first requisite mentioned above, several fragments of mtDNA were cloned in plasmids that can be used for the in vitro synthesis of RNA. These RNA precursors were tested in crude mitochondrial lysates, but we were unable to observe any specific cutting.

Unexpectedly, the partially purified mitochondrial RNase P (Doersen et al., 1985), which has a good activity on an in vitro synthesized bacterial tRNA precursor, was also unable to cut the mitochondrial RNA precursors. We are presently further modifying the precursors to elucidate this point. In parallel, the crude mitochondrial lysates will be fractionated, and the various fractions tested for any processing activity; the possibility that the RNase P enzyme is part of a large multienzyme complex (spliceosome-like) will also be investigated.

References:

154. Is There Translational Regulation in Human Mitochondria?
Anne Chomyn

Mammalian mitochondrial DNA contains genes for 13 proteins, as well as genes for two ribosomal RNA species and 22 tRNA species. The 13 proteins are subunits of enzyme complexes involved in oxidative phosphorylation, namely, the rotenone-sensitive NADH dehydrogenase, the ubiquinone-cytochrome c oxidoreductase, the cytochrome c oxidase, and the \( ^{1+} \text{H-ATPase} \). In order to learn about the regulation of gene expression at the posttranscriptional level in human mitochondria, I am conducting experiments with HeLa cells to determine the synthesis rates of the various proteins, and to relate these synthesis rates to the concentrations of the individual messenger RNAs. In one such experiment, mitochondrial translation products were labeled with \(^{35}\text{S-methionine}\) for a very short time, 15 minutes. The various translation products were found not to be labeled to the same extent. Subunits belonging to the same enzyme complex were labeled to a fairly similar extent, but the labeling rates were different for subunits of different complexes. Most importantly, these rates were not proportional to the concentrations of the various messenger RNAs [previously determined in this laboratory (Gelfand and Attardi, 1981)], the ratios of protein labeling to RNA concentration varying over a tenfold range. Since the pulse length was much shorter than the generation time of the cells (16 hr),
and much shorter also than the half-lives of the proteins [expected to be similar to the half-lives of mitochondrial translation products measured in a hepatoma cell line (Hare and Hodges, 1982)], the labeling rates presumably represent translation rates.

Other experiments, involving short labeling pulses in the absence of inhibitory drugs and utilizing peptide-specific antibodies, will be conducted to determine synthesis rates under conditions that better approximate the physiological situation.

References:

155. Synthesis of Mitochondrial Translation Products in Permeabilized HeLa Cells

Anne Chomyn, Ivan Tarle

We are using a method of labeling mitochondrial translation products in digitonin-permeabilized cells (Kuzela et al., 1981) to gain information concerning factors that may play a role in the regulation of mitochondrial protein synthesis. After permeabilization, the cells no longer exclude the dye trypan blue and most of the soluble components of the cytoplasm are released. Mitochondria continue to synthesize protein in such cells and, in fact, the labeling pattern after a 1-hour pulse with 35S-methionine is qualitatively similar to the pattern obtained by in vivo labeling of cells. Mitochondrial protein synthesis in permeabilized cells is greatly stimulated by the addition of ATP, or of ADP, succinate, and phosphate, to the incubation mixture. The system, therefore, lends itself to the study of the energetic control of mitochondrial protein synthesis, as well as to the identification of cytosolic components that may influence this process.

Reference:

1 Undergraduate, California Institute of Technology.

156. Development of a Submitochondrial Protein Synthesizing System from HeLa Cells

Serge G. Alziari

Mitochondrial mRNA cannot be faithfully translated in in vitro translation systems of cytosolic origin because of the difference in codon usage between the cytosolic and the mitochondrial protein synthesizing machinery. We are trying to develop a homologous system from human cell mitochondria partially lysed with a low concentration of a non-ionic detergent or gently sonicated.

To test such a system with an exogenous mitochondrial RNA, this must be supplied in an adequate amount, its products must be distinguishable from those of the equivalent endogenous mRNA, and it must have a structure similar to that of its natural counterpart. In fact, the mRNAs from mammalian mitochondria differ from other mRNAs for lacking a significant sequence to the 5' side of the reading frame.

For the purposes of this work, we have therefore engineered a gene for subunit II of human cytochrome c oxidase (COII) by fusing a synthetic oligonucleotide containing a 5' end proximal segment of the gene and a HindIII site, with a human mitochondrial DNA restriction fragment containing the remainder of the gene. This construct will be inserted into an Sp6-T7 expression vector. This plasmid, cut, or not cut, at different sites within the COII reading frame, will be transcribed in vitro, and the transcripts tested in the in vitro translation system for the synthesis of complete or truncated COII, as revealed by specific immunoprecipitation. The aim of developing this system is to study the regulation of mitochondrial genome expression at the translational level.

157. Effect of Senescence on Mitochondrial Gene Expression in Rat Brains

Valeria A. Gregg

Recent advances in the study of mitochondrial DNA informational content and transcriptional and translational events provide the basis for investigating the regulation of mitochondrial gene expression.

Earlier work from this laboratory measured a fivefold decrease in the incorporation of 3H-uridine into mitochondrial RNA in purified synaptosomes from brains of 30-day-old rats as compared to 10-day-old rats (England and Attardi, 1976). It is of interest to further examine the effect of senescence on organelle-specific protein and RNA synthesis in synaptic mitochondria because of their critical role in synaptic function. The brain is also of interest because its high
rate of O\textsubscript{2} consumption places a great functional demand on the oxidative-phosphorylation apparatus. Currently, we are characterizing by gel electrophoresis and specific immunoprecipitation a cultured line of rat fibroblasts, which will be used to provide the reference pattern of mitochondrial translation products. Cells are labeled with \textsuperscript{35}S-methionine in the presence of emetine and followed by analysis of the mitochondrial translation products in SDS-urea polyacrylamide and SDS-polyacrylamide gradient gel systems. Functional identification of some of the mitochondrial translation products by specific immunoprecipitation with peptide-specific antibodies will be necessary because of the different pattern observed between rat and HeLa cells. We also are assessing the purity of synaptosomal fractions obtained by centrifugation through discontinuous dextran-sucrose and Percoll sucrose gradients.

We then will analyze the rate of synthesis of mitochondrial translation products in synaptosomes from brains of rats differing in age. Further, mitochondrial RNA will be characterized from the reference rat cell line, and steady state levels of mitochondrial RNA assessed in brain synaptosomes from rats of different ages.

Reference:

158. Cloning of the Human cDNA(s) that Encode(s) the ADP-ATP Carrier of Mitochondria

Jane Houldsworth

The ADP-ATP carrier, situated in the inner membrane of mitochondria, is a protein intimately involved in mitochondrial energy metabolism. This protein exchanges cytoplasmic ADP for ATP synthesized in the matrix of the mitochondrion and thus, by regulating the supply of ADP to the matrix space, it may exert control over the rate of oxidative phosphorylation. As for the vast majority of mitochondrial proteins, the carrier protein is encoded by a nuclear gene(s). This project is aimed at cloning this gene from human cells.

A cDNA clone for the homologous bovine gene product was used as a probe to screen a human liver cDNA library in the plasmid vector pEX1 by colony hybridization. Positively-hybridizing recombinants were observed at a frequency of two in 1000 and, on rescreening, could be grouped in terms of insert size and restriction map. Five clones (pHAT 5, 8, 22, 27, and 32) were found to be overlapping, with inserts up to 1120 bp in size. Sequence analysis confirmed that the inserts code for the human ADP-ATP carrier protein, on comparison of the translated sequences to that of the homologous gene product in bovine heart. When used as probes in RNA transfer hybridizations with human poly(A)+ RNA, the inserts of these recombinants revealed the presence of two hybridizing RNA species (approximately 1200 and 1500 nt in size).

One cDNA clone, pHAT 3 (1000 bp), hybridized only to the lower molecular weight mRNA species in RNA transfer hybridizations, and sequence analysis of this clone is now being performed to confirm that the insert codes for the ADP-ATP carrier.

159. Isolation of Nuclear Genes for the Catalytic Subunits of the Respiratory Chain NADH Dehydrogenase

Anne Chomyn, Susan S.-A. T. Lai

NADH dehydrogenase is the first enzyme complex of the respiratory chain of the inner mitochondrial membrane. In animal cells, it is chimeric in its biogenesis, seven of its subunits being encoded in mitochondrial DNA and about 18 others being encoded in the nucleus, synthesized in the cytoplasm and imported into the mitochondria. The number of subunits of this enzyme, which is much larger than that described in prokaryotic systems, raises the possibility that the extra subunits have a regulatory role, possibly related to tissue-specific or growth phase-specific expression. We intend to study the regulation and tissue specificity of expression of the genes for this large enzyme complex and the coordination of the nuclear and mitochondrial genetic systems in the formation of the complex. In another context, the frequent involvement of NADH dehydrogenase deficiencies in the so-called "mitochondrial" diseases makes it important to elucidate the genetic control of this enzyme.

For the purposes described above, we have started a project aimed at cloning the nuclear genes encoding subunits of this enzyme complex, starting with the genes for the six identified catalytic subunits of the enzyme. We are using as probes antisera specific for
bovine subunits. We also plan to use synthetic oligonucleotides corresponding to amino acid sequences derived from the microsequencing of isolated bovine NADH dehydrogenase subunits. Initially, bovine cDNA libraries will be screened, and, subsequently, we will use the bovine clones to isolate the corresponding human cDNA and genomic clones.

160. Mitochondria-mediated Transformation of Human Cell Lines

Michael P. King

As an approach to understanding the molecular genetics of human mitochondria, a procedure is being developed whereby foreign DNA molecules can be stably introduced and expressed in mitochondria in vivo. The first step in developing this methodology has focused on the introduction of intact mitochondria into human cells, in particular, the microinjection of chloramphenicol (CAP)-resistant human mitochondria into CAP-sensitive human cell lines. Mitochondria from the CAP-resistant cell line CAP 23 were isolated and subsequently microinjected into sensitive cell lines. The recipient cells had been depleted of their mtDNA by pretreatment for several days with ethidium bromide, a well known inhibitor of mtDNA replication. CAP-resistant cells were selected and the transformation confirmed by nuclear drug-resistance markers and mitochondrial restriction fragment length polymorphisms which differ between the donor and recipient cell lines. The transformation frequency is estimated to be one in every three to four hundred cells injected. After optimization of the procedures, these studies will be extended to the introduction of isolated DNA into mitochondria either in vitro or in situ to achieve a similar transformation.

161. A Human Cell Line Without mtDNA

Michael P. King

To serve as an improved recipient cell line in the mitochondria-mediated transformation of human cells, a cell line without any mtDNA is being developed. It has previously been shown that the avian cell line LSCC-H32 is inherently resistant to the toxic effects of ethidium bromide when supplied with exogenous uridine (Desjardins et al., 1986). Ethidium bromide is an inhibitor of mtDNA replication. Thus, when cells are grown in its presence, the mtDNA is progressively diluted out during each successive cell generation. The cells become auxotrophic for uridine because dihydroorotate dehydrogenase, a key enzyme in the pyrimidine biosynthetic pathway, is functionally linked to the mitochondrial electron transport system.

The human cell line 143B has been grown for an extended period of time in the presence of ethidium bromide and uridine. This line is currently being analyzed for the presence of mtDNA. In addition, its growth rate and plating efficiency in varying concentrations of uridine and mitochondrial inhibitors are being determined.

Reference:

162. Identification of Novel Submicroscopic Extra-chromosomal Elements Containing Amplified Genes in Human Cells

Barry J. Maurer, Eric H. C. Lal, Barbara A. Hamkalo, Leroy E. Hood, Giuseppe Attardi

In previous work from this laboratory (Masters et al., 1982), several methotrexate-resistant variants were isolated from the human cell line HeLa BU25, which exhibited a high degree of dihydrofolate (DHFR) gene amplification (estimated to be 250- to 300-fold; Morandi et al., 1982). Some of these variants did not contain any chromosome with a homogeneously staining region and exhibited only a small average number of minute chromosomes per cell: these two types of karyotypic abnormalities generally accompany selective gene amplification. An experiment in which a metaphase chromosome preparation from one of these variants, HeLa BU25-10B3, was fractionated in a sucrose gradient, and DNA from the individual fractions was tested with chromosome-specific probes, showed that most of the DHFR-specific sequences are in structures sedimenting slower than chromosome 5, which contains the unamplified DHFR gene (Maurer et al., 1984) and also slower than chromosomes 13, 14, 15, 21, and 22, which contain the nuclear rRNA genes. This experiment suggested that the amplified DHFR genes in 10B3 cells are in extrachromosomal structures not visible in the light microscope due to their small size.

The use of electrophoretic techniques utilizing orthogonal (Schwartz and Cantor, 1984) or inverted field pulses (Carle et al., 1986) has now allowed the
separation of the putative submicroscopic structures as homogeneous DNA molecules of ~650 kb. Furthermore, electron microscopy of metaphase spreads from 10B3 cells has revealed chromatin fibers with a similar DNA content and with an apparently circular configuration, which are probably related to the above elements. These represent a novel type of extrachromosomal structure in mammalian cells.

References:

1Department of Molecular Biology and Biochemistry, University of California, Irvine.


Giovanni Pauletti, Eric H. C. Lai

Our goal is to characterize the novel submicroscopic extrachromosomal elements containing amplified copies of the dihydrofolate reductase gene (DHFR), which have been detected in several methotrexate (MTX)-resistant variants of the human cell lines HeLa BU25 and VA2-B (see Abstract No. 162). A quantitative analysis of various cell populations corresponding to successive points of the stepwise selection of one of these cell lines, HeLa 10B3, is currently under way by non-conventional electrophoretic techniques (pulse-field and field-inversion gel electrophoresis), in order to investigate the time of appearance and the possible quantitative or qualitative changes of the extrachromosomal elements under increasing selective pressure. At the same time, their structure is also being investigated with regard to its topology, the presence of sequences which may be responsible for their replication, and the possible co-amplification of other genes. To this end, a preliminary study is being carried out by long-range restriction mapping with octanucleotide-recognizing restriction endonucleases to determine the size of the amplification unit.

With these parallel lines of research, we aim to further our understanding of the mechanism of the amplification of the DHFR gene and the relationships between intrachromosomal and extrachromosomal copies of the amplified genes.

PUBLICATIONS


Summary: Our laboratory is interested in the regulation of replication of eukaryotic chromosomes. Our studies use yeast because the small genome size allows isolation of the DNA sequences essential for such regulation and since all aspects of macromolecular synthesis are otherwise typical of all eukaryotes. There are two levels of the regulation of replication in eukaryotes. First, since replication occurs only during a portion of the cell cycle, namely S phase, there must be specific events controlling the transition into S phase. In order to establish the molecular basis for entry into S phase, we are using various cell division, cdc, mutants blocked at the beginning of S phase. The second level of control occurs within S phase. At this point, regulation is mediated at the level of initiation of replication within specific replicons, presumably involving specific protein-DNA and protein-protein interactions at origins of DNA replication. Our approach to these problems is a combined genetic and biochemical one.

In the past we have used in vitro replication systems to identify the proteins involved in replication and to demonstrate that ARS sequences are specific origins of replication (Celniker and Campbell, 1982). Recently, we have purified several proteins by biochemical assay that are thought to be involved in replication. We have made antibody and cloned the corresponding genes by screening an expression vector library using the antibodies as probes. The first gene isolated was that of DNA polymerase I. This was an obvious choice, because it was not known when we began our work which of the four cellular DNA polymerases were required for replication. Gene disruption experiments indicate that polymerase I is essential for replication. During the past year we have isolated a battery of temperature-sensitive mutations in this gene and have further explored the role of DNA polymerase I in the cell. We have also used the mutants to initiate studies of the role of a second DNA polymerase, DNA polymerase II, in DNA replication.

The second gene isolated encodes a single-stranded DNA binding protein. Again, we made mutants using the cloned gene and gene replacement. In this case, surprisingly, the mutants were not deficient in DNA synthesis. Instead, we have found, using indirect immunofluorescence, that this protein is localized to the yeast nucleolus providing the first molecular probe to study this important organelle in yeast.

As to the initial events in recognition of an origin of replication, we have extended our studies of the autonomously replicating sequence ARS1 and of other ARSs. We have identified three proteins that bind to yeast ARS1. Two of these proteins bind to a consensus sequence, mutations which reduce but do not abolish ARS function. The third protein binds to a second consensus sequence, in which point mutations completely abolish ARS function. We have prepared antibody to one of these proteins and are instituting the "reverse genetics" approach described above for polymerases and SSBs to these ARS binding proteins. The availability of these proteins should not only improve the efficiency of in vitro replication systems, but isolation of the genes encoding these proteins should yield important mutants in the regulatory aspects of yeast DNA replication.

Another project has focused on the prokaryotic model of DNA replication. The dnaA protein of E. coli is analogous to the yeast ARS-specific proteins in that it binds to the E. coli origin of replication and facilitates initiation there. We have studied the initiation of plasmid replication in bacteria and are now investigating the role of the dnaA protein in that system (Conrad and Campbell, 1979). This offers a paradigm for the yeast work and is an interesting regulatory system in which a small RNA molecule
serves as a negative regulator of initiation through its action as an anti-sense inhibitor. Amplification of genes within the E. coli chromosome is also being studied.

Finally, we have instituted a program of in vitro mutagenesis of an unrelated protein, yeast iso-1-cytochrome c, in collaboration with some of our colleagues in the Division of Chemistry.

References:

164. Cell Cycle Regulation of the DNA Polymerase I Gene

Colin Gordon

Replication of DNA occurs during a discrete period, termed S phase, in all eukaryotic cell cycles. In S. cerevisiae, there are several examples of genes required for S phase showing cell cycle regulation. The histone genes, CDC9 (encoding DNA ligase) and CDC21 (thymidylate synthetase), are all cell cycle regulated. The gene coding for DNA polymerase I was recently cloned in this laboratory and because it is intimately involved in DNA replication, it would seem an ideal candidate to be periodically expressed during the cell cycle.

To test this possibility, RNA was obtained from synchronous cultures prepared by size selection from an elutriating rotor and subjected to Northern blot analysis using the cloned DNA polymerase I gene as a probe. These studies showed that the POU transcript was cell cycle regulated reaching a peak during late Gl.

Future studies will include investigating the level of POU transcript in various blocked cdc mutant strains to determine if the rise and fall in POU transcript is dependent on the completion of specific cell cycle events and the identification of any cis-acting sequences responsible for the cell cycle regulation by deletion analysis.

165. Affinity Purification of S. cerevisiae Proteins Required for DNA Replication

Colin Gordon, Karen Sitney

DNA replication is a complicated process involving interactions among a number of different proteins. Studies in prokaryotes have demonstrated that, in most cases, these interactions turn out to be relatively weak, making them difficult to detect by conventional biochemical methods. One promising approach that has been used to isolate proteins required for DNA replication in the phage T4 is affinity chromatography. Affinity chromatography is able to detect even relatively weak protein/protein interactions when the concentration of ligand protein immobilized on the column is high. This approach will be used to identify proteins involved in DNA replication in the budding yeast Saccharomyces cerevisiae using purified DNA polymerase I protein as a ligand. To aid in the purification of the DNA polymerase I protein, the cloned DNA polymerase I gene was subcloned in front of the GAL10 galactose-inducible promoter. When grown in the presence of galactose DNA polymerase, activity is increased 10- to 20-fold in strains carrying this plasmid.

166. Yeast DNA Replication Origins

Kevin S. Sweder, Judith L. Campbell

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

The functional domains of ARS1 have been dissected using Bal31 and SI mapping (Celniker et al., 1984; Kearsy, 1984) and shown to consist of three domains, which we have designated A, B, and C. Domain A consists of an 11-base-pair (bp) consensus sequence required for ARS activity on centromeric plasmids (Srienc et al., 1985). Domain B immediately flanks domain A and is between 46-109 bp. Domain B also contains an 11-bp consensus sequence 88 bp 3' to the domain A consensus. Deletion of domain B drastically reduces the stability of the plasmids. Domain C, about 200 bp in size, flanks domain A on the opposite side from domain B. Outside of the consensus region, domains B and C show little homology between different ARSs. Our model for the
function of the domains within ARS1 is that domain A is a protein recognition site crucial for DNA replication, analogous to the 9 bp dnaA protein-binding site of E.coli, and the sites recognized by the O protein of λ phage, the T antigen of SV40, or the nuclear factor I of adenovirus. Domain B contains sequences required for proper temporal initiation during S phase and/or as attachment sites to ensure proper segregation. Deletions in domain C have been shown to affect stability of centromeric plasmids but its function remains unknown.

The interaction of DNA-binding proteins with ARSs has been examined. A 55 kDa protein has been isolated which binds within ARS1. Competition binding studies using synthetic oligonucleotides and DNAase I footprinting show that binding is within the domain B consensus sequence of ARS1 and an oligonucleotide column has been used to purify the protein to homogeneity. Antibody has been raised and used to show that there are 10,000 copies of the protein per cell. A second protein, ~90 kDa, has been isolated which also binds the domain B consensus sequence. Both proteins bind to the ARS at HMR-E.

References:

167. In Vitro Initiation of DNA Replication

Etienne-Pascal Joumert

We have purified yeast DNA polymerase I using a bacteriophage T7 overexpression system—an E.coli strain carrying the yeast DNA polymerase I gene under control of the T7 RNA polymerase promoter and the T7 RNA polymerase (Johnson et al., 1985; Tabor and Richardson, 1983). Antibodies against the purified protein were prepared.

Three ARS-binding proteins have been purified in our laboratory on the basis of specific binding to ARS-DNA recognition sequences. We are currently interested in their further biochemical characterization and especially their potential enzymatic activities, such as DNA-dependent ATPases, topoisomerases I and II and helicase. Such activities are known to be associated with SV40 large T antigen and the E. coli dnaA protein, both required for the initiation of DNA replication in the respective systems. Interactions with the purified DNA-polymerase I also will be examined. Our results should help in determining further the proteins and the reaction conditions required for the in vitro reconstitution of an initiation event.

References:

168. Characterization of pollts Mutants from Saccharomyces cerevisiae

Martin Budd

A set of seven temperature-sensitive mutants of DNA polymerase I have been isolated by in vitro mutagenesis of the DNA polymerase I gene. In vivo DNA synthesis is defective in all the mutants at 36°C. A sucrose gradient of newly synthesized DNA shows that at 36°C there is no chromosomal DNA synthesis in the poll-17 mutant. The result shows that in the absence of DNA polymerase I, chromosomal DNA synthesis is blocked. The poll-17 mutant has been crossed into the background, SK-1, which allows for high frequency sporulation. At 35°C poll-17 SK-1 diploids do not sporulate, indicating that DNA polymerase I is required for sporulation. Furthermore, poll-17 spo13-1 double mutants do not sporulate, indicating that polymerase I is required for sporulation even when the first meiotic division is blocked. Repair studies have been done with the poll-17 mutant and have shown that the mutant is capable of repairing X-ray-induced single strand breaks. These results suggest that another polymerase besides polymerase I is involved in repair.

Analysis of DNA polymerase activity in crude extracts of pollts strains revealed the presence of a DNA polymerase activity distinct from polymerase I. This activity has been purified from a poll-17 strain. It is resistant to N2(p-n-butylphenyl)-2'-deoxy­guanosine triphosphate at concentrations that inhibit polymerase I. The activity also copurifies with a 3'-5' exonuclease activity, further distinguishing it from DNA polymerase I and strongly suggesting that it is DNA polymerase II. In the past, studies of the role of polymerase II have been hampered by the small amounts found in wild-type cells. Because of the ease
of purifying this polymerase from the pol1-17 mutant, "reverse genetics" can now be applied to this previously difficult-to-obtain protein.

169. Yeast Single-stranded Nucleic Acid Binding Proteins

Ambrose Jong

In our prior experiments to define the role and interrelationships of eukaryotic SSBs, we have purified three of the major, abundant, single-stranded nucleic acid binding proteins in yeast (Jong et al., 1985): SSB1 = 45 kDa; SSB2 = 50 kDa; SSBm = a mitochondrial species of 20 kDa. Also abundant are a 31 kDa species shown to be homologous to higher cell hnRNPs (Cusick and Abelson, unpublished results) and a 23 kDa species which has not been further characterized. SSB1 stimulates DBA polymerase I and antibody to SSB1 inhibits in vitro replication (Jong et al., 1985). However, we have cloned the gene for SSB1 and gene disruptions show that it is not an essential gene (Jong and Campbell, 1986). Southern analysis suggested the presence of a second, related gene in yeast that might encode a complementary function. Furthermore, Western blots indicate that two proteins of 55 and 75 kDa cross-react strongly with SSB1 antibody and both are increased in abundance in a strain containing only a disrupted SSB1 gene.

Because of the recently demonstrated relationship of higher cell SSBs and hnRNPs, we sequenced the SSB1 gene and compared the sequence of hnRNPs known in other organisms. Although the amino acid composition is similar, there is no significant primary sequence homology between the SSB1 and other proteins (Campbell et al., 1986). Two features of the sequence merit comment in themselves. In the internal portion of the gene, there is a six- to sevenfold repeat of the amino acid sequence RGGF(Y)RG. The meaning of this is unclear, but a similar sequence is found repeated in the rat HD1 gene. The second interesting feature is that the COOH terminus of the protein is very acidic, similar to several prokaryotic SSBs. Of the 18 terminal amino acids of SSB1, nine are glutamate (50%) and one is aspartate (total acidic amino acids >55%). Such an acidic structure may be characteristic of all nucleic acid binding proteins.

References:

170. Biochemical Studies of the Yeast CDC7

Hyejoo Yoon, Ambrose Jong, Judith L. Campbell

The product of the CDC7 gene of Saccharomyces cerevisiae appears to have pleiotropic effects. For instance, a cdc7 mutant arrests in the late G1/S phase at restrictive temperatures. While it is required for the initiation of mitotic DNA synthesis, it is not required for the initiation of meiotic replication. The mutant cells, however, fail to show commitment to genetic recombination during meiosis and do not form ascospores. Furthermore, the CDC7 gene product has been implicated in an error-prone DNA repair pathway, as a member of the RAD6 epistasis group.

We have cloned the CDC7 gene. The RNA transcript of CDC7 is about 1,700 nucleotides, and the size of the protein is 58 or 56 kDa, depending on which of two possible ATG codons initiates translation. It seems that the first ATG codon is used to give about 58 kDa protein, since there was almost no production of the CDC7 protein in E. coli with the disruption of the first codon.

Most interestingly, the CDC7 gene product shares homology with a number of vertebrate protein kinases including those of the src oncogene family and of cAMP-dependent protein kinases (CADPK). The recent S. cerevisiae data suggest that CADPK and the ras gene product may have a key role in cell cycle regulation. If CADPK is inactivated, cells are arrested in G1. There may well be changes in both chromatin and cytoskeletal organization at G1/S transition and these changes may be brought about by the protein kinases phosphorylating specific proteins. A cascade of protein phosphorylation can potentially be involved in cell cycle control.

In order to identify the CDC7 protein and determine its precise function, we cloned the gene into a T7 RNA polymerase/promoter expression vector. In
this system the CDC7 protein represents the major band on SDS-polyacrylamide gels when the E. coli cells are labeled with $^{35}$S-Met in the presence of rifampicin. By taking advantage of this, we have partially purified the CDC7 protein. We will raise antibodies against the purified protein to determine its subcellular locations (s), to predict the cellular proteins with which it interacts, and to further purify it.

171. Does dnaA Protein Participate in the DNA Replication of ColEl-type Plasmids?

Doreen Ma

The dnaA protein of E. coli is required for the initiation of DNA replication at OriC (origin of replication of the E. coli chromosome). This protein binds to a specific sequence within OriC and apparently creates a nucleoprotein complex which is recognized by the proteins that give rise to a DNA replication fork. The dnaA protein consensus sequence is also found adjacent to the origin of replication of the ColEl-type plasmid pBR322. This result, together with mutational and biochemical studies, strongly suggests that the dnaA protein participates in ColEl DNA replication.

Initiation of replication of ColEl-type plasmids is regulated by two trans-acting negative control elements. One of these elements, RNA I, when hybridized to RNA II, the preprimer, interferes with RNase H catalyzed processing of RNA II to produce the mature primer. The other element is the rop gene product (repressor of primer), which is a small plasmid-encoded protein. With the results of our mutational studies, we have suggested that the rop gene product acts by enhancing the RNA II:RNA II interaction and disfavoring the RNA II:DNA hybrid formation (Moser et al., 1984). We are using an in vitro DNA replication system to investigate the effect of dnaA protein on ColEl plasmid replication and the control mechanism mentioned above (Conrad and Campbell, 1979). We have shown that extracts of E. coli that contain the dnaA protein overproduced 100-fold, have similar stimulation of pBR322 replication, compared to wild-type extracts. When a pBR322 derivative, pHM38 (a gift from H. Masai), that lacks the primosome assembly site (n' site) on leading strand but retains both the n' site on lagging strand and the dnaA binding site was tested, a twofold stimulation was observed with the overproducing extract. This suggested that the role of dnaA protein in in vitro DNA replication of ColEl plasmids was related to n' site at the origin. We were also able to purify the dnaA protein to homogeneity, judging from SDS-PAGE. We tested the ability of purified dnaA protein to stimulate pBR322 replication. The amount of purified dnaA protein needed to stimulate in vitro plasmid replication is at least fivefold the amount needed to stimulate OriC replication.

We have also prepared polyclonal antibodies against dnaA protein. Western analyses revealed that anti-dnaA serum was specific towards dnaA protein while preimmune serum had no reactivity towards dnaA protein. Using the serum, we found that the stimulation of pBR322 caused by the overproducing extract was partially due to dnaA protein. Presumably, other replication proteins are limiting in the extracts which led to the result that wild-type extracts have similar stimulation. However, for pHM38, which lacks the leading strand n' site, specific inhibition occurred with anti-dnaA serum. Inhibition could be reversed by previous incubation of anti-serum with the purified dnaA protein. These results together with Seufert and Messer's results (1987), suggested that dnaA protein can bind to the dnaA consensus sequence at the origin of pBR322 and serve as primosome assembly site in place of the n' site and protein i. By binding to the dnaA site at the origin, dnaA protein directs other essential proteins to the replication complex. The interaction of dnaA protein with the two trans-acting elements mentioned above still remains to be investigated.

References:

172. Development of a Method for Improving the Stability of Amplified Foreign DNA in Bacteria

L. Elizabeth Bertani, Giuseppe Bertani 1, Judith L. Campbell

We are investigating the possibility of improving the stability of cloned foreign genes by transferring them first to the chromosome of E. coli and then amplifying them in situ. The vector is a hybrid consisting of the integration system of the temperate
phage P2 cloned into the non-integrating plasmid pBR322. When introduced into a suitable strain of E. coli, the vector integrates very efficiently at the phage attachment site. When extrachromosomal copies of plasmid DNA are eliminated, the single integrated copy continues to confer low-level tetracycline resistance on the host. Amplification of the integrated DNA can be achieved by selecting gradually for increased resistance to tetracycline. In the strains examined, at least 35-40 kilobases of surrounding host DNA are amplified, in addition to the plasmid.

Although the single copy integrants are stable at all temperatures in the absence of antibiotics, the amplified stains are unstable and must be kept under selective conditions. We have attempted to stabilize the amplification by introducing the bacterial recA" mutation into an already amplified strain. Preliminary Southern blots indicate that isolates presumed to be recA" continue to retain the amplification even under non-selective conditions. This suggests that recombination within the repeated structure may be the main cause of instability.

Reference:

1Jet Propulsion Laboratory, Pasadena, CA.

173. The Use of "Nonperturbative," Site-directed Mutagenesis for the Study of Electron Transfer Mechanisms in Metalloproteins

Stephen L. Mayo, John H. Richards1, Harry B. Gray1, Judith L. Campbell

Recent advances in genetic engineering methodology have led to the design of proteins that provide new routes to the study of electron transfer mechanisms. Using site-directed mutagenesis (Zoller and Smith, 1983), we have optimized yeast iso-1-cytochrome c (Montgomery et al., 1978; Smith et al., 1979) for studying intramolecular electron transfer mechanisms via the ruthenium modification method (Margalit et al., 1984). Each mutation was designed to maintain the structural and functional integrity of the protein while providing for the efficient study of the effect of donor/acceptor distance, intervening medium and donor/acceptor orientation on intramolecular electron transfer rates. Mutants were designed on an

Evans and Sutherland PS300 (an interactive computer graphics system) using the protein engineering stimulation capabilities of program BIOGRAPH/I (written by S. L. Mayo, B. D. Olafson and W. A. Goddard III). Currently, we have completed the generation, isolation and characterization of three isostructural/isofunctional mutants of yeast iso-1-cytochrome c. The first two mutations, Cys102/Ser and Ser102/Thr, were designed to circumvent complications in the physical characterizations caused by the cysteine sulfhydryl. The third mutation, His39/Lys (performed on the Thr102 mutant), was designed to provide convenient access to the other naturally occurring surface histidine (His33). We are now concerned with the design rationale and experimental manifestation of the above mutations and the biological and physical characterizations of these systems. The work also addresses the potential of using protein engineering and computer graphics methodologies for the design of novel, metalloprotein-based, redox catalysts (for example, see Margalit et al., 1983).

References:

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PUBLICATIONS

are sense organs such as our eyes shaped and formed during embryogenesis? How are the complex and beautiful pigment patterns generated on the wings of insects? How are they transmitted to the subsequent generations? How are sense organs such as our eyes shaped and formed during embryogenesis?

We still wonder about the same fascinating phenomena but today we can formulate additional specific questions: What sort of address-sensing molecules are present on the one million optic nerve fibers that make their way from their birthplace in the retina to the topologically correct locations in the brain? What sort of genes and chromosomal programs choreograph the movement of neuronal growth cones as they make sharp changes in direction and take a new and correct path?

About two years ago we decided that the time was right to develop a research plan and to undertake a project aimed at answering some of these questions. Based on our background and research experience in molecular biology, immunology, and microchemical instrumentation, we decided that one appropriate first aim would be to isolate cell surface protein molecules which have properties appropriate for cell guidance during pattern formation. When such protein molecules are isolated in picomole quantities, the instruments in the newly developed Caltech Microchemical Facility permit us to determine the amino-terminal sequence of each protein of interest, design and synthesize DNA probes, and clone the relevant genes—all with relative ease.

We took great care in designing the first, most critical step in this approach (others are possible too), the isolation and sequencing of the cell surface proteins. Other molecules known to be important in specific cell-cell interactions (such as Mac-I and LFA-I or the T-cell receptors) are typically present in small amounts on cell surfaces and we assumed the same would be true for address molecules. Because of the relative scarcity of such molecules, many grams of tissue are still required for their isolation in suitable quantities. We expected the same to be true for address molecules in the nervous system. We considered many possible experimental systems and decided on the chick embryo for many good reasons but most importantly because fertile eggs are easily obtainable in unlimited quantities. Three of us then arranged to spend one year at the Max Planck Institute for Developmental Biology in Tübingen, Germany, working with the chick retinal-tectal system. While there we initiated a new, long-term collaborative project with Uli Schwartz and other Max Planck scientists. As a result of our initial efforts we have
isolated and fractionated cell surface proteins, prepared a large panel of very intriguing monoclonal antibodies and isolated and sequenced the first proteins. Even these early results are extremely fascinating; one of the sized fractions of proteins yielded a number of different monoclonal antibodies that react in diverse patterns with axons in the retinal-tectal system. Some of these, "type X," react in a pattern with only some of the axons in the optic fiber layer of the retina. A second class of monoclonal antibodies generated from the same size antigen fraction reacts with most or all of the optic fibers and hence was immediately thought to resemble the L-1 type of molecule that has been implicated in neural pattern formation. Amino-terminal sequencing of the purified antigens revealed that this is the case. The latter antigen had a sequence exactly matching the published amino-terminal sequence of the chicken form of L-1. The type X antigen has a different but evolutionarily related sequence. These results suggest that it is very likely to function in a manner similar to L-1, perhaps in pattern formation.

Our panel of monoclonal antibodies contains a large number of antibodies that react in fascinating and different patterns in the developing chick neural system. The new project seems to be off to a good start, but there is a great deal more to be done.

174. Generation of Monoclonal Antibodies to Surface Proteins of the Retinotectal Tissues from Chick Embryos

Jon Faiz Kayyem, Janet M. Roman, Enrique de la Rosa, Uli Schwartz, William J. Dreyer

In designing the project aimed at generating monoclonal antibodies against cell-surface receptors on neural tissue, we have used as a model the only organ system about which we have extensive experience and detailed information regarding specific cell recognition molecules: the immune system. Given what we know about the T-cell receptor, class I and class II MHC proteins and the MAC-1 family of cell recognition molecules, we are able to make predictions regarding the isolation of specific recognition molecules on other tissues. The most important criteria predicted by prior studies are that specific cell recognition molecules will be scarce and they will be present on the cell surface. We therefore designed our protocols to optimize our chances of detecting scarce cell-surface molecules and determined that the procedures are effective on our model system, the thymus. Using the same procedures, we isolated cell-surface proteins and fractionated them into molecular weight fractions using HPLC. The multiple fractions thus obtained represent a purification of these molecules relative to their amounts on intact cells of several orders of magnitude. Thus, during monoclonal antibody production, when individual mice are immunized with only one of the 12 molecular weight fractions, the antibody response to antigens present in very low amounts will be greatly enhanced over immunization with the original tissue.

Histological distribution and biochemical analyses of proteins reactive with selected monoclonals are described in Abstract No. 175. Further analyses of these antibodies will include functional tests in vivo and in vitro assays, several types of which are in current use by our collaborators at the Max Planck Institute. One such assay is a retinal explant culture in which the effects of antibodies on axonal elongation may be observed.

1Max Planck Institute für Entwicklungsbiologie, Tübingen, Federal Republic of Germany.

175. Histological and Biochemical Analyses of Proteins Reactive with Monoclonal Antibodies of Interest

William J. Dreyer, Jon Faiz Kayyem, Janet M. Roman, Jennifer K. Kim

Our most interesting monoclonal antibodies may be grouped into two general categories based on their staining patterns on cryostat sections of chick retina and tectum. These categories have been termed "A" ("adhesion") and "P" ("pattern") types of antibodies. "A" antibodies stain molecules present on all cells and axons similarly to the group of proteins known as cell adhesion molecules (CAMs). Because of the generalized presence of these molecules on all or most cells, they are not likely to be the only mediators of cell specificity. Some of these "A" type antigens cross-react with the L1/NgCAM family, while others appear to be more like N-CAM. Selected members of this group have been subcloned for use in establishing purification and sequencing protocols; the remainder of this group (>50 hybridomas) are frozen for possible future use. The much more exciting "P" category of
molecules includes several hybridomas that stain specific subsets of axons in the tectum, diencephalon, optic tract and retina. These intriguing patterns differ from one hybridoma to the next and differ from known, published patterns of other molecules. We are currently purifying these molecules (some of which are approximately 125 to 135 kDa and appear to be members of a protein family) for further analysis, amino acid sequencing and, where appropriate, gene cloning. Additionally, we plan to generate further monoclonals against other groups of cell-surface molecules.

**PUBLICATIONS**


Professor Emeritus: Herschel K. Mitchell

Support: The work described in the following research reports has been supported by the National Institutes of Health, USPHS.

Summary: I continue with several parts of our program in developmental biochemistry that have been in progress for several years. The primary system makes use of differentiating wings in the pupal stages of *Drosophila*. This unique system was developed here several years ago in collaboration with Dr. Nancy S. Petersen and the collaboration continues. I am concerned primarily with the proteins that are involved in heat shock and the resulting changes in morphogenesis.

176. Phenocopies and Morphogenesis

*Herschel K. Mitchell, Nancy S. Petersen*¹

We have continued to examine the details of the construction of wing hairs in *Drosophila* with the objective of relating the timing of production of structural proteins and the dynamics of changes in cell shapes and positions. We have isolated in a pure form a protein (cuticulin) that appears to be the precursor to the epicuticle of hairs and cells. The epicuticle is the impervious layer that forms the exterior part of the exoskeleton of all arthropods. In initial stages of hair construction, the protein exists in fibers of approximately 80 Å in diameter. These show in cross sections of primordial hairs as well ordered sheets and bundles. The protein has a molecular weight of 50 kDa and it undergoes spontaneous degradation to yield four major smaller polypeptides. Studies on composition, sequence, and genetic origin are in progress.

For later stages in hair construction, we have refined previous methods of relating patterns of protein synthesis to structure by making use of one wing from a dissected pupa for protein synthesis and the other wing from the same animal for electron microscopy. We have observed unique accordion-like structures in hairs just at the time protein synthesis resumes following a heat shock. The results provide further evidence that abnormal structures which can be induced by heat shock are derived from loss of synchrony between interdependent developmental programs.

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177. Stress Protection in *Drosophila* by Mild Heat Shock

*Herschel K. Mitchell, Nancy S. Petersen*¹

We showed earlier that a mild heat shock administered to *Drosophila* just prior to a shock that would be lethal by itself serves to rescue the animals. This phenomenon appears to be as universal among species and cell types as the induction of heat shock proteins. We have now examined the phenomenon in greater detail with respect to conditions for protection, particularly in relation to the induction of abnormal, but non-lethal, phenotypes. For *Drosophila*, which we usually culture at 25°C, pretreatments between 29°C and 36°C can be effective in preventing several different abnormal phenotypes. Each of the
abnormal characters studied has its own specific time window for induction and each can be induced in 100% of the animals treated. In all cases, the abnormalities can be completely prevented by pretreatments within two hours of the specific time window. All protecting conditions show induction of heat shock proteins to some extent. We presume that one or more of these proteins provides protection directly or indirectly, but no definitive mechanisms of action have yet been established.

1Department of Molecular Biology, University of Wyoming, Laramie, WY.

PUBLICATIONS


Professor: James H. Strauss
Senior Research Associate: Ellen G. Strauss
Visiting Associates: Ricardo Galler, Shlomo Lustig
Research Fellow: Richard Kuhn
Graduate Students: Chang Soo Hahn, Young Shin Hahn, W. Reef Hardy1, Kangsheng Wang
Research and Laboratory Staff: Edith M. Lenches, Nina R. Patel

1Department of Chemistry and Chemical Engineering, California Institute of Technology.

Support: The work described in the following research reports has been supported by:
National Institutes of Health, USPHS
National Science Foundation
United States Army Medical Research and Development Command
World Health Organization

Summary: Our work concerns two families of human pathogens, the flaviviruses and the togaviruses. The alphavirus genus of the family Togaviridae contains about 25 members. Much of our work is with the type alphavirus, Sindbis virus, which is avirulent for man, but for which model systems of neurovirulence exist in the mouse. We are now able to transcribe infectious Sindbis virus RNA in vitro from cDNA clones. This allows us to do a large number of studies including mapping of temperature-sensitive mutants, neurovirulent variants or other markers in the virus genome, as well as site-specific mutagenesis to alter defined regions of the genome and test for the effect on viral replication. A number of the abstracts below report results of this type. Our overall aim is to understand in great detail virus replication and assembly and the interaction of the virus with the host cell; mapping of mutations of defined phenotype in the RNA genome goes a long way in aiding our understanding. Other studies involve characterization of the protease activities encoded in the virus, characterization of the nonstructural proteins of the virus, mapping of neutralization epitopes on viral glycoproteins, and studies to identify and isolate the cellular receptor to which Sindbis attaches in the preliminary stages of infection.

The family Flaviviridae contains about 70 members, many of which are important human pathogens, and is also being intensively studied by our group. The flaviviruses were once regarded as a second genus within the family Togaviridae, but recent sequencing studies from our laboratory and other laboratories have shown that these viruses are quite distinct in their genome organization, leading to their reclassification as a separate family. Yellow fever virus, the type flavivirus, is a well-known and feared virus that caused widespread and devastating epidemics for several hundred years. Epidemic yellow fever is now largely controlled through the control of the mosquito vector of urban yellow fever, Aedes aegypti, and by the use of a highly efficacious and safe vaccine, the 17D strain of yellow fever. We have now compared the nucleotide sequence and encoded amino acid sequence of the wild-type strain of yellow fever with that of the 17D strain in order to identify regions of the virus that have become altered upon attenuation. We have also completed the sequence of the RNA genome of dengue virus, another important flavivirus which annually causes millions of human cases. These comparative studies have told us much about the genome organization of flaviviruses, make possible more detailed studies of the molecular biology of these viruses, and will be useful in vaccine development.
178. Production of Infectious RNA Transcripts from Sindbis Virus cDNA Clones

Charles M. Rice1, Robin Levis1, James H. Strauss, Henry V. Huang1

We have constructed full-length cDNA clones of Sindbis virus that can be transcribed in vitro by SP6 RNA polymerase to produce infectious genome-length transcripts. Viruses produced from in vitro transcripts are identical to Sindbis virus, and show strain-specific phenotypes reflecting the source of RNA used for cDNA synthesis. The cDNA clones have been used to confirm the mapping of the causal mutation of ts2 to the capsid protein. A general strategy for mapping Sindbis mutations has been developed and used to identify two lethal mutations in an original full-length construct that did not produce infectious transcripts (substitution of Ala-382 in nsP2 by Val and Leu-438 by Pro). An XbaI linker has been inserted into the cDNA clone near the transcriptional start of the subgenomic mRNA; the resulting virus retains the XbaI recognition sequence, thus providing formal evidence that viruses are derived from in vitro transcripts of cDNA clones. These cDNA clones have many potential applications in the study of Sindbis virus, some of which are described in other abstracts.

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179. Site-directed Mutagenesis of the Proposed Catalytic Amino Acids of the Sindbis Virus Capsid Protein Autoprotease

Chang S. Hahn

Current data suggest that the precursor of the structural proteins of the alphaviruses is first processed by an autoprotease activity that maps in the C-terminal domain of the capsid protein. We have previously postulated that His-141, Asp-147, and Ser-215 of the Sindbis virus capsid protein form a catalytic triad similar to that found in serine proteases, in part because of amino acid sequence homology between the capsid protein and mammalian serine proteases near these residues. To test this hypothesis, each of these amino acids has been replaced with other amino acids using oligonucleotide site-directed mutagenesis: Ser-215 has been changed to Thr, Ile, or Cys; His-141 to Pro; and Asp-147 to His or Tyr. Each of these mutagenized plasmids, which contain the entire sequence of 26S subgenomic mRNA adjacent to the T7 promoter, was used as template for in vitro transcription using T7 polymerase followed by in vitro translation using a rabbit reticulocyte translation system.

The Ser to Cys mutant retained more than 70% of the protease activity (as measured by the percentage of polyprotein molecules cleaved) and even the Ser to Thr mutant retained up to 10% activity. The Ser to Ile and His to Pro mutants lost the protease activity completely. However, the Asp to His and Asp to Tyr mutants retained 100% activity. These results support the hypothesis that Ser-215 and His-141 are important amino acids in the protease activity, but suggest that the Asp-147 may not participate in proteolysis.

These mutagenized sequences have been substituted for the corresponding domains in the wild-type Sindbis infectious clone in an attempt to isolate mutant virus. However, RNA transcription followed by transfection with these full-length mutant clones has failed to produce viable virus. This suggests that the particular sequence of the C-terminus of capsid protein has essential functions in viral replication and maturation in addition to proteolytic activity.

180. Mapping of RNA Mutants of Sindbis Virus

Young S. Hahn, Charles M. Rice1

Several temperature-sensitive (ts) RNA mutants of Sindbis virus have been isolated, characterized and grouped into four complementation groups. They are presumably defective in one of the nonstructural proteins (nsP1, nsP2, nsP3, and nsP4) that function as the viral replicase. We are trying to determine the function of each nonstructural protein by localizing the ts RNA mutations responsible for each of the complementation groups.

cDNA for one or more ts RNA mutants in each of the four complementations (A: ts21, ts4; B: ts11; F: ts6, 110, 118; G: ts7, 18), as well as their revertants, have been constructed. To assign each phenotype to a specific locus in the viral genome, restriction fragments from the mutant cDNA clones were substituted for the corresponding regions in an infectious full-length cDNA clone of Sindbis virus. These plasmids were transcribed in vitro by SP6 RNA polymerase to produce infectious transcripts which were then assayed for the production of ts virus. The mutations respon-
sible for the group A, F and G phenotypes have been mapped by this method. Sequence analysis of ts6, ts110 and ts118 indicates that ts6 and ts110 have single base substitutions in nsP4, resulting in a replacement of glycine by glutamate at position 152 in ts6 and at position 287 in ts110. However, ts118 has a mutation in nsP2, with Leu in the parental replaced by Pro at position 424. The other mutants require finer mapping and sequence analysis in order to assign them to a specific locations.

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181. Localization and the Functional Assay of the Mutation in ts103, a Maturation-defective Mutant

Chang S. Hahn, Charles M. Rice¹, Ellen G. Strauss

ts103 is a mutant with an interesting phenotype that was isolated in our laboratory a number of years ago. Although intracellular RNA synthesis and protein synthesis are near normal, the assembly of particles is slow and inefficient and many infectious particles contain multiple nucleocapsids within a single envelope. Early in infection, aberrant nucleocapsids are present in the cytoplasm of infected cells which are later found in mature virions only in multicolored particles.

We have localized the mutation that causes the ts103 phenotype by constructing hybrid cDNA genomes between the infectious clone of the parental HRSP (heat-resistant small plaque) genome and that of ts103 and testing hybrid viruses, produced by transfection, for retention of the ts103 phenotype. In this way, the ts103 mutation has been localized between the HpaI site at 6921 nt and the BssHII site at 9804 nt, i.e., in the region extending from the end of nsP4 to glycoprotein E2. We are currently sequencing this region in cDNA clones of ts103 and its revertants.

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182. The Molecular Basis of Neurovirulence in Mice Caused by Sindbis Virus

Chang S. Hahn, Shlomo Lustig, Alan C. Jackson¹, Diane E. Griffin¹, Ellen G. Strauss

Neuroadapted Sindbis virus (NSV) has been selected by serial passage of the wild-type Sindbis virus (SV) in mouse brain. Following intracerebral inoculation, NSV causes a severe encephalomyelitis in mice with hind-limb paralysis and high mortality, whereas SV causes mild disease which is non-fatal.

We have constructed cDNA clones of these viral RNAs and then generated hybrid genomes by swapping restriction fragments into the Sindbis infectious clone. The original Sindbis infectious clone is avirulent for adult mice; to construct a virulent hybrid virus, parts of both E2 and E1 of NSV were required. Sequence comparisons between NSV and SV have shown two amino acid differences in E2 (Gln 55 in SV is changed to His in NSV and Arg 209 in SV, to Gly in NSV). There are also two amino acid differences in E1 (Val 72 in SV is changed to Ala in NSV and Gly 313 in SV to Asp in NSV). However, the HRSP strain of Sindbis virus, which is not neurovirulent, has Gly (209) in E2 and Ala (72) in E1, as does NSV. Therefore, it seems unlikely that these two amino acids are involved in virulence. Thus, His 55 in E2 and Asp 313 in E1 are probably required for neurovirulence. We have separated these two changes from each other in independent hybrid constructions and neither change alone produced the neurovirulent phenotype.

¹Department of Neurobiology, School of Medicine, The Johns Hopkins University, Baltimore, MD.

183. Site-directed Mutagenesis of an Amino Acid Important for Neurovirulence of Sindbis Virus in Adult Mice

Shlomo Lustig, Chang S. Hahn, Diane E. Griffin¹

We have localized two amino acids that are important for neurovirulence of Sindbis virus in adult mice. We have shown previously that there is a difference between wild-type Sindbis virus (SV) and neuroadapted Sindbis virus (NSV) in the E2 glycoprotein: the 55th amino acid is Gln in SV and His in NSV. Even though this amino acid substitution alone, when inserted into infectious clones, did not produce neurovirulence, the hybrid viruses produced from such infectious clones could be discriminated from SV by the same monoclonal antibody that discriminates between NSV and SV. To explore the nature of the neurovirulence further, we have replaced Gln-55 of E2 with Lys, Gly, Glu and Arg using in vitro site-directed mutagenesis. The mutagenized fragment was inserted into infectious Sindbis clones containing either a neurovirulent or non-
neurovirulent background. We plan to rescue virus from these mutagenized infectious clones and test them for the neurovirulence in adult mice.

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184. Deletion Mapping of the Putative Nonstructural Viral Protease of Sindbis

W. Reef Hardy, Milton Schelsinger

It is a common strategy among nonsegmented, single-strand RNA viruses to encode their nonstructural proteins in a polycistronic message that is translated as a polypeptide precursor which is subsequently processed to yield the mature nonstructural proteins. For example, Sindbis virus uses this scheme to produce its four nonstructural proteins, nsP1, nsP2, nsP3 and nsP4, from their precursor through the action of a putative viral protease. We are attempting to map this protease activity to a domain in one of the nonstructural proteins and to determine whether it acts predominantly in cis or in trans to effect the cleavages.

Using an SP6 transcription system to transcribe copies of full-length viral RNA from an infectious DNA clone has enabled us to assay protease function in an in vitro translation system. This affords us greater control over the conditions present during proteolytic processing as well as the ability to tailor the viral genome. Specifically, by making deletions within each of the nonstructural protein genes, we have been able to localize regions that appear to be important for protease function. Furthermore, the use of monoclonal polyclonal antibodies to each of the nonstructural proteins will help us to characterize the products resulting from proteolytic processing of these deleted constructs.

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185. Analysis of the 3′ Conserved Sequence Element of Sindbis Virus RNA

Richard J. Kuhn

Comparative sequence analysis of the 3′ end of alphavirus genomes has identified a region of 19 nucleotides that is highly conserved in all alphaviruses sequenced to date (Ou et al., 1982). This conserved sequence element, which is located at the 3′ end of the genomic RNA immediately preceding the poly(A), has been proposed to serve as the replicase recognition site. We plan to test this hypothesis by site-directed mutagenesis of an infectious cDNA clone of Sindbis virus.

We are currently introducing mutations into the 19-nucleotide conserved sequence element at various positions selected on the basis of the comparative sequence data. RNA from these mutants will be transcribed in vitro using SP6 polymerase and introduced into susceptible cells by transfection. The replication efficiencies of the resulting viruses will be studied. Revertants that restore wild-type replication efficiency will be isolated from those viruses that exhibit defective phenotypes. We believe that a subset of revertants will be second-site mutations located in the protein that interacts with the 19-nucleotide conserved sequence element. We plan to clone these revertant viruses and map the locations of the changes. Identification of the protein that binds to the 19-nucleotide conserved sequence element will be a step forward in elucidating the replication scheme of the alphaviruses.

Reference:

186. Mapping Neutralization Epitopes on Glycoproteins E1 and E2 of Sindbis Virus

Kangsheng Wang, Alan Schmaljohn

Sindbis virus contains two species of glycoproteins (E1 and E2) which are integral membrane proteins present in the viral envelope. They are the major components of viral spikes which play an important role in Sindbis virus attachment to the cell.

To investigate important Sindbis antigens and to explore mechanisms of antibody-mediated protection from viral infection, monoclonal antibodies (MAbs) have been prepared and characterized. Each MAb is reactive with one of the two Sindbis envelope glycoproteins, E1 or E2. Five neutralizing MAbs bound E2 and one bound E1.

We have constructed a λgt11 randomly primed cDNA library of Sindbis virus. Neutralizing MAbs (N-MAbs) were used as primary antibodies and 125I-conjugated sheep anti-mouse IgG as a secondary
antibody to screen a half million plaques. Clones recognized by five individual N-MAbs have been isolated. We are sequencing the insert cDNA to map the individual neutralization epitopes of E1 and E2.

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187. Isolation of the Sindbis Virus Receptor from Chicken Fibroblasts

Kangsheng Wang, Alan Schmaljohn

The entry of an enveloped virus into a host cell is reported to be a receptor-mediated endocytotic event. However, it is thought that at the first step viruses may bind to cell receptors with a low affinity. The application of anti-idiotypic antibodies as specific receptor probes has been a feasible strategy which may overcome the shortcomings of low affinity during isolation and has unique advantages over conventional ligand-based methodology (Gaulton and Greene, 1986). It has been suggested that anti-idiotypic antibodies can mimic the ligands which bind to receptors with a high affinity.

We have raised anti-idiotypic antibodies to neutralizing monoclonal antibodies (N-MAbs) made against the E1 and E2 glycoproteins of Sindbis virus. It has been demonstrated that injection of N-MAbs are sufficient to prevent lethal encephalitis caused by Sindbis virus. This suggests that the site recognized by these N-MAbs may be important for virus-receptor binding.

We have screened Xgtll libraries of cell cDNA with anti-idiotypic antibodies and positive clones have been isolated that specifically associate with these anti-idiotypic antibodies. At this time we are trying to analyze these clones to determine their relationship to cellular membrane proteins which may serve as the viral receptor.

Reference:

1 Virology Division, U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, Frederick, MD.

188. Sequence of the Nonstructural Proteins of O'Nyong-nyong Virus

Randy S. Levinson1, Ellen G. Strauss

In the previous annual report we reported the sequence of the genomes of O'Nyong-nyong virus and Ross River virus in the region encoding nsP3 and nsP4, to show that Ross River (like Sindbis and Middelburg) possesses an opal termination codon (UGA) in the otherwise open reading frame encoding these two polypeptides while Ross River (like Semliki Forest) has CGA encoding arginine at the equivalent position. We have continued the sequencing of O'Nyong-nyong. The original library has been screened by colony hybridization to isolate clones containing the 5'-terminal genome sequences. We are finding that the degree of homology among the encoded replicate proteins varies not only between the particular moieties (nsP4 is the most highly conserved) but also within a given polypeptide. The N-terminal half of nsP2 is strongly conserved; the C-terminal half is only weakly conserved. The C-terminal region of nsP3 shows no conservation; the non-conserved region varies in length from 136 to 246 amino acids among the five viruses examined to date.

1 Undergraduate, California Institute of Technology.

189. In Vitro Reconstitution of the RNA Replication Complex of Sindbis Virus

W. Reef Hardy, Richard J. Kuhn, Randy S. Levinson

The study of Sindbis virus RNA replication in vitro has been hampered by the inability to isolate replication complexes that will respond to exogenous RNA templates. Because of this, the functional identity of the four nonstructural proteins which presumably comprise the replication complex has not been elucidated. Using an infectious cDNA clone of Sindbis, we are attempting to study RNA replication by a novel method. Under the direction of a SP6 transcription promoter, virus-specific RNA will be transcribed and used to program a rabbit reticulocyte translation system. The proteins produced by this system will then be assayed for their ability to bind to specific sequences of the viral RNA and for their ability to copy an exogenous RNA template. Previous work in our laboratory has shown that the polyprotein precursors synthesized in vitro from SP6-transcribed RNA yield mature proteins analogous to those observed in infected cells. The ability to define the products synthesized in the in vitro translation system by genetic manipulation of the cDNA clone will allow us
to separate the different replicase activities that are present in infected cells. In addition, known components from uninfected cells can be added to the translation system to determine the requirements for host components in the replication process. Use of this in vitro reconstituted replication system has the advantage that we can selectively control the individual elements of the system. The system has the further advantage that it relies on the strengths of both molecular genetics and biochemistry.

1 Undergraduate, California Institute of Technology.

190. Conserved Elements in the 3' Untranslated Region of Flavivirus RNAs and Potential Cyclization Sequences

Chang S. Hahn, Young S. Hahn, Charles M. Rice1, Eva Lee2, Lynn Dalgarno2, Ellen G. Strauss

We have isolated a cDNA clone after reverse transcription of the genomic RNA of Asibi yellow fever virus in which the 3' terminal nucleotides from 10,773 to 10,862 from one strand are connected to the sequence from the complementary strand beginning at nucleotide 10,775 and proceeding upstream, that is, toward the 5' end of the genome. We propose that this clone arose by self-priming during first strand cDNA synthesis in which the 3' terminal nucleotide, U-10862, was extended by reverse transcriptase copying of the RNA strand, which would require a 3' terminal structure in which U-10862 was hydrogen bonded to A-10776. We had previously hypothesized, based upon primary sequence data, that such a structure can form in solution and the existence of this clone provides support for this 3' secondary structure. We have also isolated a clone from cDNA made to Murray Valley encephalitis virus RNA in which the first nucleotide corresponds to nucleotide 10,775 of yellow fever. We propose that this clone also arose by self-priming and that MVE RNA may have a 3' terminal structure very similar or identical to that of yellow fever. In addition, we have obtained the 3' untranslated sequence of the S1 strain of dengue 2 RNA. This RNA is also capable of forming a 3' terminal hairpin, using the 3' terminal 79 nucleotides.

These three viruses possess two 20-nucleotide sequence elements which are highly homologous to each other. One of these sequence elements is repeated in MVE RNA and dengue 2 RNA but not in yellow fever RNA. In all three viruses, which represent the three major serological subgroups of the mosquito-borne flaviviruses, the 3' proximal conserved sequence element, which is found immediately adjacent to the potential 3' terminal hairpin, is complementary to another conserved domain near the 5' end of the viral RNAs, suggesting that flavivirus RNAs can cyclize (calculated ΔG < -11 Kcal) and that cyclization is important for virus replication.

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191. Comparison of the Virulent Asibi Strain of Yellow Fever Virus with the 17D Strain Vaccine Strain Derived from It

Chang S. Hahn, Joel M. Dalrymple1, Charles M. Rice2

We have sequenced the virulent Asibi strain of yellow fever virus and compared this sequence with that of the 17D vaccine strain derived from it. These two strains of viruses differ by more than 240 passages. We found that the two RNAs, 10,862 nucleotides in length, differ at 68 nucleotide positions; these changes result in 32 amino acid differences. Overall, this corresponds to 0.63% nucleotide sequence divergence, and the changes are scattered throughout the genome. The overall divergence at the level of amino acid substitution is 0.94%, but these changes are not randomly distributed among the virus proteins. The capsid protein is unchanged, while proteins NS1, NS3, and NS5 contain 0.5% amino acid substitutions, and proteins ns4a and ns4b average 0.8% substitutions. In contrast, proteins ns2a and ns2b have 3.0% and 2.9% amino acid divergence, respectively, and the envelope protein also demonstrates a relatively high rate of amino acid divergence, 2.4% (a total of 12 amino acid substitutions). The large number of changes in ns2a and ns2b, which are largely conservative in nature, may result from lowered selective pressure against alteration in this region; among flaviviruses, these polypeptides are much less highly conserved than NS1, NS3, and NS5. However, many of the amino acid substitutions in the E protein are not conservative. It seems likely that at least some of the difference in virulence between the two strains of yellow fever virus results from changes in the envelope protein which
affect virus binding to host receptors. Such differences in receptor binding could result in the reduced neurotropism and vicerotropism exhibited by the vaccine strain.

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192. Structure of the Dengue 2 Virus Genome

Young S. Hahn, Ricardo Galler, Ellen G. Strauss

cDNA clones have been constructed that together contain the entire nucleotide sequence of the dengue 2 virus genome (S1 candidate vaccine strain derived from PR-159) except for 15 nucleotides at the 5' terminus. Inspection of this sequence reveals a single, long open reading frame of 10,164 nucleotides, which could encode a polyprotein of 3388 amino acids beginning from the first methionine residue [5' C-prM-NS1-ns2a-ns2b-NS3-ns4a-ns4b-NS5 3']. The genome organization is the same as other flaviviruses and the amino acid sequences of the encoded proteins show striking homology to those of other flaviviruses. The overall amino acid sequence similarity between dengue 2 and yellow fever is 44.7%, whereas that between dengue 2 and West Nile is 50.7%. These viruses represent three different serological subgroups of mosquito-borne flaviviruses. Comparison of the amino acid sequences shows that the homology is not uniformly distributed among the proteins; the highest homology is found in certain domains of nonstructural protein NS5 and lowest homology in the hydrophobic polypeptides ns2a and ns2b. The structural proteins are less conserved than the nonstructural proteins. Comparison of the dengue 2 PR-159 sequence with partial sequence data from other strains of dengue 2 and with dengue 4 virus reveals amino acid homologies about 97% and 65%, respectively, in the structural protein region, although the nucleotide sequence conservation is only 90% and 50%, respectively.

193. Expression of Dengue 2 Virus Structural Proteins in Recombinant Vaccinia Viruses

Young S. Hahn, Edith M. Lenches

Dengue virus is the causative agent of millions of cases of human illness worldwide every year. Although dengue fever is primarily a self-limiting febrile illness of children, dengue virus also leads to hemorrhagic fever and shock syndrome with significant mortality. To date there is no vaccine available for any dengue serotype. We hope to construct recombinant vaccinia viruses that express dengue antigen in mammalian cells as a first step towards the development of a safe and efficacious dengue vaccine.

We have obtained a vaccinia virus recombinant that contains a cDNA copy of the portion of the viral RNA encoding the structural protein of dengue 2 virus (S1 candidate vaccine strain) within the thymidine kinase gene of the vaccinia virus genome. This recombinant constitutively transcribes the dengue 2 sequences throughout infection. The dengue-derived transcripts were translationally active, giving rise to mature structural proteins of dengue virus including the capsid protein, prM(M), and the mature envelope glycoprotein. These are the same products as those translated from the 5' portion of dengue 2 genomic RNA during infection. Thus, these proteins were apparently cleaved and glycosylated in a manner analogous to that seen during authentic dengue 2 virus infection.

PUBLICATIONS


Assistant Professor: Barbara J. Wold
Research Fellow: V. Craig Bond
Graduate Students: Raffi V. Aroian, Paul A. Garrity, Paul R. Mueller, Frank Preugschat, David W. Ruff, Sean Tavtigian, Roger A. Wagner
Special Graduate Student: Jane E. Johnson
Research and Laboratory Staff: Jessie Mei Huei Jong, Steve Luber

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Summary: We are interested in the events that lead to selective gene expression. The level of expression from a given gene is determined by the combined effects of transcriptional and posttranscriptional processes. The long-term goal is to identify the cellular machinery responsible for these steps in RNA physiology and to understand how their individual functions are integrated to ultimately give rise to explicit patterns of expression. In several of these projects, we are at the stage where the key step is to define the relevant features of the substrate nucleic acids, while in others we are also studying proteins that interact with DNA or RNA.

Paul Mueller and Steve Salser have continued their investigation of metal-regulated genes and have been able to describe in some detail the in vivo pattern of protein DNA contacts characteristic of basal level or metal-induced transcription from a Met I promoter. This has led them to focus on explicit elements in this complex promoter, and to generate specific mutant promoters to test for the role played by these elements. Jane Johnson has recently joined the group and is completing her thesis work with Prof. Steve Haushka at the University of Washington. Jane has generated transgenic mice carrying either wild-type or mutant versions of the muscle creatine kinase gene in order to define those sequence elements responsible for temporal and tissue-specific regulation.

Dave Ruff, Frank Preugschat and Paul Garrity have been working on the issue of posttranscriptional events in RNA physiology. Dave and Frank use the *Xenopus* oocyte as an experimental system in which to study RNA transport from the nucleus to the cytoplasm. Progress has been made on two fronts: one involves study of the sequence requirements for export, the kinetics of the process, and the role of intranuclear RNA turnover in defining the nature and amount of RNA to be exported; and a complementary study focuses on characterization of the proteins associated with prospective mRNAs in the nucleus, the HnRNPs. Paul is studying the posttranscriptional side of regulation of the Myc gene expression in cultured cells. Myc is of interest for its postulated function as a regulator of other genes and for the means by which it is regulated. An immediate question is what features of the Myc transcript contribute to its posttranscriptional regulation, and Paul is working to define these sequences by making appropriate chimeric genes. In addition he is further characterizing the
steps in RNA maturation and utilization that are regulated by different environmental cues.

Sean Tavtigian, Roger Wagner and Craig Bond are working on Myc expression and function. Sean has generated cell lines and transgenic mice carrying genes that provide conditional overexpression of cMyc RNA and protein, and he is presently testing the effects that this expression has on transcription from other genes that are candidates for Myc-regulated targets. Roger and Craig are studying Myc in Xenopus oocytes and embryos in order to probe the function of this gene product in normal development. Frog Myc cDNA clones have been isolated for two members of the Myc family and their pattern of expression during early development determined. Initial unexpected observations include the high level of Myc mRNA in the oocyte. In all other systems, high levels of Myc RNA are characteristic of mitotically active cells. The clones together with Myc immune reagents are being used to study the accumulation and localization of the gene product at maturation, fertilization and during the early stages of embryogenesis. In several of our projects we have needed to develop new assays or techniques, and this year that has included a new generation of antisense RNA experiments being done by Steve Luber and Jeff Miner and substantial modifications of in vivo footprinting techniques by Steve Salser and Paul Mueller.

194. Genomic Footprinting of Mouse Metallothionein-I

Paul R. Mueller, Stephen J. Salser

The expression of metallothionein can be induced by a variety of agents (metal, glucocorticoids, phorbol esters, etc.), and most of this induction is controlled at the level of gene transcription. Our present understanding of transcriptional regulation suggests that this induction is dependent on the interaction of cis-controlling elements (promoters and enhancers) and trans-controlling elements (transcription factors).

The interaction of such factors with specific DNA sequences can be visualized by DNA footprint experiments. When proteins bind to DNA, they protect or enhance the sensitivity of the DNA to various nucleases (DNase I) or chemical agents (DMS). Traditional footprinting is done in vitro with purified cell extracts and bacterially-amplified DNA. We have modified a genomic sequencing procedure to footprint DNA in vivo and have applied it to the mouse metallothionein-I (MT-I) promoter. In order to perform an in vivo footprint, cells or nuclei are reacted with DMS or DNase I, which footprints the DNA in its native state, i.e., with transcription factors bound to actively-transcribing DNA. This genomic DNA is then purified, radioactively labeled by primer extension, and visualized following gel electrophoresis. At present, this technique yields high resolution when there is a minimum of ~20 copies of the sequence per mammalian genome, and in order to obtain this resolution, we have taken advantage of cell lines that contain extra copies of the MT-I promoter.

We have found two classes of interactions by this type of analysis. One class is over DNA sequences responsible for basal level expression. The metallothionein gene, in addition to being highly inducible, has an appreciable level of transcription in the absence of inducers, which is called basal level expression. This basal level class of interaction can be further divided into the two specific DNA sequences footprinted, a G-rich region (the GTG box) and the SP! recognition site. The GTG box contains a 14-bp sequence which is conserved in all higher eukaryotic metallothioneins. The SP! recognition site is found in many eukaryotic promoters including those of the metallothioneins, and represents a more general transcription element. SP! is one of the few transcription factors that has been purified to homogeneity and, by footprinting the MT-I promoter in vitro with purified SP!, we have shown that the footprint observed in vivo corresponds to the strong binding site observed in vitro. Neither of the footprints over the SP! site or the GTG box change upon in vivo metal induction.

The other class of footprint interaction we have found is only seen upon induction with metal. This inducible footprint is over conserved regions called MREs (Metal Responsive Elements). All five previously identified MREs show footprinting in vivo and in addition, we have found binding at a sixth site which is homologous to the other MREs. Although mutational analyses have shown that MREs are required for metal induction, our in vivo footprints provide the first direct evidence for metal-dependent interaction between the MREs and postulated MRE transcription factors. Finally, preliminary work suggests that there is a second inducible factor called
AP1 or EP1, which binds to a unique site in the MT-I promoter. This factor has been shown by others to be responsive to TPA, a phorbol ester.

1Undergraduate, California Institute of Technology.

195. Site-directed Mutagenesis of Mouse Metallothionein-I

Stephen J. Salser*, Paul R. Mueller

One of the most attractive features of DMS footprinting is that it permits the detection of DNA-protein contacts at nucleotide resolution. Because of this high resolution, DMS footprinting can serve as a guide to site-directed mutagenesis. In site-directed mutagenesis experiments, the idea is to interfere with the action of protein factors on a promoter by altering the sequence elements to which they bind. Comparison of promoter sequences by themselves can lead to discovery of consensus elements and provide clues as to which nucleotides to mutate. The complex, multi-element structure of most eukaryotic promoters, however, makes it difficult to decide which elements are active and how they interact. Promoter deletion studies provide important information, but they are complicated by redundant sequences and the possibility of interactions between regulatory elements. DMS footprinting experiments complement these analyses by allowing one to verify that a given site is involved in protein binding and to choose nucleotides at which close DNA-protein contact occurs.

We have used data from the in vivo footprinting of the mouse metallothionein-I promoter together with consensus sequence information to select three candidate sites for oligonucleotide-directed mutagenesis: the distal SP1 site, the G-rich conserved region, and MRE A. In each case that mutation was chosen so as to interfere both with footprinted nucleotides and with sequence homology. If modification of promoter function results from these changes, additional specific mutations can be tried later.

From the footprinting experiments, it appears that the SP1 site and G-rich region bind factors constantly while the MREs bind factors upon metal induction. By analyzing the mutants we hope to resolve whether or not the constantly bound factors play a role in metal induction. One model is that factors constantly bound to the SP1 site and G-rich region serve to maintain a high basal level of expression while another set of factors binds at the MREs and independently produces an induced level of expression. Alternately, the factors binding to the MREs could act solely through cooperation with basal factors bound at the SP1 site or G-rich region. Previous studies have assayed the functioning of mutant versions of this promoter only under uninduced or fully induced conditions. We hope to address the issue of promoter sensitivity by carrying out a more detailed dose-response analysis on each mutant.

If any of the mutants result in interesting changes in transcription, we may choose to footprint the mutant promoters in vivo. This will not only permit us to verify that the mutation interferes with local factor binding but will also allow detection of secondary effects at other protein binding sites. Such secondary effects would hint at interactions between the various factors.

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196. Titration of trans-acting Regulatory Factors Involved in Mouse Metallothionein-I Gene Expression

Paul R. Mueller

Eukaryotic genes can be regulated at a variety of levels including the transcriptional, posttranscriptional, and translational levels. All of these forms of regulation are thought to involve the interaction of cis-controlling elements with trans-controlling elements, and I am interested in understanding how these trans-controlling elements are influencing gene expression. I have specifically asked this question about metallothionein gene expression. Metallothionein mRNA is expressed at relatively high levels without induction (~200 copies/cell) and this can increase >10 fold upon metal induction. One way to understand how the trans-acting factors involved in the expression and induction of metallothionein act would be to limit their availability. I reasoned that I could create such limiting circumstances by titrating the regulatory factors with a large number of metallothionein cis-controlling sequences. To accomplish this, I transformed mouse fibroblasts with a construct that expresses dihydrofolate reductase (DHFR) under the control of the mouse metallothionein-I (MT-I) promoter. In the initial transformants, this fusion construct and the endogenous MT-I and MT-II genes
were all highly inducible. In order to make cell lines that contain a large number of the cis-controlling sequences, I selected cells containing amplified MT-DHFR genes by using methotrexate, a potent competitive inhibitor of DHFR. Because I have demanded the expression of DHFR, many of the fused cis-controlling sequences will be in an active and functional chromatin configuration. Furthermore, the endogenous MT-I and MT-II genes have remained unchanged by this selection, and can therefore be used to monitor the titration effect by evaluating the state of their expression.

In the presence of several hundred copies of the MT-I promoter, the endogenous metallothionein genes are inducible by metal to only 25% of the level obtained in the parental cell line. Lower numbers of MT-I promoters also reduce induction, but to a lesser degree, as would be expected for in vivo titration of one of more regulatory factors acting on the metallothionein promoters. The reduction in inducibility of the endogenous metallothionein genes shows that the dominant mechanism of metal induction is the action of one of more limiting positive regulatory factors. This is by contract with release from a repressed state maintained by one of more negative regulatory molecules. If the latter were true, I would have expected to see induction or constitutive expression of the endogenous genes as the copy number of the MT-I promoters increased. Basal level expression (in the absence of inducers) is unaffected by the increased gene dosage, which suggests that the factors essential for basal level expression alone are present in relative abundance.

I am presently investigating the level at which these positive trans-acting regulatory factors are interacting with the metallothionein cis-controlling elements. If the interaction is with the metallothionein promoter (as opposed to non-translated sequences in the mRNA), I would expect that the titration effect would be evident by decreased in vivo binding to the metal responsive elements (MREs) in the promoter upon metal induction. The binding of the limiting factors can be assayed by in vivo footprinting and I am using this technique to ascertain whether the binding of the factor(s) to the MREs is titratable (see Abstract No. 194).

197. Analysis of Muscle Creatine Kinase Transcriptional Regulatory Elements in Transgenic Mice

Jane E. Johnson, Steve Haushka, Barbara J. Wold

Skeletal muscle development involves an initial period of myoblast replication, followed by a phase in which some myoblasts continue to proliferate while others undergo terminal differentiation. The latter process involves the permanent cessation of DNA synthesis, activation of muscle-specific gene expression, and fusion of single cells into multinucleated muscle fibers. The muscle-specific isozyme creatine kinase (MCK) can be used as a model to study the mechanisms regulating the activation of the muscle-specific genes.

Several muscle-specific genes including MCK are known to be activated transcriptionally during myogenesis. In earlier work the regulatory elements 5' to the transcription start site of the mouse MCK gene that specify its activation during myogenesis in culture have been identified. A muscle cell type-specific enhancer responsible for high level expression was located between 1050 and 1256 nucleotides (nt) upstream of the transcription start site. Even in the absence of the enhancer, low level expression from 800 nt upstream sequence retains some muscle specificity. In addition to these positive regulatory elements, there is some evidence for negative sequence elements which act in proliferating myoblasts.

In this work we have generated transgenic mouse lines containing various MCK promoter-deleted genes that enable us to study the expression from the MCK regulatory sequences through development in many tissues. For example, do the regulatory elements identified in culture act in a similar manner when integrated into the germline of a mouse? Is the enhancer limited to activity in skeletal muscle or is it also active in cardiac muscle and smooth muscle? Are sequences responsible for tissue specificity separable from those responsible for quantitative control?

To date, we have made transgenic mouse lines containing 3300 nt or 1256 nt MCK 5' sequence fused to the bacterial chloramphenicol acetyl transferase structural gene (CAT). In seven independent mouse lines, CAT activity in skeletal muscle is approximately five orders of magnitude greater than that in non-muscle tissues such as spleen, kidney, and liver. Cardiac tissue has approximately two orders of
magnitude less CAT activity than skeletal muscle per microgram of tissue, but this amount is significantly higher than that found in nonmuscle tissues. We are presently focusing on the enhancer previously defined in cultured cells to determine the minimal sequence that will direct tissue and developmental specificity, and to ask how this is coupled to quantitative control of transcription. We recently established transgenic mice containing just the 200 nt enhancer plus 117 nt MCK basal promoter fused to the CAT gene.

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198. *Xenopus laevis* hnRNPs and RNA Transport

David W. Ruff

Gene expression is regulated at many different levels within the cell. RNA in the nucleus must be processed in order to form a functional mature product for export to the cytoplasm. My goals are to define the RNA structural requirements and begin to describe the trans-acting factors that direct the proper export of mature mRNAs or, alternatively, mediate intranuclear degradation.

Our lab has developed a microinjection assay system for studying nucleocytoplasmic transport of RNA. *Xenopus laevis* oocytes are an attractive system to use for these studies because we can readily micromanipulate the nucleus. Radioactively-labeled RNA is microinjected into the nucleus of *Xenopus* oocytes. The rate of RNA transport is monitored by manually microdissecting the oocyte at various time points and quantitating the amounts of RNA present in the nucleus and cytoplasm. Presently I am studying the effects of various modifications of the 3' end RNA structure on RNA transport. The 3' end of most nonhistone mRNA is first cleaved and then polyadenylated prior to transport. One possibility is that completion of these 3' events serves as a key in identifying molecules to be exported to the cytoplasm. Certain consensus signals present in the 3' end RNA sequence have been shown to direct the polyadenylation process. A polyadenylation signal 5'-AAUAAA-3' is required along with a downstream G+U-rich sequence. The mRNA is cleaved and polyadenylated at a site between these two signals. In these experiments I am asking several questions about the role of the 3' processing events in stabilizing and transporting mRNA to the cytoplasm: What effect does the polyadenylation of RNA have on whether the molecules will be transported? Will non-adenylated molecules be transported at all? What happens to the transport rate if the polyadenylation complex in the oocyte is presented with RNA that has been pre-cleaved at the polyadenylation site? To address these questions I am microinjecting into oocyte nuclei specific transcripts made by in vitro transcription with T7 RNA polymerase.

I am also continuing my characterization of the protein components of *Xenopus* oocyte hnRNPs. Electron microscopy studies have shown the appearance of "beads" on newly synthesized RNA that represent hnRNP assemblies. The positions of the hnRNPs along the RNA are conserved on the newly synthesized RNA molecules for each gene. The positioning of the hnRNPs is thought to play a role in preparing the RNA for processing. I can biochemically prepare hnRNPs by sucrose gradient centrifugation of nuclei extract. Our lab has cloned two of the major proteins present in the *Xenopus* hnRNP. Frank Preugschat has cloned and sequenced the C-protein gene and I have cloned the A-protein gene. A major motivation for cloning and sequencing these genes is to use sequence data to generate antibodies that recognize the hnRNP proteins using synthetic peptides and cloned hnRNP proteins produced in bacteria as antigens. In addition, I expect that the complete sequence of the frog A protein will provide some interesting insight into the evolution of these genes since it is known that there is perfect conservation of the A protein from rat to human.

199. The *Xenopus* hnRNP C Protein

Frank Preugschat

The C proteins are major components of heterogeneous ribonucleoprotein complexes (hnRNP) in nuclei of vertebrate cells. They are closely associated with newly synthesized mRNA precursors and may be required for proper excision of intervening sequences. To begin to describe their structure, expression and function, I have isolated and determined the DNA sequence of *Xenopus laevis* C protein cDNA clones. The protein predicted from the DNA sequence has a molecular weight of 30,916, although its migration in SDS-polyacrylamide gels is significantly retarded. The predicted protein is acidic as is expected from previous biochemical characterization of partially
purified C protein. The protein consists of three domains: a highly structured amino-terminal domain which is conserved among several eukaryotic RNA binding proteins, a central domain unique to C protein among the RNA binding proteins characterized to date, and a highly acidic carboxy-terminal domain. DNA sequence data show that Xenopus C protein is highly homologous with its human counterpart. Each contains within the amino-terminal domain a conserved octapeptide sequence, (ARG/lys)-GLY-(PHE/TYR)-(GLY/ALA)-(PHE/TYR)-VAL-X-(PHE/TYR), which occurs in many nuclear RNA binding proteins of diverse phylogenetic origin. While mammalian genomes contain many copies of C protein sequence, the Xenopus genome contains few. When C protein RNA was synthesized in vitro and microinjected into stage VI Xenopus oocytes, newly synthesized C proteins were efficiently localized in the nucleus. In vitro rabbit reticulocyte lysate and in vivo Xenopus oocyte translation systems both produce from a single mRNA two discrete polypeptide species that accumulate in the ratio characteristic of mammalian C1 and C2 proteins in vivo.

200. Regulation of cMyc Expression
Paul A. Garrity

Expression of cMyc RNA is regulated by both transcriptional and posttranscriptional mechanisms. In addition, unusual sequence features of this RNA suggest the possibility of significant regulation of mRNA utilization, perhaps by regulation of translational efficiency. Myc levels vary significantly in response to extracellular signals such as stimulation of 3T3 fibroblasts by platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), or in conjunction with cell differentiation as is the case in Friend erythroleukemia cells or HL60 cells. The goal of my project is to further describe posttranscriptional regulation and define the points in RNA utilization and/or turnover that are regulated. The ultimate objective is to understand in detail the mechanisms responsible.

Initial experiments have addressed two aspects of the problem. The first is to characterize the expression of Myc genes that have been liberated from the Myc promoter. These genes retain different parts of myc mRNA. The expression of such genes following their introduction into cells by standard transfection procedures should be useful in dissecting posttranscriptional control mechanisms by revealing which features of the gene product confer Myc-like regulation. The first experiment in this set asked whether the Myc protein coding sequence bounded by heterologous 3' and 5' untranslated sequences (see Abstract No. 201) would exhibit any Myc regulation. Initial measurements of expression from this gene indicate that Myc protein coding sequence alone does not confer regulation in the wild-type Myc pattern. Additional constructs carrying Myc 5' and 3' untranslated sequences joined to a heterologous protein coding sequence will be assayed next, as will constructs containing one or both of the native Myc gene intervening sequences.

I have also investigated further the pattern of wild-type Myc regulation. One interesting observation is that cMyc RNA accumulation is significantly increased in response to zinc treatment even though the gene's promoter and intervening sequences lack discernable metal regulatory elements. It is also clear that metal-mediated induction of Myc RNA is distinct from growth factor stimulation because it fails to induce cFos. Thus, metal stimulation does not provoke a round of cell division and effectively uncouples expression of the "competence" gene family. It will be of interest to find out whether the metal response is transcriptional or posttranscriptional.

201. Conditional Expression of Cellular Myc (c-myc) in Tissue Culture
Sean V. Tavitigian

Several lines of evidence indicate that the c-myc proto-oncogene has a profound effect on cellular growth mechanisms. Deregulation of c-myc by transposition, amplification, or proviral insertion has been observed in a variety of tumors. In tissue culture, c-myc overexpression relaxes the growth factor requirements of primary cell lines and increases the frequency with which they become established. Its transient overexpression can also drive quiescent cells through a round of DNA replication that would normally require growth factor stimulation. Its mechanism of action remains unknown.

In cell transformation studies, adenovirus Ela proteins behave in a manner analogous to c-myc. They
are also known to induce the expression of both viral and cellular genes including endogenous HSP 70s and α-tubulins. Similarly, several tumor cell lines express elevated levels of HSP 70. In transient cotransformation experiments, c-myc appears to stimulate the expression of one of two Drosophila HSP 70s and one human HSP 70. Furthermore, c-myc proteins are primarily nuclear in localization and bind to DNA cellulose in vitro. These data suggest that c-myc is involved, directly or indirectly, in regulation of gene expression.

Our approach is to study the effect of regulated c-myc overproduction on expression of genes including several HSP 70s, several α-tubulins, p53 tumor antigen, and EGF receptor. In order to do this, we have stably transfected NIH 3T3 cells with a gene in which the MMTV glycocorticoid regulated enhancer and MT-I metallothionein promoter have been fused to a minimal c-myc coding sequence. Solution hybridization/RNase protection analyses indicate that our myc message is induced five- to tenfold by dexamethasone and ten- to 15-fold by zinc or cadmium. The message has a half-life of about two hours and reaches much higher steady state levels than the endogenous c-myc message. In situ antibody staining experiments performed in collaboration with Ray Koski at AMGen Corp. indicate that our myc protein is strongly induced and localized in the nucleus during Zn induction. Under these induction conditions we have also shown that myc overexpression drives the cells through a round of DNA replication.

By inducing our myc construct and then monitoring the abundance of messages derived from the aforementioned reporter genes by solution hybridization/ RNase protection or Northern blot, we will determine which, if any, of these genes is actually myc inducible. Interestingly, we have noticed that during Zn induction, myc RNA levels increase from basal to steady state over a period of about four hours, while during dexamethasone induction the message abundance peaks between two and four hours of induction and then falls off. This may be a consequence of myc-mediated repression of the glucocorticoid enhancer and will receive further attention. By comparing the kinetics of transcription induction and cytoplasmic accumulation, we will determine the step at which myc acts in the production of the message. From these data, we will develop a scheme to determine the mechanism by which myc participates in the regulation of the expression of these genes.

202. Conditional Expression of Cellular Myc in Transgenic Mice

Sean V. Tavtigian, Jessica A. Dausman, Jane E. Johnson

We have prepared transgenic mouse lines containing several different myc gene constructs. Three lines have been shown to express myc RNA upon acute stimulation with cadmium. RNA from the transgene is most prominently expressed in liver, intestine, and kidney as is generally expected for metallothionein-promoted genes. We are presently characterizing the time course of accumulation, tissue distribution, and transcript abundance of the transgene product during both dexamethasone and heavy metal induction. These mice can be used to ask several questions about myc biology:

1. NIH 3T3 cells are obviously immortal, i.e., they already express one function for which c-myc is primarily recognized. Because they have been studied extensively and their growth factor requirements have been well defined, they serve as a useful experimental system; nevertheless, it seems certain that 3T3 cells limit us to observing only a subset of myc-mediated events. They also represent but one of many cell types that normally express cMyc and appear to be affected by accumulation of Myc protein during the course of proliferation and differentiation. Our transgenic mice provide a source of primary cell preparations of diverse cell types that transiently or chronically overexpress c-myc in a regulated manner.

2. If we can demonstrate myc-mediated regulation of gene expression in tissue culture, our major goal will be to recreate that effect in vitro. Transgenic mouse tissues will provide a plentiful source of myc overproducing cells from which appropriate extracts can be made.

3. Tissue-specific overexpression of c-myc in these transgenic mouse lines will allow a tissue-by-tissue analysis of relative susceptibility to immortalization and growth transformation by c-myc.
The myc family of nuclear phosphoproteins is thought to influence normal and aberrant cell proliferation and perhaps play a role in commitment and differentiation as well. It is presently thought that these proteins act, at least in part, by altering the rate of transcription from a selected subset of genes. In mammals, the gene family consists of at least three (and probably more) distinct members whose expression is specific for different cell types and tissues. Our interest in the function of these proteins in the context of normal growth and differentiation has led us to use *Xenopus* as a model system.

I have isolated cDNA clones for *Xenopus c-myc* and *N-myc*, two of the three distinct myc gene family members that have been observed in mammals. I am presently using the cloned myc genes, along with myc immune reagents, to characterize the expression and activity of the genes and their products in oogenesis and early embryogenesis. Northern blot and quantitative RNase protection assays show that maternal c-myc message is present in large quantities in stage VI oocytes and in early embryos. Its prevalence falls until mid-blastula transition, at which time newly activated embryonic transcription supplants the maternal stores. The unusually high level of c-myc message in the mitotically inactive oocyte contrasts sharply with the pattern of myc RNA expression in all cell types examined previously. In those cases, myc is expressed only in mitotically active cells, and the RNA is extremely unstable, with a half-life of less than 30 min. In contrast to this, I have shown that in early Xenopus development the myc RNA has a half-life of approximately 4 hr as a result of this unusual stability. Moreover, the oocyte contains a very high level of myc RNA absent in other non-mitotic cells. The structure of myc RNA suggests that translational control mechanisms may affect the stability of the message, and therefore the availability of the gene product. Tests of the role of c-myc, N-myc, and related genes may play in oocyte maturation and subsequent fertilization and cell division are being performed. The diversity of the myc family and the pattern of expression in early embryogenesis will be further explored in the coming year.

**PUBLICATIONS**


Summary: The bending of eukaryotic flagella and cilia is generated by active sliding between the doublet microtubules which are the dominant structural feature of these organelles. The active sliding process must be regulated in some manner to provide for the other features of flagellar motion—oscillation and bending wave propagation—and to explain the diversity of flagellar bending patterns.

Much of our experimental study of flagella exploits the ATP-reactivated movements of demembranated flagella, which can be exposed to diverse environments. These movements can be photographically recorded with high spatial and temporal resolution. Computer-assisted analysis of the photographic images allows us to obtain extensive quantitative data on flagellar behavior. Our recent work has emphasized some higher-level regulatory mechanisms involving cAMP-dependent phosphorylation, calcium and calmodulin, with the aim of finding how these regulatory mechanisms interact with critical control points in the basic mechanisms for oscillation and bend propagation.

205. Determination of the Average Shape of Flagellar Bends

Dan Eshel, Charles J. Brokaw

Unfiltered, digitized data from high-resolution photographs of ATP-reactivated sea urchin spermatozoa were converted to functions of angle vs. length and analyzed. The Simplex method was used, as in previous work, to fit model curves to the data and determine the best parameters for the model curves. Three models were compared: sine-generated bending waves, modified to incorporate a linear change in wavelength along the length of the flagellum (MSGM); bending waves containing regions of constant curvature (circular arcs) separated by transition regions with a linear change in curvature (CCM); and a modified constant curvature model in which the curvature within each bend changes linearly with length (graded curvature model, GCM). The GCM fitted the data with less residual error than the MSGM and gave a value for the length of the transition region of 0.17 x wavelength, compared to a value of 0.34 x wavelength obtained when the GCM is fitted to known MSGM curves. Parameters obtained from the fitting...
process were used to normalize the size of each bend, so that the average shape could be determined. The RMS difference between the average shape and a MSGM was large (0.05 rad) and consistent; the RMS difference between the average shape and a GCM was small (0.01 rad) and variable. Approximately 90% of the bends had a decreasing curvature, with a mean decrease of 0.007 rad/µm², so that typical bend with a length of 14 µm and a mean curvature of 0.2 rad/µm displayed a 33% decrease in curvature between the transition regions. A sine-generated bending wave is a very close approximation to the curve that would minimize the elastic energy in a bend if the flagellum has a simple linear elasticity; demonstration that this model does not fit the shapes of real flagellar bending waves provides evidence that other factors determine the bending behavior of flagella.

**PUBLICATIONS**


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**Summary:** Our laboratory has been using yeast as a model genetic system to investigate the mechanisms of protein sorting and organelle biogenesis in eukaryotic cells. Each of the many different subcellular compartments in an eukaryotic cell exhibit unique structural and functional characteristics. These distinguishing features are conferred on each organelle largely by the unique set of proteins that resides within them. Many fundamental questions still remain unanswered as to how these various component proteins are specifically routed from their site of synthesis in the cytoplasm to their correct site of function in the appropriate subcellular compartment. During the past year, we have continued to take advantage of the wide range of genetic and biochemical techniques that can be applied in both yeast and bacteria to identify and characterize, in several different organelles, the sequence signals that specify delivery to a particular target as well as the sorting machinery that deciphers these signals and executes their delivery instructions.

The secretory pathway in both yeast and mammalian cells consists of a complex set of membrane-enclosed compartments that mediate the sorting, transport, and modification of proteins destined for delivery to several different organelles (e.g., ER, Golgi, plasma membrane, and lysosomes). Secretory proteins enter this pathway at the level of the endoplasmic reticulum and then are delivered to the Golgi complex where the bulk of secretory protein sorting is presumed to occur. Within the Golgi, proteins targeted for delivery to the yeast lysosome-like vacuole are sorted from other secretory proteins destined for the cell surface. We have employed a gene fusion approach to map the sequence determinants
present in two vacuolar glycoproteins, carboxypeptidase Y (CPY) and proteinase A (PrA), that direct accurate intracellular sorting of these enzymes. Different sized amino-terminal coding segments of CPY and PrA have been fused to a large, enzymatically-active, carboxy-terminal segment of the normally secreted yeast enzyme invertase. We found that relatively short amino-terminal sequence domains derived from both CPY (50 amino acids) and PrA (76 amino acids) contain sufficient information to quantitatively divert invertase to the vacuole, indicating that these sequences contain vacuolar sorting signals. We have demonstrated that deletion of the putative vacuolar sorting signal from an otherwise wild-type CPY protein results in missorting and secretion of CPY. Interestingly, no significant primary sequence homology was found when the vacuolar sorting signal in CPY was compared to that present in PrA. Potentially, different recognition systems may participate in the sorting of these proteins or, more likely, some other feature of these sequences such as their secondary or tertiary structure may play an important role in the recognition event. We are continuing to employ the gene fusion approach to define additional vacuolar sorting signals so that a more detailed structural comparison of these sequences can be made.

The efficient delivery of CPY-invertase and PrA-invertase hybrid proteins to the yeast vacuole by strains having no other source of active invertase (a sucrose cleaving enzyme) results in the inability of such strains to grow on sucrose as their sole fermentable carbon source. This Suc" phenotype has enabled us to select mutants that "mislocalize" the hybrid proteins to the cell surface. To date, we have isolated and partially characterized 600 such mutants. We have assigned the mutations in these strains to some 35 different complementation groups. In addition, we have found that these mutants all exhibit hybrid protein-independent defects in the sorting of wild-type CPY protein. In the mutants, CPY protein is missorted and secreted from the mutant cells. However, we have evidence indicating that most of these vacuolar protein targeting (vpt) mutants are defective in the sorting of only a subset of vacuolar enzymes. Most mutants still are competent for assembly of the vacuole compartment as determined by microscopic analysis of the mutant cells. A small set of the vpt mutants exhibit extreme defects in vacuolar protein sorting and in the biogenesis of a normal vacuole. Through more detailed biochemical analysis of these mutants combined with an effort to clone certain of the VPT genes and characterize their products, we hope to be able to develop a better molecular appreciation of the mechanisms by which secretory protein traffic is regulated in yeast.

In addition to the secretory pathway, additional protein delivery mechanisms participate in the sorting of proteins to other organelle destinations. Among these, protein sorting to mitochondria has been studied in greatest detail. We and others have demonstrated that the amino-terminal transient presequence on several different mitochondrial proteins is sufficient to direct heterologous "passenger" proteins into mitochondria. No consensus primary amino acid sequence among the known mitochondrial presequences has been found, but most are rich in basic and hydroxylated residues and free of acidic residues. It has been proposed that the presequences may form amphiphilic helices containing high hydrophobic moments which could contribute to their role as import signals.

We have employed the technique of oligonucleotide-directed mutagenesis to further extend the characterization of the mitochondrial import information contained at the amino terminus of the F₁-ATPase β-subunit protein. By analyzing the import properties of a number of mutant F₁-β-subunit proteins, we have found that removal of various portions of the β presequence does not significantly affect the steady-state level of the F₁-β-subunit protein in its proper intramitochondrial location. In fact, by deleting the entire 19 amino acid presequence (except for the initiating methionine residue), we have demonstrated that the "mature" F₁-β-subunit is competent for mitochondrial import in vivo. Based upon a systematic analysis of various combinations of a set of deletion mutations, we have defined three functionally redundant regions of the F₁-β-subunit import signal. Recently, we initiated an in vitro analysis of the mitochondrial import properties of the various mutant β-subunit proteins. Together with continued genetic and biochemical studies of these and other mutants, we hope to be able to define precisely the sequence and structural features contained in this mitochondrial
protein presequence that lead to its selective recognition by the organelle.

Other efforts in the lab are directed at developing new approaches for studying protein transport. An endoprotease present in yeast Golgi is being used as a model protein to analyze the mechanism by which certain proteins are retained within a specific secretory organelle. In addition, we have cloned, sequenced, and identified the product of the yeast SEC18 gene. This gene product functions in the transfer of proteins from the rough endoplasmic reticulum to the Golgi complex. Finally, our group has maintained an interest in bacterial protein export as it serves as a simple genetic system to study the requisite signals and machinery directing protein export from the cytoplasm.

206. Yeast Vacuolar Protein Targeting: Analysis of the Carboxypeptidase Y Targeting Signal

Lianna M. Johnson

Both secreted and vacuolar proteins enter the secretory pathway through the endoplasmic reticulum (ER) and are subsequently sorted to their correct subcellular location (periplasm or vacuole). In order to identify the sorting or targeting signal recognized in vacuolar proteins, we have fused portions of the vacuolar protein carboxypeptidase Y (CPY) to the secreted protein invertase (INV). Through the study of seven hybrid proteins, consisting of amino-terminal segments of CPY fused to a large enzymatically active COOH-terminal fragment of invertase, we have found that the N-terminal 50 amino acids of CPY are sufficient to direct efficient delivery of a CPY-Inv hybrid protein to the yeast vacuole (Johnson et al., 1987). Our data suggest that this 50-amino-acid segment of CPY contains two distinct functional domains: an N-terminal signal peptide that is cleaved at the level of the endoplasmic reticulum (ER), followed by a segment of 30 amino acids that defines the vacuolar sorting signal. Deletion of this putative vacuolar sorting signal from an otherwise wild-type CPY protein leads to missorting and secretion of a proenzyme form of CPY to the periplasm and extracellular medium.

Our studies suggest the following scenario: the N-terminal 20 amino acids of CPY act as a signal sequence to direct and/or assist in translocation of the protein into the ER. After cleavage of this peptide in the ER lumen, a new N-terminal segment is exposed (amino acids 21-50) which functions as a protein sorting signal that is both necessary and sufficient to direct vacuolar delivery of this protein. Final targeting of the protein to the yeast vacuole does not occur until after CPY has traversed compartments of the Golgi complex that contain the mannosyl transferase enzymes responsible for outer chain mannose addition. Presumably, vacuolar proteins are separated from proteins destined for secretion in a terminal compartment of the Golgi complex. This model is consistent with both biochemical and immunocytochemical studies of lysosomal protein targeting in mammalian cells.

Reference:

207. Yeast Vacuolar Protein Targeting: Localization of Vacuolar Proteins in Saccharomyces cerevisiae is Due to an Amino-terminal Targeting Signal

Daniel J. Klionsky, John A. DeModena

The yeast vacuole contains a variety of hydrolytic enzymes including proteinase A and carboxypeptidase Y (CPY). Extensive work has been done on CPY to determine how this protein is targeted to the vacuole (see Abstract No. 206). We have undertaken additional studies utilizing a gene fusion approach to analyze delivery of proteinase A and alkaline phosphatase to the vacuole in Saccharomyces cerevisiae.

Fusion of the gene encoding proteinase A, PEP4, to the SUC2 gene, coding for the normally secreted yeast enzyme invertase, results in the production of a hybrid protein that retains invertase activity. PEP4-SUC2-encoded hybrid proteins containing 76 or more N-terminal amino acid residues of proteinase A are efficiently directed to the vacuole. The information necessary for directing proteinase A to the vacuole then is apparently contained within the 76 N-terminal amino acids of the polypeptide. A similar analysis of PHO8, the gene encoding alkaline phosphatase, has indicated the presence of vacuolar targeting information within the N-terminal 191 amino acids of this protein. Additional gene fusions will allow a more precise identification of these sorting signals and will enable us to carry out detailed comparisons of these
sequences to determine the important general characteristics of vacuolar sorting signals. Common sequence or structural elements in these signals will be mutated to test their functional role in directing the accurate recognition and sorting of these proteins.

208. Regulatory Determinants Affecting Oligosaccharide Processing of Yeast Glycoproteins

Thomas A. Vida

All eukaryotic cells synthesize glycoproteins along the secretory pathway. The sequential biosynthetic steps involved in producing complete oligosaccharide chains are well understood. However, regulatory determinants that control the site-specific addition of precursor sugars to immature oligosaccharides on glycoproteins await complete elucidation.

Saccharomyces cerevisiae, unlike higher eukaryotes, synthesizes oligosaccharides composed solely of mannose and glucosamine (e.g., high-mannose type). Two yeast glycoproteins, carboxypeptidase Y (CPY) and invertase (Inv), contain 4 and 10-12 N-linked oligosaccharides, respectively. In the Golgi complex, mannosyltransferases elongate the common core precursor, Man₈GlcNAc₂, on CPY and Inv to different extents. CPY receives no more than 10, while Inv receives as many as 150 additional mannose residues.

Previous studies have shown that the amount of CPY sequence encoded in CPY-Inv hybrid proteins can influence the extent of mannose elongation in the Golgi complex (Johnson et al., 1987). Hybrids with 20, 50 or 95 N-terminal CPY amino acids show oligosaccharides that are elongated like wild-type Inv. A hybrid protein containing 433 CPY amino acids shows oligosaccharides that are not elongated or only slightly elongated in the Golgi complex. These results suggest that a region of protein sequence between amino acids 95 and 433 of CPY may in some way limit or prevent the action of specific mannosyltransferases. Gene fusion and hybrid protein expression in yeast provide the framework of a model system to study this regulation of glycoprotein biosynthesis. The construction of new fusions is under way to further map the determinant(s) that affects mannose elongation in the Golgi complex. This approach will test the hypothesis that unique aspects of protein structure can determine the ultimate structure of oligosaccharides present on glycoproteins.

References:

209. Isolation and Characterization of Vacuolar Protein Targeting (vpt) Mutants in Yeast

Jane S. Robinson

The protein constituents of the many distinct organelles in yeast are synthesized in the cytoplasm before being directed to their proper subcellular locations. The secreted enzyme invertase (Inv) and the vacuolar hydrolyses, carboxypeptidase Y (CPY) and proteinase A (PrA), appear to transit together through early stages of the yeast secretory pathway. During passage through the Golgi complex, these proteins are sorted and targeted for delivery to their respective destinations.

When certain CPY sequences are fused to the sucrose-cleaving enzyme invertase, they efficiently divert this normally secreted enzyme to a new intracellular destination, the yeast vacuole (see Abstract No. 206). The efficient delivery of such CPY-Inv hybrid proteins to the vacuole by yeast strains having no other source of active invertase results in the inability of such yeast to grow on sucrose as their sole fermentable carbon source. This phenotype has enabled us to select for mutants that "mislocalize" the CPY-Inv hybrid protein to the cell surface (Bankaitis et al., 1986). Most of the mutants have been found to contain recessive genomic mutations. At present, these recessive mutations have been assigned to at least 35 different complementation groups, suggesting that accurate protein targeting to the vacuole requires a large number of trans-acting functions. These mutants, defective in protein targeting to the vacuole, have been designated vpt for vacuolar protein targeting mutants.

By radioactively labeling and immunoprecipitating wild-type vacuolar proteins, the vpt mutants have been shown to exhibit hybrid protein-independent defects in the vacuolar sorting of wild-type CPY protein. Precursor forms of CPY accumulate in the mutants and are secreted into the yeast periplasm and extracellular medium. In some of the mutants, defects are also seen in the processing and sorting of wild-type PrA. Several vpt mutants exhibit other interesting phenotypes, including defects in growth at 37°C and assembly of a normal-looking vacuole (see Abstract
Further genetic and molecular analyses of the \textit{vpt} mutants, currently in progress, should provide useful information about the mechanisms by which yeast regulate protein traffic to the vacuole.

\textbf{Reference:} \\

\section*{210. Characterization of Yeast Vacuolar Protein Targeting Mutants}

\textit{Lois M. Banta}

We are interested in the mechanism by which vacuolar proteins are sorted from other secretory proteins and then targeted specifically to the vacuole. Other work in this laboratory has identified at least 35 complementation groups defining \textit{trans}-acting functions that affect the targeting of vacuolar proteins (see Abstract No. 209). We have undertaken a phenotypic characterization of these vacuolar protein targeting (\textit{vpt}) mutants, with an emphasis on their morphology. In order to visualize the vacuoles in these cells, we have used Nomarski optics and three fluorescent dyes which are localized to the vacuole by different mechanisms (FITC-dextran, quinacrine, and a biosynthetic intermediate of adenine). In addition, electron microscopy has allowed a more detailed ultrastructural analysis of the morphology of these mutant strains. These studies have revealed at least three distinct groups of \textit{vpt} mutants. The class A mutants, comprising 28 complementation groups, resemble the wild-type parent strain in that they have a single, large vacuole which is easily observed using light microscopy. A second class of mutants, class B, consists of three complementation groups and is characterized by an altered morphology in which the vacuole is highly fragmented. Mutants in the four class C complementation groups have no discernible vacuoles. These mutants exhibit extreme morphological defects such as the accumulation of large membranous structures and small vesicles throughout the cytoplasm. Other organelles in these mutants (nuclei, mitochondria, ER, etc.), however, appear to be normal in appearance and function (nuclei, mitochondria, ER, etc.).

In addition to the above morphological classifications, the \textit{vpt} mutants have been analyzed by a number of other phenotypic criteria. Cells in one complementation group are extremely sensitive to low pH, while others are unable to grow under conditions of osmotic stress. A subset of the mutants exhibits defects in the normal physiological responses involved in yeast mating type interactions.

The large number of complementation groups and the variety of phenotypes associated with the \textit{vpt} mutants suggest that a number of gene products, acting at different stages in the targeting process, are involved in the localization of proteins to the yeast vacuole. The altered morphologies observed in certain \textit{vpt} mutants suggest that vacuole biogenesis is also impaired in some of these mutants. Efforts to clone the genes responsible for these defects are in progress. These studies should help elucidate the role played by these gene products in vacuole biogenesis and vacuolar protein targeting.

\section*{211. Cloning and Characterizing Genes Responsible for Vacuolar Protein Targeting Mutants}

\textit{Raffi V. Aroian, Paul K. Herman}

As reported elsewhere (Abstract No. 209), this lab has isolated vacuolar protein targeting (\textit{vpt}) mutants in yeast. To date, these recessive mutations have been assigned to over 35 different complementation groups. We have initiated an effort to clone certain of the \textit{VPT} genes. Selected \textit{vpt} mutants are being crossed with one another to rigorously demonstrate that mutations assigned to distinct complementation groups do indeed correspond to mutations at different genetic loci. Mutants of interest are being transformed with a yeast genomic library and the transformants are being tested for complementation of the \textit{vpt} mutant phenotype. Complementing clones will be integrated and mapped to verify that they correspond to the correct \textit{VPT} locus. Ultimately, by sequencing these genes and generating specific antibody probes for their products, we hope to be able to study the biogenesis of these proteins and get some insights into how they may be functioning in the vacuolar protein sorting pathway. Further genetic experiments also will be carried out with mutants of particular interest.

\section*{212. Mitochondrial Protein Import in Yeast}

\textit{David M. Bedwell}

Greater than 90\% of the proteins found in mitochondria are encoded by nuclear genes. Since the
protein species found in mitochondria are distinct from those found in other cellular locations, specific mechanisms must exist that facilitate their transport from the cytoplasm to their final mitochondrial location. It is known that many proteins destined for mitochondria are initially synthesized with an NH₂-terminal leader sequence that presumably facilitates the binding and import of these proteins into mitochondria. However, very little is known about either the important features of this leader sequence or the components of the import apparatus that associate with these sequences during the import process.

The NH₂ terminus of the yeast F₁-ATPase β-subunit precursor directs import of this protein into mitochondria. In order to define the functionally important components of this import signal, oligonucleotide-directed mutagenesis was used to introduce a series of deletion and missense mutations into the gene encoding the F₁-β-subunit precursor (Bedwell et al., 1987). Among these mutations were three non-overlapping deletions, two within the 19 amino acid presequence (Δ5-12 and Δ16-19) and one within the mature protein (Δ28-34). Characterization of the mitochondrial import properties of various mutant β proteins containing different combinations of these deletions showed that import is blocked only when all three deletions are combined together. All possible single and pairwise combinations of these deletions each were found to retain the ability to direct mitochondrial import of the F₁-β subunit. These data suggest that the F₁-β subunit contains redundant import information at its NH₂ terminus. In fact, deletion of the entire F₁-β-subunit presequence did not prevent import, indicating that a fully functional mitochondrial import signal is present in the mature protein. Furthermore, by analyzing mitochondrial import of the various mutant proteins in a− yeast, we have obtained evidence indicating that different segments of the F₁-β subunit import signal may act in an additive or cooperative manner to optimize the import properties of this protein. We also have initiated an in vitro analysis of the import properties of these mutant F₁-β-subunit precursors with the hope that we will be able to identify the exact step of the import process that is defective in these mutants.

Reference:

213. The Generation of a Library of Mitochondrial Sorting Signal Mutations
Gregg D. Jongeward, Kyuson Yun

The β subunit of the F₁-ATPase complex (encoded by the ATP2 gene) has been shown to contain redundant mitochondrial protein import information at its amino terminus (see Abstract No. 212). A functional F₁-ATPase complex is necessary for growth on a non-fermentable carbon source, such as glycerol. To analyze the critical sequence and structural components of this mitochondrial sorting signal, we have constructed a large (76 bases) mutagenized oligonucleotide encoding a subsequence (14 amino-terminal amino acids) of the β-subunit transient leader peptide which functions as an efficient mitochondrial import signal. During its chemical synthesis, the region of the oligonucleotide encoding this peptide was mutagenized by doping certain of the nucleotide coupling reactions with small amounts of all four base substrates (A, T, G, and C). The resultant single-stranded 76-mer was then annealed to an 11-base DNA primer complementary to its 3' end, extended with DNA polymerase, and cut at restriction sites synthetically introduced at both ends of the oligonucleotide to give a 66 base pair restriction fragment. This was cloned into a F₁-β subunit expression plasmid (cut with the same two restriction enzymes) in which the ATP2 gene was deleted for all mitochondrial targeting information. This gives rise to a library of mutations in a F₁-β mitochondrial import signal, some of which should be silent (Gly±), some of which should cause slower growth on glycerol, and some of which should be defective (Gly−). By analyzing this library, I hope to be able to determine which amino acid residues contribute most significantly to the function of this sequence. I also hope to isolate second-site revertants of some of the Gly− mutants which may allow us to identify and characterize components of the mitochondrial import machinery.

214. Investigation of the SEC18 Gene of Saccharomyces cerevisiae: Characterization of a Component of the Yeast Secretion Machinery
Kurt Eakle

A series of temperature-sensitive mutants that have defects in the process of protein secretion/localization have been described in yeast (Novick et
These mutants accumulate subcellular organelles analogous to the endoplasmic reticulum, Golgi apparatus, and secretory vesicles described in the secretion pathway for cells of higher eukaryotes. One mutant, sec18, accumulates the yeast equivalent of endoplasmic reticulum and is blocked for protein transport to the Golgi apparatus at the nonpermissive temperature. We are interested in characterizing the location and function of the product of this gene.

The SEC18 gene of *S. cerevisiae* was cloned from a genomic YEp13 plasmid library by complementation of the sec1858 defect. Gene disruption with the LEU2 gene has shown that the cloned DNA indeed maps to the SEC18 locus and that the SEC18 gene is essential for yeast cell growth. The complementing activity maps to 2.5 kb at the HindIII end of a 3.0 kb BamHI-HindIII fragment. DNA sequence analysis of the region reveals a 2271 bp open reading frame that would code for a protein of 83.9 kDa which is generally hydrophilic in nature with no evidence of a signal sequence or transmembrane spanning domain. Northern blot analysis shows that a 2500 bp polyadenylated message is transcribed from the gene in the orientation of the open reading frame. Mapping of the 5' and 3' ends of the mRNA reveals that the message includes about 30 bp upstream from the first AUG of the open reading frame and continues for about 125 bp after the termination codon of the open reading frame. *In vitro* transcription and translation of the minimum complementing fragment of our clone produced two proteins of approximately 84 and 82 kDa.

Random BAL31 deletions were used to create SEC18-lacZ gene fusions that overproduced hybrid proteins in *E. coli*. These were purified and injected into rabbits in order to produce antisera that could recognize both of the proteins produced by *in vitro* translations. Immunoprecipitation of 35S-labeled whole yeast cells identifies two proteins of the same size as seen in *in vitro* translation. Pulse-chase experiments show that the two forms of the protein are not the result of proteolytic processing. We believe that the production of two forms of the Sec18 protein seen both *in vitro* and *in vivo* represents the use of alternative AUGs about 60 bp apart at the 5' end of the mRNA. These proteins show no evidence of N-linked glycosylation, even though potential sites for glycosylation are revealed in the sequence. The exact intracellular location of Sec18 is currently under investigation; however, these results suggest that the Sec18 protein is probably located in the cytoplasm of the yeast cell.

**Reference:**

### 215. Delivery and Retention of a Golgi Protein

**Paul K. Herman**

Studies with secreted proteins in eukaryotic cells have led to the identification of a general protein secretion pathway. However, during the course of these studies, little attention has been focused upon those protein molecules that are specifically retained within the endoplasmic reticulum or the Golgi complex. The correct and efficient localization of such proteins is necessary for the establishment of the morphologically and functionally distinct environments of these organelles. We are interested in characterizing the targeting signals and the cellular machineries responsible for the specific retention of proteins within the Golgi complex.

We have chosen to study the intracellular localization of the *KEX2* gene product in the yeast *Saccharomyces cerevisiae*. Present evidence from a number of studies has indicated that the Kex2 protein resides within the Golgi. We have constructed and analyzed a series of *KEX2* gene fusions to *SUC2*, the structural gene for the normally secreted invertase protein. From these studies, we have been able to identify *KEX2* sequences that are capable of retaining the fusion protein within the cell. In order to determine the intracellular location of these fusions, we have made use of polyclonal antisera directed against α1-3 linkages between mannose residues in glycoproteins. In practice, this antiserum will recognize only Golgi-modified forms of both the Kex2 and invertase proteins. We have shown that this antiserum does recognize our fusions and therefore that our fusion proteins have, at least, arrived in the Golgi. Experiments presently under way are aimed at characterizing the signals present within the Kex2 protein that are responsible for this specific intracellular localization. We also are currently employing the fusion proteins in attempts to generate yeast mutants that mislocalize the wild-type Kex2 protein as
as well as the Suc2-Kex2 fusion protein. The analysis of such mutants will hopefully lead to a better understanding of the cellular mechanisms responsible for this Golgi localization.

216. Mapping an Internal Export Signal in LamB

Elliot Altman

An observation has been made in E. coli that the cytoplasmic synthesis and accumulation of a normally exported protein harboring a defective signal sequence interferes with the export of other secreted proteins (Bankaitis and Bassford, 1984; Bankaitis et al., 1987). This interference, which is measured by precursor accumulation, can be explained by hypothesizing the presence of a second, internal export signal (I.E.S.) in the mature portion of certain exported proteins. This I.E.S. sequence may interact with a component of the cellular export machinery and thereby limit its availability for promoting the export of other secreted proteins.

To test this model, we have mapped and partially characterized the I.E.S. in an E. coli outer membrane protein, LamB. The I.E.S. site in LamB maps between amino acid residues 320 and 370 of the mature protein (mature LamB contains 421 amino acids). The map position was determined by constructing lacZ-lamB fusions (3' coding sequences of lamB were fused to the coding sequence for the first seven amino acids of LacZ) as well as lamB-lacZ fusions. The resulting fusion proteins were expressed in E. coli and tested to see if they interfered with the export of a second secreted protein, the normally periplasmic maltose binding protein (MBP). Fusion proteins whose expression led to the cytoplasmic accumulation of precursor MBP were assumed to contain a functional I.E.S. sequence.

To further confirm the location of this sequence, the mapped I.E.S. was deleted from LamB using site-directed mutagenesis. Two related observations indicate that the I.E.S. was successfully deleted: 1) the synthesis of an export-defective LamB protein harboring an I.E.S. deletion does not interfere with the export of other secreted proteins (MBP, OmpF, etc.), and 2) the synthesis of an export-defective MBP protein no longer interferes with the export of LamB protein when it lacks the I.E.S. sequence.

Current experiments are directed at determining which components of the cellular export machinery interact with the I.E.S. and what function this interaction plays in promoting protein export in E. coli. It may play a role in maintaining LamB protein in a stable tertiary conformation which is competent for membrane translocation.

References:


Publications


Professor: John J. Hopfield

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Summary: The general area of interest is the physics of how biological processes take place. We attempt to abstract from biology important chemical and physical properties, to understand, through theory and experiments, how these properties may come about in terms of structure, dynamics and context, and to then take these results back into biological systems to test out findings. Molecular areas in which studies have been made include the relationship between protein structure and local group chemical kinetics of heme proteins and the mechanism of biological electron transfer processes. The information processing of a network of neurons is also a physical process, and the "computational" behaviors of such nets can be approached in terms that make use of concepts from statistical mechanics. We attempt to relate such ideas to processing in simple biological systems and subsystems such as taste learning, a categorization in *Limax maximus*.

217. Emergent Properties of Neural Networks

*Eric B. Baum, David Feinstein¹, John J. Hopfield, John Platt¹*

Emergent or collective properties of systems are behaviors that arise through interactions of many similar objects and are not displayed or directly anticipated from the properties of very small systems. One of us has previously given simple models of neural networks that utilize emergent behavior to perform tasks such as content addressable memory. We have more recently been extending these models, on the one hand to be more biologically realistic, and on the other hand toward practical applications in signal processing and computation. It now seems reasonable to hope for practical implementations in the near future. Applications to combinatorial optimization problems, error correction in noisy channels, data compactification, learning algorithms, and pattern recognition are being studied. This involves improved understanding of the dynamics of network decision making and the effects of hierarchy in neuronal connections. Realization of such ideas in electronic circuit hardware of novel design is under consideration. In addition to heuristic reasoning and simulation, we are also applying mathematical tools to prove rigorous theorems about these models and related physical systems such as spin glasses.

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218. Electron Transfer Processes

*David N. Beratan¹, Alvin D. Joran², Peter B. Dervan³, John J. Hopfield, Jose Nelson Onuchic¹, Burton A. Leland⁴*

Biological electron transfer reactions typically involve exchange of electrons over large distances (10-20 Å). These electron transfers are atypical chemical reactions because their rates are determined by very weak donor-acceptor interactions. A body of mostly untested theoretical ideas has evolved to relate the dependence of transfer rate on distance and driving force. With these ideas in mind we have synthesized the family of molecules where the distance (N = 0, 1, 2) can be varied. Exothermicity also can be varied by metal choice M or by peripheral substitutions on the porphyrin or quinone. Some measurements of the transfer rates have been performed. Theoretical predictions of the dependence of rate on N have been made (see figure below).

![Figure 1](image-url)

Conventional work has been done to explicitly include the structure of the intervening bonded medium in the rate calculation. Electron exchange between donor and acceptor occurs slowly relative to bond vibrations on the donor and acceptor. This may invalidate the time scale separations that are generally used in chemical physics. New theories for charge transfer have been developed without the usual time scale separations and which allow electronic coupling to fast and slow ("diffusive") vibronic modes. Fully quantum mechanical models have been used to couple the "heat bath" provided by the surrounding...
medium to the charge transfer process. Such models provide an understanding of which time scales are important in the electron transfer problem.

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Summary: My laboratory continues to be interested in the structure of gap junction channels, which permit the exchange of small molecules between adjacent cells. We are still very excited by our recent finding of multiple gap junction proteins in the same junction plaques, and have been gratified to see the result confirmed in several other tissues. We are continuing our analysis of the main intrinsic protein (MIP) of the lens: evidence is accumulating in support of its involvement as a lens gap junction protein. Barbara Yancey and Kris Koh (Undergraduate, California Institute of Technology) have made the interesting discovery that message for MIP as detected by in situ hybridization is not uniformly distributed in the lens fiber cells. In addition they have found that the distribution of message coding for MIP is different than that of message coding for a-crystallin. We are carrying out a major effort in isolating highly purified undegraded heart gap junction proteins and preparing both polyclonal and monoclonal antibodies against these proteins. This has taken much of the time of Brian Austin and Alex Battaglia. Their work will provide the basis for deepening the understanding of these interesting molecules. There is also a renewed interest in the junctions of primitive animals, particularly the mesozoans, which now seem to be the earliest multicellular organisms extant today.

219. MIP Gene Expression During Development

S. Barbara Yancey, Kristine R. Koh1, Brian J. Austin

MIP, the main intrinsic protein of the eye lens fiber cells, is believed to be involved in the formation of the cell-to-cell channels that interconnect the lens fiber cells and provide the pathway for exchanges of metabolites in this avascular tissue. MIP is the product of a single gene (Biology 1984, Abstract No. 159) which does not seem to be expressed in any tissue other than the lens. This specificity of expression and the fact that the protein is highly conserved among species suggests that MIP is of fundamental importance for the development and specialized function of the lens. We have used an antisense RNA probe (Biology 1983, Abstract No. 192) for in situ hybridization and a polyclonal antibody for immunofluorescence microscopy to study the expression of the MIP gene during development of the rat lens. The lens is unusual in that morphogenesis occurs through a single pathway of cell differentiation, from a cuboidal epithelial cell to the elongated, terminally differentiated lens fiber cell. During lens morphogenesis, both MIP and its mRNA are detected only after the epithelial cells have begun to differentiate, early in lens development as the cells
that form the posterior wall of the lens vesicle differentiate to form the primary fibers, or in more mature lenses as the anterior epithelial cells near the equator of the lens differentiate to form the secondary fibers. The gene transcripts accumulate at the base of the primary fibers and in the juxtanuclear regions of the secondary fibers. The immunofluorescence indicates that the protein is incorporated throughout the plasma membrane of the fiber cells but suggests that it is present at greater density in plaque-like areas of the membrane (gap junction?). At all stages of lens morphogenesis, protein synthesis appears to be tightly coupled to mRNA transcription, suggesting that this is the major site of regulation of MIP gene expression.

Undergraduate, California Institute of Technology.

220. Differential Distribution of MIP and ß-Crystallin Gene Transcripts in Lens Development

S. Barbara Yancey, Kristine R. Koh¹, Johnson Chung¹, Jean-Paul Revel

As described in the previous report, we have used in situ hybridization to determine the spatial and temporal pattern of expression of the gene for MIP, the protein believed to form the junctions that interconnect the lens fiber cells. In order to place this pattern in context with the sequence of events that occurs with lens fiber cell differentiation, we have hybridized the mRNA in adjacent sections of the eye with an antisense RNA probe for ß-crystallin, B-A1/A3. The crystallin proteins are the major cytoplasmic components of the lens fiber cells and along with MIP appear to be specific to the lens. The pattern of distribution of the ß-crystallin mRNA is very different from that for the MIP message. In contrast to the localized concentrations seen for the MIP gene transcripts at the base of the primary fibers and in juxtanuclear regions of the secondary fibers, the transcripts for the ß-crystallin gene are relatively uniformly distributed throughout the cytoplasm of the primary and secondary fibers. Neither gene appears to be transcriptionally active in the undifferentiated epithelial cell, but the transcripts of the ß-crystallin gene accumulate earlier in fiber cell differentiation than do those of the gene for MIP, indicating that the ß-crystallin gene is activated before the gene for MIP. The fact that the two types of mRNAs are differentially distributed suggests that the patterns reflect different pathways for synthesis of the membrane protein and the cytoplasmic one.

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221. MIP Antipeptide Antibodies, Effects on Cell Communication In Vivo and Channel Activity In Vitro

S. Barbara Yancey, Jean-Paul Revel

There is still some controversy with regard to whether or not MIP is the protein that forms the cell-to-cell channels that comprise the gap junctions which couple lens fiber cells to each other. On the basis of a hypothetical model of MIP as it might be assembled in a gap junction (Revel and Yancey, 1985), we have caused two peptides representing portions of the molecule that could be important in the function of MIP to be synthesized. One of these is a peptide located near the carboxy terminus that would lie in the cytoplasm (LP1), and the other is a peptide that would lie in the extracellular gap between the two membranes that form the junction (LP2). We have raised antibodies to these two peptides with which to test the model (Biology 1985, Abstract No. 193; Biology 1986, Abstract No. 203) and the function of MIP as a channel-forming protein and a component of gap junctions. In experiments done in collaboration with David Spray of Albert Einstein University and Ross Johnson of the University of Minnesota, it has been possible to show that injection of the antibody to LP1 causes a partial block in the transfer of fluorescent dye between lens fiber cells grown in culture. Injection of the antibody to LP2 had no effect. Neither antibody was effective when injected into lens epithelial cells in the same cultures. These are the results expected if the model were correct and MIP is a component of the gap junctions that interconnect the fiber cells but not of those that couple the epithelial cells to each other. The results are consistent with the immunofluorescence and in situ hybridization studies (see Abstract No. 219) which indicate that the MIP gene is not expressed by the lens epithelial cells. In a further test of the channel-forming ability of MIP, we have collaborated with Jim Hall at the University of California, Irvine, to determine whether either of these antibodies is effective in blocking the channel activity of MIP reconstituted in artificial bilayers. Preliminary
experiments suggest that as in the case in vivo, the antibody to LP1 is effective, while the antibody to LP2 is not.

Reference:

222. Production of Polyclonal and Monoclonal Antibodies to the Heart Gap Junction Protein

S. Barbara Yancey, Brian J. Austin, Alexander S. Battaglia

Among the goals we hope to achieve are the isolation of a cDNA encoding the gap junction protein from heart, the characterization of the protein as it is oriented in the membrane, and the identification of regions of the molecule important for function. To these ends we are preparing antipeptide (Biology 1986, Abstract No. 199), polyclonal, and monoclonal antibodies. In order to raise polyclonal antibody, about 35 µg of electroeluted 45-47 kDa gap junction protein was injected intradermally and into the popliteal lymph nodes of a single rabbit. After a boost (150 µg, subcutaneous at one month after the primary injection), the antiserum detects the 45-47 kDa band in Western blots of gap junction preparation when used at a dilution of 1/100. It appears to be less reactive to the breakdown products of the 45-47 kDa protein which are also present in the preparation. We have nearly accumulated enough electroeluted protein to give a second boost that should raise the titer. With the aid of To Ha Thai, we have injected two mice with the SDS-solubilized gap junction preparation and are now at the stage of cell fusion. The polyclonal antibodies from the mice react at a dilution of 1/10,000 and also appear to be more reactive against the parent protein than the breakdown products, suggesting reactivity to the carboxy-terminal portion of the molecule which is lost with proteolysis of the protein.

223. Further Characterization of an Antipeptide Antibody to the Heart Gap Junction Protein

S. Barbara Yancey, Brian J. Austin, Alexander S. Battaglia, Scott John

The antiserum from a rabbit injected with crude peptide representing the amino terminal 1-20 amino acids of the sequence of the heart gap junction protein reacts at a dilution of 1/10,000 with the peptide by ELISA or dot blots and with the 45-47 kDa heart gap junction and its major breakdown products (34, 31, and 28 kDa) on Western blots. Preliminary experiments by immunofluorescence microscopy on frozen sections of heart tissue show binding of the antibody in the region of the intercalated disc, a site of interaction between heart cells rich in gap junctions. In order to obtain more precise localization, we are in the process of affinity purifying the antiserum against the peptide. In collaboration with David Spray of Albert Einstein University, we are conducting experiments to determine whether binding of the antibody interferes with the ability of cardiac myocytes in culture to communicate with each other. Intracellular injection of the antibody has no effect on the ability of the cells to transfer fluorescent dyes. Experiments are now in progress to determine whether antibody applied in the culture medium can prevent the formation of gap junctions.

224. Molecular Analysis of the Gap Junction Gene Family

Jan H. Hoh, Jean-Paul Revel

Over the last few years it has become clear that the well studied 28 kDa liver gap junction protein is a member of a gene family. To characterize this gene family we have used a cDNA for the 28 kDa liver gap junction protein to perform low stringency screens of a rat genomic library. About 20 genomic clones that cross hybridize with the liver 28 kDa cDNA have been isolated by screening 4-5 genome equivalents of the genomic library. We are currently characterizing these clones to determine some of the most basic features of the gap junction gene family, such as how many genes there are and their genomic organization.


Jan H. Hoh, Jean-Paul Revel

During the last 20 years much has been learned about the structure and physiological function of the gap junction. However, the biological function of the gap junction remains an unsolved problem. The structural conservation and prevalence among metazoans leads one to believe that gap junctions serve some very basic function in multicellular organisms. One way to gain some insight into the
biological function of gap junctions is to identify mutants defective in intercellular communication. To this end, we have attempted to isolate the gap junction gene from *Drosophila melanogaster*. Using the rat liver 28 kDa gap junction of cDNA, we have screened several *Drosophila* cDNA libraries and genomic libraries at extremely low stringencies. While several clones have been isolated and characterized so far, none appear to be related to the rat liver gap junction.

226. Origin of Multicellular Animals

Jean-Paul Revel

The Mesozoa consist of an axial cell surrounded by an epithelial jacket. This simplicity could be the mark of a primitive organism; alternately, because Mesozoa are parasites, it could be that their simple organization only reflects "the luxurious life they lead" (Whitman, 1883). Ohama et al. (1984), comparing the 5S RNA of a number of primitive organisms, have suggested that mesozoans arose before flatworms, sponges or coelenterates. Their closest relatives seem to be unicellular ciliates, as was already suspected by Libby Hyman (1940). Assuming that this analysis gives the correct relationships, Mesozoa must be seen as the descendants of some of the most primitive multicellular animals. They certainly are the most primitive multicellular organism known to survive today. We have previously shown (Biology 1985, Abstract No. 133) that gap junctions link all the cells of Mesozoa to each other. Since the cells of primitive multicellular plants are also linked to each other by structures which, like gap junctions, allow direct cell-cell exchanges, we propose the hypothesis that direct cell communication may have provided one of the evolutionary advantages which gave rise to multicellularity.

References:


227. A Novel Assay for Gap Junctions

Kee-Ming Ma1, Jean-Paul Revel

Testing for communication between cells linked by gap junctions has been tedious and time consuming. Individual cells have to be impaled by microelectrodes and an electric current, or a marker dye, injected into one while the exchange is monitored in its neighbors. At present the only practical fashion to avoid testing one cell at a time is to use radioactively-labeled nucleotides. They can be exchanged and because they are trapped as RNA in the recipient cells, they are not lost during processing for autoradiography. We are trying to combine the advantages of both approaches, i.e., seeing results in real time as in the case of electrophysiology and having a statistically adequate sampling as in the case of nucleotide transfer. To this end we are learning to introduce label into all cells of a suspension. These cells will then be added to unlabeled cells and the total number of labeled cells counted after various times. When appropriate controls are done, an increase in the number of labeled cells should denote cell communication. In a first step we have been successful in introducing carboxyfluoresceine diacetate into Novikoff hepatoma cells. We are presently defining conditions for more uniform uptake and are trying to find dyes that remain in the cell longer than 90 minutes.

1Undergraduate, California Institute of Technology.

PUBLICATIONS


CELLULAR NEUROBIOLOGY

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Summary: Many brain functions such as vision, sensation, and motor control are subserved by
different specialized brain regions. It has also become
clear that the molecular composition of neurons within
these regions varies in ways that can affect their
signaling properties. An important goal in neuro­
biology is to explain the functioning of different brain
regions both in terms of the organization of their
neuronal networks and in terms of the unique
molecular structures of their neurons.

Some of the most interesting molecular differences
among neurons are those that result in different modes
of regulation of the strength and effectiveness of
synaptic connections, conductance of ion channels and
other specialized neuronal functions such as axonal
transport or dendritic shape. Transient or permanent
changes in the strength of synaptic connections, in
excitability, and in the extent and shape of dendritic
branches are thought to be important for many
mechanisms of information processing and storage in
the brain. This lab has pioneered studies of a calcium­
and calmodulin-dependent protein kinase that is a
major regulatory enzyme in many central nervous
system synapses. We have shown that it is particularly
highly expressed in the telencephalon where it is
present throughout the neurons, but appears to be
concentrated in specialized synaptic structures called
postsynaptic densities. Related protein kinases,
distinct in structure from the brain kinase, are present
in much lower quantities in other tissues.

The kinase is an oligomer composed of -12
structurally-related 50,000 and 60,000 dalton subunits.
We found that calcium-dependent autophosphorylation
of a small number of the subunits in a holoenzyme
produces a new calcium-independent activity in all of
the subunits. This activity remains after the decay of
the initial calcium signal. In a test tube, the kinase
can perpetuate its activity in the presence of ATP by
continuing the process of autophosphorylation. In a
similar way in vivo, the kinase could delay its own
inactivation by protein phosphatases. Such a
"molecular switch" seems appropriate to mediate
certain forms of regulation of synaptic strength.

This year we have used biochemical and molecular
biological techniques to learn more about the structure
of the kinase. We have selected and sequenced a
cDNA encoding the $\beta$ subunit and are sequencing
cDNAs encoding the $\alpha$ subunit.

We have shown that the two subunits are encoded
by distinct genes and that isoenzymes of the $\beta$ subunit
may be generated by alternate splicing of messages
transcribed from the $\beta$-subunit gene. In another study,
we are using biochemical methods to identify the
important autophosphorylation sites and their locations
within the primary sequence. This structural infor­
mation will allow us to develop highly specific methods
for studying activation of the kinase in vivo.

We have developed two systems in which we are
examining kinase activity and its control in intact
neurons. We are beginning to characterize the brain
phosphatases that dephosphorylate the kinase and its
substrates. Finally, we are characterizing an
homologous protein kinase and its genes in
Drosophila, an organism in which a genetic analysis of kinase
function may be possible.

228. cDNA Clones Encoding the $\alpha$ Subunit of Brain
Type II CaM Kinase

Robert F. Bulleit, Mark K. Bennett, James B. Hurley

The Type II CaM kinase is a large holoenzyme
composed of 50 K ($\alpha$) and 60 K ($\beta$) subunits. The major
forebrain isozyme is an oligomer of -9 $\alpha$ and -3 $\beta$
subunits, whereas the cerebellar isozyme contains -2 $\alpha$
and -8 $\beta$ subunits.

We have selected several cDNA clones coding for
the $\alpha$ subunit. A size-selected rat brain cDNA library
cloned into pBR322 was obtained from Gary Schull of
the University of Cincinnati. The library was screened with two probes, a 45 nucleotide "guessmer" (45 mer) based on partial amino acid sequence of the alpha-subunit and a restriction fragment from the previously cloned beta-subunit cDNA. This fragment contains the N-terminal half of the coding domain (a portion not containing the 45 mer). The beta-subunit cDNA appears homologous to the alpha-subunit message since it will hybrid select mRNAs that code for both subunits and it hybridizes at low stringency to alpha-subunit mRNA on Northern blots.

From this library a single clone was selected (ck4-la) which contains a 1.3 kb insert that hybridizes to both probes. A second size-selected forebrain cDNA library cloned into SgrI0 was screened with labeled ck4-la to identify clones containing the entire coding region. Several clones were selected. Those with the longest inserts were characterized further. They all hybridize on Northern blots to a 5.4 kb band that is recognized by the 45 mer and is more abundant in forebrain than cerebellum. Restriction maps indicate that these cDNAs are all distinct from beta-subunit cDNA and that the full coding region is represented. The longest clones are now being sequenced.

Northern analysis reveals that while the 5.4 kb message for the alpha-subunit is far more abundant in the forebrain than in the cerebellum, the 4.8 kb message for the beta-subunit is expressed at roughly equal levels in the two brain regions. This presumably accounts for the difference in subunit composition of the holoenzyme from the two brain regions.

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229. Genes Encoding the alpha and beta Subunits of Brain Type II CaM Kinase

Robert F. Bulleit, Mark K. Bennett

The alpha and beta subunits of Type II CaM kinase are structurally similar as revealed by similarities in their peptide maps and by antigenic cross-reactivity. However, characterization of cDNA clones for the two subunits indicates that they are encoded by distinct messages.

We have examined the genes that encode the two subunits by Southern blot analysis. Rat genomic DNA was digested with four different restriction endonucleases, fractionated by agarose gel electrophoresis and blotted onto charged nylon filters by alkaline transfer. The blots were probed with labeled cDNAs containing portions of the coding regions for one or the other of the two subunits. The alpha-subunit probe, which contained the N-terminal half of the coding domain, hybridized strongly to a single band in each of the four digests, suggesting that this subunit is encoded by a single gene. A probe made from the C-terminal end of the alpha-subunit cDNA also hybridized to a single band in several of the digests, again suggesting that it is encoded by a single gene. When a probe generated from the full coding domain of the beta-subunit cDNA was used, a more complex pattern was observed with two bands present in two of the restriction digests. This pattern is consistent with a single beta subunit gene interrupted in the coding domain by at least one intron. However, the interpretation of this data will await analysis of the gene structure. The bands labeled more strongly by the alpha-subunit probe were distinct from those recognized by the beta probe, indicating clearly that the two subunits are encoded by different genes.

230. Beta' Subunit of Brain Type II CaM Kinase May Be Synthesized on a Separate Message

Mark K. Bennett

A minor subunit of 58 kDa, called the beta' subunit, is often present in the forebrain form of the Type II CaM kinase and is even more prominent in the cerebellar form. This subunit is structurally very similar to the beta subunit based on its peptide map and its immunological cross-reactivity with antibodies to the beta subunit. For this reason, we and others have assumed in the past that it may have been generated from the beta subunit by proteolysis during purification.

We have re-examined whether the beta' subunit could be coded for by its own separate message. Among several cDNA clones that encode the beta subunit, we found two independent clones with an identical discrete deletion of 45 bases from the middle of the coding region. Messages complementary to these clones would encode a protein 15 amino acids shorter than the beta subunit, with a molecular weight of approximately 58 kDa. Such messages could encode the beta' subunit. To see if such messages are present in the brain, S1 nuclease protection experiments were performed in which labeled probes including the 45
bases were hybridized to forebrain or cerebellar poly(A)+ RNA, then digested with Sl nuclease. A small proportion of both labeled probes was digested to fragments of the size predicted if messages with the 45-base deletion were present. These fragments were more abundant in the cerebellar digests than in the forebrain digests, consistent with the known distribution of the δ' subunits between the two brain regions. Thus, the δ' message may be synthesized on a separate message. We propose that the source of this message may be alternate splicing of the δ-subunit gene.

231. Time Course of Incorporation of Phosphate into Tryptic Phosphopeptides of the Type II CaM Kinase

Stephen G. Miller

Ca2+-dependent autophosphorylation of only a few subunits of the Type II CaM kinase holoenzyme is sufficient to produce Ca2+-independent activity. We are interested in characterizing the sites of autophosphorylation for several reasons: 1) to determine whether the sites phosphorylated in vivo are the same as those in vitro, 2) to locate the sites within the primary structure of the kinase subunits relative to putative functional domains, and 3) to determine which sites are responsible for the observed functional changes in kinase activity. To study the third question, we have used the method of reverse phase HPLC (RP-HPLC) tryptic peptide mapping described in a previous annual report (see Biology 1986, Abstract No. 216) to investigate the kinetics of phosphate incorporation into the kinase subunits.

We generated RP-HPLC tryptic peptide maps for kinase that had been autophosphorylated in the presence of Ca2+ and calmodulin for varying times. After 5 sec of autophosphorylation, two phosphopeptides are found in the α subunit and one in the δ subunit. With increasing time, these sites continue to be filled and two additional "late" phosphopeptides appear in digests of both subunits. After 180 sec of autophosphorylation, the α subunit contains four and the δ subunit three major phosphopeptides. The kinetics of phosphorylation of each of the sites is identical for the forebrain and the pons-medullary isozymes of the kinase. The changes in kinase activity resulting from autophosphorylation are complete within 5 sec under the conditions used for the phosphopeptide mapping. Therefore, phosphorylation of the two α and single δ subunit sites occupied initially correlates with the observed changes in kinase activity; incorporation of phosphate into the four "late" phosphopeptides is not required. We have not yet observed any further changes in kinase activity that may result from phosphorylation of these "late" sites.

232. Ca2+-independent Autophosphorylation of the Type II CaM Kinase

Stephen G. Miller, Bruce L. Patton

As mentioned in the previous abstract, phosphorylation of only a few sites on the Type II CaM kinase is sufficient to produce Ca2+-independent activity toward exogenous substrates, and also additional calcium-independent autophosphorylation. We have used RP-HPLC tryptic peptide mapping to compare the sites autophosphorylated in the presence and absence of Ca2+. After autophosphorylation for 5 sec in the presence of Ca2+/CaM, free Ca2+ was chelated by the addition of EGTA. Autophosphorylation was allowed to continue. Aliquots were removed at various times and the reaction was quenched by the addition of excess EDTA. RP-HPLC tryptic peptide maps at the various time points (up to 180 sec) were compared to control maps in which Ca2+/CaM was present throughout the autophosphorylation reaction.

Within 5 sec of the removal of Ca2+, a major Ca2+-independent autophosphorylation site was observed in the δ subunit. In addition, two minor Ca2+-independent sites appeared in the α subunit. With increasing time, the two α subunit Ca2+-independent sites incorporate additional phosphate, whereas the amount of phosphate in the initial δ subunit Ca2+-independent site remains at a constant high level. A second Ca2+-independent site appears in the δ subunit, but its level of phosphorylation is variable. The amount of phosphate incorporated into several of the sites described in the previous abstract was reduced in the absence of Ca2+, while others were unaffected. When autophosphorylation was allowed to proceed for 60 sec (rather than 5 sec) before Ca2+ was removed by EGTA, the same Ca2+-independent autophosphorylation sites were observed.

Kinase that had been autophosphorylated using the method described above (15 sec + Ca2+/15 sec - Ca2+)
was assayed for activity using Synapsin I as substrate and compared to the activity of a control in which Ca\(^{2+}\) was present throughout the autophosphorylation reaction. The only difference was a small (<20%) but consistent reduction in \(V_{\text{max}}\) in the presence of Ca\(^{2+}\); Ca\(^{2+}\)-independent activity was unaffected. The apparent \(K_a\) for calmodulin was the same after each of the two phosphorylation paradigms.

### 233. Identification of the Autophosphorylation Sites of the Type II CaM Kinase by Microsequencing

**Stephen G. Miller**

We are interested in determining the amino acid sequence surrounding the autophosphorylation sites of the Type II CaM kinase for two major reasons: 1) to localize the autophosphorylation sites within the primary structure of the kinase subunits relative to putative functional domains, and 2) to enable us to synthesize peptides corresponding to specific sites for use in the generation of monoclonal and polyclonal antibodies that are specific for the phosphorylated sites. We have adapted the method of reverse phase HPLC tryptic peptide mapping described in a previous annual report (see Biology 1986, Abstract No. 216) to isolate sufficient amounts of individual phosphopeptides for gas phase microsequencing.

To maximize recovery of phosphopeptides, the forebrain isozyme (9a:3a) was used for generation of α-subunit phosphopeptides and the pons/medullary isozyme (6a:6a) was used for β-subunit phosphopeptides. Kinase was phosphorylated with \([γ-^{32}\text{P}]\text{ATP}\) for 120 sec in the presence of Ca\(^{2+}\) followed by chelation of free Ca\(^{2+}\) by EGTA and a further 15 sec of autophosphorylation. This paradigm was chosen to permit maximum incorporation of phosphate into all of the possible autophosphorylation sites (see previous two abstracts). The α and β subunits were separated by SDS-PAGE. The gels were dried and the location of the α and β subunits determined by autoradiography. Pieces of the gel containing the α and β subunits were cut out, washed to remove SDS and other contaminants, and lyophilized. The gel pieces were reswollen in a small volume of buffer containing trypsin and incubated at 37°C. The supernatant solution containing the tryptic peptides was removed and lyophilized. The peptides were resuspended and separated by RP-HPLC. Absorbance of the column eluent at 214 nm and 280 nm was monitored. At the same time, 1 ml fractions were collected and their radioactivity determined. Fractions containing the labeled phosphopeptides of interest were lyophilized and rechromatographed on the same column with a shallow gradient. The phosphopeptides purified by this technique are being sequenced by gas phase microsequencing.

### 234. Antibodies Selective for the Phosphorylated Form of Type II CaM Kinase

**Sean S. Molloy**

Monoclonal antibodies were raised against phosphorylated Type II CaM kinase using a variation on standard hybridoma technology. In order to enhance the possibility of isolating clones secreting antibody specific for the phosphorylated form of the enzyme, we adapted the immunosuppression technique of Matthew and Patterson (1983). Three RBF/DN mice were initially immunized with non-phosphorylated (control) kinase. The immune response toward the control kinase was suppressed in two of the mice by injection of cyclophosphamide. All three mice were then immunized repeatedly with a thiophosphorylated form of the kinase.

Antigen was prepared by autophosphorylating purified enzyme under conditions that result in incorporation of approximately 20 thiophosphates/holoenzyme. ATP-γ-S was used as a substrate in the preparation of antigen in order to render it less susceptible to phosphatase activity. The autophosphorylated enzyme was precipitated from the reaction mixture by ammonium sulfate and used for subcutaneous and interperitoneal immunizations.

Serum from each mouse was assayed for anti-kinase activity in a solid phase RIA. Both suppressed mice had serum reactivity considerably lower than that of the control, non-suppressed mouse. This low level of activity, however, appeared to be slightly selective for the phosphorylated form of the kinase. Spleen cells from the control and suppressed mice were fused separately with 653-1 myeloma cells. The resultant clones were screened using both phosphorylated and control kinase in the solid phase RIA.

The control mouse fusion yielded five clones that secreted antibodies that displayed a seven- to tenfold selectivity for the phosphorylated enzyme and also several non-selective clones. The fusion carried out
with spleen cells from the suppressed mice resulted in numerous clones with low degrees of selectivity. We plan to characterize the site specificity of the phospho-specific clones and to use them as probes for assaying the phosphorylation state of the Type II CaM kinase in various experimental paradigms.

Reference:

235. Protein Phosphatase Activities Against Phospho-Type II CaM Kinase and its Substrates

Bruce L. Patton

Brain homogenates contain an endogenous protein phosphatase activity that dephosphorylates auto-phosphorylated Type II CaM kinase and reverses the changes in kinase activity associated with auto-phosphorylation (Miller and Kennedy, 1986). In order to completely understand the control of the phosphorylation state and activity of the CaM kinase, as well as phosphorylation of its substrates, we are identifying the endogenous brain protein phosphatases that dephosphorylate the kinase and its substrates. Although brain phosphatase activities have not been well characterized, some evidence suggests that the phosphatases that act on phospho-CaM kinase are similar to protein phosphatases-1 and -2A in skeletal muscle (Shields et al., 1985).

We have purified protein phosphatases-1 and -2A from rabbit skeletal muscle, according to the procedure of Tung et al. (1984). Fully autophosphorylated CaM kinase is a good substrate for both phosphatases. The initial rates with phospho-CaM kinase as substrate are comparable to those for phosphorylase a, the major muscle protein substrate for protein phosphatases-1 and -2A. Another brain phosphoprotein, synapsin I, a primary substrate of the CaM kinase, is also a substrate for both purified phosphatases, although the initial velocities with phosphosynapsin I as substrate are quite different for the two phosphatases. We are characterizing phosphatases present in brain homogenates by obtaining the \( K_m \)'s and initial velocities with phosphorylase a, phosphosynapsin I, and phospho-CaM kinase as substrates and comparing them to the values established with purified protein phosphatases-1 and 2A.

References:

236. Type II CaM Kinase Activity in Hippocampal Slice Cultures

Seán S. Molloy

We assessed the expression of Type II CaM kinase in cultured hippocampal slices from rat using a variety of biochemical and immunological criteria. Cultures were prepared from 6- to 7-day-old rat pups by a roller-tube technique similar to that described by Gahwiler (1981). Hippocampal slices cultured using the roller-tube system reportedly develop functional synaptic connections in at least two fiber pathways ( mossy fibers and Schaffer collaterals) and express several proteins associated with synaptic function.

Slices maintained 3-4 weeks in vitro were homogenized in buffered medium and then assayed for \( \text{Ca}^{++} / \text{calmodulin} \)-stimulated phosphorylation of exogenous synapsin I. Such activity was readily detectable in slice homogenates. Monoclonal antibodies specific for the Type II CaM kinase inhibited this endogenous activity. The exogenous synapsin I was phosphorylated on the same site recognized by the purified forebrain enzyme.

Phosphorylation of endogenous proteins in the slice homogenates revealed two major bands on SDS-PAGE gels which co-migrated with the \( \alpha \) and \( \beta \) subunits of the kinase. The phosphorylation of these 50 and 60 kDa bands was stimulated by \( \text{Ca}^{++} / \text{calmodulin} \).

Immunoblots using a monoclonal antibody specific for the \( \alpha \) subunit revealed a single band in hippocampal slice homogenates. The relative concentration of the \( \alpha \) subunit in the homogenates was consistent with the level of \( \text{Ca}^{++} / \text{calmodulin} \)-stimulated synapsin I kinase activity. The same antibody appeared to stain pyramidal cells in CA1 and CA3 fields of intact slice cultures.

These results suggest that, unlike transformed neuronal cell lines, cultured hippocampal slices will express an active form of the Type II CaM kinase, containing both \( \alpha \) and \( \beta \) subunits. This culture system should prove useful in examining regulation of the
kinase in hippocampal cells.

References

237. In Vivo Activation of Type II CaM Kinase
Karen A. Ocorr, Sean S. Molloy

Experimental evidence has indicated roles for calcium, protein kinases, and protein phosphorylation in regulation of synaptic efficacy in the central nervous system. We are investigating the potential role of Type II CaM kinase in modulating synaptic efficacy or other neuronal functions in the hippocampus, where it is highly concentrated. We are specifically interested in demonstrating whether activation of autophosphorylation of the kinase produces a relatively long-lived, calcium-independent activity in vivo as it has been shown to do in vitro. Consequently, we have begun to examine whether the kinase can be induced to switch to a calcium-independent form in hippocampal slices.

Our initial experiments have established optimal conditions for treating hippocampal slices and for preserving in homogenates any alterations in enzyme activity that may have been induced in vivo. Kinase activity present in slices remains relatively constant for up to 4 hr if the slices are maintained in calcium-free, oxygenated saline at 32°C. We have designed a mesh-covered chamber for use in manipulating the slices. The chamber permits rapid exposure of one or more slices to saline containing various pharmacological agents. Preliminary results indicate that treatments that would be expected to increase calcium-influx into neurons result in the appearance of calcium-independent activity in slices. We have begun to address the question of whether neurotransmitters and/or depolarization can also elicit calcium-independent kinase activity in vivo. Future experiments will be designed to examine whether stimuli that are known to produce long-term potentiation (a characteristic change in synaptic efficacy in the hippocampus) can affect the activity of this kinase. Once we identify the neurotransmitters and/or stimulation paradigms capable of switching the kinase to its calcium-independent form, we will attempt to identify the endogenous protein substrates of the enzyme under each of the paradigms.

238. Characteristics of Drosophila Type II CaM Kinase
Phyllis C. Pugh

Homogenates made from heads of Drosophila melanogaster contain a calmodulin-dependent protein kinase that is homologous to rat brain Type II CaM kinase. This enzyme contains three subunits of molecular weights 60, 58 and 53 kDa (Biology 1985, Abstract No. 219). We partially purified the three subunits by methods similar to those developed for purification of the Type II CaM kinase. The purified subunits become autophosphorylated in the presence of calcium, calmodulin, and ATP. The autophosphorylated form of the purified kinase displays a new calcium-independent activity, indicating that this property of the kinase is conserved in Drosophila.

Preliminary observations made by assaying head homogenates for kinase activity had suggested that the Type II CaM kinase from the radish memory mutant was activated by a lower calcium concentration than the wild-type kinase (J. B. Wall, unpublished observations). We followed up this observation by examining the calcium dependence of purified Type II CaM kinase from both wild-type heads and radish heads. There was no difference in the calcium sensitivity of the two enzymes, indicating that the difference in the activation of kinase in radish homogenates may have been due to a difference in the calcium content of the homogenates. Enzymes purified from both the mutant and the wild type showed the same level of calcium-independent activity following autophosphorylation, indicating that there is no difference between the two kinases detectable at this level.

239. Genetic Analysis of Type II CaM Kinase in Drosophila
Debra A. S. Leonard

From the work described in the previous abstract, it is clear that several properties of the rat brain Type II CaM kinase are also properties of the Drosophila kinase. We were particularly interested that the 60 kDa subunit of the fly kinase comigrates with the α subunit of the rat brain kinase on SDS-polyacrylamide gel electrophoresis. Thus, perhaps this subunit of the fly kinase bears the strongest homology to the rat brain enzyme. Since a cDNA encoding the entire β subunit of the rat brain kinase has been cloned and
sequences (Bennett and Kennedy, 1987), it seemed reasonable to use this clone as a probe to identify and characterize gene(s) or cDNA(s) coding for the homologous kinase in Drosophila. Determination of the sequences encoding the Drosophila kinase will enable us to study kinase function in a system well suited for performing a genetic analysis of kinase functions.

We have used the cloned full length rat α-subunit cDNA as a labeled probe to identify homologous sequences in digested Drosophila genomic DNA. We found that the rat α-subunit probe hybridized strongly to a single 6.5 kb restriction fragment of HindIII-digested fly DNA under reduced stringency conditions, allowing hybridization between sequences with as much as 33% mismatch. We are currently screening Drosophila cDNA and genomic libraries for clones that encode the subunits of the fly kinase.

References

PUBLICATIONS


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Summary: We are interested in the ion channels of neuronal membranes and in the receptors that control these channels. In recent years the group has approached this topic by combining the methods of biophysics and of nucleic acid molecular biology. Individual researchers divide their time between electrophysiological experiments and molecular cloning. The current favorite preparation is the Xenopus oocyte, which faithfully translates injected mRNA, assembles channels and receptors into its membrane, and allows convenient voltage-clamp and patch-clamp experiments on these expressed channels.
We "electric cloners" have recently explored three major applications of the oocyte translation system. First, the oocyte can be viewed as a convenient reconstitution system, in which one defines those RNA species necessary to express the full range of pharmacological and kinetic characteristics of a channel or receptor expressed in native tissue. These studies have been pursued with electrically excitable Ca channels, with the Na channels that underlie the nerve impulse, and with the serotonin receptors of the central nervous system.

Next, the oocyte is used as a functional assay to screen clones for receptors and channels. As an example of this work, the group has recently described a clone for a serotonin receptor and is searching for similar molecular information about K and Ca channels.

Finally, once cDNA clones are in hand, the oocyte and similar expression systems provide a powerful tool to study the relation between the structure and the function of the proteins that underlie membrane excitability. cDNA clones for membrane proteins are mutated to test theories about the role of individual amino acids in the channel. The new clones are used to generate mRNA in vitro; this in turn is expressed and the resulting function measured with the full spectrum of biophysical methods. Acetylcholine receptors and Na channels are currently being studied in this fashion.

The group has other interests in molecular neurobiology. Unique kinetic and mechanistic information has been obtained with a series of specially designed light-activated molecules. Some of these molecules resemble synaptic transmitters, such as acetylcholine; other "caged" molecules release intracellular messengers, such as cyclic nucleotides or Ca, in response to light flashes. Experiments on these molecules thus involve the tools of photochemistry, organic synthesis, and of course, electrophysiology. In the context of the graduate option in Computation and Neural Systems, other projects have included theoretical studies of synaptic function and extensive software development.

Jerome Pine, Professor of Physics, is also associated with the research group. Professor Pine's interest is in the patterns of connections formed by neural networks and the effects of activity on connectivity. His group has developed techniques for noninvasively studying the electrical connections in cultured neural networks so that they may be studied as a function of time. Cultures are grown in dishes which embody microelectrodes for stimulation. Optical recording techniques based on the use of voltage-sensitive fluorescent dyes are used for recording from these cultures. In addition, work is in progress on a family of silicon microdevices designed to make long-term electrical contact with cultured neurons. These devices are intended to facilitate long-term studies of network plasticity using only electrical methods. Finally, the group is continuing to work with others in developing techniques for using ultra-soft x-ray microscopy to reveal synaptic ultrastructure in cultures.

240. Equilibrium Properties of Mouse-Torpedo Acetylcholine Receptor Hybrids Expressed in Xenopus Oocytes

Kiyonori Yoshii, Lei Yu, Katharine Mixter Mayne

This study utilized messenger RNA encoding each subunit (α, β, γ, and δ) of the nicotinic acetylcholine (ACh) receptor from mouse BC3H-1 cells and from Torpedo electric organ. The mRNA was synthesized in vitro by transcription with SP6 polymerase from cDNA clones. All 16 possible combinations which include one mRNA for each of α, β, γ, and δ were injected into oocytes. After allowing 2–3 days for translation and assembly, we assayed each oocyte for: 1) receptor assembly, measured by the binding of [125I]α-bungarotoxin to the oocyte surface; and 2) ACh-induced conductance, measured under voltage clamp at various membrane potentials. All combinations yielded detectable assembly (30-fold range among different combinations) and ACh-induced conductances (>1000-fold range at 1 µM). On double-logarithmic coordinates, the dose-response relations all had a slope near two for low [ACh]. Data were corrected for variations in efficiency of translation among identically injected oocytes by expressing ACh-induced conductance per fmol of α-bungarotoxin binding sites. Five combinations were tested for d-tubocurarine inhibition by the dose-ratio method; the apparent dissociation constant ranged from 0.08 to 0.27 µM. Matched responses and geometric means are used for describing the effects of changing a particular subunit
(mouse versus Torpedo) while maintaining the identity of the other subunits. The most dramatic subunit-specific effect is that of the \( \beta \) subunit on voltage sensitivity of the response: \( g_{\text{ACH}}(-90 \text{ mV})/g_{\text{ACH}}(+30 \text{ mV}) \) is always at least 1, but this ratio increases by an average of 3.5-fold if \( \beta_M \) replaces \( \beta_T \). Also, combinations including \( \gamma_T \) or \( \delta_M \) usually produce greater receptor assembly than combinations including the homologous subunit from the other species. Finally, \( E_{\text{ACH}} \) is defined as the concentration of \( \text{ACH} \) inducing \( 1\mu\text{S}/\text{fmol} \) at \(-60 \text{ mV} \); \( E_{\text{ACH}} \) is lowest for \( \gamma_M \) and for \( \delta_T \). We conclude that receptor assembly, voltage sensitivity, and \( E_{\text{ACH}} \) are governed by different properties.

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241. Voltage Sensitivity of the Acetylcholine Receptor: A Study of Unitary Currents
Lei Yu, Reid J. Leonard, Cesar G. Labarca
This work extends the study reported in the preceding abstract. We have examined voltage sensitivity at the level of single-channel recording with the following hybrid receptors:

- \( \alpha_M \beta_M \gamma_M \delta_M \gamma_T \delta_T \), \( \alpha_M \beta_T \gamma_M \delta_M \), \( \alpha_M \beta_M \gamma_T \delta_M \), and \( \alpha_M \beta_M \gamma_T \delta_T \).

In outside-out patches, all combinations showed linear single-channel current-voltage relationship, but open duration depended on voltage. Average channel duration, \( \tau \), was measured at \(-60 \text{ mV} \) and \(+60 \text{ mV} \). The extent of the voltage dependence of each combination was estimated by the ratio of \( \tau_{-60 \text{ mV}}/\tau_{+60 \text{ mV}} \) and the results were in agreement with our previous conclusions from the macroscopic measurements. The voltage sensitivity of the closed times was assessed either by directly measuring the closed duration (in favorable cases where desensitization was minimal) or by subtracting the voltage sensitivity of the equilibrium conductance from that of the open duration. According to either method, closed times had less than one-fifth the voltage sensitivity of the open times. Thus, of the three possible parameters underlying voltage sensitivity (channel conductance, opening rate, and closing rate), the closing rate makes the major contribution to the voltage sensitivity of the macroscopic conductance of the AChR.

242. Local Anesthetic Effects on Gating of Mouse-Torpedo Hybrid AChRs
Reid J. Leonard, Lei Yu, Cesar G. Labarca
cDNA clones are available for all four subunits of the nicotinic acetylcholine receptor (AChR) from several species. The mRNAs transcribed \textit{in vitro} from those cDNAs can be injected into \textit{Xenopus} oocytes; the receptors synthesized and inserted into the membrane are amenable to electrophysiological recording. This approach promises to improve our knowledge of the structural correlates of AChR function. In an attempt to identify portions of the subunits' structure that may be involved in formation of the ion channel of the AChR, we are comparing the effects of a local anesthetic (QX-222) upon the gating parameters of "hybrid" AChRs constructed by mixing the individual subunit mRNAs derived from mouse and Torpedo cDNAs. Because the site of action of local anesthetics is presumed to lie within the ion channel, the sensitivity of hybrid, chimeric, or mutated AChRs to QX-222 may prove to be an additional probe for the assignment of particular regions or even individual residues to discrete parameters of channel function. So far, comparisons of mouse-Torpedo hybrid channels indicate that some hybrids differing in mean open time in the absence of local anesthetic exhibit very similar open times in the presence of blocker, thus indicating that the forward blocking rate of QX-222 is only weakly influenced by subunit composition. On the other hand, the residence time of QX-222 (measured by the time constant of closings within an open channel burst) does appear to be influenced by subunit composition.

243. More than One RNA Species is Involved in Controlling Inactivation of Rat Brain Na Channels
Douglas S. Krafte, Terrance P. Snutch, John P. Leonard
We have injected RNA from the brains of 18-day-old rats into \textit{Xenopus} oocytes in order to study the properties of voltage-dependent Na channels from this tissue. Oocytes were injected either with un-fractionated poly(A)\(^+\) (RNA\(_u\)) or poly(A)\(^+\) RNA that was size fractionated over a sucrose gradient. We identified a fraction (RNA\(_a\)) that contained the 9 kb mRNA encoding the \( \alpha \) subunit of the Na channel both by Northern blot analysis and by electrophysiological
recording. Currents elicited by this fraction exhibited a threefold or greater slowing of the macroscopic inactivation rate as compared to currents following injection of RNAu. In addition, the steady-state voltage dependence of inactivation was shifted in the depolarizing direction (V1/2 = -45 mV for RNAu versus -34 mV for RNAa). To eliminate the possibility that these effects were an artifact of our manipulations, we pooled all the fractions from the gradient and injected the pooled RNA into oocytes. Macroscopic inactivation of Na currents elicited by pooled RNA was indistinguishable from that following injection of RNAu. The mechanism behind the slowing of the inactivation kinetics was investigated at the single channel level using both cell-attached and excised outside-out patches. In both cases a greater tendency for the channels to reopen appears to underlie the macroscopic observations. This gating behavior is illustrated in the current traces below in response to a voltage step from -100 mV to -20 mV in a cell-attached patch (left-RNAu, right-RNAa, calibration bars = 2 pA and 4 ms). The single channel conductance was approximately 13 pS for channels induced by both RNAu and RNAa injections.

244. TTX-sensitive Na Channels Expressed in Xenopus Oocytes Injected with Rabbit Heart RNA

Fedora Sutton, Kiyonori Yoshii

We are characterizing the types of sodium channels expressed in cardiac tissue. RNA was isolated from atrium, ventricle, or whole rabbit or rat heart (ages 8 to 25 days). Based on Northern blot analysis with the neuronal-specific probe pSCG-10, there was no detectable contamination with neuronal RNA. Two hundred ng of RNA was injected into oocytes in 30 nl of water. After 2 days incubation at 20°C, voltage-dependent Na currents (100-500 nA peak) were observed with a standard two-electrode voltage-clamp circuit. With either ventricular or atrial RNA, these currents were blocked by TTX; the dose response relation suggests that at least 90% of the channels were blocked by the binding of a single TTX molecule with a dissociation constant of 8 + 2 nm. This affinity equals that for blockade of the five- to tenfold larger Na currents induced by rat or rabbit brain RNA. This sensitivity to TTX contrasts with the dissociation constant of at least 1 µM observed with voltage-clamp analysis of ventricular muscle. Perhaps TTX insensitivity requires a post-translational modification that oocytes cannot perform.

245. Ca Channels Induced in Xenopus Oocytes by Rat Brain mRNA: Size Fractionation and Physiological Studies

John P. Leonard, Terrance P. Snutch

We have continued to study Ca channels induced by injection of rat brain mRNA into Xenopus oocytes and have begun to characterize the mRNA species responsible by size fractionation. RNA was isolated from fresh brains of 15- to 17-day-old rats by a lithium chloride-urea procedure. Oocytes were injected with 70 ng samples of poly(A) mRNA and membrane currents were recorded under standard two-microelectrode voltage clamp after allowing 2-7 days for expression. Ba currents through Ca channels exhibited the following properties: relatively slow partial inactivation (τ = 630 ms), peak current activation at +15 mV, insensitivity to dicyclopyridines, insensitivity to α-CgTX, 50% inhibition at <10 µM cadmium, insensitivity to forskolin, and enhancement by phorbol esters. Many of these properties are shared by Ca channels involved in neurotransmitter release. Preliminary recordings from excised outside-out patches revealed a single channel conductance of 10-20 pS in 70 mM BaCl2. Reconstruction of macroscopic currents from single channel records shows a current with little inactivation during a 60 ms pulse at +20 or +40 mV. Size fractionation on a 6-20% sucrose density gradient has provided the initial characterization of the mRNA species encoding the induced Ca channels. Surprisingly, Ca channel activity is found in two peaks: (8.5-10 kb) and (4-7 kb) MW fractions. The I_Ba from high MW fractions shares many features with currents from unfractonated mRNA including relatively slow inactivation, peak at +15 mV, high sensitivity to cadmium, and insensitivity to nifedipine. Further physiological characterization of I_Ba induced by low and high MW mRNA is under way.

246. cDNA Cloning of a Serotonin 5-HT<sub>1C</sub> Receptor using Electrophysiological Assays of mRNA-injected Xenopus Oocytes

Hermann Lübbert, Beth J. Hoffman<sup>1</sup>, Terrance P. Snutch, Terry van Dyke<sup>1</sup>, Arnold J. Levine<sup>2</sup>, Paul R. Hartig<sup>1</sup>

We have designed a strategy for the cloning of
neurotransmitter receptor and ion channel cDNAs that is based on electrophysiological assays of mRNA-injected *Xenopus* oocytes. This procedure circumvents the purification of these membrane proteins which is hindered by their low abundance and their hydrophobic nature. It involves methods for RNA fractionation by high resolution gel electrophoresis, directional cDNA cloning in a single-stranded vector, and screening of the cDNA library by voltage-clamp measurements of currents induced by serotonin in mRNA-injected oocytes. We used this approach to isolate a serotonin receptor cDNA clone from mouse choroid plexus papilloma. The clone was identified by hybrid depletion and hybrid selection procedures. The receptor expressed in oocytes injected with hybrid selected RNA is fully functional, indicating that it is composed of a single subunit encoded by a 5 kb RNA. The pharmacology of the hybrid-selected receptor confirms that we have successfully cloned a 5-HT<sub>1C</sub> receptor cDNA.

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247. Analyses of Cloned Serotonin 5-HT<sub>1C</sub> Receptor cDNAs

Beth J. Hoffman<sup>1</sup>, Hermann Lübbert, Hieu T. M. Nguyen, Paul R. Hartig<sup>1</sup>

Serotonin (5-HT) induces conductances in *Xenopus* oocytes injected with rat brain mRNA. Although there are at least six subtypes of serotonin binding sites in the mammalian central and peripheral nervous systems, we have shown that only the 5-HT<sub>1C</sub> receptor is functionally expressed in mRNA-injected oocytes. This receptor opens an endogenous Ca-dependent Cl channel in the oocyte membrane via inositol trisphosphate (IP<sub>3</sub>) release. We have shown that 5-HT<sub>1C</sub> receptors mediate an increase in phosphoinositide turnover in intact rodent and pig choroid plexi. The richest source of 5-HT<sub>1C</sub> binding sites is the choroid plexus while other regions of the brain, for instance, hippocampus, cortex and striatum, express lower densities of these receptors. Using serotonin-induced currents in mRNA-injected oocytes as a bioassay, we have isolated a cDNA clone for the serotonin 5-HT<sub>1C</sub> receptor. We are obtaining sequence data for the 5-HT<sub>1C</sub> receptor and are comparing our data to other receptors and transmembrane proteins. Using Northern blot analysis, we have determined the abundance and size distribution of serotonin 5-HT<sub>1C</sub> receptor mRNA in various brain regions and in several peripheral tissues which receive serotonergic innervation. Since these receptors are present in all mammalian species examined, we have analyzed the homology between 5-HT<sub>1C</sub> receptor genes in rat, mouse, pig and human by Southern blotting.

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248. Diversity of Voltage-dependent Potassium Channels Induced in *Xenopus* Oocytes by Total and Fractionated Rat Brain mRNA

Jeffrey H. Hoger, Chulbul Ahmed, Bernardo C. Rudy

K channels show a high degree of diversity, varying in voltage dependence, kinetics, pharmacology, and single-channel properties. The types of K channels present in the brain are incompletely characterized due to the difficulties of applying present electrophysiological techniques to many cell types and regions of the cell. In order to characterize brain voltage-dependent K channels, we have prepared poly(A) RNA from rat brains by a LiCl-urea procedure. This poly(A) RNA was injected into prepared *Xenopus* oocytes and expression of voltage-dependent K channels was measured by two-microelectrode voltage clamping. This technique may allow the study of the source of diversity of K channels, because all the different channels are expressed in the same system. The K currents observed include a fast transient component with very similar characteristics to the "A" current and a "delayed rectifying" current. The delayed rectifying current may be composed of more than one component as determined by inactivation properties, activation and deactivation kinetics, and sensitivity to externally applied 4-aminopyridine (4-AP), tetraethylammonium (TEA), and toxin I from Black Mamba. We have fractionated the mRNA by centrifugation on linear sucrose gradients (6-20%). A delayed rectifier-type current was expressed by mRNA comigrating with the sodium channel α-subunit mRNA (7.5-9.5 kb). This delayed rectifier-type current may still contain more than one component; it is not completely blocked by TEA and it still contains different deactivating components. However, the "A" current is not expressed or greatly reduced in this fraction. A fraction of
smaller size (6-8 kb), in contrast, expresses an "A" current and a "delayed rectifier" component. These results indicate that different voltage-dependent K channels are encoded by different mRNAs and are therefore composed of different polypeptides which may in part account for the diversity of voltage-dependent potassium channels.

249. Expression of Different "A" Currents in *Xenopus* Oocytes Injected with Total or Fractionated Rat Brain mRNA

*Bernardo C. Rudy, Jeffrey H. Hoger*

*Xenopus* oocytes injected with rat brain mRNA express a fast transient K current with very similar properties to the "A" currents observed in molluscan and mammalian neurons. The current has a relatively low threshold, activating at around -60 mV; for small depolarizations, the time constant is about 5 msec at room temperature. The activation time constant decreases as the depolarization is increased. The current inactivates with a relatively voltage-independent time constant of about 30-40 msec. Steady-state inactivation by long prepulses is complete at -50 mV and half inactivation occurs at around -65 mV. The current is completely blocked by 5 mM 4-AP and half blocked at 2 mM. The degree of block at a given 4-AP concentration decreases with increasing depolarization and decays during sequential pulses at frequencies higher than 0.5 sec⁻¹. The "A" current is insensitive to external TEA at concentrations of up to 40 mM. We have observed the expression of this current to be of variable magnitude with the same RNA, suggesting that other factors probably related to oocyte variability are important for its expression. Oocytes injected with sucrose gradient fractionated RNA express a fast transient K current when injected with a fraction of 6-8 kb. This current differs from that observed when total mRNA is injected as follows: (a) its activation and inactivation kinetics are faster; and (b) it is less sensitive to 4-AP. Steady-state inactivation of this current by prepulses remains unchanged. We propose that the altered pharmacology is the result of the change in kinetics, as can be expected for a state-dependent block. This current may represent a distinct, fast transient K current or its altered kinetics may result from the fact that the channel contains more than one polypeptide. We are now attempting to reconstitute the original kinetics and pharmacology.

250. Structure and Function of Glutamate Receptors from Rat Brain

*Tung Ming Fang*

Excitatory amino acid neurotransmitters such as glutamate and aspartate induce depolarization in many brain regions. Two major types of glutamate responses have been found at rat brain neuronal membranes. One involves the glutamate-gated cation channel(s) and the other seems due to the coupling of a glutamate receptor to a second messenger which increases intracellular Ca++. The study of glutamate receptors is complicated by the fact that there appear to be several subtypes of glutamate binding sites with different anatomical localization as revealed by different glutamate analogs; little is known about the molecular nature of these glutamate receptors. To characterize the functions of glutamate receptors, poly(A) mRNA has been isolated from the whole rat brain and from several regions. Functional glutamate receptors can be detected on *Xenopus* oocyte membranes by mRNA injection and voltage-clamp methods. Responses to L-glutamate, quisqualic acid, kainic acid, and N-methyl-D-aspartic acid are all detected, although the response amplitudes are quite different for different ligands. Preliminary results indicated that mRNA from hippocampus encodes more cation channel(s) while mRNA from cerebellum encodes more second messenger-coupled receptor(s). In order to elucidate the molecular properties of glutamate receptors, poly(A) mRNA is size-fractionated by velocity sedimentation and present efforts are devoted to characterizing the glutamate receptor activity in each fraction. In addition, the partially purified mRNA-encoding glutamate receptors will be used to construct a cDNA library which can be screened by hybridizing with the original mRNA population and assaying the hybridization-depleted mRNAs. The cloning of glutamate receptor will ultimately unravel some of the mysteries observed physiologically.
251. Development and Plasticity of Small Neural Networks

Chi-Bin Chien, W. David Crank, Becky G. Tanamachi, Jerome Pine

Neural networks can be grown in culture. We are interested in studying the patterns of synaptic connections that develop normally and the effects of controlled patterns of electrical stimulation. Initial studies are being made with sympathetic neurons from the rat superior cervical ganglion, after which central neurons from brain or spinal cord will be used.

To study the electrical connectivity of networks without damaging the cells, and to apply chronic stimuli, we have developed a system that utilizes voltage-sensitive fluorescent dyes in combination with extracellular electrical stimulation. Microcircuit culture dishes incorporating an array of 61 extracellular stimulating electrodes in an area 0.5 mm in diameter have been fabricated. A culture system has been developed for isolating a microculture containing a small number of neurons over this array. A circle of silastic adhesive confines the cell processes, and the surrounding area is used for a mass culture which biochemically conditions the medium. For recording, the cells are stained with a fluorescent dye that dissolves in the cell membranes and whose fluorescence varies with membrane potential. The microculture is imaged on an array of 256 optical fibers, which are viewed by photodiodes to record the fluorescent signals. This electro-optical system is now being assembled and tested.

To determine the network connectivity, each cell will be stimulated individually so that it generates an action potential, and the monosynaptic connections will be determined by optically recording the postsynaptic response of all the other cells. The connectivity patterns that result from normal development will be determined as a function of time in culture. The effects of activity will be studied by applying selected patterns of chronic stimulation through the extracellular electrodes while the cultures are developing.

252. Synapse Elimination at Cultured Neuromuscular Junctions

John R. Gilbert, Jerome Pine

Synapse elimination at the neuromuscular junction is the most well studied example of synaptic plasticity, yet the basic principles that control this phenomenon are only poorly known. In culture, neuromuscular junctions can be formed between chick ciliary neurons and skeletal muscle, and it is known that polyinnervation and synapse elimination occur in these cultures. To study the course of this phenomenon in detail and the effects of different patterns of activity, we plan to use microcircuit culture dishes for chronic stimulation.

Culture dishes have been fabricated incorporating 61 electrodes, which can be used to extracellularly stimulate selected neurons growing in the dish. Muscle cells are first plated in these dishes, and after about one week, when muscle fibers are well developed, ciliary neurons are added. The neurons make synapses on the muscle, and fibers are typically innervated by more than one neuron. The electrodes make it possible to individually stimulate different neurons that drive a given muscle fiber. The strength of each synaptic input can be determined by intracellular recording of postsynaptic potentials in the muscle fiber, and this may be done repeatedly without permanently damaging the muscle. One or more of the neurons that are identified as inputs to a particular muscle can be stimulated chronically, and the change in relative synaptic strength of the driven and nondriven inputs can be observed. In this way we plan to study how activity influences synaptic change in this system.

253. Neuron-Microdevice Connections

Wade Regehr, Tina J. Kramer, Becky G. Tanamachi, Jerome Pine

New techniques for maintaining long-term, two-way electrical communication with cultured neurons are being explored. The goal of this work is to provide purely electrical methods for noninvasively stimulating and recording from cultured neural networks. The basic idea is to emulate with a silicon device the intimate contact that a patch electrode makes with a cell membrane. The device being used for initial studies is called a "diving board" electrode because it consists of a base that supports a long flexible arm. At the end of the arm, a silicon dioxide cup is formed, which contains a gold electrode. An insulated lead connects this electrode to the base and from there, a thin insulated wire provides a connection with external
electronics. The electrode is manipulated into position so that the cup sits atop a neuron, and the base is then glued to the bottom of the culture dish with an ultraviolet polymerizing resin. Initial tests have shown that the diving board electrode works effectively for both stimulation and recording with rat superior cervical ganglion neurons and identified snail neurons (kindly supplied by Dr. S. B. Kater). The first experiments will explore the effects of chronic stimulation on the morphological development of individual snail neurons.

Since diving board devices are awkward to use for more than a small number of neurons simultaneously, a "neurochip" is also being developed, which consists of a silicon structure containing wells in which neurons grow. Chips with 16 wells are being fabricated and cultured neurons have been found to grow normally when contained in the wells. Work is now in progress on the technique for fabricating electrodes in each well which will connect each cell to external electronics. The first experiments with neurochips will be aimed at studying development and plasticity of networks of small numbers of superior cervical ganglion cells. Further experiments are planned in which identified cell types are used, labeled by retrograde transport in the embryo. Initial studies of cell-labeling in chick and rat are now being made.

254. Soft X-Ray Microscopy

Jerome Pine, Becky G. Tanamachi

It is not presently possible to visualize the synapses of a cultured neural network without destroying it by thin-sectioning and electron microscopy. With that technique it is not practical to see more than a small fraction of the synapses in a given network. There is a possibility that soft X-ray microscopy may make it possible to see all the synapses of an intact culture, which would be a very important adjunct to electrophysiological measurements which are the primary interest of our laboratory. To explore this possibility, we have been working with a group from SUNY Stony Brook and the IBM Watson Laboratories who have built a scanning soft X-ray microscope at the National Synchrotron Light Source. The microscope forms images by transmission at an X-ray wavelength of about 3 nm. The resolution is defined by the size of the scanning spot, which in turn is defined by the fineness of the pattern of a focusing zone plate. Presently, the smallest spot size is about 50 nm, but further work is expected to reduce this considerably in the coming year or two. The X-ray wavelength is such that the X-rays are very weakly absorbed by water and are strongly absorbed by carbon. As a result, imaging of the structure of whole, wet, unstained cells is feasible, at a resolution that is ultimately expected to be ten times better than light microscopy.

Thus far we have imaged wet, fixed neurons at a resolution comparable with phase-contrast light microscopy. We also have transported live nerve and muscle cells to the synchrotron laboratory and have imaged them wet and unfixed. These images have required exposures of over one hour, and it has not been possible to maintain an environment that enabled the cells to remain alive for that time. However, in the coming year the intensity of the X-ray beam will be increased by at least a factor of 1000, and the exposure time will drop to seconds. The resolution is also expected to improve greatly, and we expect to make images of live cells that could reveal synaptic structures.

PUBLICATIONS


DEVELOPMENTAL NEUROBIOLOGY

David J. Anderson
Seymour Benzer
James M. Bower
Masakazu Konishi
Paul H. Patterson
Mark A. Tanouye
Chromaffin cells of the adrenal medulla constitute the facial bones, all develop from the neural crest. This transient precursor population, therefore, generates a diverse array of both neuronal and non-neuronal differentiated derivatives. Further, many of these differentiated cell types retain the potential to express alternate developmental fates, in response to specific extracellular signals. The repertoire of these alternative fates is not infinite, but rather, is highly restricted, in a way that reflects the developmental history of these cells.

At the moment, we have focused on the development and plasticity of two major neural crest-derived cell types: chromaffin cells of the adrenal medulla (which are endocrine cells), and sympathetic neurons. Using a battery of molecular markers (both cDNA probes [Anderson and Axel, 1985] and antibodies), which discriminate these two cell types, we have traced the migration and differentiation of these cells in intact embryos and reconstituted their development in primary cell culture. These studies have identified an embryonic neuroendocrine precursor cell with the potential to develop into either a sympathetic neuron or a chromaffin cell (Anderson and Axel, 1986).

Apparently, these precursors develop into neurons unless they migrate into the adrenal gland, where the high, local concentration of glucocorticoids diverts them to an endocrine pathway of differentiation. Importantly, the differentiation of a chromaffin cell is not an irreversible development event: work by Paul Patterson and his colleagues has clearly demonstrated that individual chromaffin cells can, in the absence of cell division or DNA synthesis, convert into cells indistinguishable from sympathetic neurons when exposed to Nerve Growth Factor (NGF) (Doupe et al., 1985a). (Once made, this switch appears irreversible; however, these noradrenergic sympathetic neurons are able to undergo a further conversion to cholinergic neurons if exposed to a glycoprotein secreted by heart cells. Taken together, therefore, these data support the notion that chromaffin cell plasticity may be viewed as the expression of an alternative developmental fate originally available to the embryonic precursor (Doupe et al., 1985b).

Current studies are concerned with two principal aspects of this developmental process. At the cellular level, we wish to understand the lineage in more detail and the factors that control the timing of this decision. To what extent is the developmental schedule governed by the time of appearance of extracellular signals (such as glucocorticoids), as opposed to the time of responsiveness of precursor cells to these signals? At the molecular level, we wish to understand the intracellular regulatory circuits which control the expression of these two alternative cell fates during development and the conditions that permit plasticity later in life. To do this, we have had to analyze in detail the structure and regulation of expression of some of the genes we have used as markers of cell fate.

References:

255. Structure of the SCG10 Transcription Unit

Nozomu Mori, David J. Anderson, Benton N. Yoshida, Reuven Stein

A neuron-specific cDNA probe, SCG10, was first isolated in a differential screen that selected for clones encoding mRNAs expressed in sympathetic nervous system neurons (Mori et al., 1985). Subsequent analysis indicated that SCG10 transcripts are more abundant in the peripheral autonomic nervous system than in the central nervous system, suggesting that this allele of Neuron-Specific Enolase (NSE) is expressed only in the posterior paravertebral sympathetic ganglia (Mori et al., 1986). However, no significant enrichment of SCG10 mRNA could be found in the adrenal medulla or in the noradrenergic sympathetic neurons of the peripheral nervous system, which suggests that SCG10 is not expressed in these noradrenergic sympathetic neurons (Mori et al., 1986). In contrast, SCG10 is expressed in the chromaffin cells of the adrenal medulla (Mori et al., 1986a). It is possible that SCG10 is expressed in a subset of chromaffin cells, such as those that innervate the adrenal medulla, which suggests that SCG10 may play a role in the development of these cells (Mori et al., 1986a).

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ganglia but not in the adrenal medulla. SCG10 first becomes expressed in embryonic central and peripheral neurons at the time they begin to differentiate morphologically. SCG10 is not detectably expressed by cultured chromaffin cells, but becomes expressed when these cells transdifferentiate into neurons, in response to NGF. The expression of SCG10 is up-regulated by NGF in the pheochromocytoma line, PC12. This induction can be partially inhibited by glucocorticoids. Therefore, SCG10 shows at least three types of regulations: initial, lineage-specific expression; induction by NGF; and suppression by glucocorticoids.

To understand the complex regulation of this gene, we have first begun to elucidate its structure. Two mRNAs, which apparently differ in their choice of polyadenylation site, are transcribed from the SCG10 gene. One is 2.5 kb, the other 1.1 kb. Most of the sequence of the longer mRNA has been determined and indicates a 495 bp open reading frame. The sequence in this open reading frame has not been found in any database we have searched. 75-80% of the mRNA appears to be 3' noncoding sequence. Primer extension and S1 protection experiments indicate four major and six to seven minor transcription initiation sites clustered within a 150 bp region. The relative utilization of these sites does not change as a function of age, cell type or hormonal regulation. This pattern of multiple mRNA start sites and long 3' untranslated regions is characteristic of many brain-specific genes, but its significance is unknown.

Genomic sequences encompassing most or all of the SCG10 cDNA have been isolated as four adjacent or overlapping lambda clones. These clones span a region approximately 40 kb in length. The gene contains at least five exons. In an attempt to facilitate the manipulation of this gene for expression studies, one of us (Yoshida) has begun to screen for cosmids clones in a mouse library generously provided by Dr. Brian Popko (Hood laboratory).

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256. Regulation of SCG10 Expression

Nozomu Mori, David J. Anderson, Reuven Stein1, Carol Wuenschell

In order to identify sequences in the SCG10 gene controlling its expression, the regulation of the endogenous gene has first been examined in some detail using various cell lines as model systems. SCG10 is expressed only in cell lines of neuroendocrine origin, such as neuroblastoma and PC12 cells. Other neural crest-derived lines, such as melanoma, do not express the gene. In some neuroblastomas, such as SY5Y, SCG10 is constitutively expressed at high levels. In PC12 cells, there is low-level basal expression, but SCG10 mRNA levels are induced five- to tenfold within 24 hours of exposure to NGF. This induction can be blocked by protein synthesis inhibitors, indicating that SCG10 is not a "primary response" gene, such as c-fos, but part of a set of tissue-specific "late genes" whose expression during differentiation may be controlled by the "primary" response genes. Nuclear run-on studies indicate that SCG10 induction by NGF is, at least in part, transcriptional. SCG10 is induced by FGF as well as NGF but not by EGF or dibutyryl cAMP. Glucocorticoids depress both NGF induction and basal expression of SCG10 approximately twofold.

We have identified and sequenced the SCG10 promoter. This promoter contains consensus "CAAT" and "TATA" elements and drives transcription of a heterologous gene (bacterial CAT) in an orientation-dependent manner in transfected cells. SCG10 promoter-CAT fusions have been made containing 0.5 and 4.1 kb of 5' flanking DNA and their activity analyzed after transient expression in various cell lines. These constructs behave similarly, in that they show some level of expression in all cell types tested (neuronal and non-neuronal), and are induced up to tenfold by NGF and FGF, but not EGF, in PC12 cells. Induction requires the high-affinity NGF receptor, as it does not occur when these constructs are transfected into melanoma cells or fibroblasts bearing the low-affinity receptor. Apparently, however, the NGF induction is not sequence-specific, as many "control" promoters (such as RSV-CAT, tk-CAT) show a qualitatively similar induction. One of us (Wuenschell) has now begun to determine whether these same 5' constructs will show appropriate expression in transgenic mice. Cell transfection studies using a cosmids are planned to determine whether expression is controlled by intragenic sequences.

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257. Analysis of the SCG10 Gene Product

Keith A. Matthews, David J. Anderson

In order to learn whether SCG10 expression is additionally regulated at the translational or post-translational levels and to gain insight into the possible function of the gene product, we have prepared an antibody against the protein encoded by this gene. The complete SCG10 coding sequence (165 amino acids) was expressed as a fusion protein in the vector pATHl, with the 37 kDa bacterial TrpE polypeptide at the NH2 terminus (Spindler et al., 1984). A polyclonal antiserum to this fusion protein was successfully raised in rabbits. In embryo sections, anti-SCGlO stains exclusively nervous tissue, with staining more intense in processes than in cell bodies. Staining of dissociated sympathetic neurons in primary culture reveals a punctate pattern in the perinuclear region of the cell body, and particularly in nerve terminals and varicosities, suggesting that SCG10 may be a component of synaptic vesicles. However, in vitro translation experiments using pancreatic rough microsomes indicate that SCGlO is neither an integral membrane nor a secretory protein, and, therefore, is most likely peripherally associated with the vesicle membrane. On Western blots of brain tissue, the antibody detects a protein of 20 kDa, in agreement with the sizes of the hybrid-selected translation product, the translation product of a synthetic message transcribed from the cDNA, and the polypeptide predicted from the DNA sequence. Unexpectedly, however, the antibody detects a protein of similar size in cell lines that do not express SCG10 mRNA, as well as a third protein of approximately 68 kDa. The significance of these cross-reactive species is unknown but is being investigated by affinity purification and absorption experiments.

References:

258. Molecular Basis of Chromaffin Cell Plasticity

David J. Anderson

A satisfactory molecular explanation of chromaffin cell plasticity must account for two main aspects of this property: the persistence of the potential to express the alternative, neuronal phenotype and the latency of this phenotype in mature chromaffin cells. We have first asked, using our neural-specific cDNA probes, whether we can reveal the neuronal potential of chromaffin cells as any overt, detectable indication of neural-specific gene activity. When Northern blots of adult adrenal medullary RNA are annealed with high specific-activity SP6 probes and overexposed, a small amount of SCG10 and neurofilament 68 kDa mRNA is detectable, at a level 50- to 100-fold lower than that found in sympathetic ganglia. This trace amount could be due to low levels of expression in all medullary cells, or could be contributed by the small number of bona fide neurons present in the adrenal medulla. Nuclear run-on transcription experiments were performed on adrenal medullary and ganglionic nuclei. These data indicate that both SCG10 and NF68 are actively transcribed by adrenal nuclei and at rates only 3-5X lower than in ganglionic neurons. This relatively high level of transcription cannot be accounted for by the small number of neurons in the adrenal and indicates that most, if not all, chromaffin cells are transcribing neural-specific genes at a detectable rate. Thus, the persistence of the neuronal potential in chromaffin cells may be explained by the fact that neural-specific genes, which are "transiently" expressed in embryonic neuroendocrine precursors, do not undergo complete transcriptional inactivation during chromaffin cell differentiation and maturation. Furthermore, the relatively large discrepancy between the steady-state mRNA measurements (50-100X difference) and the run-on data (5X difference) suggests that at least some part of the latency of the neuronal phenotype in chromaffin cells may be due to a relative instability of neural-specific transcripts, compared to their counterparts in bona fide neurons. Direct measurements of mRNA turnover will be necessary to test this hypothesis.

259. Embryonic Extra-Adrenal Chromaffin Cells Express Neural-Specific Markers

Arie M. Michelsohn, David J. Anderson

Using classical histochemical techniques, neuroanatomists have extensively documented the appearance of collections of "chromaffin" cells in embryos at locations outside of the adrenal gland (Lempinen, 1964). Although often transient, the development of this extra-adrenal chromaffin tissue presents a paradox: if high concentrations of glucocorticoids are necessary for intra-adrenal chromaffin cell develop-
memt, how can chromaffin cells form outside of the adrenal gland? Our earlier observations that, in vitro, chromaffin precursors express neural-specific markers in the absence of steroids suggested a resolution to this paradox: extra-adrenal "chromaffin" cells would be different from intra-adrenal, true chromaffin cells, by the criterion of expression of neural-specific markers.

Using several different antibodies, we have recently confirmed this. Monoclonal antibody B2, which detects a surface antigen present on ganglionic sympathetic neurons but not chromaffin cells, and anti-SCG10 both stain extra-adrenal chromaffin tissues such as the para-aortic body at E14.5 and E18.5. The levels of staining are equivalent to those observed in bona fide sympathetic ganglia of the same ages and contrast sharply with the absence of staining inside the adrenal. Conversely, a series of monoclonal antibodies raised by Josette Carnahan (Patterson lab), which stain postnatal chromaffin cells but not neurons, fail to stain most extra-adrenal chromaffin cells in late-gestational animals but stain intensely those inside the adrenal. Thus, these studies for the first time indicate that intra- and extra-adrenal chromaffin cells are phenotypically distinct and are consistent with the hypothesis that glucocorticoids act on chromaffin precursors not only to promote the development of endocrine properties but also to inhibit the expression of neuronal properties.

Reference:

260. Temporal Specificity of PNMT Expression in Cultured Embryonic Adrenal Chromaffin Cells

Arie M. Michelsohn, David J. Anderson

Although chromaffin cells in the adrenal medulla and neurons in the sympathetic ganglia derive from a common embryonic progenitor, most chromaffin cells release epinephrine, whereas sympathetic neurons release norepinephrine. Epinephrine synthesis depends upon expression of the enzyme PNMT (phenylethanolamine N-methyl transferase). This enzyme is induced with striking temporal specificity in the development of adrenal chromaffin cells but not in sympathetic neurons. Using a bovine PNMT probe, we have cloned a rat PNMT cDNA and demonstrated that the mRNA encoding this enzyme is first detectable in the embryonic adrenal gland at day 16.5 of gestation (E16.5); expression increases dramatically thereafter. The induction of PNMT has been shown to be dependent upon glucocorticoids (GCs) (Bohn et al., 1981; Teitelman et al., 1982). The timing of this induction is well correlated with the burst in corticosterone synthesis by the adrenal cortex, which occurs at E16.5; the placement of chromaffin cells in the adrenal medulla would then expose them to high concentrations of the hormone. However, embryonic chromaffin cells are phenotypically distinguishable from their neuronal, extra-adrenal counterparts as early as E14.5, and these differences can be reconstituted in vitro by exposure of E14.5 cultures to GC. This suggests that GCs control the differentiation of chromaffin cells at least 2-3 days prior to the appearance of PNMT, and therefore that the timing of induction of the enzyme must be controlled by factors other than the appearance of the hormone or the responsiveness of chromaffin cells to it. In order to identify these factors, we have first asked whether the schedule of PNMT appearance can be reconstituted in dissociated cultures of embryonic adrenal glands. Using antibodies to PNMT, we have learned from our initial experiments that the in vivo timing of expression can indeed be reconstituted in vitro: cultures of cells obtained from embryos at E14.5, E15.5, or E16.5, if maintained in culture in the presence of GC, all express significant numbers of PNMT-positive cells at the equivalent of E17.5 (3, 2 and 1 day in culture, respectively); staining for the enzyme is negligible before E16.5. Cultures maintained in the absence of GC develop PNMT-positive cells at the appropriate time but the number of positive cells is significantly reduced. Thus, GCs are unable to induce precocious expression of PNMT. If we are able to demonstrate, by independent criteria, that these immature chromaffin cells are nevertheless responsive in other ways to GC, the implication is that a timed process controls the ultimate accessibility of PNMT gene to the hormone-receptor complex. Using specific antibodies and a fluorescence-activated cell sorter, we hope in the future to determine whether this timed process is governed by a cell-autonomous developmental clock. If it is, we would like to know the factors that activate and terminate such a clock in
chromaffin cells, and also those that prevent its functioning in sympathetic neurons.

References:

261. A Factor that Induces Adrenergic Differentiation in Avian Neural Crest Cells

Derek L. Stemple

One of the major pathways of differentiation for neural crest cells is the adrenergic pathway. Cells in this pathway consist of a subpopulation of sympathetic neurons, chromaffin cells of the adrenal medullae and SIF cells. Howard and Bronner-Fraser (1985) have shown that extracts made from central nervous system of 11-day chick embryos contain a factor that will induce adrenergic differentiation in cultured neural crest cells. In addition, these workers have found that the activity is soluble, and is also produced by 2-day-old neural tube in culture. One possibility is that as neural crest cells migrate over the neural tube toward their final destination, they are instructed to become adrenergic by this diffusible signal. This hypothesis is consistent with the timing and source of production of this putative factor. However, in order to directly assess the role of this factor in vivo, it is necessary to purify it, characterize its mechanism of action in vitro and develop probes for examining and perturbing its expression and function in the intact embryo.

There are several approaches one might take towards the isolation of factors such as these. Direct biochemical purifications have been employed successfully for a number of growth factors and hormones. These strategies generally are time- and material-intensive and produce only the purified factor, rather than anything that may be directly used as a specific molecular probe. Moreover, the small quantities of most such factors present in tissues make sequencing and antibody production extremely difficult. As an alternative strategy, therefore, I am employing an expression cloning procedure for the identification of this factor (Noma et al., 1986). As an initial step, I have purified RNA from the brains of 11-day chicken embryos and injected the purified RNA into Xenopus oocytes. In preliminary experiments, the oocytes thus injected appear to be capable of secreting the adrenergic activity as assayed in vitro. This functional assay in principle can permit us to screen a cDNA library by expression of biological activity, rather than by using antibodies or nucleic acid probes. Positive cDNA clones would in turn provide specific molecular probes for the activity, which may be used to address the importance of the factor in vivo.

References:

Publications


Professor: Seymour Benzer
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Senior Research Fellow: Utpal Banerjee
Research Fellows: Dennis G. Ballinger, Roger W. Hackett, David R. Hyde, Kirk L. Mecklenburg, John Archie Pollock, Bruce A. Rabin
Graduate Students: William M. Leiserson, Patricia J. Renfranz, Erich M. Schwarz
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Summary: The Benzer group is interested in the problem of how the information in the genome is utilized, during development, to determine specific cell types and their interconnections to form a neural network. Monoclonal antibodies (MAbs) enable one to identify the molecules that endow neurons with their individual specificities, while recombinant DNA methods, together with classical genetic techniques, make it possible to isolate and study the corresponding genes and their functions. If hybridomas are made, using unpurified brain material, each hybridoma produces a MAb against only one specific antigen of the mixture. Professor Benzer and his colleagues have isolated a panel of varied MAbs against the Drosophila nervous system to use in studying developmental problems (Fujita et al., 1982).

A simple neural system that is amenable to analysis is the developing Drosophila eye. The adult eye is a modular structure of some 800 similar ommatidia, each containing only eight photoreceptor neurons of three kinds: R1-6, R7, and R8. They differ in their precise positions, their action spectra, and in the way their axons terminate. R1-6 neurons send axons to the first optic ganglion (the lamina), whereas the axons of R7 and R8 pass through the lamina to synapse in the second optic ganglion (the medulla). This is, in effect, a nervous system of eight neurons of three specificities, reiterated 800 times. Genetic mosaic experiments have shown that the eight cells in each ommatidium are not derived from a single mother cell; they appear to arrive at their specific identities by epigenetic interaction. Development of the cluster occurs in the eye imaginal disc, during the third larval instar, in the wake of a morphogenetic furrow that sweeps from posterior to anterior across the disc. Ahead of the furrow, the cells are unpatterned; behind it, they form into a regular array of cluster of five (R2, 3, 4, 5 and 8). R1, 6 and 7 are added shortly afterward (Ready et al., 1976).

This system is being approached on several levels: 1) characterization of mutations that affect the initiation of the array, the formation of its pattern, and the sequential activation of associated gene products; 2) starting with a specific mutation such as sevenless, in which photoreceptor R7 is absent in each group of eight, cloning the gene and studying the timing and localization of its expression, using in situ hybridization and monoclonal antibodies to a fusion protein (Banerjee et al., 1987); 3) the reverse procedure, i.e., starting with a monoclonal antibody that identifies a photoreceptor-specific antigen, using the antibody to purify the protein, sequence it, clone the corresponding gene, map its site, and induce local mutations to identify the function (Zipursky et al., 1984; 4) isolation of mutants in which the projections of the photoreceptor axons to the optic ganglia are altered; and 5) isolation of cDNA clones that are specifically expressed in the eye and the optic lobes.

An exciting development in the work has opened up wide possibilities, namely, the discovery that a large fraction of the MAbs against the Drosophila nervous system also cross-react with various components of the human nervous system (Miller and Benzer, 1983; Miller et al., 1987; Hinton et al., 1987). The group is currently investigating homologies at the gene level between the two species. These experiments are being done in collaboration with Dr. Carol Miller, who is a visiting associate at Caltech and, as chief of neuropathology at the University of Southern California School of Medicine, has ready access to human brain material.

References:

262. Molecular Characterization of the sevenless Gene in Drosophila

Utpal Banerjee, Patricia J. Renfranz, Seymour Benzer

When the sevenless (sev) gene in Drosophila is mutated, photoreceptor cell number R7 is specifically absent in each ommatidium of the compound eye. It has been demonstrated that the sevenless phenotype is cell autonomous (Harris et al., 1976; Campos-Ortega et al., 1979), implying that a putative R7 must itself contain a normal sev+ gene in order to develop.
properly. Since the neuronal types in the fly’s eye do not use clonal relationships to specify identities, the sev\(^*\) gene may play a role in the developmental decisions that cells make in the eye disc.

DNA from the sev region was cloned by P element transposon tagging. Three P element-induced alleles were isolated and DNA from the sev region was cloned from the allele sev\(^{P1}\). This was in turn used to isolate over 120 kb of wild-type fly DNA (Banerjee et al., 1987a).

DNA mapping has shown that the allele sev\(^{P1}\) resulted from a complete deletion of the sevenless gene, and thus represents a null allele. DNA from the sevenless region mapped to a smaller, 11 kb deletion in another allele, sev\(^{P3}\), and identified a restriction site polymorphism in an EMS-induced allele, sev\(^{f1}\).

Northern blot analysis showed that the sev transcript is 8.2 kb in length, and is expressed in late third instar eye discs, in early pupae, and in adult heads. It was not detected in late pupae nor in adult bodies. Several cDNA clones corresponding to this transcript were isolated, the longest being about 5 kb. Altered transcripts were detected in the P allele sev\(^{P3}\).

Northern analysis of sev mutants showed that the transcript is present in most of these alleles, despite the lack of cell R7, suggesting that the message is not restricted to cell R7 in wild type. The expression in adult head was persistent in eyeless mutants, implying a second role of sevenless in the adult brain.

A fusion protein of sevenless with bacterial a-galactosidase was generated. This was used to immunize mice and generate monoclonal antibodies. The MAb staining pattern showed that the sev protein is expressed immediately behind the furrow on the apical tips of many cells. Posteriorly, as the cluster is being organized, the staining pattern becomes periodic, exhibiting a doughnut shape coincident with each cluster. These doughnuts appear to consist of fibrillary material. Finer localization of the protein was achieved, in collaboration with David Hinton, by immunoelectron microscopy. The sev protein was found to be localized in the apical tips of cells within each cluster, restricted to the membranes of the most apical parts of the cells and in the membranes of microvilli (Banerjee et al., 1987b). The expression is seen in all photoreceptor and cone cells, and is not restricted to cell R7.

The developmental decisions leading to the formation of photoreceptor R7 seem to be made at the apical tips of the photoreceptor cells, and by some mechanism not yet clear, the presence of sev protein seems to be important for the development only of cell R7.

References:

263. Expression of the sevenless Gene

John Archie Pollock, Seymour Benzer

Gel-isolated restriction fragments of the sevenless genomic DNA and sevenless cDNA were tritium-labeled and used for in situ hybridization to eye imaginal discs from late third instar larvae and early pre-pupae.

During the third larval instar, the sevenless gene was found to be transcribed in the eye-imaginal disc, beginning at the morphogenetic furrow, where the mRNA became apparent through the height of the disc epithelium. Within a few hours, the mRNA appeared to become consolidated in the apical region of the disc, persisting for approximately two days. The expression then diminished, beginning first at the posterior end of the disc. By the time of pupation (11–12 hours after puparium formation) and eversion of the disc to its adult configuration, the sev transcript was apparently turned off. Northern analysis confirmed this; mRNA bands were seen in 8-hour but not in 25-hour pupae (Banerjee et al., 1987).

Northern analysis clearly indicated that a sev\(^+\) transcript of the same size as that seen in the larval/prepupal stage remains in the adult head. The mutant eyes absent (eya) lacks adult compound eyes (see Abstract No. 266). Using poly(A)\(^+\) mRNA from eya heads for Northern analysis showed that the 8.2 kb transcript was present. This indicated that the transcript is not restricted to the compound eyes. In situ hybridization with sev\(^+\) cDNA on cryostat sections of the head indicated the transcript to be present at a low level in the cortex of the brain and optic lobes, but not in neuropil. To see this signal required 4- to 6-
week exposures. A second method of in situ hybridization employing 1 µm plastic sections and biotinylated DNA probe stained with anti-biotin also indicated that transcript to be present in the cortex.

Reference:

264. The sevenless Gene Transcript is Ectopically Expressed in the Salivary Gland of Certain Alleles

John Archie Pollock, Seymour Benzer

Eighteen different mutant alleles of the sevenless were analyzed by both Northern analysis and in situ hybridization. The sevP1 allele lacked a detectable transcript by both methods. This mutation involves a 55 kb deletion including the entire sev+ gene (Banerjee et al., 1987). In other alleles, there were varying effects on in situ hybridization, from normal to reduced expression.

The most striking results involved the ectopic expression of sev transcript in the salivary glands of two independent mutant alleles. The expression seemed to follow the developmental time course normally seen in the eye disc. These results are being checked by Northern analysis of RNA from dissected salivary glands. Since it is possible that the sev promoter has been affected in these mutants, we are in the process of identifying their specific DNA alterations to identify sequences that direct tissue and temporal specificity of expression of the gene.

Reference:

265. Identification and Characterization of 60 Optic Pathway-specific cDNAs from a Pool of 500 "Head"-specific Clones

David R. Hyde, Kirk L. Mecklenburg, Michael J. Palazzolo, K. Vijay Raghavan, Elliot M. Meyerowitz, Seymour Benzer

We are analyzing pattern formation in the Drosophila compound eye and the axonal wiring specificity of the photoreceptors to the optic lobes. cDNA clones, representing messages specific to the optic pathway may identify molecules required for ommatidial pattern formation or may be used as cell-specific markers for studying development. We have isolated approximately 500 unique cDNAs whose corresponding mRNAs are expressed in adult heads, but are not detected by Northern analysis in embryos (see Abstract No. 103). The cDNAs corresponding to optic pathway-specific messages were identified by Northern analysis utilizing a Drosophila mutant deficient for specific tissues. Flies homozygous for the mutation eyes absent (eya) completely lack the compound eyes and have greatly disorganized and reduced optic lobes, yet the remainder of the head appears anatomically complete (see Abstract No. 266). Poly(A)+ mRNA was isolated from Oregon-R heads, bodies, eye heads, and embryos. The mRNA was electrophoresed on formaldehyde gels, transferred to Hybond-N paper, and individually hybridized to antisense cDNA from each cDNA. Using this screen, we have identified 60 unique cDNAs that are optic pathway-specific, in that they recognize an RNA species present in Oregon-R heads that is absent in both eya heads and Oregon-R bodies. The chromosomal locations of these cDNAs are currently being mapped to determine whether they correspond to known loci which exhibit mutant eye phenotypes. The cDNAs are also being hybridized in situ to tissue sections for temporal and spatial analysis of expression. Antibodies to cDNA fusion proteins are being made.

266. Characterization of eyes absent, A Mutation that Eliminates the Compound Eyes

Roger W. Hackett, Seymour Benzer

The adult integument of Drosophila derives in part from cells that are sequestered during larval development into structures called imaginal discs. One pair of discs, the eye-antennal discs, gives rise to the adult compound eyes, antennae, ocelli, and much of the remaining adult head cuticle. Discrete portions of each eye disc generate a particular adult structure. A new recessive and highly penetrant mutation, eyes absent (eya), has been described (Sved, 1986) in which the compound eyes fail to develop, but other adult structures derived from the eye-antennal discs (as well as from the other imaginal discs) appear normal. This mutation may identify a gene product required for proper development of only that region of the eye-antennal disc whose epithelial cells are destined to contribute to the various differentiated cell types of the compound eye. The high specificity and penetrance of the mutant phenotype distinguish eya from other...
"eyeless" mutants of Drosophila which cause variable loss of eyes and/or noticeably affect other structures derived from the eye discs.

We have compared eya and wild-type eye discs dissected from mature larvae and find: 1) the eye portion of mutant discs is significantly smaller than normal; 2) no expression of eye-specific antigens can be detected in mutant discs using several monoclonal antibodies, including antigens expressed very early during cellular differentiation in normal discs; and 3) mutant discs nevertheless retain a connection to the larval brain (the optic stalk), and the larval photoreceptor nerve traverses the optic stalk en route to the brain, as it does in wild type.

Only one spontaneous allele of eya exists, associated with an inversion on the second chromosome (Sved, 1986). In order to more fully define the function(s) identified by eya and to determine a genetic map position, new EMS-induced alleles are being isolated that fail to complement eya. Electron microscopic examination of eya eye discs should indicate whether extensive cell degeneration occurs in the mutant. A molecular analysis of eya has been initiated by isolating P factor–containing alleles of eya.

References:

267. Developmental Biology of glass

Erich M. Schwarz, Seymour Benzer

Over 200 mutations of Drosophila melanogaster have been discovered that roughen, flatten, or eliminate its eye surface. glass grossly disorders not only the eye exterior, but also the retina and two underlying optic ganglia to which the retina projects axons (Meyerowitz and Kankel, 1978). Furthermore, it abolishes expression of an antigen that normally appears in the developing compound eye (Lebovitz and Ready, 1982).

Although glass was first isolated in 1918, no developmental studies of it have been published since Medvedev (1935). We have begun a further analysis of glass development, using the monoclonal antibodies against developmentally-specific antigens isolated in this laboratory, to determine at which point in development glass first deviates from normal, and what anatomical and antigenic differences it shows as development progresses. Screens have begun to isolate temperature-sensitive alleles to determine at what time glass's effects on the visual system are exerted.

In normal development, future eye cells early on express Ag22C10 and Ag72H5, antigens restricted to neurons and photoreceptors, respectively; 20-30 hours later, the photoreceptor-specific antigen Ag24B10 appears. glass eye discs show a normal furrow and express 22C10 and 72H5; however, the cells expressing these antigens are ragged and disarrayed and do not express 24B10 later on. Thus, glass+ activity may be required for normal neural organization by the time neurons differentiate.

References:

268. Studies on the Synchrony and Symmetry of Eye Development in Drosophila

William M. Leiserson, Seymour Benzer

The way in which pattern formation is specified in the Drosophila eye is poorly understood. Some progress has been made in understanding the mechanism by which cells in an ommatidium are arranged, once the basic lattice of the eye has been formed. But little is known about how the spacing of the lattice is set up, how the morphogenetic furrow is initiated, and what the requirements are for its propagation. Furthermore, little is understood about how the mirror symmetry of the eye about its equator is generated. Using a mutant that places "physical barriers" in the way of pattern formation, we are attempting to answer questions concerning the synchrony and symmetry of eye development.

The mutant denoted Cephalotergite (Cet) is a homeotic mutation that causes inappropriate tissue (thought to be related to tissue found in the tergites) to differentiate in the eye (M. Schardin and E. B. Lewis, personal communication). Patches of this tissue can be of varied size, shape, and location in the eye. In extreme cases, the tergite tissue causes patches of ommatidia to become isolated. The tissue can be readily observed in imaginal discs of larvae in the late third instar. Discs containing the defect can have approximately normal shape, unlike some other
mutations that cause ectopic eyes. Experiments are under way to determine whether normal symmetry is maintained in isolated patches of ommatidia. In addition, a culture system will be set up to directly observe the progress of the morphogenetic furrow and the equatorial groove (Ready et al., 1976). If differentiation proceeds synchronously in the eye disc, independent of the geometric configuration of the tergite patch, we can conclude that either: 1) direct cell-cell contact is not necessary for the synchrony of differentiation, or 2) the tergite patch is capable of transmitting the necessary information to developing eye cells.

Reference:

269. Isolation of Monoclonal Antibodies Specific for the Nervous System of Drosophila

Bruce A. Rabin, Seymour Benzer

As an extension of our interest in obtaining monoclonal antibodies (MAbs) specific for the developing nervous system (Fujita et al., 1982), third larval instar imaginal discs and adult head homogenates were fractionated into subcellular components and subsequently used as immunogen for the generation of MAbs. The class of MAbs obtained that was of most interest contains MAbs directed against specific regions of the compound eye and nervous system of Drosophila. MAb135B9, for instance, recognizes an antigen in the adult eye localized to the apical tips of photoreceptor cells, at the point where they are juxtaposed to the cone cells. These tips are thought possibly to play some role in pattern formation within the developing eye. However, Ag135B9 does not appear to be present in third instar eye imaginal discs. It therefore represents one of the proteins elaborated during terminal differentiation of the eye and may be an important tool with which to probe this process. In contrast to MAb135B9, MAb139E5 is present at all stages of development and appears to recognize a molecule common to many neurons. Ag139E5 is particularly evident in fiber tracks of the larval cerebral hemispheres and adult brain. This MAb recognizes two proteins on Western blots with apparent molecular weights of approximately 38,000 and 120,000. Unlike many of the other MAbs previously isolated which demonstrate similar specificities (Fujita et al., 1982), MAb139E5 does not stain imaginal discs of third instar larvae and may, therefore, be recognizing a CNS-specific molecule. Many other eye-specific antibodies have been identified in this screen including those specific for pigment cells, photoreceptor cells, rhabdomeres, cones, and lens.

Reference:

270. Photophobe (Ppb): A Mutation in Drosophila that Reverses the Phototactic Response

Dennis G. Ballinger, Seymour Benzer

Wild type (C-S) D. melanogaster are strongly phototactic towards both ultraviolet (UV) and visible light. In a T-maze, when flies are given a choice between UV light (350 nm) and green light (550 nm), under the conditions we use, they choose UV about 10 to 1. Flies carrying a mutation in the sevenless (sev) gene lack one photoreceptor cell (R7) from among the eight found in each normal ommatidium. Such mutants make the opposite choice, preferring green over UV about 10 to 1. This is true for any one of over 20 independently-isolated mutant alleles of the X-chromosomal sev gene. The dominant, second chromosomal mutation, Photophobe (Ppb), was isolated as a suppressor of the green preference of the sevenless allele sev<sup>L</sup>Y<sub>3</sub>, i.e., the double mutant sev<sup>L</sup>Y<sub>3</sub>;Ppb/+ once again prefers UV over green. This preference is demonstrated in spite of the continued absence of cell R7.

When tested, in the same T-maze, for their phototactic preference for green light over darkness, sev<sup>L</sup>Y<sub>3</sub>;Ppb/+ flies prefer darkness. This is contrary to the positive phototactic responses of either sev<sup>L</sup>Y<sub>3</sub> or normal flies. Ppb is, therefore, of interest concerning the genetic control of decision making in the neural network of the Drosophila visual system. Flies of the genotype sev<sup>L</sup>Y<sub>3</sub>;Ppb/+ are negatively phototactic over at least a 4-log range in green light intensity. Electron microscopic examination of sev<sup>L</sup>Y<sub>3</sub>;Ppb/+ flies shows that the first optic ganglion, the lamina, is structurally altered in comparison to sev<sup>L</sup>Y<sub>3</sub> flies. The electroretinogram shows an enhancement of a specific component that develops
after a series of light flashes. This enhancement is even more prominent in the presence of two doses of the \( \text{Ppb} \) mutation.

The effect of \( \text{Ppb} \) varies when the mutant gene is combined with eight different \( \text{sev} \) alleles, indicating an unexpected complexity in the \( \text{sev} \) phenotype and its suppression.

### 271. Nervous System-specific Genes Conserved between \textit{Drosophila} and Mammals

Kirk L. Mecklenburg, David R. Hinton, Carol A. Miller, Seymour Benzer

A variety of genes and proteins have been shown to be highly homologous between \textit{Drosophila} and man. We are employing several strategies in order to identify conserved genes which are nervous system specific.

We have previously described a panel of monoclonal antibodies (MAbs) made with \textit{Drosophila} as the primary immunogen which show a high degree of cross-reactivity with the human nervous system (Miller and Benzer, 1983). Among them are neural-specific MAbs which react with similar cell types and show similar reactions on Western blots in \textit{Drosophila} and man. We are attempting to clone the corresponding gene from human and fly cDNA expression libraries.

Recently, the \textit{Drosophila} sevenless (\( \text{sev} \)) gene was cloned in our lab (Banerjee et al., 1987). We have used a \( \text{sev} \) cDNA as a probe to search for homologous sequences in genomic DNA of various species. Discrete bands observed on genomic Southern blots suggest that these may be present in many organisms, including man. Currently, additional portions of this gene are being used as probes to determine whether the complete gene, rather than just a fragment, is conserved.

In addition, a wider search for homologies is being made using a collection of cDNA clones that represent \textit{Drosophila} nervous tissue-specific genes. Recently, we have isolated over 500 \textit{Drosophila} cDNAs that are expressed in the \textit{Drosophila} head but are not detected in the early embryos (see Abstract Nos. 103 and 263). These clones are being screened against mouse brain and kidney poly(A)\(^+\) RNA to identify which of the nervous system-specific clones are highly conserved between species. The conserved clones will be characterized in \textit{Drosophila}, and the transcripts localized by in situ hybridization in \textit{Drosophila}, mouse, and human brains.

### References:

### 272. Neuronal Subsets in Human Spinal Cord and in Murine Spinal Cord-Muscle Cultures Identified by Monoclonal Antibodies

Celia Williams, David R. Hinton, Carol A. Miller

The human spinal cord contains a heterogeneous population of functionally organized neurons. A major limitation in defining the interaction of neuronal subsets has been the paucity of molecular markers that both identify the neuronal subtypes and trace the individual neurites from the neuronal perikarya to their corresponding terminals. Monoclonal antibodies (MAbs) offer the means to recognize neuron-specific antigens. MAbs can be applied to primary spinal cord-muscle cultures providing a two-dimensional model system in which the formation and maintenance of neuronal and neuromuscular interconnections can be analyzed.

A panel of 16 MAbs was prepared by using either \textit{Drosophila melanogaster} heads (Fujita et al., 1982) or human spinal anterior grey tissues as immunogen. The MAbs were comparatively tested by immunoperoxidase microscopy on human spinal cord tissues (Miller and Benzer, 1983) and murine primary cultures, established from fetal muscle and spinal cord tissues. Cultures harvested on days 5, 12, and 19, and cryostat sections of cervical, thoracic and lumbar spinal cord were acetone-fixed and stained using the avidin-biotin immunoperoxidase method.

The MAbs showed neuronal or glial specificity in the spinal cord and a majority were similarly reactive in the mouse primary cultures. MAb reactivity was present in the cultures harvested on day 5, and most of the antigens continued to be expressed thereafter. In the spinal cord, MAbs 44.1, 2F12, and 3F12 stained the perikarya and axons of most neurons, and MAbs 3B4 and 10G4 stained neuronal perikarya only. MAbs 6A2 and 5A11 reacted solely with perikarya and proximal dendrites of the Nucleus Dorsalis of Clarke. Axons in the spinal cord roots were reactive with MAb 3A4.

In the cultures, MAbs 44.1, 2F12, and 3F12 reacted with only a few neurons and their processes, while MAb 3B4 identified a larger number of neurons and their corresponding processes. A minor population of
neuronal perikarya only were reactive with MAb 10G4. There was no neuronal staining in the cultures with MAbs 6A2 or 5A11. MAb 3A4 reacted with a small population of neurites, particularly those making contact with muscle fibers in the cultures. Astroglial cytoplasm was reactive with MAb G12, and MAb 23E9 distinguished probable oligodendroglial cells, both in vivo and in vitro (Williams et al., 1986).

These results suggest that some antigens recognized by our MAb panel are neuron-subset or glial-specific both in the human spinal cord and in primary cultures. This experimental system may be useful in defining the functions of the antigens identified by the MAbs, and the roles of certain neuronal subsets in neuronal and neuromuscular interactions.

References:

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273. Neuronal Subset Degeneration in Alzheimer's Disease

Carol A. Miller, Maria Rudnicka1, David R. Hinton

Alzheimer's disease (AD) is a dementing neurodegenerative disease with the histologic hallmarks of neuronal loss, neurofibrillary tangles and neuritic plaques. While selective vulnerability of neurons of certain subcortical nuclei such as the nucleus basalis and locus ceruleus have been identified, loss of neurons within the cortex is less well defined. Using a panel of four monoclonal antibodies (MAbs) as immunohisto­logic markers, three of which were originally isolated using Drosophila tissues as immunogen (Fujita et al., 1982), we have examined autopsy tissues from the hippocampus, primary motor and premotor cortices (Brodman's area 4 and 6) from five patients with AD and five normal controls. We have identified three discrete neuronal subsets within the neocortex and hippocampus that are reactive with MAbs 44.1, 3F12, and 6A2.

In all AD tissues examined, a subset of pyramidal projection neurons reactive with MAb 3F12 is selectively lost within the hippocampus, especially in CA-1, subiculum and superficial layers of the entorhinal cortex. There is also loss of 3F12 reactive neurons primarily in layers 3 and 5 of the associated cortex (Brodman's area 6) (Miller et al., 1987).

MAb 3A4 identifies intracytoplasmic material only in AD hippocampus and cortex and is distributed in a pattern coincident with neurons containing 3F12 antigen. Thioflavin S staining also identifies neurofibrillary tangles and neuritic plaques within the regions containing MAb 3F12-positive cells. MAb 3A4 does not generally colocalize in cells containing thioflavin S-positive material. However, in rare cells, 3A4 reactive material can be seen around the periphery of thioflavin S reactive intracytoplasmic deposits. MAb 3A4 is non-reactive after incubation of tissue with alkaline phosphatase. MAb 3A4 does not react with neurofilament triplet proteins on immunoblots of tissue homogenates or purified phosphorylated and nonphosphorylated neurofilaments.

Our results have defined a target cell intrinsic to the hippocampus and neocortex in AD. We have also identified an intracellular antigen that may be unique to AD and also a possible precursor form of neurofibrillary tangles.

References:

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274. Axonal Transport of Neuronal Antigens Characteristic of Subpopulations of CNS Neurons

Roscoe D. Atkinson1, Carol A. Miller, Judith A. Garner

Certain monoclonal antibodies (MAbs) identify nervous system antigens that are localized to neuronal subpopulations which undergo change during the course of neurodegenerative diseases, such as Alzheimer's disease (Miller et al., 1987). We have now examined the axonal transport of four of the corresponding antigens for two reasons. First, neuronal forms of the antigens can be unequivocally identified by their axonal transport. Second, each of the three major axonal transport rate-components is thought to convey a subcellular structure within the axons: the fast
component (FC) provides membranes and membrane proteins, while the two slow components (SCb, SCa) convey the cytoplasmic matrix and cytoskeleton, respectively. Association of an antigen with a particular rate-component may give insight into its intracellular functions and its structural nature.

Radioactive amino acids were unilaterally injected into the vitreous of guinea pig eyes. Radiolabeled proteins synthesized in retinal ganglion cells were axonally transported into the optic nerve and tract. After appropriate intervals permitting separation of the rate-components in the axons due to their unique transport rates, animals were sacrificed. Optic nerve containing radioactive proteins characteristic of single axonal transport rate-components, and contralateral control nerves, were removed and subjected to immunoadsorption with each of the four MAbs. Visualization of radiolabeled antigen was by gel electrophoresis followed by fluorography.

All four MAbs identified antigens specifically associated with single axonal transport rate-components. Three MAbs (3F12, 44.1, and SC3) specifically identified polypeptide bands from tissue containing radioactive SCb proteins. Another MAb (2C210) identified two polypeptide bands from tissue that contained radioactive SCa proteins. None of the MAbs identified antigens from tissue containing radioactive FC proteins (Atkinson et al., 1987).

Assignment of these antigens or antigen subunits to specific subcellular systems has permitted their characterization as putative cytoplasmic matrix proteins (3F12, 44.1, SC3) or cytoskeletal proteins (C22C10). Three of these MAbs were originally isolated using Drosophila tissue or immunogen (Fujita et al., 1982). The roles these antigens may play in the nervous systems of fly and mammal during normal axonal function and during neuropathogenesis can now be further examined.


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PUBLICATIONS

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Summary: Work in our laboratory over the last year has involved numerous diverse but related projects. In general, our efforts can be divided into two overall areas of focus. First, several projects are aimed at understanding how real neural networks compute. Two specific structures are studied, cerebellar cortex and piriform (cerebral) cortex. In both cases, our work includes structural simulations of these networks, intracellular electrophysiological investigations of neuron and network properties to provide parameters for the models, multi-single unit recording as a means of linking the output of models to the real activity of the networks, and recently the development of behavioral experiments to look at overall network function.

The second general area of research involves the development of the pattern of peripheral projections to cerebellar cortex using genetic, immunocytochemical, and electrophysiological techniques. Over the last year considerable progress has been made in each of these areas as our diverse research group has continued to expand.

Neural Networks—Structural Simulations of Neural Networks Using a General-Purpose Neural Network Simulator Based on a Parallel Supercomputer

Upinder S. Bhalla, Wojtek Furmanski, Inna C. Harvath, Mark E. Nelson, Matthew A. Wilson, James M. Bower

Central to our efforts to understand the functional organization of neural networks is the ability to explore the dynamics of these networks using structural simulations based on their detailed anatomy and physiology. For the last year and a half, we have been developing a general-purpose neural network simulator to allow arbitrary networks to be modeled rapidly using the computational power of parallel supercomputers. This project involves, in general, three areas of effort: 1) the development of a graphically-based user interface to easily specify the network to be modeled; 2) the development of a simulation base code running on a hypercube class parallel computer into which the graphical description of the network can be compiled; and 3) the development of rapid, animated, graphical output from the modeled networks to evaluate network dynamics. In the last year, considerable progress has been made in all three areas. We now have a working user interface which allows rapid specification of connectivity for arbitrary networks composed of different types of neurons. We have also developed a very efficient hypercube base code. The network simulator currently is being used to model cerebellar cortex and piriform (cerebral) cortex. For a large piriform cortex simulation (3,000 fully interconnected neurons of two types; see next abstract), the hypercube base code simulates 1 msec real time in 100 msec computer time.

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Neural Networks—A Computer Simulation of a Three-Dimensional Model of Piriform Cortex with Functional Implications for Storage and Recognition of Spatial and Temporal Olfactory Patterns

Matthew A. Wilson, James M. Bower

Using parameters obtained from anatomical and physiological experiments (Haberly, 1986), a computer model of piriform (olfactory) cortex has been developed that simulates physiological responses obtained using a variety of recording methods (Wilson et al., 1986). Over the last year, we have extended this one-dimensional model of 70 neurons to a two-dimensional array of several thousand pyramidal cells interconnected by a highly divergent/convergent system of associational fibers. Excitatory input is now delivered to the cells along an afferent pathway containing multiple independent fibers. These fibers are capable of carrying complex spatial/temporal patterns with characteristics similar to the lateral olfactory tract projection to piriform cortex from the olfactory bulb. A more detailed dendritic model is now included which allows for spatial interaction of activity generated along different segments of the pyramidal cell dendrite. As in the original model, the two populations of interneurons included are each responsible for different forms of inhibition: a feedforward, long latency, long duration hyperpolarizing potential, which simulates the K+-mediated potential
observed in piriform cortex, and a feedback, short latency current shunting-type inhibition simulating the well-known Cl⁻-mediated process. The simulation incorporates the different propagation velocities of the separate fiber pathways, synaptic delays, refractory periods, time constant estimates for inhibitory and excitatory events, and spatial variations in the distribution of different excitatory and inhibitory influences. The previous model has been shown capable of simulating a variety of known intracellular and extracellular responses including odor-like oscillatory bursts, characteristic shock responses, and epileptic activity. Simulation results have revealed the presence of propagating waves of activity across the cortex which are driven by afferent input and carried by both afferent and association pathways. The current model has been extended to explore the significance of these simulated cortical dynamics in the processing of olfactory information. Using connections consistent with the known circuitry of piriform cortex and a learning rule that modifies synaptic strengths as a function of physiological variables, the model can successfully store and recall spatially- and temporally-encoded patterns. Discrimination of such patterns is hypothesized to be the basis for olfactory processing in the cortex.

References:

277. Neural Networks—Analysis of Multi-Single Neuron Data: Optimal Mapping of the Configuration Space of a Network of Neurons

George J. Carman, Brian Rasnow, James M. Bower

A major focus of ongoing efforts in our laboratory concerns the development of methods to record and analyze the activities of many neurons recorded simultaneously. This approach is based on the assumption that describing the collective properties of populations of neurons is essential for understanding the processing and representation of information within biological and simulated networks. Such analysis should identify the structure within the distribution of the state variables of the network over time, where by structure we mean the configurations, or sequences of configurations, to which the network is attracted. Since each neuron represents one potential degree of freedom, we have in principle a search for structure in a space of N+1 dimensions, where N is the number of neurons in the network, and we add an extra dimension for time. However, the network may be such that its configurations lie on manifolds of smaller dimension. If this is the case, then it may be possible to find an embedding of these manifolds which preserves the topology and geometry of a useful subset of the configuration space in an observation space of fewer dimensions.

We have used the technique of simulated annealing in an attempt to obtain such a nonlinear mapping (Kirkpatrick et al., 1983; cf. Carman and Van Essen, 1985). In our application of this optimization technique, we represent each of the $2^N$ possible configurations or states for N binary neurons by a point in N dimensions whose components describe the activity of each neuron. For each such point in the configuration space, we create a corresponding point in an observation space of three dimensions. For a given choice of a metric in each space, we minimize the square of the difference between the distance from a point to each of its neighbors in configuration space, and the corresponding distance in observation space, over all points and neighbors. The final positions of the points in the observation space describes one of what will, in general, be a degenerate set of optimal mappings.

For several such mappings, we chose as metrics the Hamming distance in a configuration space of seven or eight spatial dimensions, and Euclidean distance in an observation space of three spatial dimensions. The optimal arrangement of points (states) in the observation space found by our mapping approximates a spherical surface in which nearby states on the sphere are also nearby in configuration space. This is the result of a hierarchical clustering of states on the map, which emerges as a consequence of the approximately ultrametric structure of our configuration space with Hamming distance (Baldi and Baum, 1986).

This mapping has an N-factorial degeneracy such that, given any one map, all others can be generated by permuting the assignment of the neurons to the N binary components associated with points on the map. Thus, if an optimal representation exists for a given set of observed configurations and trajectories joining them in time, it can be found by a second annealing to
select an optimal permutation. For example, one can minimize the length of the tour specified by the trajectories, so that sequences of states that have $M$ components in common will span at most $2^{-M}$ of the map. The resultant assignment of neurons to components of the $N$-dimensional configuration provides a compact description of the hierarchical organization of the network. This mapping is currently being used to describe the patterns of activity recorded with multi-single neuron techniques.

References:

278. Neural Networks—New Silicon-Based Multi-Single-Neuron Recording Devices Applied to Cerebellar Cortex

Jasho J. Banik, Mark E. Nelson, Brian Rasnow, James M. Bower

The ability to record the simultaneous activity of a large number of neurons is invaluable to the experimenter who is trying to understand the dynamics of biological neural networks. For example, correlations in activity can provide information about the underlying network architecture which cannot be deduced from the activity of neurons recorded singly. Furthermore, information that is coded in distributed representations across many neurons is difficult to extract unless a representative fraction of those neurons can be observed simultaneously.

We have been using multi-neuron recording techniques in our laboratory to study the network properties of the cerebellar cortex. As a major part of this effort, we have been developing a relatively new type of multichannel microelectrode which takes advantage of semiconductor microfabrication techniques to achieve small probe size and a high density of recording sites. The basic geometry of our probes can best be described as a comb-like structure with the shank of each tooth approximately $20 \mu \times 20 \mu$ in cross section, tapering to a sharp point at the end of its $1.5-2.0$ mm length. The spacing of the teeth and the positioning of recording sites on an individual tooth can be optimized for the particular structure one wishes to study. For recording from the cerebellum, the teeth are typically spaced $100-200 \mu$ apart and can have up to six recording sites on a single tooth. The recording sites are created by cutting holes in insulating layers using a plasma etching process to expose gold pads which are subsequently plated with platinum-black for recording. The size and impedance of the recording sites can also be optimized for a particular recording situation. Our sites are typically $80-100 \mu^2$ in area and have impedances of less than $1 \text{M} \Omega$ at $1 \text{kHz}$ after platinizing. Having a silicon-based recording probe also provides us with the opportunity to incorporate signal processing electronics onto the same silicon substrate as the recording array. To this end, we are beginning to use CMOS technology to fabricate circuitry for preamplification and multiplexing of the neural signals. This eliminates the need for the tedious wire bonding required for passive probes, significantly decreases the size and rigidity of the "wiring harness," and decreases the susceptibility of the system to external noise.

A significant amount of hardware and software is also required to fully utilize the recording capabilities of the silicon-based probes. We have developed a system for precisely positioning the probes using a 6-axis stepping motor-controlled microdrive with $2 \mu$ spatial resolution and 2 mrad resolution in azimuth and elevation. We also have developed a 100-channel system of computer-controlled differential amplifiers which were designed and built for the readout of the silicon probes. Each channel has a computer selectable gain from 40 to $-10,000$ in 256 steps and computer selectable high-pass ($100, 300, \text{or} 600 \text{Hz}$) and low-pass ($1 \text{k}, 3 \text{k}, \text{or} 8 \text{kHz}$) filter stages.

These technical advances made in the last year have now placed us in a position to record from tens and possibly hundreds of cerebellar neurons simultaneously.

279. Neural Networks—A Hopfield Network Approach to Discriminating the Spike Waveforms of Simultaneously Recorded Neurons

Issac Wong, Amir Atiya*, James M. Bower

The experimental neurobiologist is faced with a variety of technical challenges when exploring the functional properties of biological neural networks. The previous abstract describes the approach we are taking to record neural waveforms from multiple cells
at once. However, once these data are obtained, the experimentalist is still left with the task of identifying (discriminating) the voltage signals of each recorded neuron and storing the resulting spike trains. Recording from many neurons at a time greatly increases the complexity of this task without diminishing the experimenter's obligation to assure high fidelity in spike train discrimination. With large numbers of recording channels, it is impractical to continuously record or even monitor the full waveform for each channel. Instead, we typically route the analog signals through a discriminator/monitoring processor which converts the analog waveforms into digital spiketrains and monitors the data for possible error conditions.

Over the last year, we have been developing techniques for automated real-time analysis and classification of neural waveforms. There are two cases in particular, where the data acquisition system must provide an automated and accurate classification of waveforms. The first is the case where a single recording site is picking up signals above threshold from more than one neuron. The second is the case where a single neuron has more than one characteristic response shape (e.g., simple and complex spikes from cerebellar Purkinje cells). In both cases, we would like to classify the waveform appropriately and provide a label to be recorded along with the spiketrain data.

Over the last year, we have developed a first version of a particular implementation of a waveform classifier which uses a Hopfield-type network to solve these tasks. The current software implementation of the classifier rapidly distinguishes different waveforms and classifies them as originating from one or another neuron. The classifier even recognizes the case of temporally overlying spikes from different neurons. We are currently designing a hardware implementation of the classifier which will be used in our multi-single experiments on cerebellar cortex.

Over the last year, we have pursued both results using two different physiological techniques. First, we have used our multi-single unit extracellular recording methods to sample activity in numerous Purkinje cells at the same time. These techniques have allowed us to use tactile stimuli to restrictively activate small regions of the granule cell layer and explore subtle influences of parallel fibers on distant Purkinje cells. Second, we have used intracellular in vitro brain slice techniques to examine in detail the influence of granule cell layer activation on Purkinje cells. For example, using a slice cut parallel to the plane of the Purkinje cell dendrite and multiple stimulating electrodes positioned under the cell, we have found that brief (100 μsec) activation of the granule cell layer can evoke a prolonged (200-300 msec) plateau potential that is graded with respect to stimulus intensity and sums with stimuli delivered simultaneously at different underlying granule cell layer locations. The additional fact that this potential can induce prolonged increases in simple spike activity suggests that it may underlie the long duration responses of Purkinje cells to short tactile stimulation in vivo.

References:
281. Neural Networks—The Interaction of Object Discrimination, Body Position, and Self-Generated Electric Fields in the Weakly Electric Fish Gnathonemus petersii

Brian Rasnow, Gary Grant, Mark E. Nelson, James M. Bower

The teleost fish Mormyridae Gnathonemus petersii are unusual among teleosits in that these fish use self-generated electric fields to detect objects in the near field. Neurobiologically, these fish are distinguished by their greatly enlarged cerebellar cortex. We have recently begun to study these fish in the interest of understanding the role of this enlarged cerebellum in the fish's behavior. Specifically, we are interested in knowing the interaction between the animal's body position and its use of its electric field to discriminate nearby objects. Based on our physiological experiments in the rodent, we have proposed that this sort of motor/sensory coordination during exploratory behavior may be one of the functions of cerebellar cortex. To test this hypothesis in these fish, it will be necessary to detect both the animal's body position and electric field with high resolution. We have recently begun to construct a detection device that will eventually consist of 2,000 closely-spaced (1 cm) detectors in a specially designed Y maze tank within which the fish will be asked to make an electrical discrimination. Concurrent with the development of this behavioral apparatus, we have also begun to use computer modeling to try to understand the consequences of fish position for sensory discrimination. Eventually, we hope to be able to record from cerebellar regions during these behavioral tasks.

282. Cerebellar Development—Monoclonal Antibodies Raised Against Specific Physiologically-defined Regions of Cerebellar Cortex

Joan L. Roach, Jami Frost, Girija Gundappa-Sulur, James M. Bower

A major and continuing issue in developmental neurobiology is the way in which complex, fine-grained sensory maps are constructed during development. Much work has gone into the general question of how growing axons establish appropriate connections in simple nervous systems and in selected regions of the developing vertebrate CNS such as the retinal-tectal system. Yet, beyond a basically descriptive accounting of fiber movements, identifying the underlying mechanisms that guide the highly ordered wiring patterns remains very much an unsolved problem in CNS development.

We have undertaken several projects designed to address this issue using the known pattern of tactile projections to cerebellar cortex of the rodent. These tactile projections have been made in great detail (Shambes et al., 1978; Bower et al., 1981) and have been shown to vary little in their arrangement from rat to rat, even though the map itself is highly disjunctive with respect to the body surface. We have taken several experimental approaches to determine if cell autonomous factors influence the establishment of this map (see next two abstracts also). In this particular experiment, we have attempted to generate monoclonal antibodies against physiologically-defined regions of cerebellar cortex looking for inhomogeneous labeling patterns. Of 1200 original clones, we have identified 12 with sufficient specificity to warrant further study. One such MAb selectively labels specific fibers in the trigeminal system which appear to terminate preferentially in cerebellar cortex. This antibody and several other antibodies are currently being further characterized at the light and EM levels.

References:

283. Cerebellar Development—Cell Lineage and the Organization of Tactile Maps in Cerebellar Cortex

Karl Herrup1, George J. Carman, James M. Bower

A second approach we are taking to understanding how the tactile maps in cerebellum are established developmentally involves the use of mouse chimeras whose two Purkinje cell lineage lines can be distinguished with a-glucuronidase histochemistry. The relationship between Purkinje cell lineage and the tactile maps is being determined by comparing the maps of the tactile projections to the granule cell layer with lineage maps of overlying Purkinje cells.

A computerized microscope is used to reconstruct a three-dimensional lineage map from a series of sagittal sections. A chi square test is used to determine any associations between the distributions
of the two phenotypes as a function of spatial location within the sample of data. This is accomplished by dividing the lineage map surface into \( N \) equal-sized tiles in a grid and creating a contingency table for each phenotype for each row and column in the grid. Expected frequency was then calculated and compared to observed frequency for each tile. The result was that the distribution of phenotypes was highly non-random in 10 of the 11 cases analyzed—indicating that cells of like lineage were grouped into patches of variable size, similar to those found with physiological mapping techniques. Experiments to directly compare the lineage and tactile maps in the same animal are currently under way.

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284. Cerebellar Development—Invariance in the Overall Patchy Organization of Tactile Projections to Cerebellar Cortex Following Peripheral Nerve Lesions Made Early in Cerebellar Development

Jo Beth Schlottman, James M. Bower

The organization of mossy fiber tactile projections to the granule cell layer of the cerebellar hemispheres results in a patchy somatotopic map representing selective parts of the body surface (Shambes et al., 1978). The patches are defined by sharp boundaries between projections from different body regions (Woolston et al., 1981). Comparisons of these maps between individuals of one species (rat) or even between closely-related species (rat-mouse) reveal a remarkably invariant pattern of patches in any one cerebellar folium. For example, the map in crus I1a of the rat or mouse is characterized by a large ipsilateral upper lip patch surrounded by smaller patches representing other peri-oral surfaces (e.g., lower lip, lower incisor, upper incisor). This invariance suggests that the general organization of this patchy pattern may be somewhat inflexibly specified during development. This suggestion is in keeping with our recent Purkinje cell lineage experiments showing, in chimeric mice, that cells are grouped in lineage domains comparable in size and distribution to the physiologically-defined patches (Herrup and Bower, 1986).

To investigate further the establishment of this patchy organization during development, we have lesioning specific peripheral nerves at a time before cerebellar connections are fully established. Specifically, we severed (under anesthesia) trigeminal nerve branches innervating the upper lip of 9-day-old rats. After these rats reached adulthood, we mapped the area of crus I1a usually containing a large representation of the now denervated upper lip. The resulting maps reveal that the overall patchy organization was virtually unchanged, i.e., there remained a very large central patch similar in shape and dimension to the one normally receiving upper lip projections. This large patch was now completely occupied by projections from two small areas bordering the upper lip, namely the corner of the mouth and an area directly below the nose. The outlying small patches representing other body surfaces in these animals were in all respects normal. These results are consistent with the view that the boundaries between patches in the tactile projection to cerebellar granule cell layer may be established inflexibly during development, for example by Purkinje cell lineage.

References:


285. A New Approach to Teaching Science to Elementary School Children

James M. Bower, Brian Rasnow, Mark E. Nelson, Jerome Pine

In addition to the scientific work described above, the lab is also involved with a project to implement a new approach to elementary school science education in the Pasadena Public School District. The approach, which is based on a hands-on approach to teaching science, has recently been introduced to one test school in the district. The basic hands-on materials that are now being used will be augmented by personal computer-based animated simulations derived from the hands-on materials. Several students from our lab will be involved in developing these simulations.

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PUBLICATIONS


Summary: We study the neural mechanisms of sound localization in the barn owl, vocal learning, and brain sexual differentiation in songbirds. Each of these projects has developed from initial behavioral observations into the systems and cellular level of analysis. The owl's auditory system takes us far beyond the level of analysis that prevails in the orthodox study of sound localization.

Learning is one of the major topics in neuroscience today. Advances are being made in the study of cellular and molecular events that occur at single synapses in simple reflex circuits. Changes in single synapses code for simple information such as go or no-go. Higher forms of learning involve complex information such as the acoustic characteristics of birdsong. Our study of song learning concerns the neural representations of learned and vocalized song in the brain of the white-crowned sparrow.

Gender differences in the brain are known in several animals including man. We found that sexual dimorphism in the song control areas of the zebra finch brain is due to cell growth in the male as well as to cell death in the female. Administration of estrogen can masculinize the female brain by preventing the programmed cell death. Thus, the zebra finch provides one of the most tractable systems for the study of brain sexual differentiation. This system also allows us to go beyond the level of analysis that characterizes most work in this field. We have been able to culture dissociated cells as well as brain slices from young zebra finches.

286. Hodological Organization of the Barn Owl's Inferior Colliculus

Terry T. Takahashi

The barn owl determines the directions from which sounds emanate by computing the interaural differences in the timing and intensity of sounds. These cues for sound localization are processed in independent channels originating at nucleus magnocellularis (NM) and nucleus angularis (NA), the cochlear nuclei. The cells of NM are specialized for encoding the phase of sounds in the ipsilateral ear. The cells of NA are specialized for encoding the amplitude of sounds in the ipsilateral ear.

Our data suggest that NM projects solely, bilaterally, and tonotopically to nucleus laminaris (NL). NL and NA project to largely non-overlapping zones in the central nucleus of the inferior colliculus (ICc). The terminal field of NL occupies a discrete zone in the rostromedial portion of the contralateral ICc, which we have termed the "core" of ICc. The terminal field of NA surrounds the core of ICc, and thus forms a "shell" around it.

287. Axonal Delay Lines Create Maps of Interaural Phase Difference in the Owl's Brainstem

Catherine E. Carr, Mark Konishi

Sensitivity to the interaural temporal disparities that underlie sound localization in the barn owl first appears in nucleus laminaris (NL), a brainstem auditory nucleus which is also the first recipient of binaural input. Eighth nerve afferents enter the brain and
divide to innervate the angularis and magnocellularis cochlear nuclei. Nucleus magnocellularis (NM) codes for phase information and projects bilaterally and tonotopically to NL.

The organization of NM afferents in NL provides a morphological substrate for a map of interaural delays. Incoming axons from the ipsilateral NM enter the dorsal surface of NL, while contralateral axons enter from the ventral surface. The afferents interdigitate in NL and innervate NL neurons through the dorsal-ventral extent of the nucleus. If NM afferents act as delay lines, physiological recordings should reveal an orderly change in conduction delay with depth.

Period histograms were used to measure the timing of phase-locked spikes in these afferents in over 200 intracellular recordings in NL. Systematic changes in phase were observed with depth; a gradual increase in delay was observed for ipsilateral afferents, and a decrease for contralateral afferents. Outside the dorsal or ventral borders of the nucleus, the phase shifts are less ordered and the best frequency no longer constant. The conduction time through the nucleus reflects the time difference between the two ears (about 180 µsec) independent of frequency. Thus, these afferents act as delay lines to create maps of interaural temporal disparities.

288. Flow of Information in the Network that Computes Azimuth in the Barn Owl

F. Hermann Wagner, Terry T. Takahashi, Mark Konishi

The central nucleus of the barn owl’s inferior colliculus (ICc) is a major source of afferents to the neural map of contralateral space found in the external nucleus of the inferior colliculus (ICx). ICc itself consists of a centrally located "core," defined by the terminal field of nucleus laminaris (NL), surrounded by a "shell," defined, in turn, by the terminal field of nucleus angularis (NA). Interaural time difference (ITD), the binaural cue for the location of a sound source along the horizontal axis, azimuth, is systematically represented in ICc. ITDs corresponding to loci in the ipsilateral hemi-field are represented in the core, and ITDs corresponding to loci in the contralateral hemi-field are represented in the lateral portion of the shell. The representation of ipsilateral space in the core is simply explained. NL of the left, for example, which represents predominantly the right, or contralateral hemi-field, projects contralaterally thereby conferring upon the core of the right ICc selectivity for right or ipsilateral space. NA does not contain cells sensitive to ITD. How then, does the lateral shell acquire selectivity for ITDs corresponding to loci in the contralateral hemi-field?

Small quantities of horseradish peroxidase were iontophoresed into the lateral shell under guidance of extracellular recordings (four cases), and the distribution of retrogradely-labeled somata was assessed by the tetramethyl benzidine method. Ipsilateral to the injection site, retrogradely-labeled somata were scattered throughout ICc, with the greatest density occurring in the immediate vicinity of the injection site. The functional significance of this labeling pattern is difficult to interpret. Interestingly, however, contralateral to the injection, retrogradely-labeled somata were concentrated in the core of ICc. This suggests that the core of the right ICc, for example, which contains a representation of right or ipsilateral space, projects to the lateral shell of the left ICc, thus presenting to it a representation of right or contralateral space. Finally, from the lateral shell, the representation of contralateral space is transmitted to the ipsilateral ICx.

289. Development of Auditory Selectivity during Song Learning

Susan F. Volman, Mark Konishi

In adult white-crowned sparrows, auditory responses in the song-control nucleus HVc are selective for an individual bird’s own song (Margoliash, 1986). Juvenile birds (4–8 months old) that have been exposed to an appropriate song model (“tutor song”), but have not yet sung, show no evidence of neuronal selectivity for familiar song (Volman and Konishi, 1986). We have now quantified song selectivity in 8- to 10-month-old birds during the plastic-song phase of song development. Plastic song has many of the phrase elements of full adult song but is more variable and often lacks some of the fine structure. We recorded the vocalizations of these birds every 2–4 days and recorded electrophysiologically from HVc as soon after development of plastic song as possible (approximately 2–10 days). Song selectivity was clearly apparent by at least two criteria: 1) Forward song elicited a greater neuronal response than reverse song at 95% of the
recording sites when a bird's plastic song was played and at 93% of the sites to the tutor song. 2) Alien song, from another subspecies of white-crown, was a poorer stimulus than plastic song at 97% of the sites and then tutor song at 76%.

In adult birds a small proportion of neurons in HVC can be classified as "song specific" in that they respond almost exclusively to particular combinations of phrases present in a bird's song (Margoliash, 1983). Song-specific units, tuned to the plastic song and/or the tutor song, were also present in birds singing plastic song. In adult and plastic-song birds, clear auditory responses can be recorded in almost any electrode penetration through HVC. In juvenile birds, by contrast, auditory responses are more likely to be inhibitory or weak and inconsistent. Thus, the onset of singing is coincident with an increase in responsiveness as well as with the appearance of selectivity in HVC.

References:

290. A Critical Period for Estrogen Action on Neurons of the Song Control System in the Zebra Finch

Eugene Akutagawa, Mark Konishi

The brain nuclei that control song in the zebra finch show marked sexual dimorphism. The gender differences do not exist at birth but arise later from a decrease in cell size and cell death in the female nuclei as well as from an increase in cell size in the male. Administration of estrogen to a female chick induces the development of masculine cell size in her song control system. The masculinizing effects of estrogen are limited to the first 45 days after hatching. The degree of masculinization is graded and is inversely proportional to the extent of atrophy. Thus, the end and intensity of hormone action are correlated with the timing of cellular atrophy.

291. Sexual Differentiation of the Avian CNS: An In Vitro Approach

Richard D. Mooney

The brain of the estrildid finch, Poephila guttata, contains a discrete chain of nuclei involved in the acquisition and production of song. These song nuclei are sexually dimorphic in the adult; although quite hypertrophied in the male, they are greatly reduced or absent in the female. This difference is due to neuronal atrophy and death in the female song system which begins approximately 30 days after hatching. The female brain can be masculinized if subcutaneous implantation of estrogen occurs before 35 days post-hatching (d ph). Estrogen acts either directly or indirectly on the CNS to prevent normally occurring cell death and to promote cell growth and differentiation.

We are using in vitro approaches to study the locus and mechanism of estrogen action in the songbird brain. An important step in the masculinization of the song system is the development of connectivity between two telencephalic song nuclei, HVC and RA. Axons from HVC gather at the border of RA between 15 and 25d ph in both sexes. Starting at 25d, HVC terminals enter RA in the male, completely filling it by 35d. This contrasts with the situation in the female, where HVC terminals are restricted from RA even in adulthood. Single 400 µm parasagittal slices of zebra finch forebrain contain most of HVC, RA and the interposed HVC fiber tract. Slices prepared from 15-20d birds are embedded in a collagen gel and cultured in a roller tube apparatus. Initial results indicate that this preparation will survive up to two weeks in culture, as evidenced by intracellular recording from neurons within the cultured slice. Slice cultures permit direct manipulation of the environmental milieu and access to structures of interest. Thus, we will directly monitor the development of connectivity between HVC and RA while manipulating steroid concentrations in the culture medium.

A second approach involves the use of dissociated cell cultures prepared from the various song nuclei. Long-term cultures (up to 4 weeks) from 15-25d ph finch telencephalon have been prepared. Immunostaining with a polyclonal antisera to the 160 kDa subunit of neurofilament reveals extensive elaboration of neuritic processes, along with occasional synaptic specializations. In vivo labeling of song system neurons with fluorescent retrograde tracers, followed by the preparation of dissociated cultures from such labeled nuclei, will permit the identification of individual neurons in vitro. We will examine steroid effects at the single cell level and also further characterize the physiology of song system neurons.
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Summary: The interests of this laboratory concern two aspects of the development of the nervous system: factors controlling the choice of phenotype, and identification of molecules involved in axonal growth and the formation of specific synaptic connections. Much of our effort in this first area has centered on sympathetic neurons developing in cell culture. These neurons can differentiate synapses that utilize catecholamines or acetylcholine as the neurotransmitter, and a glycoprotein secreted by heart cells can control this transmitter choice without affecting neuronal growth or survival. We have begun work on cloning the gene for this differentiation signal, and antibodies that block its function are being injected into neonatal rats in an attempt to perturb development. The role of hormones and growth factors has been extended to other phenotypic choices in this lineage, and interconversions of adrenal chromaffin cells, SIF (small intensely fluorescent) cells and neurons have been demonstrated in culture. The control of these interconversions is now being studied in vivo using antibody injection and hormone perturbation. Thus, this area of work involves the identification, purification, and study of the molecular action of cues in the embryonic environment that influence phenotypic choices in various lineages of the developing nervous system. We also are making use of the experimental control of phenotype in producing cholinergic neurons from adrenal chromaffin cells for injection into lesioned and cognitively impaired brains of adult and aged rats.

We have identified a number of neuronal macromolecules, surface-bound and secreted, which mediate axonal growth, and several of these are being studied in antibody perturbation experiments and with mutant cell lines. The NILE (Ng-CAM) protein may play a role in axon outgrowth and fasciculation, and mutant PC12 cell lines deficient in this protein are being characterized. Another neuronal surface glycoprotein, Thy-1, was originally found on lymphocytes. Since it shares sequence homology with immunoglobulins, Thy-1 has been postulated to play a role in intercellular recognition in the nervous system. We have found that anti-Thy-1 antibodies can enhance neurite outgrowth and regeneration. Further work involves generating mutant cell lines deficient in Thy-1, characterizing their outgrowth and regeneration behavior, and transfection of the Thy-1 gene back into the cells that lack the protein. The properties of cells transfected with the gene for Thy-1 antisense message also are being studied. We previously obtained evidence for a role of plasminogen activator and its naturally occurring inhibitor in axonal outgrowth in culture. An in vivo project will assess the role of these proteins in nerve regeneration in the adult and embryonic animal.

A novel method for producing monoclonal antibodies against rare antigens is being used to investigate the molecular basis of the specificity of synaptic connections in the spinal cord innervation of sympathetic neurons. This innervation displays a positional bias, with rostral cord neurons preferring to innervate...
rostral sympathetic neurons, as well as rostral skeletal muscle. In a search for surface molecules that might mediate this positional bias, monoclonal antibodies were generated that bind preferentially to membranes from rostral neurons. The cellular localization of the corresponding antigens is being investigated. Thus, this second area of work involves the identification of molecules involved in intercellular interactions leading to axon outgrowth, regeneration, and specific synapse formation.

292. Molecular Cloning of a Neuronal Cholinergic Differentiation Factor
Sigrun Korsching, Tetsuo Yamamori

We are cloning the cDNA of a neuronal differentiation factor that changes the neurotransmitter phenotype of cultured sympathetic neurons from noradrenergic to cholinergic (Fukada, 1985). Eleven amino acids of the N-terminal sequence were determined by R. Aebersold, working with S. Kent and L. Hood, and the corresponding synthetic peptide, as well as several oligonucleotides were synthesized by S. Horvath. Antibodies made against the peptide precipitate the native protein (Fukada, 1986) but do not detect a specific signal in in vitro translation experiments using the rabbit reticulocyte lysate and the oocyte systems, with poly(A)+ RNA from several different tissues. This suggests the cholinergic factor is present in low abundance.

A cDNA library was made from poly(A)+ RNA of cultured heart cells, using a random primer to ensure extension of the cDNAs to the N-terminal region and standard methods for synthesis of double-strand cDNA. The cDNA was size-fractionated, and fractions of more than 400 bp were pooled, ligated to λgt10 arms, packaged in vitro and plated without further amplification. The library was screened with two sets of labeled eightfold mixtures of 32-mer oligonucleotides, which were based on mammalian codon usage and contained two inosine substitutions. We obtained 200 positive clones and isolated the 50 strongest candidates as single plaques. These were grouped according to stringency of hybridization with the 32-mers and four sets of 14-mers containing all possible nucleotide sequences of the central five amino acids in our probe sequence. So far, we have sequenced several of the clones with the highest stringencies and found in-frame homologies of 5-7 (out of 11) amino acids.

References:

293. The Role of the Cholinergic Factor In Vivo
Story Landis1, Paul H. Patterson

A minority population of neurons in sympathetic ganglia use acetylcholine as their transmitter, and they innervate particular target organs such as the sweat glands of the rat foot pad. These neurons switch their transmitter phenotype from noradrenergic to cholinergic after their axons innervate the sweat glands during the second postnatal week (Landis and Keefe, 1983). In culture, noradrenergic sympathetic neurons can be converted to the cholinergic phenotype by growth in the presence of a 45 kDa glycoprotein produced by cultured heart cells. Antisera that block the activity of this protein have been produced and affinity purified. We are injecting these antibodies into neonatal rats in order to determine if the differentiation factor discovered in the culture work also plays a role in the noradrenergic-to-cholinergic switch observed in the innervation of the sweat gland.

Reference:

1Case Western Reserve Medical School, Cleveland, OH.

294. Precursor and Mature Tyrosine Hydroxylase RNA Levels in Cholinergic Sympathetic Neurons
Dona Chikaraishi1, William V. Bleisch, Paul H. Patterson

When noradrenergic sympathetic neurons are converted to the cholinergic phenotype, a reduced amount of the catecholamine synthetic enzyme tyrosine hydroxylase (TH) remains as a remnant of the previous transmitter identity. This occurs when the conversion takes place during normal development in vivo, as well as when the transmitter phenotype is switched in culture. In order to determine if the reduced catecholamine synthesis seen in the cholinergic neurons is reflected in the TH mRNA levels, total RNA was prepared from noradrenergic and cholinergic sympathetic neuron cultures. Using an RNA dot blot assay, the cholinergic cultures had two-
to fourfold less than the noradrenergic cultures, and this corresponded to the reduction in catecholamine synthesis observed in the cells. To assess if the change in RNA was due to changes in transcription, we determined the amount of the precursor (nuclear) TH RNA by measuring the level of unprocessed TH RNA using a probe containing intron sequences. In preliminary experiments, the level of precursor TH RNA showed no difference between the noradrenergic and cholinergic cultures, suggesting that transmitter switching may be due to post-transcriptional regulation of mRNA levels. Possible mechanisms for this include (i) increased stabilization of TH mRNA in noradrenergic cells, (ii) preferential processing of TH mRNA from a constant amount of nuclear mRNA, or (iii) parallel increases in the rates of both transcription and RNA processing in noradrenergic cells such that although more TH RNA is transcribed, it is also processed more efficiently, generating an equivalent steady state TH precursor pool in noradrenergic and cholinergic cultures. The transcription mapping procedure used to identify precursor TH RNA sequences also allowed simultaneous quantitation of processed (mature) TH RNA, which was two- to fourfold less in the cholinergic cultures, in agreement with the RNA dot blot analysis.

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295. Control of Phenotypic Interconversions In Vivo
Josette F. Carnahan

Adrenal chromaffin cells, sympathetic neurons and small intensely fluorescent (SIF) cells are all neural crest derivatives and show a number of features in common, but they can be distinguished by morphological, biochemical, and immunological criteria. Since cultured SIF cells can be converted into adrenal chromaffin cells or neurons by manipulation of corticosteroid and nerve growth factor (NGF) levels, it has been suggested that SIF cells may be the central intermediate in the sympathoadrenal lineage, giving rise to these other cell types during normal development. This hypothesis is being tested by observing the consequences of altering the number of SIF cells during development by treating neonatal rats with anti-NGF antibodies or glucocorticoids. Injection of anti-NGF antibodies for 7 days yielded the expected decrease in neuronal number, but a significant increase in the number of SIF cells was also found. This result is consistent with a precursor role for SIF cells in that their number would depend, in part, on the availability of NGF in order to undergo the transition to the neuronal phenotype. Hydrocortisone injections also increase the number of SIF cells, as expected from the culture work.

Another approach in testing this hypothesis involves selective killing of SIF cells during development and observing the consequences on the number of neurons that differentiate. To this end, we would like to generate monoclonal antibodies that bind selectively to SIF cells and not neurons and inject these for the purpose of killing the putative precursor cells of this lineage. Since it is very difficult to obtain significant numbers of SIF cells, we are making use of the fact that chromaffin cells share a number of properties in common with SIF cells. Mice are first injected with a membrane homogenate from adult sympathetic neurons, and the response to these antigens is suppressed with cyclophosphamide. Membranes from neonatal chromaffin cells are then injected and hybridomas produced. Several antibodies have been obtained that bind to adrenal chromaffin cells and a subpopulation of small cells in the embryonic sympathetic ganglion, but not to sympathetic neurons. We are currently characterizing the cells stained in the ganglia with regard to other known phenotypic markers, as well as the response of the cells to altered hormonal conditions.

296. Adrenal Chromaffin Cell-derived Cholinergic Neurons for Brain Transplants
Paul H. Patterson, Fred Gage

It has recently been reported that transplanting a patient's own adrenal chromaffin cells into a particular location in his brain can very effectively ameliorate the symptoms of Parkinson's disease (Madrazo et al., 1987). It is presumed that the catecholamine-containing cells of the graft are replacing the transmitter lost in the death of the dopaminergic neurons in the brain of such patients. Similarly, considerable work with rats has shown that surgical or age-related deficits in cognitive function can be markedly improved by implantation of fetal cholinergic brain tissue. Some of these behavioral deficits and lesions are reminiscent of those seen in
humans with Alzheimer's disease. Since grafting fetal brain tissue into such patients would involve a number of legal and ethical difficulties, we are investigating the possibility of implanting autologous adrenal chromaffin cells that have been converted into cholinergic neurons into adult rat brains that have lesioned surgically or through aging. We have previously shown that chromaffin cells from the adult rat adrenal gland can, in fact, be converted into such neurons in culture. Therefore, the remaining questions are whether such converted neurons will survive in the host brain and form appropriate connections.

Reference:

1 University of California at San Diego Medical School.

297. Function of Neuronal Thy-1 as Probed with Antibodies and Mutant Cell Lines
Nagesh K. Mahanthappa, Paul H. Patterson

Thy-1 is a cell-surface glycoprotein that was first identified in mouse thymus and brain; more recently, homologues have been purified from brains of rat, human, frog, chicken, and tunicates. In the rat, Thy-1 is found on many, but not all, neurons and shows a complex developmental modulation of expression, yet its function remains unknown. Antibodies against Thy-1 can induce mitosis and lymphokine secretion in T cells (Gunter et al., 1984). In the case of neurons, a significant enhancement of neurite regeneration is observed when dissociated rat retinal ganglion cells are cultured upon a surface on which anti-Thy-1 MAbs have been coated (Leifer et al., 1984). This effect may, however, be simply ascribed to providing a better substrate for neuronal adhesion.

In order to pursue the function of Thy-1 in neurons, we are currently engaged in antibody perturbation experiments with dissociated superior cervical ganglion neurons, adrenal chromaffin cells, and the neuron-like, pheochromocytoma cell line, PC12. Simple addition of anti-Thy-1 MAbs to the culture medium enhances neurite outgrowth from sympathetic neurons in the presence of NGF. Furthermore, these MAbs can also promote neurite initiation from adrenal chromaffin cells and PC12 cells in the absence of NGF. These effects are not observed in experiments where the substrate is pre-incubated with the MAbs, and suggest a role for Thy-1 in neurite outgrowth.

In an effort to examine this idea with a different approach, we are producing mutant PC12 cell lines deficient in Thy-1 expression with ethylmethane sulfonate mutagenesis and selection by MAb-mediated complement lysis. Preliminary observations indicate that Thy-1-deficient cells show a very high incidence of spontaneous neurite extension in the absence of NGF. Thus, Thy-1 may serve to stabilize neural processes and the MAbs may be blocking this function in our experiments.

References:

298. Analysis of Thy-1 Function by Modulation of Thy-1 Expression
Hirayuki Nawa, Benton N. Yoshida

In order to obtain a better understanding of the function of Thy-1 in the nervous system, we are attempting to alter Thy-1 levels in neuronal cell lines by molecular biological techniques: (i) mutant cell lines are established from a Thy-1-positive line (PC12) by blocking Thy-1 production through expression of its antisense RNA, and (ii) Thy-1-negative mouse neuroblastoma cells (NIE115 and N18) are transformed with the Thy-1.2 gene to establish Thy-1.2-positive lines. The method for establishing mutant cell lines in which gene expression is specifically inhibited by its antisense RNA is currently under development. In order to optimize expression and stability of the antisense RNA, we have determined the strongest promoter in PC12 cells using the CAT assay and have examined the most suitable gene construct by transfection with several plasmid constructs. Eleven clones expressing the antisense message are now growing. In the second approach, we have established several Thy-1.2-positive mouse neuroblastoma lines by transfection with pSV2 neo-Thy-1.2 gene. We will study the differences between the transformed cells and their parental lines in terms of their neuronal properties such as neurite outgrowth, cell adhesion, and synapse formation.
299. Functions of a Neuronal Surface Protein, NILE

Michael DeFreitas, William V. Bleisch

We are continuing to study the function of the neuronal surface protein, NILE, also known as NgCAM and LI. NILE is a 230 kDa glycoprotein found on the surface of all neurons and localized to the neurites of neurons in culture. NILE has been implicated in neuron-neuron and neuron-glia adhesion and in the formation of neurite bundles. The role of cell adhesion molecules in the development of the nervous system has traditionally been studied by using antibodies to perturb their function. As an alternative, we are studying NILE-deficient mutants, the PC12 cell line. Using the ASCS4 monoclonal antibody against NILE, Bill Matthew (Harvard Medical School) selected for PC12 cells that are deficient in the ASCS4 epitope. We have cloned cells that are normal in morphology and in their complement of other surface proteins (such as N-CAM and NGF receptors) but do not stain with anti-NILE antibodies. Some of these mutants have reduced NILE on their surface, while others have a mutant form of the NILE molecule. We now are looking for alterations in the ability of these mutants to perform neuronal-specific functions. One difference is that mutants lacking NILE extend fewer long, thin processes in response to NGF, compared to wild-type cells. This approach should give us a better understanding of the functions and mechanism of action of this neuron-specific surface protein.

300. Rostro-Caudal, Position-specific Monoclonal Antibodies

Joann Imrich, Toshihiko Suzue, Paul H. Patterson

One of the best studied systems of specific synapse formation in mammals is the spinal cord innervation of sympathetic ganglia. The spinal axons display a positional bias in their connections with sympathetic ganglia such that neurons in the rostral cord prefer to connect with neurons in rostral sympathetic ganglia, while neurons in the caudal cord prefer to connect with neurons in caudal sympathetic ganglia. This specificity continues to be displayed even when adult ganglia are transplanted to novel sites; if a caudal sympathetic ganglion is moved to a rostral location, it is still preferentially innervated by more caudal spinal cord neurons. That this rostral-caudal bias may be an example of a more general positional marking system is suggested by a heterologous transplantation experiment in which skeletal muscle is substituted for a sympathetic ganglion. These novel neuromuscular synapses display the same positional bias as the normal interneuronal synapses, in that rostral muscle is preferentially innervated by axons from rostral spinal cord and caudal muscle by caudal cord (Wigston and Sanes, 1985). These phenomena suggest the possibility of a positional information system for synaptogenesis which is shared between neurons and muscle.

In an effort to discover the molecular basis of such a system, we would like to produce monoclonal antibodies that display a rostro-caudal bias in their binding to both neurons and skeletal muscle. The discovery of such antibodies would support the notion of a positional marking system, and they could be used in perturbation studies of selective synapse formation. Our approach has been to inject mice with homogenates of neonatal rat caudal dorsal root ganglia (or caudal sympathetic ganglia), suppress the response to these antigens with cyclophosphamide, and then inject membranes from rostral dorsal root or sympathetic ganglia. This method produced many antibodies that bind specifically to membranes from rostral but not caudal ganglia, both sympathetic and sensory. The histological localization of antibody binding within the ganglia is currently being determined, as is their binding to rostral versus caudal muscle membranes. An early, and surprising, result is that several of the rostral-specific antibodies bind to glial (Schwann and satellite) cells rather than to neurons.

Reference:

301. Role of Plasminogen Activator and its Inhibitors in Axonal Outgrowth and Regeneration In Vivo

Ana I. Millaruelo

A role for plasminogen activator (PA) and its inhibitors in neurite outgrowth has been proposed, based on results from cell culture experiments. Briefly, sympathetic and sensory neurons in culture release PA from their distal processes and growth cones, and inhibition of this PA activity by natural or synthetic inhibitors (PA-I) increases the rate of neurite outgrowth (Pittman and Patterson, 1987).
The involvement of PA and PA-I in neurite outgrowth in vivo is being tested using the model of reinnervation of the adult rat iris after destruction of its sympathetic fibers by systemic injection of 6-hydroxydopamine. Regeneration is monitored by specific uptake of 3H-noradrenaline by the iris, and glyoxylic acid-induced fluorescence of catecholamines in the tissue. Fifty percent of control 3H-noradrenaline uptake is recovered by 14 days after its complete abolishment, with total recovery achieved by 10 weeks. Regeneration proceeds most rapidly during the first few days after 6-hydroxydopamine injection; during this period, antibodies against plasminogen activator or its natural inhibitors will be injected into the anterior chamber of one eye and control immunoglobulins into the other.

References:

PUBLICATIONS


Assistant Professor: Mark A. Tanouye
Cornelius Wiersma Visiting Professor of Neurobiology: Martin Heisenberg
Research Fellows: Medha Gautam, Linda E. Iverson, Jonathan D. Poilock, Julie C. L. Tseng-Crank
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Summary: We are concerned with basic molecular and genetic mechanisms underlying nervous system structure and function. Our approach is to use a combination of methodologies—including classical and molecular genetics, electrophysiology, and neuroanatomy—to study genes necessary for proper nervous system function in Drosophila melanogaster. The abstracts presented in this year's report focus on two types of genes: 1) genes responsible for the development and stable maintenance of neuronal connections, and 2) genes underlying the generation of electrical signals.

K+ channels are a heterogeneous group of ion channels that contribute significantly to several physiological functions, for example, action potential repolarization, cardiac pacemaking, neuron bursting, and possibly learning and memory. Despite their physiological importance, virtually nothing is known about K+ channel molecules due to technical difficulties associated with biochemical purification. Given these difficulties, we undertook the molecular cloning of the first K+ channel gene using genetic criteria alone. In abstracts by Linda Iverson and Alexander Kamb, we report the molecular cloning and structure of the Drosophila Shaker (Sh) gene which encodes a type of voltage-sensitive K+ channel called the A channel. Additional abstracts by Medha Gautam and Julie Tseng-Crank further characterize Sh and the region containing it.

Mutations in several Drosophila loci are known to affect voltage-gated Na+ channels. Although these loci interact genetically, the genetics remain unclear
because it is not known which loci encode channel proteins. The abstract by Mani Ramaswami describes the isolation of several Drosophila genomic clones homologous to the mammalian Na\(^+\) channel.

The giant fiber system of Drosophila is a favorable preparation for examining the developmental genetics of neuronal connections. The giant fiber is a command interneuron that drives an escape response. The interneuron receives input from visual centers and has two major outputs. The first is to the jump muscle via a monosynaptic connection with the jump muscle motoneuron. The second is a disynaptic pathway to the wing depressor muscle.

Mutations that disrupt transmission between the giant fiber and the jump muscle motoneuron are relatively easy to select. A behavioral screen selects for jumpless mutants. Giant fiber system defects may then be determined using simple electrophysiological assays. By isolating a large series of mutations that disrupt the connection between the giant fiber and the jump muscle motoneuron, one has a reasonable chance of determining how the genome acts to build neuronal connections, using this particular identified synapse as an example. One mutation that affects this connection is bendless (ben). The abstract by Tanouye et al. describes a genetic analysis of ben.

302. Cloning of the Shaker Gene

Linda E. Iverson, C. Alexander Kamb

Mutations of the Shaker (Sh) gene in Drosophila melanogaster cause severe leg-shaking during ether anesthesia. The Sh locus is defined genetically by a number of semi-dominant ethylmethane sulfonate-induced alleles (Sh\(^{KS133}\), Sh\(^{102}\), Sh\(^{E62}\), Sh\(^{5}\), Sh\(^{rKO120B}\), B55), and one mutant that shows alterations in channel kinetics and voltage sensitivity (Sh\(^{5}\)).

To understand the molecular basis of the K\(^+\) channel abnormalities of Sh mutants, we have cloned a major portion of the X chromosome region containing the Sh gene by chromosome walking. A bidirectional walk was initiated using the cDNA clone, adm135 H4 (courtesy of Dr. M. Wolfner), that hybridizes to a 3–8 band interval defined by the B55 (16F1–3) and JC153 (16E2–4) translocation breakpoints (the JC153 translocation breakpoint does not cause Sh behavioral or physiological abnormalities and hence provides a distal boundary for the Sh gene). Using this method we have isolated 350 kb of flanking genomic DNA sequences from recombinant phage and cosmid libraries.

We have mapped the three Sh translocation breakpoints (B55, LC, W32) by in situ hybridization to polytene chromosomes and/or Southern (DNA) blot analysis. The translocation breakpoints are distributed over about 65 kb of DNA. Mapping of the breakpoints provides an approximate location for the Sh gene. However, because the location of the Sh point mutants relative to the breaks is unknown, and because translocation breaks are known to affect the expression of genes located 50 kb or more from the site of the break, it was necessary to delimit the Sh gene using independent means.

We generated wild-type recombinants between two Sh viable alleles (Sh\(^{E62}\) and Sh\(^{102}\)) and between a Sh allele and an allele of the adjacent lethal gene (Sh\(^{KS133}\) and I\(_3^{583}\)). The Sh\(^{E62}\) and Sh\(^{KS133}\) mutations were originally isolated in a Canton-S strain. The Sh\(^{102}\) and I\(_3^{583}\) mutations were originally isolated in an Oregon-R strain. These two wild-type strains show frequent restriction fragment length polymorphisms in their genomic DNA. Recombinants between mutants in polymorphic strains will show a restriction endonuclease cleavage pattern similar to one parental type that shifts to a pattern similar to the other parental type at the crossover point. We have mapped the crossover sites in these recombinants to a 60 kb interval that includes the sites of the B55 and LC translocation breakpoints. This analysis provides a distal boundary for the Sh\(^{KS133}\) and Sh\(^{102}\) mutations, and a proximal boundary for the Sh\(^{E62}\) and I\(_3^{583}\) mutations (see molecular map of Sh).
303. Structure of the Shaker Gene

C. Alexander Kamb, Linda E. Iverson

We have used subcloned fragments from the cloned region identified above as hybridization probes to detect transcripts on Northern blots and to screen cDNA libraries (courtesy of Dr. L. Kauvar). Using this approach, four candidate Sh genes have been identified in the proximal half of the cloned region. One of these genes (F-a) gives rise to a family of transcripts related to the adm 135 H4 cDNA. One (F-b) gives rise to an abundant, 4.5 kb transcript that maps just distal to the B55 breakpoint. The two other genes are defined only by single cDNA clones (however, see below). One of these (F-c) maps between the B55 and LC breakpoints, the other (F-d) maps between the LC and W32 breakpoints.

Using P element-mediated germline transformation, we were able to show that the gene defined by the adm 135 H4 cDNA (F-a) corresponds to the recessive lethal 2 (l2) gene. The mapping of recombinants described in the previous abstract indicates that the ShE62 and l3583 mutations must map distal to the F-d gene. Therefore, the F-d gene cannot encode the ShE62 mutation. Both the F-b and F-c genes map to the same interval defined by our recombinant analysis. However, DNA blot analysis of the spontaneous mutant, ShM, indicates that this mutation contains a 3 kb insert in the F-c gene. Because many spontaneous mutations in Drosophila arise from the insertion of transposable elements into a gene, this result provides strong evidence that the F-c gene encodes Sh.

Further examination of the F-c cDNA clone revealed interesting features about the genomic organization of the gene. The F-c gene encompasses a total of about 95 kb and is split by a major 85 kb intron. Both the LC and the W32 translocation breakpoints are located within the large intron and must physically interrupt the gene. The B55 translocation may break in or just outside the gene. The structure of the F-c gene provides a plausible explanation for why the LC and W32 translocations completely eliminate A current but the B55 translocation only reduces the level of A current by about 50%. Although the F-d gene is encoded within the large intron of the F-c gene, we think it unlikely that these two genes are related, either structurally or functionally.

DNA sequence analysis of the 938 nucleotide F-c cDNA clone revealed a single open reading frame containing at least one putative transmembrane domain. The cDNA clone is incomplete since the open reading frame extends its entire length. Sequencing of genomic DNA has revealed two additional membrane spanning regions. The amino acid sequence shows no strong homology with any of more than 4,000 published protein sequences including known kinases or phosphatases, which argues against the possibility that Sh encodes a K+ channel modifier. The sequence shows only weak homology to other membrane proteins, including the acetylcholine receptor and the Na+ channel. However, the sequence is extremely rich in arginine residues, and it has been suggested that arginine rich-sequences in the Na+ channel may constitute a voltage-sensing domain.

Preliminary evidence from RNA blots suggests that Sh encodes an extremely rare 4 kb mRNA that is not expressed in either the LC or W32 translocation mutants, consistent with the interpretation that these translocations break within the gene. In addition, we have recently isolated a 3.5 kb cDNA clone of Sh from an adult head cDNA library (courtesy of Dr. P. Salvaterra). Characterization of this cDNA clone is in progress, and its sequence may yield the complete sequence of the Sh gene.

304. Identification of the F-c Transcription Unit as the Shaker Gene of Drosophila with P Element-mediated Germline Transformation

Julie C. L. Tseng-Crank, C. Alexander Kamb

Two transcriptional units, F-c and F-d, have been identified in the vicinity of Shaker (Sh) translocation breakpoints. A combination of genetic and molecular evidence has indicated that F-c is the best candidate for the Sh gene (see above). Direct evidence, however, should come from P element-mediated germline transformation experiments.

To construct the appropriate plasmid clone for transformation, a full-length cDNA has to be used to supply the F-c gene sequence. F-c genomic DNA spans a wide range of at least 100 kb, and therefore is not practical for this purpose. Isolation of a full-length cDNA is currently under way. Screening a Drosophila head cDNA library made by Dr. Paul Salvaterra has already given us a cDNA insert close to the size of F-c
transcripts. The *Drosophila* heat shock protein gene hsp70 promoter will be fused to the 5′ end of the F-c cDNA to provide convenient transcriptional control, and a poly(A) site will be fused to the 3′ end. The *Drosophila* rosy+ gene for xanthine dehydrogenase will be linked to F-c cDNA as the positive selection marker for stably transformed lines. The hsp70 promoter-F-c-rosy+ DNA will be flanked by the P element-inverted repeats. A helper P factor, p25.7wc, together with the P[Sh1-rosy+] plasmid, will be co-microinjected into ry- embryos to provide the integrase necessary for integration. Transformed lines with high xanthine dehydrogenase expression will be crossed to the Sh- mutant strains. Larval muscle fibers of the heterozygotes will be voltage-clamped to test for the fast K+ channel activities. Because the Sh mutations are dominant, a gene dosage effect manifested by partial restoration of the fast K+ channel activity would imply that the F-c transcription unit corresponds to the Sh gene.

### 305. Generation of Antibodies Against the Shaker Gene Product

*Linda E. Iverson*

We will attempt to generate antibodies to the Sh gene product using two different approaches. In the first approach, we will construct expression vectors in which Sh cDNAs have been fused in frame to the dihydrofolate reductase (DHFR) gene of *E. coli* (courtesy of Dr. R. Ogden). The DHFR gene was chosen because of the expectation that the protein will be less antigenic than the more commonly used α-galactosidase protein. Appropriate restriction sites will first be generated in the Sh cDNA clone by oligonucleotide-directed mutagenesis. Sh cDNA fragments will then be inserted into the DHFR gene. The DHFR-Sh fusion proteins will be used to raise antibodies against the Sh gene product. In the second approach, several peptides corresponding to different regions of the Sh gene product will be synthesized by Dr. S. Horvath. The chosen peptides contain sequences that may be located on the exterior of the plasma membrane based on their proximity to putative transmembrane domains and glycosylation sites. The peptides will be used alone and also coupled to either keyhole-limpet hemocyanin or ovalbumin to raise antipeptide antibodies.

Depending on the results obtained using polyclonal antisera, we may generate monoclonal antibodies for further analysis. Monoclonal antibodies will be produced by To Ha Thai in the Institute's Monoclonal Antibody Facility. Some of the potential uses of polyclonal and/or monoclonal antibodies include: SDS gels of immunoprecipitates or Western blots for identification of the Sh gene product, immunoabsorption chromatography for purification of the Sh protein (and possibly other K+ channel-associated proteins), immunofluorescent cytochemistry for the localization of the Sh protein and, perhaps, inhibition of biological activity (i.e., blockage of K+ channel conductance).

### 306. Characterization of Lethals in the Shaker Complex Locus of Drosophila

*Medha Gautam*

The 16F region on the X chromosome in *Drosophila* appears to contain several loci that affect neurological functions. Two loci have been reasonably well characterized: *Shaker* (Sh) and *minibrain* (mnb). Mutant studies suggest that the Sh gene encodes a structural component of the A-type potassium channel (reviewed in Tanouye et al., 1986). A mutation in mnb reduces brain volume by 40-50% with a drastic reduction in cell number (Fischbach and Heisenberg, 1984). The two loci are separated by about 35 kb (Heisenberg, Kamb, and Fischbach, unpublished).

In addition to Sh and mnb, several other 16F loci have been identified as lethal complementation groups flanking the Sh locus (Ferrus, unpublished). Although these loci have not been well characterized, preliminary observations suggest that they encode neurological functions, perhaps related to membrane excitability (Tanouye, unpublished).

Many of the earlier observations suggesting that the various 16F genes may be organized into a complex genetic locus (Tanouye et al., 1981) can now be explained by the discovery that Sh is a large split gene with an intron of about 80 kb (Kamb and Iverson, unpublished). However, the clustering of neurological loci in the region and the lack of correlation between the severity of the leg-shaking defect and the alteration in A-current in mutants suggests that there may be additional leg-shaking functions flanking the Sh locus.

We will examine two loci that lie between mnb and Sh, called *lethal complementation group-2* (L2) and...
lethal complementation group-3 (l3). I2 has been mapped to an 8.8 kb genomic fragment by P element-mediated gene transfer (Kamb and Iverson, unpublished). A 4.5 kb transcript corresponding to the expected position of I3 has been mapped to within 18 kb in that region. The transcript appears to be developmentally regulated. We will confirm that it corresponds to I3 by P element-mediated transformation using the genomic region corresponding to the transcript. In situ hybridization to determine the spatial and temporal distribution of I2 and I3 transcripts, and sequencing of their corresponding cDNA clones will be done in order to find the possible functions of these genes. Escaper males carrying a mutant copy of one of the lethals, and flies heterozygous for both Sh and lethal genes, will be examined for abnormalities in electrophysiology and leg-shaking behavior as an approach to finding the exact functions of these genes. Since they lie between mnb and Sh, detailed analysis of I2 and I3 has the best chance of determining a common function which might link these loci together into a complex locus.

References

307. Screening for Ion-Channel Genes in Drosophila
Mari Ramaswami

In keeping with the laboratory's interest in the molecular biology of ion channels, I have been screening the genome of Drosophila melanogaster for sequences homologous to a mammalian sodium channel gene. The cDNA probe I used was a gift from Al Goldin and Andy Dowset of Norman Davidson's lab at Caltech. After several independent screens, I have obtained six non-overlapping sets of clones from Drosophila genomic libraries. They each contain one or more restriction fragments that hybridize to the rat sodium channel probe and also are strongly conserved in a distant species of Drosophila, D. virilis. By these criteria, we expect the majority of them to contain functionally important sequences of DNA.

In the course of Drosophila genetics, several mutations that affect neural excitability have been isolated. Of these, nap<sup>ts</sup>, para<sup>ts</sup>, seizure<sup>ts</sup>, and tipk<sup>ts</sup> are mutations that have been proposed to affect sodium channel function in the fly. All four of them undergo rapid and reversible paralysis at "restrictive temperatures" and interact strongly in some double mutant combinations. Cloned sodium channels in Drosophila would, in principle, allow the study of the genetic interactions between these loci and their participation in sodium channel function at a finer level than genetic techniques permit by themselves.

In collaboration with Kate Loughlin and Barry Ganetzky of the University of Wisconsin at Madison, we have shown that one of our clones maps molecularly to sequences near chromosomal rearrangements which uncover the para<sup>ts</sup> mutation. Another one of our clones maps cytologically to the site of a putative sodium channel which has been cloned and partially sequenced in another laboratory. At present we are focused on further characterizing all the different clones that we have obtained. We hope to have sequenced about 400 nucleotides from each of these clones in the next two months. We expect to be able to identify other good candidates for ion channel genes with this limited sequence data.

308. Genetiic Analysis of bendless
Mark A. Tanouye, C. Alexander Kamb, Ross J. McMahon, Susannah J. Hannaford<sup>1</sup>, Ann J. Lewis<sup>1</sup>

The giant fiber system mediates a jump/flight escape response in Drosophila. The first interesting giant fiber system connectivity mutant is bendless (ben) (Thomas and Wyman, 1982). The mutation was isolated in a behavioral screen designed to select for mutants with a defective jump response. Electrophysiological analysis showed that the pathway between the giant fiber and the jump muscle did not function properly. Anatomically, the giant fiber failed to make a bend which would normally bring it into proximity with the jump muscle motoneuron. Additional analysis showed that the giant fiber output to the wing depressor flight muscle is anatomically and functionally normal. Thus, the ben mutation has very specific effects. It can apparently function differentially on a single nerve cell to alter some outputs and not others.

We mapped ben by recombination to map position 45.8. Because of difficulties in scoring the ben
phenotype, mapping was done by electrophysiological analysis of 59 recombinant lines derived from heterozygous females (genotype: ben/g sd f). This analysis localized ben 1.42 cM proximal to g (44.4; 12B6-7). These results complement previous deletion mapping of ben that showed it was uncovered by Df(1)HA92 = 12A6-7;12D3 (Thomas and Wyman, 1982). We confirmed that the ben defect affects the giant fiber output pathway to the jump muscle but not the pathway to the wing depressor. In addition, we showed that three other giant fiber outputs, to each of the three wing elevator muscles, are unaffected by the ben mutation. We have also examined mutant output to the jump muscle in more detail and have shown that it is driven by a functional, albeit highly abnormal, connection from the giant fiber. We have determined that this is a de novo connection in the mutant that has no wild-type antecedent. We observed that ben flies have an abnormal phototaxis response. These observations have been followed up by Seymour Benzer's group, who have shown that in a photochoice test, ben flies abnormally choose visible light over UV; the choice is opposite in normal flies. Anatomically, ben photoreceptors appear to have abnormal terminal projections in the medulla and perhaps the lamina.

We isolated five new EMS-induced alleles of ben after screening about 42,000 mutagenized chromosomes. All of the new mutations are less extreme than the original ben allele. The new alleles cause non-jumping behavioral phenotypes that do not complement the original ben allele. Interestingly, the behavioral defect is less extreme over the deficiency (genotype: ben/Df(1)HA92), with each of the new alleles giving rise to some progeny that are capable of jumping. In electrophysiological tests, the giant fiber system appears normal in the new ben alleles; i.e., the giant fiber is capable of driving the jump muscle normally. Thus, the giant fiber motor output defect does not appear to account for behavioral jumplessness in these alleles. Perhaps altered visual inputs to the giant fiber may account for the abnormal behavior.

We isolated 20 EMS-induced phenotypic suppressors of ben after screening about 15,000 mutagenized chromosomes. Behaviorally, jumping ability is restored in all of the suppressors; electrophysiologically, the giant fiber is capable of driving a normal jump muscle potential. Eighteen of the suppressors are dominants and have not been analyzed in detail; two are recessives, second site, and allelic.

Reference:

1 Undergraduate, California Institute of Technology.

309. Regulation of Potassium Channels in PC-12 Cells by Nerve Growth Factor

Jonathan D. Polloock, Bernardo Rudy

The PC-12 cell line is derived from a transplantable rat pheochromocytoma tumor. Nerve growth factor (NGF) transforms these cells from a chromaffin-like to a neuronal-like cell that develops neurites and action potentials. Previous work by Rudy et al. (1982) showed that NGF increases the number of sodium channels in PC-12 cells. We therefore sought to assess whether NGF alters the density of potassium currents.

Using the technique of whole cell recording, we report here that the treatment of PC-12 cells with NGF for 14 days in addition to increasing sodium current density also augments the density of a potassium current. This current is sensitive to TEA, activates at -40 mV, and reaches a steady state in about 25 msec. Experiments are currently under way to determine the calcium dependence and the sensitivity of this current to potassium channel blockers other than TEA such as charybotoxin and 4-amino pyridine.

Furthermore, we have been characterizing apamin binding in PC-12 cells. Apamin is an 18 amino acid neurotoxin isolated from honeybee (Apis mellifera) venom that blocks a calcium-activated potassium channel with a $K_D$ in the picomolar range. Evidence suggests that this potassium channel (IAHP) mediates the slow after hyperpolarization and the number of action potentials fired during a depolarizing stimulus (i.e., the amount of accommodation). PC-12 cells have been shown to manifest the highest number of apamin binding sites of any tissue thus far analyzed (Schmid-Antomarchi et al., 1986). We have observed an increase in the number of apamin binding sites as a function of days in culture of intact NGF untreated cells. A similar observation of increased apamin binding as a function of time has been made by Schmid-Antomarchi et al. (1986) in isolated PC-12 cell membranes. Schmid-Antomarchi et al. (1986) also reported that NGF inhibits the time-dependent
increase of apamin binding. We are currently examining the mechanism of this increase and its inhibition by NGF.

In conclusion, PC-12 cells seem to be an attractive and tractable system for studying changes in ion channel expression. Moreover, the large number of apamin binding sites in PC-12 cells may permit the eventual isolation and cloning of the apamin-sensitive potassium channel from this source.

References:

PUBLICATIONS
Summary: In the past year we have continued our exploration of the human visual cortex. In nonhuman primates, the Middle Temporal Visual Area (MT) is a densely myelinated cortical area devoted to the analysis of direction of motion. We have found evidence for the location of MT in the human visual cortex both on the basis of stimulus-evoked changes in cerebral blood flow monitored with positron emission tomography (PET) and on the basis of myelin-stained histological sections (see Abstract Nos. 310 and 311). We also have found an area in superior parietal cortex activated during visual fixation or eye movements (Abstract No. 312).

We have generated a series of lamina-specific monoclonal antibodies in the visual cortex of the newborn macaque monkey. We also have a series of antibodies that label well-defined strata in the underlying white matter. The antibodies may be useful in exploring the development of cortical connectivity.

We have continued our physiological studies of the organization of the visual cortex in owl monkeys and have found new evidence for functional and topographic differences among areas (Abstract Nos. 314 and 315).

Dr. Leslie Brothers has joined our laboratory and has undertaken a neuro-ethological study of the mechanisms of social communication in the temporal lobe of monkeys (Abstract No. 316). Finally, Dr. Pat Wright has continued her studies of the evolution of behavior in primates both at Caltech and in the field in Madagascar. In the course of the studies in the eastern rainforest in Madagascar, she rediscovered a species of primate, the greater bamboo lemurs (*Hapalemur simus*), that had previously been considered to be extinct.

310. Responses to Low Contrast Moving Random Dot Patterns in Human Visual Cortex

Francis M. Miezin, Peter T. Fox, Marcus E. Raichle, John M. Allman

The magnocellular system of the visual pathway is characterized by its sensitivity to low contrast moving patterns. The ascending stages in the magnocellular system are the P-alpha retinal ganglion cells, the magnocellular laminae of the LGN, layer 4C-alpha and 4B of striate cortex, and the middle temporal area (MT). Neurons in the magnocellular laminae of the LGN are sensitive to stimulus contrast of less than 10% while the neurons in the parvocellular laminae are much less sensitive (Kaplan and Shapley, 1982). In deoxyglucose experiments Tootell (1987) found that low contrast stimuli selectively activated layers 4C-alpha and 4B of striate cortex and area MT with relatively little activation of adjacent cortical structures. We have used stimulus-evoked changes in cerebral blood flow monitored with a positron emission tomograph at the Washington University Medical School to map the portions of the human visual cortex responsive to low contrast stimuli. Subjects were instructed to fixate on a small dark fixation point in the center of a random pattern of dots with a local contrast of 8% and a 10% dot density. Data were generated by subtracting the response to a control stimulus condition from a test stimulus condition (see Fox et al., 1987). In the control condition the dots were stationary; in the test condition the array of dots moved at 16 degrees per second with the direction of movement for the array changing every 2 seconds. Eight movement directions presented in pseudorandom order were used. There were a total of 17 test-control comparisons in six subjects. The data were combined using a stereotactic system (Fox et al., 1985) and averaged together to reduce the background noise level. The largest response was centered in the fundus of the temporal-occipital-parietal pit (TOPP) (Polyak, 1957; Talairach and Szikla, 1967). This region is included in the site of a bilateral lesion found in a
patient with a selective impairment of perception of motion (Zihl et al., 1983) and is a possible candidate for the location of area MT in human visual cortex.

References:

Departments of, 1 Neurology and Neurosurgery (Neurology), and 2 Radiology, and 3 McDonnell Center for Studies of Higher Brain Function, Washington University School of Medicine, St. Louis, MO.

311. Anatomy of Human Visual Cortical Areas

Martin I. Sereno, Colin T. McDonald, John M. Allman

Physiological and anatomical studies of extrastriate visual cortex in New and Old World monkeys have uncovered a large number of distinct areas, many of which contain whole or partial maps of the visual field. Human extrastriate cortex is presumably organized similarly. However, the only visual area in humans whose borders are surely known is VI. We have undertaken to describe the myeloarchitecture of human visual cortical areas using both normal and flat-mounted tissue.

Standard sections were cut from human brains post-fixed for several weeks in 4% paraformaldehyde. For flat-mounted sections, whole occipital lobes were first immersed in 0.4% paraformaldehyde for 2 hours. This resulted in the grey matter becoming lightly fixed but left unexposed white matter unaffected. Exposed gyri were outlined with cresyl violet and numbered with neutral red, and then the entire occipital lobe was unfolded by digging out the white matter with a blunt probe while viewing the operation from the pial surface. The loose cortex was cut into 3 by 4 inch pieces and flattened between slides in 4% paraformaldehyde for a month before tangential sections were cut and stained with the Gallyas silver method for myelin.

The border of striate cortex is especially obvious in the flat-mounted sections. A mottled banding pattern with a repeat distance similar to ocular dominance columns is present in the middle layers of VI. The rostral border of VI is smooth, except for a distinct protruding point just dorsal to the posterior end of the lateral occipital sulcus. There is a small, ellipsoidal, coarsely-mottled, heavily myelinated cortical area in the rostral end of the lateral extension of the parieto-occipital sulcus. This area is about 9 cm rostral to the V1/V2 border, measured on the flattened cortex, and is about 2.2 cm long dorsoventrally and 1.6 cm wide rostrocaudally. There is no other area that is as densely myelinated within a 14 cm band surrounding the V1 border. This densely myelinated zone may correspond to the middle temporal visual area (MT) in non-human primates (Allman and Kaas, 1971).

If the densely myelinated zone corresponds to MT, then humans have proportionally more cortex in between MT and VI than monkeys do. It is interesting that low contrast flashing stimuli selectively activate a human extrastriate area near or coincident with the myelinated zone as measured by positron-emission tomography using oxygen-15-labeled water to detect regional cerebral blood flow and a stimulus/control subtractive image paradigm (see Abstract No. 310).

Reference:

312. Superior Parietal Cortical Activation during Visual and Oculomotor Tasks Measured with Averaged PET Images

Peter T. Fox1,2, Steven E. Petersen1, Francis M. Miezin, Marcus E. Raichle1,2,3, John M. Allman

Positron emission tomography images of regional blood flow change in normal human subjects were used to identify focal activation during: 1) simple fixation (versus eyes closed), 2) non-targeted, volitional saccades in darkness (versus eyes closed), and 3) three distinct eccentricities (macular, perimacular, peripheral) of patterned visual stimulation during target fixation (versus target fixation alone). As expected, the saccadic task activated frontal eye fields and supplementary motor cortex while the fixation target and patterned stimuli activated striate and extrastriate visual cortex. Unexpectedly, superior parieto-occipital (SPO) cortex was activated by all five conditions.

SPO activation was greater with a macular stimulus than with eccentric stimuli, although the
relative sizes of the stimuli produced the reverse order in striate cortex (peripheral > macular). This could indicate that SPO is a predominately macular retinal representation, an interpretation also supported by the response to a fixation target alone. Activation by non-targeted saccade generation, however, makes this interpretation less likely. As a unifying formulation, we postulate that SPO activation was induced by demands on visual attention (focused, shifted, or enhanced) present in all conditions.

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313. Monoclonal Antibodies as Developmental Markers in Primate Cerebral Cortex

Colin T. McDonald, To Ha Thai, John M. Allman

Last year, we reported on a monoclonal antibody (MAb), 6F2, generated against adult macaque striate cortex which shows an altered staining pattern in the newborn primate (McDonald et al., 1986).

In the hope of identifying other antigens present at a time soon after most cortical neurons have completed their radial migrations (Rakic, 1974), we have recently performed another mouse x mouse fusion, this time using the striate cortex of a newborn macaque monkey (postnatal day 0) as the primary immunogen. Employing a modification of the immunosuppression protocol of Matthew and Patterson (1983), we have generated several MAbS with specificities toward either various laminae of the newborn macaque cerebral cortex or to elements in the underlying white matter.

MAb16B10 appears to recognize extracellular components in layers 4 and 6 of the striate cortex in neonates: layers 4 and 6 are the thalamic input layers from the lateral geniculate nucleus; additionally, layer 6 contains cells that feed back onto the lateral geniculate nucleus. Other monoclonal antibodies from this fusion recognize: cells in the middle layers of cerebral cortex (MAb 19D3); extracellular elements in the upper and lower cortical layers, but not the middle layers (MAb 17B6); and cells from only the upper cortical layers, possibly including a transient layer 1 neuron, the Cajal-Retzius cell (MAb 13C3). MAb 17B10 recognizes fibers in the white matter closely associated with layer 6. MAb 20C1 recognizes elements in deeper white matter, including a well-defined stratum running below striate cortex.

Since the cortex develops in a laminar progression and since strata in the white matter appear to contain specific fiber pathways (Poliak, 1932; Weller et al., 1984), the identification of antibodies that bind to elements in these structures may be crucial to understanding the mechanisms underlying the formation of cortical connections.

References:

314. Transient and Sustained Responses in Four Extrastriate Areas of the Owl Monkey

Francis M. Miez, Steven E. Petersen1, John M. Allman

Single neuron responses to stationary flashed bars were recorded in four visual areas of the owl monkey: the middle temporal area MT, the dorsal lateral area DL, the dorsal medial area DM, and the medial area M. Data were collected at the optimum bar size and orientation for each cell and each post-stimulus histogram was normalized to its maximum bin height. A cumulative histogram was produced for each area by adding together all the corresponding cell histograms. The cumulative histograms reveal a short latency, transient component and a distinct longer latency, sustained component to the response for each of the areas (see Figure 1 on following page).

<table>
<thead>
<tr>
<th>Extrastriate Visual Area</th>
<th>MT</th>
<th>DL</th>
<th>DM</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transient Component</td>
<td>33</td>
<td>63</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>Onset Latency (msec)</td>
<td>59</td>
<td>84</td>
<td>64</td>
<td>59</td>
</tr>
<tr>
<td>Transient Component</td>
<td>196</td>
<td>129</td>
<td>111</td>
<td>172</td>
</tr>
<tr>
<td>Peak Latency (msec)</td>
<td>229</td>
<td>139</td>
<td>146</td>
<td>234</td>
</tr>
<tr>
<td>Sustained Component</td>
<td>24%</td>
<td>68%</td>
<td>48%</td>
<td>28%</td>
</tr>
<tr>
<td>Onset Latency (msec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Sustained Component</td>
<td></td>
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<tr>
<td>Peak Latency (msec)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ratio of Sustained to</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transient Response</td>
<td></td>
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The short latency of the transient component of the response in MT may be related to the densely myelinated architecture of this area and to its input from layer IVb of striate cortex. Layer IVb receives its input from layer IVc-alpha of striate cortex, which
in turn receives its input from the magnocellular neurons of the dLGN which have rapid conduction velocities. In area DL the latency of the transient component is 30 msec longer than in area MT. Area DL is much less densely myelinated than is MT and does not receive a direct projection from striate cortex. The sustained component of the response may reflect a second excitatory input in each of these areas and is largest in area DL, intermediate in DM, and smallest in M and MT.

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Figure 1. Each panel displays the averaged peristimulus histogram for the response to a flashed oriented bar. The different panels correspond to the four cortical areas recorded from. The histogram bins are 5 ms wide. Time since stimulus onset is represented on the horizontal axis with responses before 0 ms being spontaneous activity. The vertical axis is normalized to the peak response for each panel. Superimposed on each histogram is the best fitting curve obtained by using a random search algorithm. The number of cells recorded from each area is listed above each histogram.

315. Multiple Visual Areas Between V2 and MT in the Owl Monkey

Martin I. Sereno, Colin T. McDonald, John M. Allman

Previous mapping experiments in owl monkey extrastriate cortex suggested that the region between the representation of central vision in V2 and area MT was occupied by a single visual area, DL (dorsolateral crescent). Degeneration and HRP studies of the interhemispheric connections of owl monkey visual areas (Newsome and Allman, 1980; Cusick et al., 1984), however, revealed a prominent labeled band located about halfway between V2 and MT. Since callosal con-
nections in the initial stages of the hierarchy of visual areas are roughly correlated with vertical meridian representations, which usually define borders between areas, we decided to reexamine this region in detail.

The visuotopic organization of this region varies more from animal to animal than does the organization of V2, MT, and DM. An additional vertical meridian representation was usually found in the dorsal half of the region between V2 and MT, approximately at the location of the callosal band described above. It separates two strip-like, mirror-image representations of most of the lower field. The posterior area congruently adjoins the horizontal meridian of DL proper. For a given dorsoventral level, receptive fields in the anterior area are more eccentric than receptive fields in the posterior area, which, in turn, are more eccentric than receptive fields in V2. Medially, both areas adjoin DI (dorsointermediate area). Thus, there are three representations of the lower visual field between V2 and MT. The most anterior area, DL proper, closely resembles V4t in macaque monkeys. The homologies of the other two areas are presently less clear.

The visuotopic organization of the ventral half of the region between central V2 and MT is more complex. There are multiple, small representations of parts of both upper and lower fields. The lower field representations are not continuous with the ones described above. The topography here does not seem consistent with the idea of there being thin, parallel, dorsoventrally continuous strip-like areas between V2 and MT, containing only dorsal lower fields and ventral upper fields.

References

316. Single Neuron Responses to Socially Relevant Stimuli in Monkeys
Leslie Brothers, Evelynn McGuinness, John M. Allman

The Primate Order has evolved complex social structures, necessitating a highly developed ability to perceive and respond correctly to social signals of conspecifics. Neurons in the superior temporal sulcus and amygdala of macaque monkeys have been shown to respond selectively to pictures of specific physical features of other monkeys (Leonard et al., 1983; Perrett et al., 1983), especially faces. The extent to which such neural response characteristics are present in other regions of the temporal lobe is unknown. Because of limitations existing until now in the ability to present a wide array of realistic socially-relevant visual stimuli, the nature of responses to a range of conspecific appearance and behavior is also unknown. Furthermore, cells that have responded to pictures of faces and bodies of other monkeys have not been tested for response to vocalizations.

We are training a macaque monkey (M. radiata) to attend to a pseudorandom sequence of visual and auditory segments of conspecific behaviors presented on a monitor. Stimuli were recorded on videotape from a large outdoor-housed colony of M. radiata. A laser disk has been prepared from the edited tape and contains a variety of postures, expressions, movements, and physical features of monkeys of various ages and both sexes. Under software control, segments are able to be presented in reverse, at varying speeds and as freeze frames. A variety of typical vocalizations are included.

During stimulus presentation, single unit activity will be recorded in the amygdala and medial temporal areas primarily. Analysis of the data will focus on selectivity for particular stimulus features, including emotional expression.

References:

PUBLICATIONS


Visiting Professor: Bela Julesz
Research Fellow: Ben J. A. Kröse
Graduate Student: James Fox
Research and Laboratory Staff: Udo Wehmeier

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Summary: Our group is interested in the processing of information by the human preattentive visual system. Psychological experiments over 25 years at Bell Laboratories and now simultaneously at Caltech are conducted to study early human vision using computer-generated patterns with controlled geometrical and statistical properties. Investigations cover four main topics: 1) the study of binocular depth and monocul
movement perception using dynamic random-dot stereograms and cinematograms; 2) psychoanatomical studies tracing the information flow in the visual system using dynamic random-dot stereograms; 3) studying the development of stereopsis in human infants with cyclopeanly-evoked potentials on the scalp; and 4) the development of a theory of pre-attentive texture discrimination, using textures of constrained stochastic properties.

While most of these studies are done psychophysically in our group at Caltech and at Bell Laboratories, some of the psychoanatomical studies use PET-Scan techniques (while the observer looks at random-dot stereograms) in collaboration with Prof. John Allman's group, and the texture discrimination work extends to the searching for texton-gradient detectors in various cortical areas of the monkey in collaboration with Prof. David Van Essen's group.

317. Texton Theory of Preattentive Vision

Bela Julesz, Ben J. A. Kröse, James Fox

In recent years we showed (Julesz, 1981, 1984, 1986; Sagi and Julesz, 1985) that preattentive (i.e., scrutiny-free) discrimination of textures is often based on the spatial density variation of a few conspicuous local features (called textons). These textons are elongated blobs, particularly line segments, and under some simple conditions, their ends-of-lines and crossings behave as textons too. The preattentive system cannot perceive the positional relations between these textons, but instead detects differences between adjacent textons (texton-gradients) and directs a narrow search-light of attention to these gradients, provided that these gradients are large. Only in this narrow aperture of focal attention are the textons "glued together" forming shapes. Current interest is to refine this texton theory of preattentive vision by determining the interaction distances between textons and the size of the focal aperture. It seems that these dimensions are scaled by the average texture element size. Experiments are being carried out to study the aperture of the preattentive system and the aperture of focal attention in relation to the texton-gradient.

We are also extending these studies from static textons to moving ones. Our main interest is whether the detection of the direction of motion is characterized by a parallel (preattentive) process or a serial (attentive) process.

Recently, we started a series of experiments in which the stimulus is stabilized on the retina. With this technique, long presentation times can be used, without interference of eye movements. The (serial) search for the target has to be carried out, under these conditions, entirely by shifting the locus of attention. We hope to determine the search times for different texton-gradient strengths.

References:

PUBLICATIONS


Assistant Professor of Biology & Engineering and Applied Sciences: Christof Koch
Research and Laboratory Staff: Matthew J. Avalos, Mark H. Foxwell, James M. Hutchinson, Jin Luo, David W. Ring, Walter M. Yamada III

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Summary: Work in my laboratory is concerned with two different but related topics. Work on the "biophysics of computation" seeks to enumerate,
simulate, and understand the different biophysical mechanisms underlying information processing in the nervous system. For instance, detailed computer simulations of simple vertebrate cells, based on voltage- and current-clamp data, lead to a better understanding of how these cells process the incoming synaptic signals and pattern their output accordingly. Studying specific computations in the nervous system, for instance, direction selectivity in the retina, LTP in the hippocampus or the function of the cortico-geniculate feedback, via computer simulations leads to a number of predictions that can be tested physiologically. Collaborations with experimentalists are therefore essential for this approach, lest it loses its biological relevance. Current projects include studying the integrative properties of single vertebrate cells (bullfrog sympathetic ganglion cells, rodent CA1 and CA3 hippocampal pyramidal cells and both in vitro and in vivo cells in the cat lateral geniculate nucleus) with special emphasis on free, intracellular calcium; we are currently building models of single cells on parallel machines of the Hypercube type (with up to 1024 very powerful processors). At the system level, we are studying the computations underlying motion and disparity selectivity in the cat and primate cortex and studying the function and implementation of selective visual attention (via psychophysics, physiology, and models).

The second major research effort in my laboratory involves the analysis of early vision from a computational point of view. Tasks such as detecting motion and computing depth and color seem effortless to us, but are quite difficult to reproduce on a machine. Based on earlier work done at MIT's Artificial Intelligence Laboratory (regularization theory), we are extending the use of the line processes technique to the problem of computing optical flow in the presence of numerous moving objects. On the basis of a very powerful mathematical technique (Markov Random Fields), it can be shown that these problems can always be formulated in terms of optimization problems which can be mapped onto simple electrical networks with local connections and a mixed analog and hybrid hardware. We are currently starting a collaboration with Prof. Carver Mead in Computer Science (Division of Engineering and Applied Science) to implement these networks in VLSI circuits using subthreshold cMOS technology. We are also starting a robotics laboratory (with special purpose, real-time image processing hardware and a roving robot). Its major emphasis is on designing a network-based vision system for navigation of an autonomous vehicle. The Jet Propulsion Laboratory is drawing up detailed mission plans for such a vehicle to navigate semi-autonomously the surface of the planet Mars in the late 1990s.

318. The Action of the Corticofugal Pathway on Sensory Thalamic Nuclei: A Hypothesis

Christof Koch

The NMDA receptor has recently attracted great interest due to its nonlinear current-voltage behavior. In order to evoke a large depolarizing postsynaptic current, the synaptic-induced conductance change must be paired with a postsynaptic depolarization. This temporally highly-tuned AND gate could underlie a number of different operations throughout the nervous system. We propose that the synapses made by the optical nerve onto projection cells in the mammalian lateral geniculate nucleus are of the NMDA type. About half of all synapses in these cells—located almost exclusively in the peripheral two-thirds of the dendritic tree—are associated with axons originating in the layer VI of visual cortex. It then follows that the corticogeniculate pathway can control the gain of the retinogeniculate pathway via its action on the NMDA receptors. Thus, near simultaneous activation of the retinal and the cortical input will transiently enhance the geniculate cell response. In conjunction with the corticogeniculate circuitry, this mechanism helps explain the conflicting and ambiguous results seen upon inactivation of cortex and implicates the massive topographic corticothalamic feedback in attention-related phenomena. Experimental evidence indicates that a similar mechanism controls the gain of the somatosensory input to the ventrobasal thalamic nucleus.

319. A Network Model for Cortical Orientation Selectivity in Cat Visual Cortex

Christof Koch

The vast majority of cells in the cat's visual cortex are orientation selective. Two classes of models have been proposed to account for this phenomena. Hubel
and Wiesel (1962) have orientation selectivity arising from a row of geniculate neurons with appropriately aligned receptive fields. However, various experiments (e.g., Sillito, 1975) underscore the crucial nature of intracortical inhibition in shaping orientation selectivity. Thus, a number of models have been proposed (e.g., Heggelund, 1981), such that inhibitory non-oriented interneurons prevent the cell from responding at non-optimal orientations. However, no population of non-oriented cells has been found in area 17 of the cat.

I propose a model of orientation selectivity, based primarily on the interactions between inhibitory interneurons, in which cells of the same orientation inhibit each other in such a manner that the entire network acquires orientation selectivity. Thus, interneurons in the "vertical" column inhibit their neighbors to the "left" and "right," but not those to the "top" and "bottom," while horizontal cells inhibit each other along the top-bottom axis (relative to the diagram plane). In addition, cross-inhibition between cells of differing orientation enhances the orientation tuning. This network model explains how orientation selectivity arises within the input layer to striate cortex and predicts cross-inhibition between neighboring interneurons of similar orientations. It can be tested using multiple-electrode techniques. A fundamental property of the model is its reliance on massive feedback among its elements, in contrast to previous models which are of the feedforward type. Excitatory-oriented afferent input, according to the Hubel and Wiesel model, can easily be included in this model. In order to investigate these and similar circuitries, we are currently simulating, in collaboration with David Van Essen, the foveal X-system of the cat.

References:

320. Roles of Voltage-dependent Currents in a Vertebrate Neuron

Christof Koch, Paul Adams

Most attempts to understand neuronal excitability are based on Hodgkin and Huxley's analysis of action potential initiation and propagation in squid axon in terms of two currents. It is only within the last few years that we are beginning to unravel the much more complex biophysical events underlying electrical activity in vertebrate neurons. Using a variety of different techniques, seven distinct voltage-, time- and calcium-dependent currents have been characterized in type B bullfrog sympathetic ganglion cells. Computer simulations of this spherical dendritic neuron lead us to a satisfactory understanding of the role of the different currents in shaping the voltage trajectory during and after action potentials, spike frequency adaptation and in slow synaptic modulation.

1Department of Behavior and Physiology, State University of New York, Stony Brook.

321. The Dynamics of Free Calcium in Dendritic Spines in Response to Repetitive Synaptic Input

Edward Gamble, Christof Koch

Increased levels of intracellular calcium at either pre- or postsynaptic sites are thought to precede changes in synaptic strength. Thus, to induce long-term potentiation in the hippocampus, periods of intense synaptic stimulation would have to transiently raise the levels of cytosolic calcium at postsynaptic sites—dendritic spines in the majority of cases. Since direct experimental verification of this hypothesis is not possible at present, calcium levels have been studied by numerically solving the appropriate electro-diffusion equations for two different postsynaptic structures. Under the assumption that voltage-dependent calcium channels are present on dendritic spines, free intracellular calcium in spines can reach micromolar levels after as few as seven spikes in 20 milliseconds. Moreover, a short, but high-frequency burst of presynaptic activity is more effective in raising levels of calcium and especially of the calcium-calmodulin complex than sustained low-frequency activity. This behavior is different from that seen at the soma of a typical vertebrate neuron.

1Center for Biological Information Processing, Massachusetts Institute of Technology.

322. Computing Motion Using Neuronal Networks

Christof Koch, James M. Hutchinson

Computing the velocity field of a moving scene is an ill-posed problem, since the aperture problem limits any measurement of velocity to the velocity component normal to the contour (Poggio et al., 1985). The problem can be made well-posed by imposing a
smoothness condition. Thus, the solution must be 1) compatible with the measured, noisy data, and 2) the smoothest in a precise sense. This allows the problem to be cast into an optimization form with a quadratic energy functional. Such schemes have been used successfully to recover smooth motion over areas (Horn and Schunck, 1981) or along contours (Hildreth, 1984). Moreover, the associated energy functionals can be mapped into linear resistive networks having only local connectivity.

However, these intensity-based schemes for recovering the velocity field fail or perform badly in the presence of more than one object moving in different directions, as for instance when an object moves over a stationary background (figure-ground problem), since these methods assume continuity in the motion field. I will show that this difficulty can be overcome by the use of "motion line processes," similar to the ones introduced by Geman and Geman (1984). If the local gradient between neighboring values of the flow field becomes too large, the appropriate line process is turned on, segmenting the velocity field at that location. Inside the motion boundary, the algorithm performs as before and computes the smoothest velocity field compatible with the data. This problem can be formulated using Markov Random Fields, equivalent to minimizing a non-convex energy function. A priori knowledge concerning the motion discontinuities (e.g., motion discontinuities occur along extended contours, they rarely intersect, etc.) can be included into this very general framework. The resulting algorithm can be mapped onto both analog or analog/digital networks with only local connectivity, similar to the ones proposed for piecewise smooth surface interpolation (Koch et al., 1986). The binary line processes correspond to simple switches making or breaking linear resistive connections between neighboring grid points. I will discuss possible CMOS VLSI implementations.

References:

PUBLICATIONS

Faculty Associate: Marianne E. Olds
Research Staff: Jeffrey D. Carpenter, Engineer
Laboratory Staff: Chun Ling Lat, Thomas Sze

Summary: The experiments performed this past year were part of a long-term study of the role of the dopamine (DA) neurons in the mammalian brain in the regulation of positive reinforcement and motor activity. Positive reinforcement was investigated using the self-stimulation model, and the dopaminergic influence on motor activity was investigated in the animal expressing hypermotility and oral stereotypy. In the self-stimulation model, the preparation is the rat chronically implanted with stimulating electrodes in lateral hypothalamus. With electrodes in this portion of hypothalamus, the animal can be trained to press a lever or poke its nose in a hole, or to perform any behavior belonging to its repertory to obtain a
brief, low intensity electric shock to lateral hypothalamus via the implanted electrodes. In the hypermotility and oral stereotypy model, the animal is treated with agents that induce specific types of movements which are aimlessly repeated for as long as the agents are effective, generally more than 60 minutes. The movements include those seen during locomotion and those of chewing, gnawing, licking, and swaying of the head, collectively referred to as oral stereotypy. The available evidence has led to the view that increased dopaminergic activity is responsible for the reinforcement produced by lateral hypothalamic stimulation and for the motor activity induced by these agents. In the case of self-stimulation behavior, it is assumed that the DA which is released by stimulation of the hypothalamus stimulates DA receptors which set in motion a pattern of information flow leading to the animal experiencing reinforcement. The question here is where must DA be released to produce this effect, and is the release of DA critical to the effect? In the case of hypermotility and stereotypy, it is assumed that DA is released in nucleus accumbens and in neostriatum, and that the action of DA receptors in these two forebrain structures is responsible for the behavioral effects of the DA agonists.

In the experiments dealing with positive reinforcement, we are investigating two forebrain regions where DA is released during self-stimulation behavior maintained by lateral hypothalamic stimulation. One is the frontal cortex, and the other is the nucleus accumbens. Each receives a dense dopaminergic input and each contains rewarding sites. The question we are asking is whether it is possible to increase the amount of reward produced by the electric stimulus applied at a low intensity when DA agents are applied locally in n. accumbens and frontal cortex. The evidence obtained to date in n. accumbens is positive. Our next task is to find out whether DA synapses in frontal cortex can be similarly manipulated to facilitate behavioral effects.

In the experiments dealing with hypermotility and oral stereotypy, we have previously shown that neurons in ventral midbrain which receive input from n. accumbens and neostriatum show an excitatory response during the expression of these behaviors. Our current project deals with the respective contribution of each forebrain structure to their neural responses.

323. The Neural Basis of Reinforcement: The Role of the Dopamine Synapses in Nucleus Accumbens

Marianne E. Olds, Chung Ling Lat

Anatomical, biochemical, and behavioral evidence implicates the brain DA neurons in self-stimulation behavior. For example, it is well established that electrodes implanted in brain structures giving rise to DA projections, containing DA axons or terminals, will prove capable of supporting self-stimulation behavior. Also, it has been shown that DA is released during this behavior, and that pharmacological manipulation of DA metabolism influences responding for the brain reward in the predicted ways: procedures that increase dopaminergic activity increase responding to obtain the brain reward while procedures that decrease dopaminergic activity depress responding. But, as there are several DA neuronal groups in the brain, each projecting to different forebrain regions and each sending axons to the forebrain via hypothalamus, there is a problem regarding which is involved in reinforcement. And within each group, it appears that the DA population is functionally heterogenous. In the experiments of this past year, we have investigated the role of the DA projection to nucleus accumbens. This projection has its origin in the ventral midbrain, in a DA cell group referred to as A10, and the axons ascend to forebrain via a bundle that traverses lateral hypothalamus, referred to as the medial forebrain bundle. Electrodes located in the A10 region, or in medial forebrain bundle, or in n. accumbens or frontal cortex, support self-stimulation behavior.

The subjects of the experiments were rats implanted with electrodes in the medial forebrain bundle at the level of the lateral hypothalamus, and also with bilateral cannulae aimed at the n. accumbens. After the subjects were trained to press a level to obtain the brain reward, the current intensity used to maintain responding was lowered to threshold level. After responding had stabilized at this intensity, DA, or DA + d-amphetamine, or DA + d-amphetamine + pargyline (an agent preventing the breakdown of DA) were injected into n. accumbens to find out whether the increased DA available at n. accumbens DA synapses could increase the rate of responding for the brain reward to the levels seen at maximal intensities of stimulation. The evidence is unequivocally positive. We have tested 20 animals and of
these, 12 showed facilitation of responding for the brain reward when more DA was made available in n. accumbens. We have also tested which combination of DA agonists is most effective. Our next task is to do similar experiments but with the injections being given in frontal cortex, another recipient of a projection from the A10 neurons. Comparing the effects of potentiating dopaminergic transmission in these two forebrain structures on self-stimulation behavior of lateral hypothalamus should help identify the projection of the A10 DA neurons that is responsible for the rewarding effect of hypothalamic stimulation.

324. Striatal Influences on the Firing Pattern of Reticulata Neurons during Hypermotility and Stereotypy

Marianne E. Olds

The activation of postsynaptic dopamine (DA) receptors in n. accumbens and in striatum is believed to underlie the behavioral effects of amphetamine and apomorphine. In a previous study, we showed that there was a positive correlation between these behavioral effects and excitatory responses of non-DA neurons in ventral tegmental area (VTA) and in substantia nigra (SN). In the present study, the contribution of striatal dopaminergic activity to these behavioral and electrophysiological effects was investigated utilizing haloperidol injected into striatum to interfere selectively with dopaminergic activity induced in that structure by systemic amphetamine and apomorphine. The experiments were carried out in the behaving rat with motor activity and discharge rates of non-DA neurons in VTA and SN measured concurrently.

The preparation was the chronically implanted rat. Unit activity was recorded simultaneously from seven fine wire electrodes (62 µm), and motor activity was recorded with the open-ended wire technique. The signals selected for study met the electrophysiological criteria deemed to characterize the output of non-DA neurons in VTA and SN. These requirements specified that the action potentials had to be biphasic, of short duration (<2 ms), and with rates of firing greater than 20 spikes/s. The signals from 5-6 of the electrodes used to record unit activity in a given session were the output of small families of cells lying close to each other (5-15 cells), and the signals from 1-2 of the electrodes used in the same session represented the output of single neurons.

Recording sessions lasted 240 min, of which the first 90 were used to measure baseline motor and unit activity. At 90 min, amphetamine (5 mg/kg) or apomorphine (3 mg/kg) was injected by the intraperitoneal route. Haloperidol (12 µg/4 µl/side) was injected bilaterally into striatum immediately before the start of the session at a rate of 1 µl/min.

In the haloperidol-pretreated animals, the behavioral response to amphetamine was attenuated compared to controls, and the excitatory unit response to the drug was attenuated in SN, but not in VTA.

In the haloperidol-pretreated animals, the behavioral response to apomorphine as measured with the open-ended wire technique showed no effect of the pretreatment, but the excitatory unit responses in both VTA and SN were attenuated.

These results show that striatal dopaminergic activity contributes significantly to the behavioral responses and the unit responses in VTA and SN elicited by systemic amphetamine and apomorphine. The influence exerted by striatum differed, however, depending on the localization of the non-DA neurons and the DA agonist given.

PUBLICATIONS


Summary: Experimental work remains focused on hemispheric specialization and interhemispheric communication in human subjects and rhesus monkeys with surgical disconnection of the cerebral hemispheres. The discovery of complementary specialization in monkeys received additional confirmation this year, and it seems increasingly likely that the functional hemispheric differences in monkeys and humans are homologous, a finding that would greatly facilitate the study and understanding of the fundamental basis of cerebral lateralization. Other experiments with partially split-brain monkeys demonstrate differential transfer of visual information through small segments of the cerebral commissures validating the usefulness of this preparation for study of the functions of specific visual areas.

Visiting investigators from Canada, New Zealand, Japan and elsewhere in the U.S. also have continued to test our population of split-brain patients examining particularly the differential capabilities of the two hemispheres and latent forms of interhemispheric transfer that appear to survive commissurotomy. In particular, progress has been made in separating genuine transfer from illusory transfer based on cross-cueing strategies, uncontrolled eye fixation, and possible right hemispheric speech. Combining dichotic with visual and motor tests continues to provide a useful approach to understanding how the two hemispheres share the control of behavior.

An analysis of the factors influencing the recent shift of theory in psychology from behaviorism to a new mentalist framework supports the interpretation of a genuine Kuhnian shift of paradigm involving an overthrow of traditional epiphenomenal views of consciousness and the subjective in favor of a new explanatory framework that gives mental states a causal functional role in brain processing.

325. Visual Transfer Through Different Commissural Segments

Charles R. Hamilton, Betty A. Vermeire

Experiments that were described last year (Biology 1986, Abstract No. 272) to determine if different segments within visual regions of the cerebral commissures transfer different kinds of visual information are nearly completed. Ten partially split-brain monkeys that each had only a small segment of their commissures left intact were tested for interhemispheric transfer. For convenience, three adjacent segments within the splenium are termed V1/V2, V4 and MT, and one within the anterior commissure, IT, because these are the visual areas most conspicuously interconnected by each of these four segments (Biology 1979, Abstract No. 205). In addition, four monkeys with section of just the optic chiasm were tested to evaluate transfer through the entire commissural system, and numerous split-brain monkeys were tested to determine if residual subcortical transfer was present. Transfer was measured by the percentage of savings on reaching a criterion of 90% correct with the hemisphere tested second. We tested transfer of discriminations based on pattern, orientation, movement, and facial features.

As expected, all problems transferred well in split-chiasm monkeys and, except for movement, did not transfer in split-brain monkeys. To eliminate a 25% savings for movement in split-brain monkeys, we had to also section the commissures of the midbrain. For the four visual segments, patterns transferred best through the IT segment and progressively less well through MT, V4, and V1/V2, in keeping with the important role of inferotemporal cortex in learning to discriminate patterns. In contrast, orientation transferred best through the V1/V2 segment and progressively less well through V4, MT, and IT, in accord with the lowered interest in orientation shown by cells farther into the visual hierarchy. Movement transferred fairly well through all segments, although the greatest transfer was through MT. Facial features also transferred through all segments, but these data are not complete enough for adequate interpretation. Although the results remain tentative until more monkeys are tested and histological evaluation of the selective disconnection is available, differential transfer does appear to occur and to follow an orderly set of expectations derived from physiological and behavioral studies. Therefore, this novel approach to identifying the functional role of cortical areas in perception without having to ablate them seems practical.
326. Hemispheric Specialization for Spatial Discrimination in Monkeys

Betty A. Vermeire, Charles R. Hamilton

Previously (Biology 1986, Abstract No. 273), we reported finding complementary superiorities of the two halves of the brains of rhesus monkeys. On the average the right hemisphere of 26 monkeys was dominant in learning, remembering, and generalizing discriminations of monkey facial identity and expression, while the left hemisphere was significantly better in discriminating the orientation of tilted lines. The left hemisphere, in addition, excelled in several other discriminations which involved spatial relationships, thus suggesting that, in general, certain spatial abilities are more lateralized to the left hemisphere of monkeys.

We are now determining how similar the monkeys' spatial abilities are to those lateralized in human subjects, and we are attempting to examine the extent and magnitude of spatial lateralization in monkeys. Two different sets of tasks are being used which are refined versions of those that have previously shown lateralization of spatial abilities. In each case, the monkeys learn the easiest version of the task first, then the difference between the relevant stimulus cues is decreased in order to determine the discrimination threshold for each hemisphere. One test requires discriminating the relative orientation of a line positioned within a circle and the other involves discriminating the relative position of a dot within a square frame of reference. Both tasks are difficult for the monkeys to perform which should accentuate any lateralized ability. To date, only a few monkeys have begun the tests, but the data already indicate that these newer tests are performed better by the left hemisphere. However, more data are needed before we can determine thresholds of spatial judgements for each hemisphere and vary parameters that are known to affect human spatial judgements.

327. Hemispheric Specialization for Facial Discrimination in Monkeys

Betty A. Vermeire, Charles R. Hamilton

As mentioned in the previous abstract, the right hemisphere of rhesus monkeys is better than the left in discriminating the facial features of conspecifics. This result suggests that the right hemisphere of monkeys may be homologous to the right hemisphere of human beings in its superior ability to process facial and emotional information. We are undertaking two further investigations to see how close the homology might be.

Inversion is especially disruptive to visual recognition in human subjects, an effect that is much more pronounced with faces than with other classes of stimuli that are usually seen upright, such as houses or cars. We are now testing the monkeys for their ability to discriminate inverted monkey faces with each hemisphere. Two different tests are being used. In the first, monkeys that have already learned upright discriminations are tested with the stimuli inverted, and in the second, the monkeys must learn inverted discriminations de novo. If monkeys are similar to humans, the right hemisphere should be more disrupted by these inversions than the left.

Six monkeys that have performed inverted discriminations of individual identity show no hemispheric advantage, in contrast to the right hemispheric advantage found when learning or performing discriminations with upright faces. Thus, it appears that the right hemisphere was more disrupted by inversion than the left. When discriminations of expression are tested upside-down, eight monkeys so far show a right hemispheric advantage in performance, which is similar to the advantage shown when upright expressions are tested. This is presently difficult to interpret because we do not know the effect of inversion on the recognition of expressions by each hemisphere of human subjects.

328. Facial Processing by Split-Brain Patients

Charles R. Hamilton, Betty A. Vermeire, Justine Sergent

Processing facial information is often claimed to utilize neural mechanisms specifically organized for facial analysis. One line of evidence is the reported large difficulty in recognizing inverted faces compared to the relatively smaller difficulty in recognizing inverted objects. We have begun to test each hemisphere of four split-brain patients for the ability to match a sample stimulus to one of five possible choices. Stimuli are 40 human faces, 40 monkey faces, and 40 houses, tachistoscopically presented either upright or inverted to one hemisphere at a time. While
only two subjects have been tested on half of the trials, it is already clear that each hemisphere can perform the task quite well, and that both subjects find inverted stimuli more difficult to recognize than upright ones. However, we cannot yet realistically interpret the performance differences associated with the key experimental variables of hemisphere, inversion, and stimulus, and their interactions because extraneous variables will not be balanced until all the tests are completed.

1 Montreal Neurological Institute.

329. Testing Split-Brain Subjects with Precise Control of Visual Fixation
Morihiro Sugishita1, Charles R. Hamilton

It is essential to present stimuli to only one hemisphere at a time when testing subjects for lateralized abilities. If we flash stimuli at positions two or more degrees lateral to the point of visual fixation, we can assume fixation is adequate as long as lateralized results are obtained. But if the results suggest bilateral processing, if quantitative estimates of laterality are sought, or if it is necessary to present many trials in a row in one position, then better control of fixation is required. One of us (M.S.) has developed an accurate method to monitor and record the position of the fovea while tachistoscopically presenting stimuli through a modified funduscope.

Four experimental questions have been investigated in our split-brain patients with this method. Preliminary analysis of the data suggests the following conclusions. 1) Macular sparing for letters, if present at all, must involve less than 1° of the ipsilateral hemifield for patient NG. LB and AA could not be as adequately evaluated because of the following result. 2) LB, sometimes AA, but not NG could name letters, words, and pictures presented to the right hemisphere as well as to the left. This must be due to cross-cueing, subcortical transfer, or right hemispheric speech because fixation was excellent. Cross-cueing is somewhat favored because of the long and variable reaction times for correctly identifying stimuli flashed to the right hemisphere. 3) Same/different judgements of two letters, one flashed to each hemisphere, were very difficult for all three subjects despite the fact that LB and sometimes AA could name the letters flashed to each side. It appears that they made the same/different judgement before the slower cross-cueing mechanisms could operate. 4) Finally, comprehension of written words by either hemisphere of LB and by only the left hemisphere of NG (AA not tested) was indicated by the ability to draw pictures of the meaning of the flashed words. Overall, this new technique provides a powerful method to investigate laterality with precise control of stimulus position.

1 Tokyo Metropolitan Institute for Neuroscience.

330. Ipsilateral Report of Melodies in Commissurotomy Subject Suggests Switching Mechanism
Polly Henninger Pechstedt

This study continues an investigation of the hypothesis that left hemisphere knowledge of stimuli presented in dichotic testing facilitates interhemispheric transfer and ipsilateral report (Biology 1986, Abstract Nos. 278 and 279). It does so by comparing the right hemisphere performance of a complete commissurotomy subject (RY) on a dichotic melodies test before and after the left hemisphere had knowledge of the stimuli. The test consisted of 22 dichotic trials of hummed nursery tunes with a variety of response modes (finger tapping, pointing with the left and right hands, oral report, drawing) and response arrays. Initial testing with pointing in the left visual field resulted in identifying left ear tunes but performance was low. When tested with response arrays in which a right ear choice was unavailable, left ear performance increased. Right ear report increased on subsequent tests of pointing in the left visual field when the test was preceded by pointing in the right visual field. Tests in which the subject reported orally led to near ceiling right ear responses in subsequent testings of pointing in the left visual field. In sum, testings in the left visual field prior to those utilizing a left hemisphere response evoked left ear report; testings after those utilizing a left hemisphere response increasingly evoked right ear report. Drawings done by each hand of the right ear melodies differed, suggesting that those reported in the left visual field reflected report of the ipsilateral input. These results suggest a switching mechanism within the right hemisphere enabling it to process verbally-encoded material. They also suggest a more dynamic view of hemispheric dominance than usually assumed.
in which control of processing varies along a gradient modulated by, in this case, the left hemisphere.

331. A Possible Relationship Between Split Personality and a Functionally Split Brain

**Polly Henninger Pechstedt**

TG, a nineteen-year-old woman with clinically diagnosed multiple personality disorder, was tested to see if her creative, artistic, little girl personality, Ta. 9, was a "right brain" personality and her adult, college student personality, Te. 19, was a "left brain" personality. Each "subject" was seen for two hours during which time one personality was maintained. Comparison of interest questions and self portrait exercises, and the absence of any sign of recognition of the investigator or testing room during the second testing, supported the diagnosis of separate personalities. Both personalities were given handedness tests and questionnaires, verbal and nonverbal dichotic tests of lateralized central processing and a drawing test designed to show developmental differences.

Ta. wrote with her left hand, first one line from left to right in normal orientation followed by a line written from right to left in mirror image. Te. wrote with her right hand in normal orientation. Handedness questions and a paper cutting test indicated that Ta. was ambidexterous with a strong preference and greater skill in her left hand and that Te. was right handed with minimal skill in her left hand. Language tests showed left hemispheric specialization for both personalities but consistently more right hemisphere involvement for Ta. with scores suggestive of creativity. Musical tests showed a left hemisphere advantage for Te. and a right hemisphere advantage for Ta. Examination by trained raters of the pictures drawn by each subject indicated that both drawings had been done by an adult and that no developmental difference distinguished the drawings.

The clear identification of each personality with opposite handedness and differences in the dichotic results suggest that these two personalities are associated with opposite hemispheres. It is possible that as each formed, it became associated with one hemisphere, and that when it split off from the other, it became increasingly localized. The finding of left hemispheric specialization for language in both personalities and the absence of a developmental difference in the drawings indicate a functionally intact brain. However, the tendency of the ambidextrous personality to write in mirror image indicates some anomaly in brain organization. These findings raise the question of whether ambidexterity increases the potential for developing multiple personalities.

332. Verbal Creativity Suggests Balance between the Two Cerebral Hemispheres

**Polly Henninger Pechstedt, James Kelly**

Popular sentiment maintains that the right hemisphere is responsible for creative behavior, but verbal creativity could be in the domain of the left hemisphere or require efficient use of both hemispheres. This study investigates whether verbal creativity, as measured by a standard pencil and paper creativity test, is associated with the right or left hemisphere or balanced dominance on a verbal dichotic test of central auditory processing. Fifteen college students, 13 right-handed and two left-handed, were tested. Left, right and total ear scores, an absolute, and a relative ear difference score (R-L/R+L) were correlated with creativity measures of originality, fluency and flexibility, and a composite score combining these components.

Significant negative correlations were found between originality and the absolute index (p = 0.02), indicating that greater originality was associated with smaller ear differences, and with the relative index (p = 0.01), indicating that originality was associated with a greater left ear/smaller right ear score. Originality was negatively associated with right ear performance (p = 0.01), indicating that originality increased as right ear performance dropped, and did not correlate significantly with left ear or total performance. Originality correlated significantly with fluency and marginally (p < 0.1) with flexibility. Fluency and flexibility were intercorrelated but neither correlated with a laterality or a performance measure. The composite score correlated marginally with the absolute index but not significantly as originality had.

These associations suggest that the creative function that makes lateralized demands is originality. They suggest that verbal creativity results from balanced hemispheric activity and that this balance results from lessened left hemisphere rather than increased right hemisphere involvement. It appears
that verbal creativity is achieved by suppression of the left hemisphere. Furthermore, greater processing capacity, measured here by total performance, was not associated with creativity, suggesting that intelligence and creativity are separate abilities.

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PUBLICATIONS


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Summary: Our studies of the visual system are aimed at understanding how information is processed in the mammalian visual cortex. We use a variety of anatomical and physiological approaches in order to identify different visual areas, trace the interconnections between these areas, and study the receptive field properties of their constituent neurons. Recently, we have begun to apply computational approaches as an additional basis for analyzing how information is represented and transformed at the level of single neurons and of neural ensembles.

In the macaque monkey there are now two dozen cortical areas known to be largely or exclusively visual in function. The number of identified pathways between visual areas is well over one hundred. Based on the pattern of connections, we have assigned the different visual areas to an orderly, 12-stage hierarchy, beginning with primary visual cortex (area VI) and ultimately leaving neocortex and entering the hippocampal formation. We suspect that hierarchical relationships of this type represent a major feature of primate cortical organization.

Another important organizational feature is that of
parallel processing. Several distinct processing streams originate within the retina and continue through cortical areas V1 and V2 and several of their higher-order targets. Comparisons of the receptive field properties of neurons in different streams have revealed a number of interesting differences, in terms of selectivity for basic stimulus properties such as wavelength, orientation, and velocity of movement. Information of this type provides valuable clues concerning possible roles in visual perception, but it does not indicate precisely which tasks are carried out by any particular neuron, area, or processing stream. We are currently switching to the use of more complex visual stimuli than simple bars and edges in order to obtain more refined assessments of neuronal functions. In addition, we have put considerable effort into understanding the psychophysical and computational issues that constrain the way in which low-level visual information (e.g., retinal velocity and binocular disparity) leads to useful inferences about specific attributes of the external world (e.g., shape, motion, and depth). We are also interested in assessing the degree to which computations associated with different functions, such as the perception of motion and depth, may share common processing strategies.

Vision is a highly dynamic process, because images are constantly shifting across our retinas and because our attention is constantly shifting from one place to another in the visual field. To deal with these issues, the visual system must have precise control over the routing of information from one processing stage to the next. We are intrigued by the possibility that there may be a general type of circuitry that subserves a variety of dynamic routing processes, relating to motion perception, depth perception, and the control of visual attention. The mechanism that we have hypothesized leads to specific, testable predictions about the dynamic properties of neuronal receptive fields.

Although we study visual cortex primarily in the macaque monkey, we are interested in cortical organization in other species as well. Last year we reported on the use of positron emission tomography (PET) as a means of studying visual cortex in the human (Biology 1986, Abstract No. 289) we reported that when inactive and active synapses are in direct competition during the period of synapse elimination, the active synapses are not at an advantage, contrary to the predictions of several previous studies. This year we have extended the analysis to show that inactive synapses actually have an outright advantage over their active counterparts. Thus, neuromuscular synapses behave opposite to classical "Hebbian" synapses which have been invoked as a cellular basis for learning within the central nervous system.

### 333. Area PIP: An Extrastriate Visual Cortical Area in the Posterior Intraparietal Sulcus of Macaque Monkeys

**Daniel J. Felleman, Andreas Burghalter**

We have identified PIP, the posterior intraparietal area, as a visual cortical area that is located at the posterior end of the intraparietal sulcus. PIP is located in cortex originally identified as part of V3A by Van Essen and Zeki, but in a region they reported as having complex topographic organization. PIP can be distinguished from V3A and other nearby cortical areas on the basis of its topographic organization and laminar patterns of connections with several well defined visual cortical areas.

We studied some of the cortical connections of PIP by making injections of anatomical tracers (HRP, $^3$H-proline, and various fluorescent dyes) into physio-
logically and anatomically identified sites in areas V3 (n = 3), VP (n = 3), and V4 (n = 13). The locations of injections and transported label were analyzed with respect to the pattern of interhemispheric connections revealed by degenerating axons and terminals following section of the splenium of the corpus callosum.

Injections of tracers into visual cortical areas V3, VP, and V4 each led to two distinct foci of labeling within a restricted region of the lunate sulcus and adjacent intraparietal sulcus. Although this overall region is delineated by a single callosal-free zone, surrounded by a callosal-recipient ring, the separation between the two projection foci is sufficiently large (2-10 mm) and consistent (16 of 19 cases) that there is a sound basis for considering these as distinct visual areas. The lateral focus, on the anterior bank of the lunate sulcus, corresponds to V3A as previously described, while the medial focus corresponds to PIP. Projections from regions representing lower versus upper parts of the visual field terminated in separate portions of PIP, indicating that this area contains at least a crude representation of visual topography.

PIP receives feedforward connections from areas V3 and VP that are reciprocated by cells primarily in the infragranular layers of PIP. Upper and lower-field V4 provide feedback projections to PIP that are reciprocated by cells in both the supragranular and infragranular layers of PIP. These data constrain the location of PEP in the hierarchy of cortical visual areas discussed in the following abstract.

Anatomical Hierarchy of Visual Cortical Processing in Macaque Monkeys

Daniel J. Felleman

The notion of a strict hierarchical organization of visual cortex of macaque monkeys was first hypothesized by Maunsell and Van Essen (1983), based on the observations that cortical areas are reciprocally connected with one another and that the laminar patterns of labeled axon terminals and cell bodies of origin can describe complementary feedforward and feedback connections.

We have substantially extended the original hierarchy by adding many newly identified cortical areas as well as by incorporating information on numerous additional pathways now known to link previously identified areas. In particular, our recent studies of the projections of area V4 have afforded new insight into the organization of visual areas in the temporal lobe. Rather than the two or so traditionally defined visual areas in the inferior temporal cortex, we found evidence for at least five subdivisions within IT proper plus two additional areas, located medially on the parahippocampal gyrus. This new information, coupled with previously published data on temporal lobe connections, has enabled us to delineate a visual processing hierarchy originating in the retina and passing through at least 14 levels before reaching the hippocampal formation.

The current anatomical hierarchy, although heavily laden with serial processing stages, contains parallel processing streams as well. A dorsal system, directed mainly toward parietal cortical areas, has been implicated in processing spatial relationships. In contrast, a ventral system, directed towards the temporal lobe, has been implicated in pattern discrimination and visual memory. Another anatomical dichotomy is based on the segregation of inputs arising from either the magnocellular or parvocellular layers of the lateral geniculate nucleus. Separate streams have been identified in areas V1 and V2, and they continue further into extrastriate cortex, where some areas appear to be dominated by magnocellular or parvocellular inputs. For example, areas V3 and MT are dominated by the magnocellular stream while areas VP and V4 are more closely linked to the parvocellular stream. The magnocellular stream projects most heavily to the parietal lobe, whereas the parvocellular stream projects mainly to the temporal lobe. However, the segregation between streams is by no means absolute. Area V3, for example, is located in a potentially strategic location to relay magnocellular-dominated information to area V4 and on into temporal lobe processing areas. Similarly, area V4t receives convergent inputs from magnocellular-dominated MT and parvocellular-dominated V4, which might provide for an important synchronization of the two major processing systems.

Reference:
A central problem in vision research is to understand the functional significance of the many different areas, subdivisions, and pathways that constitute the central visual system. Is each subdivision dedicated to the processing of a single sensory cue, relating to the perception of color, form, motion, or depth, as some investigators have suggested? Other investigators have advocated a complementary viewpoint, which focuses on the relative contribution of each subdivision to the perception of "what" versus "where," i.e., object identification versus spatial localization.

Neither of these viewpoints adequately represents the known anatomical and physiological complexities of the visual system, nor do they reflect the rich processing capacities of human vision as judged from psychophysical and computational perspectives. Accordingly, we have begun to formulate a hybrid conceptual framework that provides a more comprehensive treatment of the transformation from low-level sensory information to high-level perceptual inferences.

Our analysis emphasizes that each low-level sensory cue normally contributes to a variety of perceptual inferences. For example, motion cues not only tell us about the trajectory of a moving object, but also about its intrinsic shape and about its depth relative to other objects. Each of these inferences represents a particular computational task that needs to be implemented by a particular processing algorithm, such as the determination of "structure from motion" or the analysis of "motion parallax." Altogether, there are perhaps dozens of such tasks involved in early vision.

Viewed from this perspective, our challenge is to determine the particular set of computational tasks any given neuron or neural ensemble is involved in. Despite the wealth of existing physiological and anatomical data on the mammalian visual cortex, there are few examples in which precise assignments of specific tasks to particular neural ensembles can unequivocally be made. The conceptual framework put forward here points the way to a variety of experiments which should improve this situation markedly in the next few years.
337. Reduction of Early Visual Cortical Processes to a Common Algorithm

George J. Carman

Early visual processes use differences in the images formed on the retinas over time in order to derive information about the visual world. The motion process obtains two images from one viewpoint at two times, while the stereo process obtains two images from two viewpoints at one time. Such pairs of images contain a series of local regions that correspond, separated by intervening "warps" where one image contains information that is absent in the other. These warps will occur at the points of projection of depth edges in the visual world, and their sign will depend on whether depth increases or decreases across those edges. Thus motion and stereo, which are usually thought of as different processes, use the same retinal information, suggesting that they might be instances of a common algorithm.

Here I describe an associative algorithm, which can be formally described as an optimization of a graph. This algorithm finds the regions in a pair of images which correspond, and in so doing, derives a representation of the edges and relative depth of surfaces of occluding objects. This algorithm accepts as input two sets of tokens, T1 and T2, with each token giving the attribute(s) for a position. For example, in motion or stereo, the tokens could give the sign of the local luminance contrast as a function of position along local, one-dimensional samples from a retinal image. The algorithm determines which token(s) i of T1 are associated with which token(s) j of T2 by an optimization of an objective function subject to applicable constraints. For example, the optimization could seek to minimize the difference in retinal position, or the difference in retinal spacing, of corresponding tokens. If there are N tokens in each set, then there are $2^{N \times N}$ possible distinct associations between two arbitrary sets of tokens, requiring on the order of $N^2$ neurons. Regularities in the retinal images constrain these associations so as to require consideration of only a fraction of the solution space and use only on the order of N neurons to represent the optimal solution. This solution gives a new local set of tokens T3 which, together with another set T3' produced in parallel, provide the input to the next level in this process of "folding together of samples."

The constraint on the size of the representation used by this algorithm allows for iteration without the combinatorial explosion expected for general association, permitting the construction of progressively more abstract representations of the visual world.

Simulations of this algorithm on random dot images demonstrate that it can combine two samples over time or space into a single representation of edges and surfaces with relative depth. A hypothetical implementation of this algorithm given the input to striate cortex leads to the formation of orientation selectivity and its loss at the center of local cortical domains, similar to that seen within the cytochrome oxidase blobs in striate cortex. Iterative application of this algorithm can in principle generate a series of representations that lead to hierarchical, object-centered representations which are thought to be implemented within extrastriate cortex.

338. Physically Unfolding and Flattening the Cerebral Cortex of the Macaque Monkey

Jaime F. Olavarria

In spite of its apparent homogeneity, the cortical mantle of mammals has been shown to be subdivided into many functional areas that are richly interconnected with each other. The complexities in the overall organization of these areas and connections are best appreciated in two-dimensional views of the cortex. Unfortunately, the deeply folded nature of the cortex in animals like the macaque monkey makes it very difficult to obtain an overall view of the cortex from a series of conventional histological sections through the brain. One solution to this problem was presented by Van Essen and Maunsell (1980), who developed a method for manually reconstructing two-dimensional maps of monkey cortex from serial sections. However, the alternative method of physically unfolding and flattening convoluted brains and sectioning them parallel to the surface offers the prospect of revealing complex patterns of tangentially arranged connections more directly and therefore with greater accuracy and detail. For instance, Olavarria and Van Sluyters (1985) showed that it is possible to physically unfold and flatten the entire cortex of the cat, and that only a few tangential sections are required for accurately reconstructing complex
cortical systems, such as callosal connections and ocular dominance patterns, in their entirety.

We have successfully applied this method to the macaque's brain, in which the sulci are more numerous and deeper than in the cat. The technique described to preserve the gyral pattern of the intact brain on the surface of the flattened cortex also yielded good results in the macaque brain. Although virtually the entire cortex of the macaque can be unfolded and flattened as a whole, it is more convenient to cut it in smaller, more manageable pieces. Examination of cytochrome oxidase stained tangential sections through VI of long-term monocularly enucleated monkeys shows that the complete pattern of ocular dominance columns can be revealed in a single tangential section. Similarly, the stripped nature of V2 was readily revealed in sections through extrastriate cortex. These results indicate that even regions as convoluted as the calcarine and lunate sulci can be unfolded and flattened without breaking and with minimal distortion, and that it is possible to cut single tangential sections that stay within the same cortical layer through extensive regions of originally convoluted cortex. These observations highlight the potential of this method for studying cortical patterns of connectivity in the macaque monkey.

References:

339. Anatomical Mapping of the Organization of Extrastriate Visual Cortex in the Rabbit and Rat Using Multiple Tracers
Jaime F. Olavarria, Daniel J. Felleman, David J. Bruning

Unlike larger mammals such as cats and monkeys, area 17 (striate cortex, VI) in rodents is bordered laterally by several visual areas rather than a single area V2. In an attempt to test the generality of the rodent visual cortical plan, we reinvestigated the organization of extrastriate cortex in the rabbit and rat by mapping the distribution of interhemispheric and striate-extrastriate connections in tangential sections of physically flattened cortex. Interhemispheric connections passing through the corpus callosum are known to be distributed preferentially to the vertical meridian representations that commonly separate adjoining extrastriate visual areas, thus providing useful landmarks for the border of visual areas in previously unexplored cortical regions. The internal topographic organization of areas defined by the callosal pattern can then be explored in more detail by analyzing the patterns of striate-extrastriate connections.

The callosal pattern of one hemisphere was demonstrated by the transport of horseradish peroxidase injected massively into the opposite hemisphere, while ipsilateral striate-extrastriate connections were revealed by injecting 3H-proline, Rhodamine-Beads, Fast-Blue, and Fluoro-Gold into discrete loci of VI.

In the rat, we confirmed the existence and internal topography of visual areas anterolateral (AL), latero-medial (LM), posterolateral (PL) and posterior (P), which are located immediately lateral to area 17. Area laterolateral (LL), and several subdivisions in 18b were also recognized. Similarly, in the rabbit, single VI injections produced multiple labeled fields distributed throughout a belt of cortex surrounding VI. Furthermore, the distribution of differentially-labeled cells from separate VI injections indicates that the majority of these fields correspond to separate, topographically organized visual areas. Analysis of both callosal and VI connections indicates that the overall pattern of visual areas in the rabbit is strikingly similar to that in the rat. For example, based on location and internal topography, areas perhaps homologous to areas AL and LM of the rat are present in the rabbit, and the location of additional fields suggests that they may correspond to areas LL, PL and P of the rat. Thus our data suggest that a similarly complex cortical organization appeared in a common ancestor of these two species, early in mammalian phylogeny.

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340. Optical Studies of Cortical Connectivity in Rat Visual Cortex
Harry S. Orbach

Changes of absorption and fluorescence of certain membrane-bound dyes are linearly proportional to changes in membrane potential. Based on this, optical methods can be used to measure activity in the mammalian brain. The mean electrical activity in many adjacent areas can be monitored by projecting an image of the brain onto an array of about 100 photo-
detectors and measuring the photodetector outputs.

We have set up such an optical system and are currently examining the response of rat visual cortex to electrical stimulation with the aim of showing that the technique can be used to examine cortical connectivity. A large expanse of striate and extrastriate cortex was stained with a potential sensitive dye and imaged onto the array. Fluorescence changes were monitored in response to short trains of electrical stimulation applied through either of two electrodes placed in striate cortex. Biphasic pulses in the range of 15-400 µamps x 0.2 msec produced a focal response at the electrode in striate cortex plus foci in lateral extrastriate cortex. Focal projection fields were found which we believe correspond to excitations in extrastriate areas LM and AM. The size of the focal response (full width at half height) was typically 1-2 mm in the striate region and 0.5-1.5 mm in the extrastriate regions. Topographic organization within and between extrastriate regions was demonstrated by shifts in the peak of the response focus of appropriate direction and magnitude given the separation of the stimulating electrodes. The extrastriate responses showed a significant delay in onset and time to peak relative to that at the stimulation site (ca. 5 msec). These results, together with the prospect of being able to trace many connections in a single experiment, indicate to us that optical monitoring of focal electrical stimulation provides a valuable means for analyzing structural and physiological aspects of cortical circuitry.

341. Neonatal Reinnervation of Rabbit Soleus Lacks Specificity by Fiber Type

James M. Saha, Edward M. Callaway

Neonatal rabbit soleus muscle contains a mixture of fast-contracting and slow-contracting muscle fibers. Previous studies have suggested a pattern of innervation which is highly specific by muscle fiber type, even while polyinnervation remains widespread. Chemospecific recognition of appropriate muscle fibers by motor neurons during synaptogenesis is one possible mechanism by which this specificity could be established. To study this possibility, we have looked for specificity during the reinnervation that follows experimental crush of the muscle nerve in 4-day rabbits, using the distribution of single motor unit twitch tension rise times as an in vitro assay.

To minimize the opportunity for conversion of muscle fiber contractile type, muscles were analyzed during the early stages of the reinnervation process. Single motor units were isolated by graded stimulation of teased ventral root filaments. The distribution of rise times for twitch tensions from a population of motor units in normal muscles was broad and bimodal, indicative of two distinct types of motor units. In contrast, the distribution of twitch tension rise times for all 10 reinnervated muscles was narrow and unimodal, suggesting that all motor units were composed of a similar combination of fast and slow contracting fibers. Rise times for reinnervated units were intermediate to those of normal control fast and slow motor units, indicating that the unimodal distribution did not arise from specific reinnervation by only one population of motor neurons. To verify that the short interval of inactivity before reinnervation did not markedly affect muscle contractile properties, an additional set of muscles was analyzed following transmission blockade for an equal interval induced by botulinum toxin poisoning. Single motor units from these muscles displayed a normal bimodal distribution of twitch rise times.

From these experiments, we conclude that there is little if any specificity by fiber types during reinnervation of early postnatal rabbit soleus. While our findings do not rule out chemospecific recognition during initial synaptogenesis, they do enhance interest in alternative mechanisms.

342. Monoclonal Antibodies Specific to Fast or Slow Motor Neurons

E. Karina Schimmerling

Sometime before birth, fast and slow motor neurons come to innervate primarily their corresponding muscle fiber types within mixed fast/slow muscle. However, the mechanisms underlying the embryonic development of this specificity are unknown. In order to examine the establishment of these connections in more detail, it would be useful to have histological markers to identify motor neuron types.

To this end, we are attempting to generate monoclonal antibodies that can distinguish between fast and slow motor neurons. Slow motor neuron homogenates
for immunization are obtained from young rabbit soleus nerves. Fast motor neuron homogenates, used for immunosuppression, are obtained from young rabbit extensor digitorum longus (EDL) nerves. Preparation of hybridomas is done by To Ha Thai in the Biology Division's Monoclonal Antibody Facility. We screen hybridoma supernatants on frozen sections of soleus and EDL muscle, which are also labeled with rhodaminated α-bungarotoxin to mark endplates, or on nerve homogenate dots bound to nitrocellulose. In our first attempt, we obtained monoclonal antibodies that label muscle, but staining of nerve terminals was faint or absent in both types of muscle.

For the next immunization, we are preparing homogenates of soleus nerves, which will be ligated near the entry point to the muscle, to collect transported materials destined for the terminal region. Immunosuppression will be against normal EDL nerve homogenates. In addition, older rabbits will be used because at later ages the muscle fiber composition of the soleus is mostly of the slow type. This protocol will hopefully increase our chances of generating antibodies that stain specifically slow, but not fast, motor neuron terminals.

### 343. Differential Loss of Neuromuscular Connections According to Spinal Position

**Edward M. Callaway**

During normal maturation, mammalian muscles undergo an orderly loss of synapses; each muscle fiber loses all but one of the multiple inputs it has at birth. One method for monitoring the loss of multiple inputs is to measure the number of muscle fibers each motor neuron innervates (motor unit size). We have examined the distribution of motor unit sizes as a function of spinal position in soleus muscles of rabbits at different postnatal ages. Motor unit twitch tensions and rise times were measured in vitro. The majority of the soleus motor axons (about 65) exit through the S1 root, while a minority (mean of 4–6 per root) leave through the L7 and/or S2 roots. Units were separated into fast and slow groups based on rise times, and twitch tensions were normalized to the median fast or slow value (as appropriate) from the S1 population for that animal in order to facilitate detection of differences in size between L7/S2 and S1 root motor units. Motor unit sizes were measured from animals in three age groups: early (4–5 days), when the rabbit soleus is heavily polyinnervated; intermediate (8–9 days), when elimination is about half completed; and late (11–15 days), when the soleus muscle is singly innervated.

At the early age, when polyinnervation is substantial, we found that L7/S2 neurons on average innervate at least as many muscle fibers as S1 neurons. For the slow population, L7/S2 units were actually about 20% larger than S1 units; for the fast population, there was no significant difference. However, by 8–9 days L7/S2 units were 20–25% smaller than S1 units for both the slow and fast populations; these differences were significant at the p < 0.001 level. The difference persisted in 11–15-day animals, where L7/S2 motor units were about 20% smaller than S1 units for both the slow populations (p < 0.05). No significant differences were observed between motor units from L7 versus S2 extremes at any age, confirming the absence of a rostro-caudal bias reported by Gordon and Van Essen (1983).

We conclude that motor neurons from extreme positions in the rabbit's soleus motor pool lose significantly more synapses than those from the middle. The differential loss of synapses appears to occur primarily during the earlier stages of synapse elimination, day 4 to day 9, when the rate of loss by extreme neurons is 50-80% faster than for middle neurons.

**References:**


### 344. Competition Favoring Inactive Over Active Motor Neurons During Synapse Elimination

**Edward M. Callaway, James M. Soha**

Synapse elimination at the neuromuscular junction is generally considered to be a competitive process, in which the probability of survival of any one synapse is dependent on competitive interactions with other synapses at the same endplate. Several studies have demonstrated that changes in overall activity levels alter the rate of synapse elimination. However, none of these studies has convincingly demonstrated that differential activity among a group of synapses confers any advantage according to the level of activity. Would a difference in activity among the terminals at the same endplate affect the outcome of the competition and not just its rate?

We investigated this issue by blocking activity
during the period of maximal synapse loss in a small fraction of the motor neurons innervating the neonatal rabbit soleus. Activity was blocked for 4 days by implantation of a tetrodotoxin-laden plug into either the L7 or S2 spinal nerve of 4- or 5-day-old rabbits. Motor unit sizes were subsequently measured in vitro for two groups: 1) 8- to 9-day animals, in which the implanted units were inactive from day 4 or 5, and 2) 14- to 15-day animals, in which implanted units were inactive from day 4 or 5 until day 8 or 9, when activity resumed. Animals implanted with control plugs (containing no tetrodotoxin) and normal animals were also analyzed. Motor units were separated into fast and slow groups and their sizes expressed as percent of the median value from the SI root (see preceding abstract).

In the control experiments, motor units in the implanted roots were on average 20-30% smaller than those from the SI root, and in this respect they were indistinguishable from normal L7/S2 motor units (see preceding abstract). In contrast, implantation of tetrodotoxin laden plugs into L7 or S2 roots resulted in motor unit sizes that on average were about 50% larger than the active units in control implanted animals. These differences were highly significant for both the 8- to 9-day group (p < 0.001) and the 14- to 15-day group (p < 0.001). We conclude from these results that inactivity increases the competitive ability of a motor neuron's synapses relative to those of active synapses.

In adult mammals there is a correlation between motor unit size and recruitment order. This presumably allows for optimal coordination during movement: small motor units are recruited most easily to mediate fine movements, while progressively larger units are recruited for tasks requiring greater force. If a motor neuron's activity level at birth is related to its adult recruitment threshold, then an advantage to less active neurons during synapse elimination could help to sculpt the adult configuration from polyinnervated newborn muscles. We are presently developing a method for determining both the recruitment threshold and size of individual motor units. These measurements might allow us to determine whether the differences in activity that normally exist between motor neurons lead to a differential loss of synapses as we observed in the tetrodotoxin-implanted animals, and whether differential synapse loss is responsible for the development of recruitment threshold to motor unit size matching observed in adult mammals.

345. Computer Modeling of Neuromuscular Synapse Elimination

James M. Soha, Edward M. Callaway

Our current understanding of synapse elimination is based upon numerous observations describing both normal development and the effects of various experimental perturbations. Several different cellular and molecular interactions have been proposed to account for certain aspects of the process, and it appears likely that many such interactions will be involved in a complete description of this complex developmental phenomenon. To aid in understanding the dynamics of these interactions, we have generated a computer model that simulates various aspects of the synapse elimination process.

Our model focuses upon competition among presynaptic motor terminals for limited synaptic space at individual muscle fiber endplates. Each terminal has a bias for either growth (sprouting) or retraction that is based on a combination of local influences and neuron-wide conditions. Actual growth or retraction of individual terminals is determined stochastically at each iteration using the calculated bias value and the bias is updated with each cycle. The local component of the growth-retraction bias is based upon the concentration of a hypothesized extracellular scaffolding molecule. This molecule is synthesized by the postsynaptic muscle fiber based upon its level of activity, and subsequently acts to stabilize presynaptic terminals and increase their probability of growth. The neuron-wide component of the bias value is determined stochastically at each iteration using the calculated bias value and the bias is updated with each cycle. The local component of the growth-retraction bias is based upon the concentration of a hypothesized extracellular scaffolding molecule. This molecule is synthesized by the postsynaptic muscle fiber based upon its level of activity, and subsequently acts to stabilize presynaptic terminals and increase their probability of growth. The neuron-wide component of the bias value is determined by the metabolic load associated with a motor neuron's axonal arbor, and its response to trophic influences. Other proposed mechanisms, such as activity-dependent release of proteolytic enzymes by presynaptic terminals, can be readily incorporated in the current framework. In addition to simulating normal development, the model permits several 'experimental' perturbations, including overall or selective activity changes, and partial denervation.

This model has successfully simulated several of the key aspects of synapse elimination. Detailed studies using increasingly refined versions of the model are currently in progress. One issue of particular
relevance concerns the mechanisms that might account for the interesting competitive effects of activity recently observed in this laboratory (see preceding abstract).

PUBLICATIONS


DEVELOPMENTAL GENETICS
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Summary: In Drosophila two large gene clusters, the Bithorax Complex (BX-C) (Lewis, 1978) and the Antennapedia Complex (ANT-C) (Kaufman et al., 1980) act as master controls for the formation of the head, thorax and abdomen of the fly. DNA sequence homology of highly conserved regions, or homeoboxes (McGinnis et al., 1984; Scott and Weiner, 1984), within many of the genes of these complexes indicates that such clusters arose by a process of tandem gene duplication accompanied by a gradual divergence in function as the result of mutation. Furthermore, the genome of vertebrates such as mice and human beings (reviewed by Gehring and Hiromi, 1986) have clusters of genes that code for homeobox regions that are remarkably similar to those of the BX-C and the ANT-C, suggesting a common origin of these complexes that antedates the appearance of the vertebrates. Evidently such homeobox-containing genes play a vital role in controlling the body plan of many groups of higher organisms. We are studying the BX-C to determine the rules that govern the way these genes control development. A simple rule seems to hold for many if not all genes of the complex; namely, their order along the chromosome parallels their order of derepression, or of activation, along the body axis of the organism. We are using genetic and molecular approaches to try to determine the mechanisms responsible for this rule. In our molecular studies reported below, we are concentrating on two regions of the abdominal or distal half of the BX-C: the region controlling the development primarily of the seventh abdominal (A7) segment, which we designate as infra-abdominal-7, (iab-7); and the corresponding gene for segment A4, or iab-4. Mutants within many of the genes of the complex result in a reduction in function or cis-inactivation (CIN) of one or more genes located more distally in the chromosome; and certain mutants result in a cis-overexpression (COE) of the gene located immediately proximally (Lewis, 1985). We are concentrating on obtaining as many rearrangements as possible that have breakage points within the BX-C in order to pinpoint the DNA regions within the complex that are involved in initiating the CIN and COE effects.

References:

346. An Improved Screen for Detecting Chromosomal Rearrangements in the Bithorax Complex

Rollin H. Baker, E. B. Lewis

We reported last year (Biology 1986, Abstract No. 304) on our development of an improved global screen for rearrangements having one breakage point in the bithorax gene complex and the other breakage point located elsewhere in the genome. This screen, designated GR2, is able to detect virtually every breakage point that occurs within the BX-C regardless of the nature of the effect of that breakage point on gene function within the complex. It is only necessary that the rearrangement disrupts somatic pairing. We x-ray males carrying a third chromosome with an inversion bringing a wild-type (+) copy of the BX-C within a few bands of the centromere region; the males are mated to females carrying a very similar inversion but containing the mutants Contrabithorax (Cbx) and Ultrabithorax (Ubx). Flies that are Ubx Cbx/+ and essentially homozygous for the inverted sequence have spread and slightly reduced wings,
whereas Ubx Cbx/+ flies which have a newly induced rearrangements with one breakage point in a short region extending from the centromere up to and including the BX-C region have completely normal wings. Using this GR2 screen, we have recovered not only numerous Ultrabithorax mutations but in addition 10 new mutations within the distal half or iab region of the complex. The latter are more or less randomly distributed, as far as can be judged at this time, as follows, based upon phenotypic analysis: one in iab-2, one in iab-3, one in iab-4, three in iab-5 and four in iab-6. COE effects are exhibited by all except the iab-2 mutant. We are collaborating with Dr. Welcome Bender and his colleagues of Harvard Medical School who are locating the molecular lesions associated with these mutations.

347. The Structure and Expression of the infra-abdominal-7 (iab-7) Gene
Susan E. Celniker, Scott L. Rosenfeld

Three homeobox-containing genes are located within the bithorax complex: Ultrabithorax (Ubx), iab-2 (abd-A), and iab-7 (Abd-B). Our genetic studies with the mutant Transabdominal (Celniker and Lewis, 1987) have led us to concentrate on the most distal homeobox-containing gene, iab-7. The Tab mutation results in overexpression of the iab-7 and/or iab-6 genetic functions creating a sexually dimorphic pattern of segmental transformation. In order to determine how this misregulation is achieved, we have initiated molecular studies of the iab-7 gene.

Using a 1.2 kb DNA restriction fragment from the 3' end of an iab-7 cDNA (previously described in Biology 1986, Abstract No. 302), we have detected two transcripts, 4.75 kb and 3.25 kb in length, that are present primarily in embryos (peaking at 4-8 hours of embryonic development) but are also expressed at low levels in third instar larvae, pupae and adults. To determine the sequence and structure of the iab-7 mRNA, we have isolated and characterized seven additional iab-7 cDNAs. By restriction enzyme analysis and preliminary sequence data, the cDNAs can be divided into two classes. We have almost complete sequences for two of the cDNAs belonging to class I: cDNA 5 is 2939 bp in length, has a consensus polyadenylation signal at 2910 and a 17 bp poly(A) tail; and cDNA 3 is 2.5 kb in length, but the 3' end terminates 0.8 kb from the poly(A) tail and consequently the 5' end of cDNA 3 has 0.38 kb of sequence not contained in cDNA 5. We have verified that iab-7 has a 160 bp homeobox identical with that reported for this gene by Regulski et al. (1983). We have also determined that the M repeat, located 5' to the homeobox spans 0.5 kb. By Southern analysis and preliminary genomic sequencing, we have identified two introns, the 5'-most is 4 kb in length and the other 0.8 kb in length. The gene spans at least 17 kb by our analysis of the cDNAs. As of yet we have no full-length cDNA representing the 4.7 kb transcript, but we are in the process of screening new cDNA libraries that should contain a higher percentage of full-length cDNAs. Analysis of mutants that disrupt the iab-7 gene suggest the gene may be as large as 40 kb.

To determine the spatial expression of the iab-7 gene, we have used radioactively-labeled probes on tissue sections from unstaged embryos. Transcripts can be detected in embryos that are undergoing germ band elongation (stages 10 and 11 or 4–7 hours) and germ band retraction (stages 13-14 or 9–11 hours). The transcripts have thus far been detected only in the ventral nerve cord and ventral ectoderm. In stage 11 embryos, the transcript appears to be localized to parasegments 13 and 14, but by stage 14 the transcript is more widely distributed including parasegments 11, 12, 13 and 14. We are now in the process of collecting staged embryos for sectioning to determine the earliest pattern of expression of the iab-7 gene and to determine more precisely its expression in older embryos as well.

Reference:

348. Molecular Analysis of the iab-4 Region of the Bithorax Complex
Sarah Smolik-Utlaut

We are interested in the regulation of the BX-C genes and their roles in the differentiation of mesodermal as well as ectodermal structures. We are concentrating on the iab-4 region of the complex. A deletion of this region (and the adjoining iab-5 region) known as iab-4,5DB (Karch et al., 1985) is viable when
homozygous. The homozygotes lack the somatic gonads, which are of mesodermal origin, and have the ectoderm of A4 transformed toward that of A3. Homozygotes for chromosomal rearrangements having breakage points in the iab-4 region also have this type of phenotype and some show a transformation of A2 toward A3, i.e., the COE effect. We have begun a molecular analysis of the iab-4 region by screening a cDNA library constructed by Larry Kauvar. For this purpose we used probes derived from genomic DNA that span the region disrupted by known breakpoints in the iab-4 region (Karch et al., 1985). Thus far we have isolated a single cDNA that is approximately 1.8 kb in length. We are in the process of screening additional libraries. No major introns are detected by restriction and hybridization analyses with genomic sequences. We have begun sequencing the cDNA.

Reference:

349. Genetic Dissection of the Abdominal Region of the Bithorax Complex

Leila M. Posakony

We are employing genetic techniques to study the functions in the BX-C responsible for establishing the identity of the abdominal segments of the larval and adult forms of Drosophila melanogaster. We are preparing to generate tandem duplications of the BX-C that will contain only portions of the abdominal region of the complex. This will enable us to determine the phenotypic consequences of over-expression of wild-type functions of the abdominal genes; specifically, we are particularly concerned with the non-homeobox containing genes, such as iab-3.

We are constructing chromosomes bearing mutant alleles of the abdominal genes iab-2 in cis, and iab-4,5 on the one hand and iab-4,5 and iab-7 on the other hand. In the process of generating these chromosomes by crossing over, we expect to recover the complimentary wild-type crossovers. These crossovers will enable us to perform cis-trans tests for the corresponding regions of the complex and therefore will provide the basis for dissecting the CIN and COE effects.

We are developing screens to recover mutations in genes regulated by the products of the BX-C. Such genes have been proposed to be responsible for implementing the segmental identity function of the BX-C genes.

PUBLICATIONS


THE FOLLOWING REPORTS ARE BY GRADUATE STUDENTS IN THE DIVISION OF BIOLOGY WHO ELECTED TO DO THEIR THESIS WORK IN THE DIVISION OF CHEMISTRY AND CHEMICAL ENGINEERING.

350. The Saccharomyces and Drosophila Heat Shock Transcription Factors Are Identical in Size and DNA Binding Properties

Greg Wiederrecht 1, David J. Shuey, Warren A. Kibbe 1, Carl S. Parker 1

Support: National Institutes of Health, USPHS Helen Hay Whitney Foundation

The heat shock transcription factor (HSTF) has been purified to apparent homogeneity from S. cerevisiae and D. melanogaster by sequence-specific DNA-affinity chromatography. A synthetic oligonucleotide containing an hsp 83-like heat shock element (HSE) was prepared and ligated into concatamers and covalently coupled to Sepharose. This DNA-affinity resin allowed the rapid isolation of a yeast and a Drosophila protein with the same apparent molecular weight (70 kDa). The yeast HSTF will bind to both its own and the Drosophila HSEs. Similarly, the Drosophila HSTF will bind to both its own and the yeast HSEs. The yeast and Drosophila HSTFs were subjected to preparative SDS-gel electrophoresis, and the 70 kDa polypeptides were eluted, renatured, and observed to generate the identical footprint pattern as the native HSTFs. Affinity-purified Drosophila HSTF was further shown to stimulate specific HSE-dependent transcription from a Drosophila hsp 70 gene in vitro.

Reference:

1Division of Chemistry and Chemical Engineering, California Institute of Technology

351. Novel Protein-DNA Interactions at the Drosophila hsp 83 Gene Promoter

David J. Shuey, Carl S. Parker 1

Support: National Institutes of Health, USPHS

Many of the heat shock protein (hsp) gene promoters contain multiple heat shock elements (HSEs) that have been shown to bind the positively-activating heat shock transcription factor (HSTF). These HSEs often overlap and share consensus sequence residues with neighboring sites. A dramatic example of this arrangement is found at the Drosophila hsp 83 gene promoter. In this report we have investigated the spatial distribution of HSTF bound at this promoter. Native agarose gel electrophoresis was used to resolve three distinct HSTF-DNA complexes of increasing apparent molecular weights. Analysis of these complexes with enzymatic and chemical probes revealed that the central HSE (site 1) is occupied at low concentrations of HSTF, followed by the sequential binding to the flanking HSEs (sites 2 and 3) and higher HSTF concentrations. We postulate that a dimeric HSTF binds first at site 1 with subsequent monomeric HSTF binding of site 2 and site 3 to form a stable complex consisting of four HSTF monomers and the promoter.

We have previously shown that HSTF induces a bend in the hsp 70 gene promoter upon binding (Shuey and Parker, 1986). As the monomeric repeat unit of the hsp 83 promoter is 10 bp, this DNA bending would be additive, perhaps resulting in some type of nucleosome-like structure. It seems conceivable or even likely that protein-DNA superstructures, possibly involving the bending or looping of the DNA, may form at these promoters facilitating the binding and initiation by RNA polymerase II.

Reference:

1Division of Chemistry and Chemical Engineering, California Institute of Technology
1. Cell Cycle Analysis for Flow Cytometry

Rochelle A. Diamond, Patrick F. Koen

The Flow Cytometry/Cell Sorting Facility has developed a protocol to routinely analyze cell cycle compartments in both live and fixed mammalian cells. The procedure is a modification of that of Darzynkiewicz et al. (1980) using acridine orange staining in situ. Acridine orange monomers intercalate into double-stranded nucleic acids that emit a maximum green fluorescence at 530 nm. Single-stranded nucleic acids form an electrostatic polymeric complex between the phosphates of the nucleic acid and acridine orange, shifting the maximum absorbance of the dye to shorter wavelengths and the emission maximum to the red near 640 nm. Therefore, when the stained cells are measured on the flow cytometer with blue excitation of 488 nm by the argon laser, the green fluorescence provides an estimate of the amount of DNA, while the red fluorescence gives an estimate of RNA. These two parameters are plotted against each other on a cytogram. Compartments can be drawn to define cell-cycle stages based on this plot. The quiescent or resting stage, G0, has a diploid or 2c amount of DNA (green) with very low RNA content (red). The activated state, G1, has a diploid amount of DNA, but the RNA content is increased. S phase, the stage of actual DNA synthesis, is marked by increases in both the DNA and RNA content. G2+M, the stage just prior to cell division and cell division itself, has the highest amount of DNA (4c) and large amounts of RNA.

Proper staining conditions—ionic strengths, dye concentrations, cell permeability, pH, chemical equilibrium of bound vs. unbound dye, etc.—are all critical to this method. Each cell type must be evaluated for its own idiosyncrasies. This procedure is especially suited for optics of the Facility's Ortho Cytofluorograf. The Facility has successfully analyzed chicken erythrocytes, mouse thymocytes and splenocytes, and various cell lines in culture.

Reference:

2. Two-color Cytometric Analysis

Rochelle A. Diamond, James P. Lugo, Patrick F. Koen

The detection of cell-surface antigens to identify and classify mammalian cells is now the most common use of the flow cytometer. We have been routinely measuring single antigen fluorescence in combination with light scatter. With the recent introduction of phycobiliprotein-conjugated reagents, the facility now has the capability to do multiparameter analysis, i.e., measure two cell-surface antigen fluorescences as well as light scatter simultaneously. Both fluorescein isothiocyanate (FITC) and phycoerythrin (PE) can be excited at 488 nm with a single argon laser. Their emission spectra can be separated using dichroic mirrors and the correct band pass filters. Although there is a small overlap of the red fluorescence into the green channels, this can be effectively corrected by computerized fluorescence compensation. Monitoring two antigens and labeling respectively with FITC (green) and PE (red), one can see up to four types of populations in a mix of cells: the two single-positive populations, the double-positive population, and the double-negative population. Quantitative differences in expression of either or both of the antigens allow additional subpopulations to be discerned. This effectively gives the researcher far more information about his cell populations than performing successive single-color parameter experiments.

Labeling reagents are extremely critical in producing good results. The reagents may either be directly coupled to the antibodies of interest, or second-stage reagent labeling steps (e.g., reaction with a fluorochrome-labeled antibody directed against the primary antibody itself) may be used with the antibody of interest. In either case, purity of reagents, elimination of cross reaction, and titration of antibody concentration to eliminate aggregation is of extreme importance. Washing procedures and blocking reagents also are important to generate a good sample.

One also can achieve two-color analysis using Texas Red for the red fluorochrome. This requires the use of two lasers for excitation and different optics and computer manipulation. This is available also to Facility users. The same specifications for reagent purity and lack of cross reactivity apply.
MICROCHEMICAL FACILITY

Supervisor: Suzanna J. Horvath
Staff: Christine A. Acklin, Peter C. Gaines, Jacqui A. Kraus, Shelby R. Newton

We have continued to provide sophisticated chemical synthesis and structural analysis of biologically active molecules to the scientific community of Caltech by using a variety of state-of-the-art automated microchemical and analytical techniques. Through a number of successful collaborative research projects we have made a special effort to maximize the performance of these techniques and to find novel, creative utilization of synthetic biopolymers in biological and chemical research.

Our four DNA synthesizers have the capacity to synthesize eight different DNA fragments simultaneously, and we have provided about 2000 deoxyoligonucleotides for a large variety of applications. In addition to the small (92 nM), regular (1 nM), and large (10 nM) scale synthesis, we routinely synthesize mixed probes (probes containing deoxyinosine residues), and we have also introduced the synthesis of "doped" and biotinylated deoxyoligonucleotides. During automated synthesis, random mutations can be introduced into the DNA by "doping" each of the four nucleotide phosphoramidites (A, G, C and T) with a low level, equimolar mixture of the other three. In this way, complete mutational libraries can be constructed. Currently the most useful non-radioactive labeling system can be constructed by synthetic biotinylated DNA probes. These probes can detect approximately 2-5 pg of target DNA. After hybridization, the probe is incubated with streptavidin, which has high affinity for biotin, and conjugated to an enzyme or other marker. The results of assays in which non-radioactive probes are used can be scanned after incubation of the blot with substrate, or can be quantified by densitometer.

Using our two automated peptide synthesizers with full capacity, we have chemically synthesized several hundred small peptides and conjugated them to carrier proteins (KLH, ovalbumin). These peptide-protein conjugates have been used, as previously reported, to immunize rabbits or mice to give conventional heterogeneous antisera or monoclonal antibodies with desired specificities. Since each of our automated peptide synthesizers operates with only one reaction vessel synthesizing one peptide at a time, followed by a time-consuming HF-cleavage and work-up procedure of the product, only 16 peptides (15-20 amino acids in length)/month can be synthesized. In order to meet increasing interest, we have started to explore alternate methods for rapid, simultaneous preparation of large numbers (25-100) of discrete small peptides. Even more exciting is the development of recent techniques that permit the automated synthesis of long polypeptide chains. The extended protein synthesis opens up a new world of potential structure-function studies.

MONOCLONAL ANTIBODY FACILITY

Supervisor: Paul H. Patterson
Staff: To Ha Thai, Julianna Horn

Over the past year we have successfully generated MAbs for the following groups: Scott Fraser (UC, Irvine), MAbs specifically against particular quadrants of the adult Xenopus tectum (using the cyclophosphamide suppression method); Elias Lazarides, MAbs against variants of the chicken erythrocyte 4.1 protein; Carl Parker, MAbs against yeast heat shock factor that cross-react with Drosophila h.s.f.; Bill Dreyer, MAbs against specific surface determinants on embryonic chick tectum; Mark Konishi, MAbs against chick calcium binding protein (which recognize time-coding elements of the owl auditory system).

In addition, we are also working with Jim Bower, David Van Essen, and Seymour Benzer.

SEQUENCING FACILITY

Supervisor: David B. Teplow
Staff: John Raes, Haul Wong

A new facility has begun operation at Caltech in 1987, providing protein sequencing and amino acid analyses to the Caltech community. Instrumentation includes an Applied Biosystems pulsed-liquid protein sequenator with on-line PTH analysis and automated sequence identification, as well as an automated amino acid derivitizer/analyzer with on-line data acquisition/analysis capability. Besides instrumental support, the facility assists researchers with problems or questions in protein chemistry and separation methodology. Research is done in the facility on both service and collaborative bases. In the future, we hope to expand the capabilities of the facility to include peptide production, separation and analysis, automated DNA sequencing, and other highly technical areas of macromolecular structure analysis.
GRADUATES

Twenty-one students in Biology were awarded the B.S. or Ph.D. degree in June 1987. Names, degrees conferred and titles of doctoral theses are as follows:

Bachelor of Science

Hwai Wen Chang, B.S.
William Douglas Cutrell, B.S. with honor
Michael Francis DeFreitas, B.S. with honor
Donna Faye Evans, B.S.
Stephen Mark Gómez, B.S.
Susannah J. Hannaford, B.S. with honor
Bharathi Jagadeesh, B.S.
William Harold Newman, B.S. with honor
Katrin M. Rodriguez, B.S.
Stephen James Salser, B.S. with honor
Jo Beth Schlottman, B.S. with honor
Michael Moonsup Song, B.S. with honor
Darin Katsumi Takemoto, B.S. with honor
Ivan Tarle, B.S. with honor
Joan Marie Tetrault, B.S.
Kenneth Kenji Yoshimoto, B.S.

Doctor of Philosophy

Ngozi Emmanuel Erondu, Ph.D. Regional distribution and subcellular associations of Type II calcium and calmodulin-dependent protein kinase in rat brain.


Beverley Bond Matthews, Ph.D. Structure and expression of the actin gene family of Drosophila melanogaster.

John J. Ngai, Ph.D. Expression of the genes encoding the cytoskeletal proteins vimentin and protein 4.1.

Lei Yu, Ph.D. The nicotinic acetylcholine receptor: Gene expression and ion channel function.
FINANCIAL SUPPORT

The financial support available for the work of the Division of Biology comes from many sources: the Institute's general budget and endowment and special endowment funds; from gifts, grants or contracts from individuals, corporations, foundations, associations, and U.S. government agencies.

Abbott Labs
Alberta Heritage Foundation for Medical Research
Rita Allen Foundation
American Cancer Society
American Foundation for AIDS Research
American Heart Association
Earle C. Anthony Fellowship
Apple Computer Corporation
Arthritis Foundation
Australian Medical Foundation Fellowship
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The Donald E. and Delia B. Baxter Foundation
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Ethel Wilson Bowles and Robert Bowles Memorial Fund
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Alice Hicks Burr Fund
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Louisa Jane Church Fund
Norman W. Church Foundation
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Leukemia Society of America
Life Sciences Research Foundation
Lockheed Corporation
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