FRONT COVER

High-resolution mapping of human brain activity with positron emission tomography (PET)
The cover illustration shows blood flow changes seen in an observer's brain when he viewed three different visual patterns. The PET technique used to acquire the data provides a non-invasive method for monitoring brain activity, since blood flow changes in a small area of brain tissue correspond to local changes in the firing rate of neurons in that area. The left column displays the patterns viewed; each pattern consists of a flashing checkerboard ring with a small spot in the center which the observer fixated on. In the right column the corresponding blood flow changes are shown as seen from a midline view of the right hemisphere of the brain, along with an outline of the brain shown in white. The back part of the brain is to the right and the top of the brain is up. The magnitude and location of the changes in blood flow are plotted as colored areas, with white indicating the most intense response, yellow an intermediate response and black comparatively little response. All responses are centered in primary visual cortex and the response location moves toward the front of the brain and up as the distance between the checkerboard ring and the central fixation point increases. The short vertical line near each response represents 2 centimeters. It is apparent that small response location changes on the order of a few millimeters can be detected with this technique (see Abstract No. 252).

BACK COVER

Complete elucidation of the human mitochondrial genome
With the functional assignment of seven unidentified reading frames as genes for subunits of the respiratory chain NADH dehydrogenase, the determination of the genetic content of this genome, which was started at Caltech 18 years ago (see Biology 1968, Abstract No. 99), has now been completed. The diagram represents the genetic and transcription maps of HeLa cell mitochondrial DNA. The two inner circles show the locations of the two rRNA genes (12S and 16S) and the positions and identities of the reading frames and tRNA genes. In the outer portion of the diagram, the mapping positions of the rRNAs and mRNAs and their precursors are shown. ND1, ND2, ND3, ND4, ND4L, ND5 and ND6 are genes for subunits of NADH dehydrogenase; COI, COII and COIII are the genes for subunits I, II and III of cytochrome c oxidase; ATPase 6 and 8 are the genes for subunits 6 and 8 of the H⁺-ATPase; O₈ and O₇ are the origins of mtDNA heavy (H) and light (L) strand synthesis (see Abstract No. 24).
A REPORT FOR THE YEAR 1985-86
ON THE RESEARCH AND OTHER ACTIVITIES
OF THE
DIVISION OF BIOLOGY
AT THE
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA
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 June 1985-June 1986, are listed separately at the end of the research reports of each group.
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The Faculty

New this year is David Anderson, Assistant Professor of Biology. David did his undergraduate work at Harvard University and obtained his Ph.D. from The Rockefeller University in 1983, studying the biosynthesis of the acetylcholine receptor in Dr. Gunter Blobel's laboratory. Since 1983 he has been a postdoctoral fellow with Dr. Richard Axel at Columbia. His research seeks to understand the development of the mammalian central nervous system, using both cellular and molecular genetic approaches. Recent work in his laboratory has resulted in the isolation of several genes that are expressed when embryonic neurons first differentiate. One of these genes has been found to be regulated by an important signal in the cellular environment called nerve growth factor (NGF). David completed his move to Caltech on July 1 and will continue this work in his newly refurbished laboratories on the first floor of the Mabel and Arnold Beckman Laboratories of Behavioral Biology. His work has already been recognized by the award of a 1986 Presidential Young Investigator Award from the National Science Foundation.

Norman Davidson formally joined the Division of Biology as Professor Emeritus of Biology and Chemistry, upon his retirement this year as Professor in the Division of Chemistry and Chemical Engineering. This designation not only recognizes Norman's long association with molecular biology and members of our Division, but also his plan to continue active research as a member of the research group of Professor Henry Lester.

Derek Fender retired this year from his joint professorships in the Divisions of Biology and Engineering and Applied Science. Derek came to Caltech in 1961 and joined the Biology Division in the following year. His research has emphasized the use of his engineering and physics background to analyze information processing in the human brain, including such topics as the relationship between the control of eye movements and the processing of visual information. Over the years, he brought a valuable outside perspective to Biology committees and discussions of neurobiological questions.

Mary B. Mitchell died in Temple City, California, on May 16, 1986. She began work on Neurospora with George Beadle while he was at Stanford and came with Beadle to Caltech in 1946. Her entire scientific career was devoted to the genetics of Neurospora, and she made important discoveries in the areas of gene conversion and extranuclear inheritance. She retired from the Division of Biology in 1970 as a Research Fellow working in association with Professor Sterling Emerson. Mary's friends have established a memorial fund for book purchases for the Biology Library at Caltech; contributions may be sent to the Mary Mitchell Fund, Office of Memorial Funds, Caltech 1-36.
Honors and Awards

Professor Seymour Benzer was awarded the Thomas Hunt Morgan Medal in recognition of a lifetime’s contribution to genetics, by the Genetics Society of America. He also received the Rosenstiel Award for Basic Medical Research from Brandeis University.

Professor Giuseppe Attardi was awarded a 1986 Fellowship from the John Simon Guggenheim Memorial Foundation, and will be spending a sabbatical year at Biozentrum der Universitat, Basel, Switzerland.

Roy Britten, Distinguished Carnegie Senior Research Associate, was elected to fellowship in the American Academy of Arts and Sciences.

Professor Leroy Hood, who enjoyed a 9-month sabbatical from his duties as Chairman of the Division of Biology during the past year, was awarded an honorary D.Sc. degree from Montana State University. He received the Analytical Prize from the German Society for Clinical Chemistry for “the development of microchemical facilities for high-sensitivity protein sequencing.” Lee was also identified by Science Digest as one of the 100 Top Innovators of 1985—for the development of highly sophisticated instruments for the synthesis and analysis of genes and proteins.

Professor Henry Lester was named a Senator Jacob Javits Investigator by the National Institute of Neurological and Communicational Diseases and Stroke, assuring 7 years of uninterrupted research support.

Professor Paul Patterson’s work was recognized with the award of a McKnight Foundation Neuroscience Research Award.

An annual prize for the best Ph.D. thesis in Biology has been established by Lawrence L. and Audrey Ferguson. This year’s recipient was Robert Pruitt, who completed his Ph.D. this year under the supervision of Professor Elliot Meyerowitz, with a thesis on “Characterization of the genome of Arabidopsis thaliana.”

Professor Emeritus Norman Horowitz was honored by a special 70th birthday symposium on the "Origin of Life," held at Caltech on October 14 and 15, 1985. Norman’s book, To Utopia and Back, an authoritative, first-hand account of the search for life on Mars, has now been published by W. H. Freeman & Co. It is available in both paperback and hardcover editions from the Caltech bookstore and many other booksellers.

Professor Emeritus and Nobel Laureate Roger Sperry was honored by two symposia: "The Dual Brain" was held at UCLA, and the proceedings, edited by D. Frank Benson and Eran Zaidel, have been published by the Guilford Press, New York (1985); "Two Hemispheres - One Brain" was held at the University of Montreal, and the proceedings, edited by Lepofe, Ptito and Jasper, have been published by Alan R. Liss Inc., New York (1986).
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MOLECULAR BIOLOGY AND BIOCHEMISTRY

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A new method for the preparation of the tRNA splicing substrate has been developed in which T7 RNA polymerase is used to transcribe a synthetic tRNA\textsuperscript{Phe} gene yielding intact, functional precursor. This method allows the rapid preparation of substrate and the characterization of precursor variants. By this procedure, we hope to understand the basis by which the precursor is recognized and correctly cleaved.

mRNA splicing proceeds by a completely different mechanism and the machinery is much more complicated. Here the process takes place on a large particle termed the spliceosome. Our present efforts are largely devoted to working out a method for the purification of this particle allowing the determination of its composition. The spliceosome probably has a molecular weight of \( \sim 2.5 \times 10^6 \) daltons. It is composed of RNA and proteins. Preliminary studies in collaboration with Christine Guthrie at UCSF have identified three small nuclear RNAs as constituents of the spliceosome. These are called SnR7, SnR14 and SnR20. The latter is \( \sim 1100 \) nucleotides in length. Manual Ares at Yale has discovered that it contains sequences homologous with mammalian U2, U4, U5 and U6 snRNAs. Work in the laboratories of Steitz, Sharp, Maniatis and Keller has established that these RNAs are all involved in mRNA splicing. Thus, yeast has fused these RNAs into a single molecule. It will be very interesting to learn which proteins associate with this RNA and whether they form a correspondingly larger SnRNP.

Characterization of the proteins involved in mRNA splicing has been greatly facilitated by the existence of a set of genes termed RNA2-II. During the past year, we have shown that the protein products of at least the RNA2, 3, 4, 5, 7, 8 and 11 genes are directly involved in mRNA splicing. Studies on the formation of the spliceosome in heat-inactivated extracts of mutant \( \text{rn}a2 \) strains have revealed that only the \( \text{rn}a2 \) heat-inactivated extracts can form the spliceosome. Thus, we can conclude that either the RNA4, 5, 7 and 11 gene products are part of the spliceosome or are required for its formation. Clearly, the genetic potential of yeast is once more being realized in the study of mRNA splicing.

We have continued with our construction of synthetic \( E. \text{coli} \) tRNA genes. This work, in collaboration with Jeffrey Miller of UCLA, has as its
goal the synthesis of active amber suppressors for each of the 20 amino acids. We are nearing that goal. At present, strong suppressors are available for Tyr, Ser, Leu, Gln, Gly, Phe, Cys, Arg, His, Ileu, Ala, Glu, Gly and Val. The Pro, Thr and Asp suppressors function but are weak. The Asn suppressor has not yet worked and the Met and Trp suppressors insert Gln. At present then, 17 amino acids can be inserted at the site of an amber mutation. We believe that these suppressors will prove a facile tool in protein engineering. Synthetic tRNA genes are also being used to study the rules for recognition of a tRNA molecule by its cognate amino acyl tRNA synthetase.

1. Saccharomyces cerevisiae tRNA Ligase Gene

Shawn Westaway

tRNA ligase is one of the proteins in Saccharomyces cerevisiae responsible for splicing tRNA precursors containing introns. As previously reported, tRNA ligase has been purified and the gene has been cloned (Phizicky et al., 1986). During the course of sequencing the clone, three open reading frames (ORFs) were discovered in the region of tRNA ligase. One corresponds to tRNA ligase, is 827 amino acids, and codes for a polypeptide of 95 kd. The second ORF occurs on the same strand as ligase but is out-of-frame and overlaps the beginning of the ligase gene by 8 nucleotides. We are presently determining if this ORF codes for a protein in vivo. The third ORF is on the opposite strand as ligase and begins just upstream of the ligase gene. A 2.1 kb poly(A)+ RNA containing this ORF is produced in vivo (E. Phizicky and J. Abelson, unpublished results). Its transcription start is 100-200 base pairs upstream from the tRNA ligase transcription start, indicating that the two genes may share a common promoter. Based on unusual complementation properties of a disruption in tRNA ligase, it seems likely that a second gene in the vicinity of ligase is required for viability. We have shown that a small disruption of this third ORF is lethal to the cell. Therefore, there is a second essential gene in the region of tRNA ligase.

Does the expression of this gene require sequences in tRNA ligase as would be predicted from the complementation properties? To answer this question, we are defining the sequences necessary to complement the disruption in the third ORF. Is this protein co-regulated with ligase or does it participate in some way in tRNA or other RNA processing events? By producing a conditional mutant for this ORF (under the control of the GAL1-GAL10 upstream activation sequence from the galactose operon in yeast), we will be able to control the amount of this gene's product and analyze what effects the absence of this transcript has on the cell and if those effects are related to RNA processing. We are also making (g)alactosidase fusion proteins to this upstream gene in order to study its cellular location and to purify any protein produced by this gene.

Reference:

2. The in vivo Role of tRNA Ligase Protein in Saccharomyces cerevisiae

Eric M. Phizicky

As mentioned above, tRNA ligase is the protein in yeast that catalyzes the ligation step of tRNA splicing in yeast after a membrane-bound endonuclease has removed the intron. Evidence from studies in Xenopus laevis suggests that splicing is the last nuclear step in tRNA splicing (Melton et al., 1980) and evidence from this lab suggests that tRNA ligase is located near the inner nuclear membrane (see Abstract No. 3). It is therefore attractive to assume that ligase resides near the inner nuclear membrane at a site adjacent to the endonuclease and there is evidence supporting this claim: endonuclease and ligase form a kinetically-defined complex in vitro in the presence of precursor tRNAs (Greer, 1986).

We are interested in defining the role of tRNA ligase protein in the cell. In a cell that conditionally loses ligase protein, what tRNA species accumulate and how are they modified? Does a cell that is conditional for ligase perturb the processing of other tRNA molecules that do not require splicing? Does a lack of ligase either directly or indirectly affect the processing of mRNA or rRNA? With what proteins does tRNA ligase interact in the cell that causes its localization?

In order to answer these questions, we have constructed a ligase-conditional strain. This strain carries a deletion of the chromosomal ligase gene and
a plasmid carrying ligase under control of the GAL10 promoter. In the presence of galactose, which is inducing for galactose-controlled genes, the cell is healthy, but in the presence of glucose, which is repressing for galactose-controlled genes, the cell eventually dies. Thus, ligase is an essential gene in yeast, and we can study its in vivo role by growing the cells in galactose, switching the cells to glucose and monitoring the effects.

We have so far discovered two effects of cells lacking ligase. By two-dimensional gel analysis of RNAs labeled during conditions of ligase deprivation, we have obtained evidence that both precursor tRNAs and tRNA half-molecules accumulate in the absence of ligase without gross effects on the synthesis of 5S, 5.8S, or other tRNA molecules. We are in the process of proving these assertions by an analysis of the eluted tRNAs and a determination of their modifications. We will also look for indirect effects of ligase-less cells on mRNA metabolism.

In addition, we are in the process of looking for temperature-sensitive or cold-sensitive tRNA ligase mutants by transforming mutagenized plasmid into the above mentioned strain, removing the resident plasmid with a drug and screening the survivors for conditional growth. These conditional mutants will be used to look for suppressors of the ligase gene in order to identify interacting gene products.

References:

3. The Cellular Location of tRNA Ligase in Saccharomyces cerevisiae

Michael W. Clark

Some of the tRNA genes in the yeast Saccharomyces cerevisiae contain introns. The mechanism that removes the introns from the yeast precursor tRNAs, referred to as the tRNA splicing process, has at least two distinct activities: a membrane-bound endonuclease that removes the intron, and a soluble ligase that joins the two resulting tRNA halves. By immune cyto logical techniques, we have determined the exact cellular location of the tRNA ligase.

Indirect immune fluorescence on wild-type yeast cells has localized the tRNA ligase in a sector of the cell nucleus. Similar studies with a strain of yeast that contains a Cen IV plasmid containing the tRNA ligase gene under galactose-inducible GAL10 promoter control (Phizicky et al., 1986) show that after galactose induction, the tRNA ligase accumulates in a sector of the nucleus. At a higher resolution, indirect immune electron microscope studies on wild-type yeast show this nuclear location to be two sites: one on the nuclear envelope, most likely the nuclear pore, and a second site in the nucleoplasm, about 100-200 nanometers from the nuclear envelope. Immune electron microscope studies on the galactose-inducible tRNA ligase strain of yeast show accumulations of tRNA ligase at the nuclear pore, while the increase of ligase in the nucleoplasm causes a distinct pattern of tRNA ligase staining. This ligase pattern follows the contour of the nuclear envelope at the region of 100-200 nanometers into the nucleoplasm.

From the tRNA ligase locations, we can make the following conclusions. At least, at some point in tRNA splicing, the ligase is associated with the nuclear pore. Considering that the endonuclease is a membrane protein and biochemical evidence of a ligase-endonuclease complex (Greer, 1986), this nuclear pore-associated tRNA ligase location is most likely the site of tRNA splicing. Also, considering that tRNA splicing is one of the last events to occur to the tRNA before it is transported out of the nucleus, this location for tRNA splicing implicates the nuclear pore as the exit site from the nucleus for the mature tRNA. The nucleoplasmic tRNA ligase location indicates two possible events. One, a newly discovered activity for the tRNA ligase protein, that is, the binding of the precursor tRNAs (Belford and Greer, personal communication), is occurring at this site with a presumed accompaniment of the pre-tRNA to the endonuclease. Also, the distinct nucleoplasmic pattern seen in tRNA ligase overproduction indicates a nuclear substructure holding the ligase in that specific region. The mechanism of this subnuclear compartmentalization is unknown.

References:
4. Highly Purified tRNA Splicing Endonuclease from Saccharomyces cerevisiae

Phillip R. Green

The tRNA splicing endonuclease and ligase enzymes catalyze the two-step splicing reaction of yeast tRNAs that contain intervening sequences (Peebles et al., 1979). Whereas the ligase has been purified and its structural gene has been cloned, the membrane-bound endonuclease (Peebles et al., 1983) has resisted purification. To understand in detail the physiological role of the splicing reaction, the endonuclease must be purified.

Cells were disrupted using a Dyno-Mill in buffer containing 1.0 M ammonium sulfate. Crude membranes were isolated following low and high speed centrifugation. They were subjected to a series of washes in buffer containing high salt, low salt plus low detergent, and high detergent without salt. Activity was extracted from washed membranes with 0.9% Triton X-100 plus 0.1 M ammonium sulfate. The extract was chromatographed twice over heparin agarose and then over blue agarose and hydroxylapatite. The activity was then eluted from CM-Sepharose using tRNA. At this step, the activity was purified 1000 fold over Triton X-100 extract and greater than 30,000 fold over crude cell extract. Three polypeptides with apparent molecular weights of 52,500, 41,000 and 29,500 are estimated by silver staining to be present in nearly 1:1:1 ratios and co sediment with endonuclease activity in a glycerol gradient. If all three polypeptides are part of the endonuclease, they comprise approximately 10% of the protein in the tRNA elution fractions and 50% of the protein in the highest purity glycerol gradient fraction. Experiments are under way to determine which of these polypeptides comprise the endonuclease.

References:

5. tRNA Splicing in Yeast: Effects in vitro of Specified Base Substitutions in the Precursor tRNA

Visente M. Reyes, Marjorie C. Strobel-Fidler

tRNA splicing in Saccharomyces cerevisiae is mediated by an endonuclease and a ligase. However, not enough is presently known about the requirements of these splicing enzymes on the precursor tRNA. Genetic studies have shown that mutations that affect processing largely map to the conserved features of the mature domain of the pre-tRNA. These changes either affect the intragenic promoters or some structural feature critical for end maturation. Consequently, it has been difficult to obtain sufficient quantities of a precursor tRNA in vitro to study the effects of these mutations on splicing. To circumvent these problems, a yeast pre-tRNA\textsuperscript{Phe} gene under the control of a strong bacteriophage T7 promoter has been constructed from synthetic oligonucleotides. Using the cognate T7 RNA polymerase, large amounts of pre-tRNA\textsuperscript{Phe} have been made. This precursor is accurately spliced in vitro by the purified yeast enzymes, immediately suggesting that base modifications on the precursor are not necessary for the activity of these enzymes.

We have constructed two sets of coordinate base changes in pre-tRNA\textsuperscript{Phe} involving two tertiary base pairs known to occur in tRNA\textsuperscript{Phe}. The C\textsubscript{19}-C\textsubscript{56} bp forms the vertex of the L-shaped tRNA molecule and is the only tertiary interaction that is of Watson-Crick type. Changing G\textsubscript{19} to C or C\textsubscript{56} to G destroys this pairing, but it should be restored in the double mutant having both changes. Our results reveal that the G\textsubscript{19}-C mutant is cut and ligated as well as wild type. The C\textsubscript{56}-G mutant and the double mutant are cleaved at approximately one-third efficiency as wild type but are ligated at a much lower efficiency, suggesting that the ligase might be recognizing some primary sequence in the T loop. Presently, we are studying the Levitt pair mutants involving the tertiary base pair between G\textsubscript{15} and C\textsubscript{48}. The location of this tertiary base pair in the interior of the tRNA and the trans orientation of its backbones impose strict requirements for the nature of the bases at these two positions if structural integrity of the molecule is to be maintained. Our preliminary results show that the double mutant (G\textsubscript{15}+A/C\textsubscript{48}+T) is spliced as efficiently as wild type, but the two single mutants are spliced at an efficiency that is about 2.5 times lower. These results support the idea that this tertiary base pair in the mature tRNA is also present in the precursor and serve as evidence for the structural recognition by the yeast splicing enzymes.

Our next goal is to construct two more site-
specific mutants, one having a U to C change at position 8, and the second having a GC base pair insertion in the anticodon stem (between the second and third secondary base pair from the top in the anticodon stem). The residue U₈ is interesting because besides being in a tertiary base pair interaction with A₁₁₄, it is known to form a transient covalent bond with the cognate synthetase during amino acylation in vitro. The GC insertion will serve to lengthen the anticodon helix, and its splicing analysis will shed light on the previous observation in this laboratory suggesting that the accuracy of splice junction recognition by the endonuclease is influenced by the length of this helical stem.

6. tRNA Splicing in Yeast: A Kinetic Study of tRNA Precursor Cleavage by the Endonuclease

Vicente M. Reyes

tRNA splicing in yeast proceeds via two distinct and separable steps: precise excision of the IVS by the endonuclease, and joining of the two tRNA halves thus generated by the ligase. We have attempted to study the kinetics of the cleavage reaction by the endonuclease. To do this, we employ a synthetic T7 polymerase transcript of the tRNAPhe precursor of yeast as substrate and we analyze the appearance of exons and intron in the absence of ligase. This synthetic precursor starts with a 5' triphosphate and is totally devoid of base modifications, in contrast to the natural (end-processed) precursor (synthesized in vivo or in cell extracts in vitro) which begins with a 5' monophosphate and contains certain base modifications present in the mature tRNA. However, we assume that these differences have little effect on the reactivity with the endonuclease or on the cleavage kinetics. Our preliminary results reveal a Kₘ on the order of 10⁻⁸ - 10⁻⁹ M, suggesting a very tight binding of the precursor by the endonuclease. G. Knapp and coworkers at the University of Alabama doing a similar kinetic study on the same substrate but approaching from a different angle, namely, analysis of precursor utilization, obtain a Kₘ in the same order of magnitude. Our present efforts are directed at further optimizing the reaction conditions all aimed at improving the reaction profiles, reliability of the kinetic parameters calculated, and ease of the experimental protocol.

Our next goal is to do a similar kinetic study on the various site-specific mutants of pre-tRNAPhe that we already have (see previous abstract) to determine whether these specific base changes have an effect on the reaction parameters. Another key question we want to address is whether the presence of ligase has any effect on the cleavage kinetics. This question arises from the recent results of C. Greer and coworkers at the University of California, Irvine, suggesting the assembly of a stable complex during tRNA splicing. To answer this difficult question, we must look at the kinetics of precursor utilization with and without the addition of ligase.

A long-term goal is to carry out a similar kinetic study of the second step of the splicing reaction, namely, the joining of the two tRNA halves by the ligase, for which sufficient quantities of tRNA half molecules (with appropriate 5' and 3' ends) will be needed. Finally, formal biochemical proof of the tRNA splicing mechanism in yeast proposed several years ago by this laboratory (Greer et al., 1983) will have to be ultimately done by a rigorous kinetic study similar to the above.

References:

7. Function of the Intron in tRNA: Ser

Calvin K. Ho

We are interested in the role of introns in tRNA gene expression and tRNA function. Approximately one-fifth of yeast tRNA genes contain introns. Their presence bears no obvious correlation with various aspects of their associated tRNAs, such as gene copy number and codon usage. For two suppressor tRNAs, SUP6 (Tyr + ochre) and SUP53 (Leu3 + amber), precise deletion of the intron results in tRNAs deficient in base modifications in the anticodon. This demonstrates that, in these two cases, the intron is required for anticodon base modifications. However, not all tRNAs associated with introns contain anticodon base modifications, so this function may not be a general one.

We are testing the effects of deleting the intron from tRNA:Ser, which lacks base modifications in the
anticodon. In contrast to SUP6 and SUP53, both of which are multicopy, the gene coding for tRNA$^{\text{Ser}}_{\text{UCG}}$ is unique and essential; suppressors of this tRNA (SUP61) are recessive lethals. The intron will be deleted from the cloned genes for tRNA$^{\text{Ser}}_{\text{UCG}}$ and its ochre suppressor, SUP61, using synthetic oligonucleotides. The mutant genes will be integrated into a yeast diploid genome. Sporulation to yield haploids that have inherited the mutant genes will establish whether the intron-less gene has any phenotypic effects. This system thus provides a test for the role of the intron on the natural function of a tRNA as well as on its function as a suppressor.

In addition to tRNA$^{\text{Ser}}_{\text{UCG}}$, yeast contains a highly homologous (96%) related tRNA, tRNA$^{\text{Ser}}_{\text{UCA}}$, differing only in the wobble base, which changes its codon specificity, and a base pair in the anticodon stem. The two mature tRNAs are produced in vivo in amounts precisely proportional to their respective gene dosages and possess identical base modifications. It is therefore intriguing that tRNA$^{\text{Ser}}_{\text{UCG}}$ contains an intron whereas tRNA$^{\text{Ser}}_{\text{UCA}}$ does not. A demonstrated effect of the intron deletion on tRNA$^{\text{Ser}}_{\text{UCG}}$ function thus would implicate a novel role for this intron.

8. The Role of the RNA Genes in Yeast mRNA Splicing

Arthur J. Lustig, Ren-Jang Lin

The yeast RNA genes have been suggested to be involved in the process of mRNA splicing. Temperature-sensitive mutations in these genes (ma2-ma10/11) result in the accumulation of intron-containing precursors at the restrictive temperature. To evaluate the nature of the splicing defects in these mutants, we have developed an in vitro assay for temperature sensitivity. Extracts isolated from the ma mutants, grown at the permissive temperature, were subjected to heat inactivation at 32°C prior to initiation of the in vitro splicing reaction. Extracts isolated from seven mutants (ma2, 3, 4, 5, 7, 8, and 10/11) were found to be temperature sensitive and appeared to be blocked at an early step in splicing. In contrast, most wild-type extracts were stable under these conditions. Pairwise combinations of extracts isolated from different mutants resulted in heat stability in most cases. This in vitro complementation demonstrates that specific exchangeable splicing components are being inactivated under these conditions. In vitro complementation, tetrad analysis and reversion analysis have indicated that these in vitro defects are indeed the consequence of the ma mutations. We also have performed in vitro complementation of the heat-inactivated mutant extracts with wild-type extract fractions and have begun to localize the RNA gene products in specific fractions of the splicing system.

Our data demonstrate that the ma mutants cause specific intrinsic defects in the yeast mRNA splicing system. We are currently engaged in isolating the RNA gene products by in vitro complementation and are investigating interactions among these components by both biochemical and genetic techniques.

9. Spliceosome Formation of ma Mutant Extracts in vitro

Ren-Jang Lin, Arthur J. Lustig

We have shown, in the preceding abstract, that extracts prepared from most temperature-sensitive ma mutants are heat sensitive for mRNA splicing in vitro. In addition, this lab has previously suggested that mRNA splicing takes place on a 40S particle (the spliceosome). Therefore, the spliceosome formation activity of the ma mutant extracts was studied by glycerol gradient sedimentation.

Extracts prepared from ma4, 5, 7, and 11 mutants are heat sensitive both for catalytic and spliceosome formation activities, while that of ma2 is only heat sensitive for catalytic but not spliceosome formation activity. The spliceosome formed by the heat-inactivated ma2 extract is indistinguishable from that of wild-type extract as indicated by: 1) sedimentation coefficient in the gradient, 2) ATP dependency, and 3) requirements of pre-mRNA containing spliceable intervening sequences.

The spliceosome-associated pre-mRNA could be chased into splicing products by addition of complementary heat-inactivated ma mutant extracts. The chasing reaction requires the RNA2 gene product and ATP, but it does not utilize newly added pre-mRNA. Two additional factors were identified, which are required for splicing, when diluted extract from an RNA2 overproducing strain was used in the chasing reaction. The two factors, called c$^n$ and b$^n$, became limiting before the RNA2 gene product did, as
determined by the titration experiment. These two factors, and probably the RNA2 gene product, are extrinsic to spliceosome. An intrinsic factor, called Cm, was identified when the biochemically fractionated extracts were used in the chase reaction. A model for the mechanism of mRNA splicing in yeast, based on these studies, is presented in Figure 1.

A working model for the mechanism of mRNA splicing

Figure 1. A proposed three-step mechanism of mRNA splicing in yeast. Lower case letters (a, b, and c) indicate factors required for corresponding step. Numbers in subscript indicate as follows: a) Arabic for the product of corresponding RNA gene; b) Roman for the factor present in corresponding fractionated extract. For example, b2 is the product of the RNA2 gene, whose activity is required for step B.

10. Cloning of the RNA5 Gene of\nSaccharomyces cerevisiae\n
Gloria Dalbadie-McFarland\n
The RNA5 gene of S. cerevisiae has been implicated in splicing the transcripts of nuclear protein coding genes. 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 (see Abstract Nos. 8 and 9). Cloning the RNA5 gene will greatly facilitate study of its gene product and the role it plays in mRNA splicing.

I have isolated a DNA fragment from a yeast genomic library by genetic complementation of a temperature-sensitive growth defect of an ma5 mutant strain. The cloned fragment complements the ma5 temperature-sensitive defect in single copy. It complements only ma5 and does not complement temperature-sensitive defects in other RNA genes, suggesting it is indeed the RNA5 gene rather than a nonspecific suppressor. Characterization of the cloned fragment as well as the encoded gene product(s) is in progress.

11. Characterization of the Yeast Spliceosome\n
Susan E. Goetz, Michael W. Clark\n
The molecules that facilitate the removal of introns from eukaryotic mRNA are, as yet, unknown. We are using yeast (Saccharomyces cerevisiae) as a system in which to study mRNA splicing in general and these molecules in particular. Previously, it was shown that a macromolecular complex, called the spliceosome, sedimented at approximately 40S in glycerol gradients. It contained the well characterized splicing intermediates, intron-exon 2 in a lariat configuration and exon 1, as well as precursor mRNA, intron, and mature mRNA. We have extended these studies to show that there are at least three distinct particles with which 32P-labeled mRNA transcript is associated. The fastest sedimenting is the spliceosome. The slowest particle contains primarily the mature message (product) and free intron, although transcript unassociated with protein runs slower still. The intron-containing mRNA precursor sediments between the spliceosome and the product complex. The S value for all three particles is salt sensitive: in low salt (25 mM KCl), they sediment much faster than in high salt (800 mM KCl). In addition, the spliceosome appears to be as stable in 800 mM KCl as in 25 mM KCl. That the salt shift is due to a conformational change in these particles is indicated by experiments where particles purified from gradients containing 400 mM KCl sediment faster (i.e., as expected) in gradients containing 25 mM KCl. The shift seen for the splicing particles is much more dramatic than that for 40S ribosomal subunits. Thus, we are using sequential glycerol gradients to get relatively pure spliceosomes and are studying their morphology by electron microscopy. The micrographs show that the spliceosome does not have a particularly distinctive shape, but is almost
spherical with dimensions of 160 Å x 180 Å at 25 mM KCl. The electron micrographs corroborate the sedimentation data in that at low salt one sees a small, compact particle and at high salt one sees a rather more extended particle.

In collaboration with Nora Riedel in Christine Guthrie’s laboratory at UCSF, we are also using this salt shift to ascertain which snRNAs are associated with the splicing particles. Preliminary studies indicate that snRNAs 20, 14 and 7 are associated with the spliceosome.

12. Studies on the Assembly of Splicing Complexes
Soo-Chen Cheng

The assembly of splicing complexes was examined by sedimentation analysis on the glycerol gradient. Kinetic studies of complex formation using an actin precursor RNA revealed rapid formation of a 16S complex upon addition of the splicing extract in the presence or absence of ATP. In the presence of ATP, a 32S complex, which contains precursor RNA at early time course and precursor, splicing intermediates and lariat IVS at later times (between 5 and 10 minutes), appears with the disappearance of the 16S complex. Accompanying the 32S complex is a 10S complex later in the time course, which contains predominantly spliced message. In the absence of ATP, a 20S complex appears as the 16S complex disappears. No splicing reaction takes place under such conditions. When a transcript of the same length containing no splicing signals was used, only a 16S complex was seen. This indicates that formation of the 20S complex, although ATP independent, is dependent on splicing signals and may represent an intermediate in the pathway of spliceosome assembly.

Formation of splicing complexes was further analyzed by polyacrylamide gel electrophoresis. The 32S complex can be resolved into two complexes, A and B, upon electrophoresis. Complex B shows up at early times of the splicing reaction. Complex A, which migrates slower than B, appears only later with the disappearance of B. Complex A contains precursor RNA, spliced intermediates and lariat IVS, whereas complex B contains only precursor RNA. Precursor RNA with a point mutation at the 5' splice site or TACTAAC sequence did not form either complex, indicating both the 5' splice site and TACTAAC sequence are involved in formation of at least complex B. Examination of the involvement of small nuclear RNAs in these complexes by Northern hybridization is underway.

13. mRNA Splicing Reactions
Stephanie W. Ruby

We are studying the mechanism of mRNA splicing using the yeast in vitro splicing system. Work in this laboratory has previously shown that splicing occurs in two stages (Lin et al., 1985). First, the 5' splice junction is cleaved and the 5' nucleotide of the intron is covalently joined to the last A (the "branch point") in the conserved TACTAAC sequence near the 3' end of the intron. The bond formed is a novel 2'-5' linkage and creates a "lariat" intermediate. The other intermediate formed is free exon 1. Second, the 3' splice junction is cleaved, the two exons are joined, and the lariat intron is released. These stages are identical to those in mammalian systems.

It has previously been proposed that the two stages of mRNA splicing occur via two transesterification reactions (Padgett et al., 1984). To test this hypothesis, we have designed and used a substrate to assay formation of the branch in the first stage of splicing. The substrate is a linear transcript consisting of only the intron and exon 2 of the actin gene. It is made in vitro as a run-off transcript from a plasmid with a T7 phage polymerase promoter. This promoter directs transcription to begin with the first nucleotide of the intron. We considered that if branch formation is separate from 5' splice site cleavage, then the linear substrate should be converted to the lariat intermediate in the in vitro splicing system. We found, however, that although the linear substrate forms a spliceosome, it does not form a lariat. Our results are consistent with the hypothesis that 5' splice site cleavage and branch formation are concerted, a property characteristic of a transesterification reaction.

We are also using the linear substrate to test whether the branch in the intermediate is necessary for the second stage of splicing to occur. We are assaying for the ligation of exon 1 to exon 2 when free exon 1 and the linear substrate are present together in in vitro splicing reactions.
14. Splicing of Mat 

Usha Vijayraghavan, Ren-Jang Lin

Mating type in yeast is determined by alleles of the mating type locus MATα or MATa, believed to code for regulatory proteins that control unlinked genes. The MATα locus produces two transcripts, only one of which, Matα, corresponds to a known Mata function. The Matα primary transcript contains two introns, the only nuclear-encoded RNA in yeast known so far to do so. Although these introns are very small (54 and 52 bases), they contain the conserved splicing signals found in the introns of other nuclear structural genes. This is therefore a useful system to study the splicing mechanism in a naturally occurring, two-intron-containing pre-mRNA.

Splicing of the Matα pre-mRNA synthesized using an SP6 promoter was tested in the yeast in vitro system. Preliminary results indicate that the removal of only the first intron is observed from an RNA molecule containing both introns. The levels of the spliced product were low, indicating that the efficiency of the reaction was poor. The reason behind the inability to detect splicing of the second intron is unknown.

Transcripts containing only the first intron or only the second intron were made and assayed to test the splicing of each of the introns independent of each other. The data so far indicate that the splicing of the first intron is inefficient, while the removal of the second intron takes place efficiently. Experiments to test the formation of the "splicing complex" with these transcripts and with pre-mRNA containing both introns are under way. It would be interesting to see if two complexes are assembled in transcripts containing two introns. The splicing patterns of the three different types of transcripts mentioned above have been tested in extracts from three different cell types—a, α, and α/α diploids. Efficiencies of intron removal remain unaltered in these extracts, arguing against a cell type-specific effect on splicing.

15. In Search of the Yeast hnRNP Protein

Michael E. Cusick

Precursor mRNAs in the cell nucleus assemble with a simple set of proteins to form the complex known as heterogeneous nuclear ribonucleoprotein (hnRNP). All subsequent steps in mRNA metabolism, including splicing, occur on hnRNP. hnRNP protein is presumably a major component of the spliceosome.

We have purified to homogeneity a yeast 30,000 dalton protein (p30) identified last year as a candidate hnRNP protein. The steps used were flow through double-stranded DNA cellulose, then chromatography on single-stranded DNA cellulose, heptyl agarose, and poly(U) agarose. Purified p30 is a single-stranded, RNA-dependent ATPase. It hydrolyzes ATP to ADP and P_i only in the presence of a single-stranded RNA. DNA of any form does not activate p30. The enzyme also hydrolyzes dATP but no other nucleotide, and it prefers poly(U) RNA two to threefold over any other RNA.

Although the heptyl agarose fraction containing p30 forms hnRNP-like complexes with added RNA, pure p30 does not. Nor does pure p30 have an amino acid composition resembling that of known hnRNPs. Possibly we have purified a protein close in molecular weight to the true yeast hnRNP. When RNP complexes formed in splicing extracts on biotinylated RNA are purified by chromatography on avidin agarose, a major protein of about 30,000 daltons is seen, but it migrates slightly slower than the p30 ATPase on SDS-PAGE.

Recently we have developed a gel electrophoretic technique that assays the in vitro formation of hnRNP complexes. We will use this method in another attempt to purify the true yeast hnRNP protein, employing the purification scheme already devised for p30 ATPase. In addition, the gene for the p30 ATPase will be isolated, mutagenized, and inserted back into yeast so that its function in RNA metabolism can be determined.

16. Changing the Identity of a tRNA

Jennifer Normanly, Richard C. Ogden

Previously, we reported that we had converted an E. coli leucine-specific tRNA into a serine-specific tRNA by making 12 base changes in a synthetic leucine amber suppressor tRNA gene (Biology 1985, Abstract No. 14). The mutant suppressor (tRNA<sub>Leu-Ser</sub>)
specifically inserts serine in response to amber nonsense codons by two criteria: 1) tRNA^{Leu-Ser} suppresses a serine-specific amber mutation in the active site of the *E. coli* beta-lactamase gene, *amp*, allowing growth of the host in the presence of the antibiotic ampicillin, and 2) tRNA^{Leu-Ser} inserts serine into an amber mutation positioned at the tenth residue of *E. coli* dihydrofolate reductase, verified by protein sequencing.

While tRNA^{Leu-Ser} is clearly a functional serine suppressor, it is very weak, with a suppression efficiency of 0.5-1%. (tRNA^{Phe} is 60+% efficient.) Presently, we are attempting to improve the suppression efficiency of tRNA^{Leu-Ser} while retaining its serine identity. To accomplish this, we are utilizing an *E. coli* host with three amber mutations that will enable the isolation of more efficient serine suppressors after site-specific or random chemical mutagenesis to the suppressor gene. This strain carries an amber mutation in the *argE* gene, which requires a suppression efficiency of at least 3% in order to allow growth in the absence of arginine. Secondly, there is a *lacZ* amber, which when suppressed in the presence of an indicator dye yields blue colonies, the shade of which vary depending upon the level of suppression. Furthermore, the *lacZ* gene product, a-galactosidase, can be assayed spectrophotometrically, enabling quantitation of the level of suppression. Lastly, the strain carries the serine-specific beta-lactamase amber mutation.

By site-directed mutagenesis we have reverted two of the original 12 base changes resulting in a serine-specific suppressor with a twofold increase in efficiency, according to assay of a-galactosidase activity. Nitrosoguanidine mutagenesis to the amber strain carrying tRNA^{Leu-Ser} has yielded a point mutant of the suppressor that is tenfold more efficient, while retaining serine specificity. Interestingly, the alteration occurs at a position in the tRNA that is not highly conserved among *E. coli* serine tRNAs and is a change that we would not have predicted. Perhaps this mutation compensates for the effects of the 12 changes that we made to the leucine tRNA. By combining site-directed mutagenesis based upon our predictions about the importance of certain residues in defining tRNA identity and random chemical mutagenesis, we can systematically determine the minimal number of alterations required to effect the transition from leucine to serine specificity.

17. Construction of Amber Suppressor tRNA Genes in *E. coli*

Jennifer Normanly, Jean-Michel Masson¹, Lynn G. Kleina¹, Jeffrey Miller¹

In order to facilitate protein structure-function studies, we have set out to expand the available collection of amber suppressor tRNAs in *E. coli*. Five amber suppressor alleles (corresponding to Leu, Ser, Tyr, Gln, and Gly tRNAs) have been isolated genetically by others, and we previously reported the construction of amber suppressor alleles corresponding to Phe, Cys, Pro, His, Lys, Arg and Thr tRNAs (Biology 1985, Abstract No. 15). These tRNA genes, constructed from synthetic oligonucleotides, are expressed constitutively from a strong promoter on multicopy plasmids. Recently, we have added to this collection the amber suppressor alleles of tRNAs corresponding to Glu, Ala, Val, Asn, Met, and Ile. We have found that the suppressors vary in efficiency: 50-100% (Phe, Cys, Arg, Ala, Val, Ile), 20-49% (His, Glu, Lys, Met) and <1% (Pro, Asn, Asp, Thr).

We have employed site-directed mutagenesis to "boost" the efficiency of some of these suppressors. For example, by altering the second nucleotide 3' to the anticodon of the His suppressor tRNA gene to an A, the efficiency range of tRNA^{His}CUA was increased from 0.8-35% to 15-100%. In another case, the anticodon stem and loop sequence of tRNA^{Phe}CUA (50-100% efficient) was superimposed upon the same region of tRNA^{Pro}CUA resulting in the transformation of an essentially nonfunctional suppressor into a highly efficient one.

One important consideration in the construction of these suppressor genes is that altering the anticodon of a tRNA, as we have done, may affect the specificity of that tRNA. For example, by employing a glutamine-specific amber in the *lacZ* gene, we have determined that tRNA^{Met}CUA is mischarged by the glutamine synthetase. Therefore, it is necessary to verify that these suppressors insert the predicted amino acid. Previously, we described an assay whereby these tRNAs are utilized to suppress an amber mutation in a protein-coding sequence (Biology 1985, Abstract No.
15). Isolation and sequencing of the resultant protein reveal which amino acid the suppressor inserts in response to the amber codon. tRNA\textsubscript{Phe} and tRNA\textsubscript{Cys} have been characterized in this manner and presently we are assaying the remainder of the synthetic suppressor genes to determine their specificity.

18. Applications of Designed Amber Mutations

Roger E. Koepp, Jennifer Norman

Aspartic acid #27 is an essential amino acid at the active site of dihydrofolate reductase (DHFR). We have used oligonucleotide-directed mutagenesis to put an amber codon (TAG) at position 27 in the cloned gene of E. coli DHFR in order to carry out two types of experiments: 1) to test the activities of a number of mutant DHFR molecules having different amino acids at position 27, and 2) to select for a high efficiency aspartic acid suppressor tRNA that is derived from a synthetic tRNA gene.

A plasmid carrying the amber-27 DHFR gene was used to transform four suppressor strains of E. coli that insert glutamine, tyrosine, serine or leucine, respectively, in response to an amber codon. The transformed strains were then tested for their ability to grow in the presence of trimethoprim; ability to grow would indicate the (over)production of a functional DHFR molecule. Of the four amino acids tested, only serine at position 27 of DHFR gave rise to any resistance to trimethoprim, and then only when the level of the drug was reduced by a factor of five from the level at which cells carrying the same plasmid with a wild-type DHFR gene were resistant. Thus, we have an in vivo method of screening a number of amino acid replacements at a single position in the sequence of an enzyme.

A synthetic gene corresponding to the gene for E. coli tRNA\textsuperscript{Asp} but having an amber anticodon (CTA) was constructed using six synthetic oligonucleotides. The gene was cloned together with a lac promoter and rrnC terminator into the plasmid that carried the amber-27 DHFR gene. The designed suppressor tRNA was inefficient at suppressing amber mutations, and E. coli that harbored the constructed plasmid were nevertheless unable to grow in the presence of trimethoprim. The suppression efficiency was in fact so low that the resistance to trimethoprim was much inferior to that of the serine suppressor strain (see above). In an attempt to improve the suppression efficiency, in vivo mutagenesis was performed using methyl-nitro-nitrosoguanidine. Survivors were subjected to a triple selection in which amber mutations in the argE, lacZ and DHFR genes had to be simultaneously suppressed in order to produce viable blue colonies. Six of the survivors have been chosen for further study. All of these are plasmid-encoded mutants. We are testing which if any of these owe their viability to one or more mutations within the tRNA\textsubscript{Asp} (amber) gene.

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Summary: In the past year, the elucidation of the genetic content of the human mitochondrial genome, which was started in our laboratory about 18 years ago (Biology 1968, Abstract No. 99), has been brought to completion with the functional assignment of the last remaining "unidentified reading frame" (URF), URF6. In particular, by the use of antibodies directed against synthetic peptides predicted from the DNA sequence, the mitochondrial product corresponding to URF6 has been identified. Furthermore, by the use of antibodies directed against the respiratory chain NADH dehydrogenase from bovine heart or against individual, well characterized subunits of this enzyme complex, it has been shown that the product of URF6, like those of six other URFs previously identified (Biology 1985, Abstract No. 24), is a subunit of the enzyme. Thus, almost 60% of the protein coding capacity of the mammalian mitochondrial genome is utilized for the assembly of the first enzyme complex of the respiratory chain. These findings, besides their obvious physiological significance and their implications with regard to the genetic basis of mitochondrial diseases, are interesting from the evolutionary point of view. In fact, no genes homologous to the mammalian mitochondrial DNA URFs that encode subunits of the NADH dehydrogenase occur in the mitochondrial genome of yeast, although several of them occur in mitochondrial DNA of other lower eukaryotic cells.

The mitochondrial DNA-encoded subunits of the respiratory chain NADH dehydrogenase, which are all hydrophobic proteins, are most probably components of the hydrophobic shell that surrounds the catalytic subunits of the enzyme. Such an assignment has already been made for three of the URF products by using antibodies specific for human URFs in immunoblot assays on the beef heart enzyme. In further investigations on the biogenesis of the NADH dehydrogenase, it has been shown by the use of subunit-specific antibodies that the two iron-sulfur proteins of the flavoprotein fragment and four iron-sulfur proteins of the iron-protein fragment are synthesized in the cytoplasm.

Another area of mitochondrial research that has been actively pursued in our laboratory in the past year concerns the mechanisms of mitochondrial DNA transcription and RNA processing and the energetic control of mitochondrial gene expression in HeLa cells. In vitro systems involving isolated mitochondria or soluble submitochondrial fractions have been utilized in these studies. Thus, a new RNA species derived from the leader of the mRNA for cytochrome c oxidase subunit I and containing the origin for light strand DNA synthesis has first been identified in the RNA synthesized in isolated organelles and subsequently shown to occur also in vivo. Besides the intriguing questions raised by the mapping position of this RNA species in connection with its possible function in light strand synthesis, this RNA has revealed an unusual electrophoretic behavior in denaturing polyacrylamide gels, which is similar to that described for branched or lariat RNA structures. An in-depth analysis of the structure of this RNA has shown that this behavior is due to its highly structured configuration.

The recent development of a highly efficient and faithful transcription system utilizing isolated mitochondria from HeLa cells (Biology 1984, Abstract No. 8) has offered the opportunity of dissecting out the energetic requirements of mtDNA transcription and RNA processing by changing the environment of the organelles in a controlled way. Thus, a strikingly different dependence of mRNA synthesis versus rRNA synthesis and L-strand transcription upon the concentration of ATP, either provided exogenously or generated by oxidative phosphorylation, has been observed.

In other studies, an efficient and very specific
soluble in vitro transcription system from HeLa cell mitochondria is being used to analyze the mechanisms that control initiation of transcription at the heavy and light strand promoters, as well as termination at the 3' end of the rDNA region. In still another line of investigations, the mechanism of action and the structure of the mitochondrial RNase P (Biology 1984, Abstract No. 10), and in particular the nature of its RNA component, are being actively investigated.

In another area of interest in our laboratory, the unusual chromosomal structures that are characteristic of mammalian cells with amplified dihydrofolate reductase genes have been further investigated in methotrexate-resistant human cell lines isolated in this laboratory. In particular, by reverse-field electrophoresis, it has been possible to isolate for the first time the intact DNA of very small double minute chromosomes from one such line. This achievement opens the way for the characterization of the structure, gene organization and expression of these minute chromosomes.

19. The Excised Leader of Human COI mRNA, which Contains the Origin of mtDNA Light Strand Synthesis, Accumulates in Mitochondria and is Polyadenylated

George L. Gaines, Cesare Rossi

We have identified an RNA species that is transcribed from the region of the heavy strand of human mitochondrial DNA containing the template sequence for initiation of light strand DNA synthesis. This RNA very probably derives from the 5'-end proximal stretch of ~320 nucleotides of RNA 6, which is excised leaving RNA 9 as the functional mRNA for cytochrome c oxidase subunit I. Accordingly, it has been designated as RNA 9L (9 leader). Although RNA 9L contains the sequence complementary to four tRNAs encoded in the light strand, its most stable potential configuration, as determined by computer analysis, exhibits a long, central base-paired segment with a terminal and some interstitial loops, as well as several other stem and loop structures, but no cloverleaf structure. The high degree of secondary structure is probably responsible for the anomalous behavior that RNA 9L exhibits when electrophoresed through denaturing polyacrylamide gels. The mapping position of RNA 9L, together with the observation that this RNA accumulates in mitochondria in vivo and is in part polyadenylated, suggests a possible physiological role in connection with the initiation of light strand synthesis.

20. Markedly Different ATP Requirements for rRNA Synthesis and mtDNA L-Strand Transcription versus mRNA Synthesis in Isolated Human Mitochondria

George L. Gaines, Cesare Rossi

In isolated mitochondria from HeLa cells, the ATP requirements for mitochondrial DNA (mtDNA) transcription and RNA processing can be satisfied by either endogenous synthesis, mainly through oxidative-phosphorylation, or by exogenous supply. The pattern of RNA synthesis changes dramatically depending upon the level of ATP available. At the low intramitochondrial ATP levels produced from endogenous ADP in the presence of an oxidizable substrate and phosphate, the mRNA species are labeled to a substantial extent, whereas there is only a marginal labeling of the rRNA species and L-strand transcripts. By contrast, high ATP levels, either provided exogenously or produced endogenously in the presence of an oxidizable substrate, phosphate and exogenous ADP, strongly stimulate rRNA synthesis (about 10 fold) and L-strand transcription (>10 fold), with only a slight increase in mRNA synthesis.

21. In vitro Transcription of HeLa Cell Mitochondrial DNA

Nalini Narasimhan

We are interested in studying the mechanisms involved in the control of initiation of transcription on the heavy strand and light strand of the mitochondrial genome and the potential termination of rDNA transcription at the 3' end of the 16S rRNA gene on the heavy strand.

For this purpose, a soluble in vitro transcription system has been developed in this laboratory (Shuey and Attardi, 1985). This in vitro transcription system is a soluble fraction released from HeLa cell mitochondria upon detergent/salt treatment. The mitochondrial lysate possesses an RNA polymerase activity capable of accurately initiating transcription from the heavy strand rRNA promoter and the light strand promoter, using an exogenous mitochondrial DNA template containing the heavy and light strand initiation sites.
At present, we are investigating in detail the effects of salts, Mg++, pH, nucleotide, template and lysate concentration, as well as of the topological structure of the template on the rate of initiation of transcription on either strand. We are also interested in testing various conditions for their capacity to activate the downstream H-strand promoter, which has been detected in vivo, but not as yet in vitro.

Clones containing the 3' end of the 16S rRNA gene fused to either heavy strand or light strand promoter have been constructed in this laboratory. These clones are being utilized to study the potential termination of heavy strand transcription at the 3' end of the 16S rRNA gene in vitro.

Reference:

22. Characterization of RNase P from HeLa Cell Mitochondria

Ram Sharma 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 coding 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 the mitochondrial processing enzyme(s) 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.

A ribonuclease P-like activity has been partially purified from HeLa cell mitochondria (Doersen et al., 1985). This enzyme has been shown to process a precursor to E. coli suppressor tRNA Tyr at the same site as the E. coli RNase P, producing the mature 5' end of tRNA Tyr. This enzyme has RNA and protein components as judged by its sensitivity to micrococcal nuclease and pronase treatment, respectively.

Work on improving the purification procedure to achieve a homogeneous preparation and on the identification of the RNA and protein components is in progress.

Reference:

23. RNA Processing in Mammalian Mitochondria

Cesare Rossi

One of the central features of the mammalian mitochondrial genome organization is that the coding sequences for the rRNAs and the mRNAs are close to one another and separated in nearly all cases by one or more tRNA genes with no or few intervening nucleotides. This information, coupled with the polycistronic nature of the primary transcripts, implies that the 5' and 3' ends of tRNA sequences must be recognized and precisely cut in order to yield the mature RNAs. The mechanism of this processing is currently being investigated.

Previous work from this laboratory has shown that HeLa cell mitochondria possess an RNase P activity capable of correctly cutting a tRNA precursor from E. coli at the 5' end of the mature tRNA. To further characterize this activity and to study the 3' end processing, we have constructed plasmids containing suitable regions of the mitochondrial genome under the control of the SP6 and T7 polymerase promoters. The transcripts from these plasmids can be used as substrates either with the partially purified RNase P or with crude lysates. We can also test the hypothesis that transcription and processing are temporally and spatially coupled by using plasmids containing the mitochondrial RNA polymerase promoter instead of the bacterial promoter. (The activity of the mitochondrial RNA polymerase in crude lysates has been demonstrated in this and other laboratories.)

24. Functional Identification of the Last Unassigned Reading Frame of Human Mitochondrial DNA

Anne Chomyn

The polypeptide encoded in URF6, the last unassigned reading frame of human mitochondrial DNA, has been identified by the use of antibodies
directed against peptides predicted from the DNA sequence. The migration of this translation product as an identifiable band on SDS-urea polyacrylamide gels was sensitive to the source of SDS (sodium dodecyl sulfate) and to heat treatment of the mitochondrial fraction. This made the identification of the URF6 product particularly difficult.

Antibodies raised against highly purified bovine respiratory chain NADH dehydrogenase, or against individual, well-characterized subunits of this complex, precipitate from a Triton X-100 lysate of mitochondria the URF6 product, as well as six other URF products previously identified as subunits of NADH dehydrogenase (Biology 1984, Abstract No. 24). Thus, more than half of the protein coding capacity of human mitochondrial DNA is utilized for the assembly of the first enzyme complex of the respiratory chain.

25. Determination of the Site of Synthesis of Individual Subunits of the Respiratory Chain NADH Dehydrogenase from HeLa Cells

Anne Chomyn

The respiratory chain NADH dehydrogenase, or complex I, consists of about 25 polypeptide subunits, according to C. Ian Ragan and other workers. Most of these are encoded in nuclear DNA, synthesized in the cytosol and imported into mitochondria, where they assemble with the seven mitochondrial DNA-encoded subunits. The enzyme can be resolved into three fractions by NaClO4 treatment: the insoluble hydrophobic shell; the hydrophilic flavoprotein fragment, consisting of three polypeptide subunits; and the iron-sulfur protein fragment consisting of at least six polypeptide subunits. Ragan has prepared antisera specific for individual subunits of the flavoprotein fragment and of the iron-sulfur protein fragment consisting of at least six polypeptide subunits. We are using mitochondria from HeLa cells labeled in vitro. Permeabilized HeLa cells are labeled with 35S-methionine in the presence of cycloheximide (an inhibitor of cytoplasmic protein synthesis). The mitochondria containing 35S-methionine-labeled polypeptides are lysed and the lysate is incubated with anti-peptide antibodies directed against an amino-terminal peptide of ATPase 8 or antibodies against a carboxy-terminal peptide of ATPase 6. These antibodies are known to be able to precipitate ATPase 8 and ATPase 6 (Chomyn et al., 1983; Mariottini et al., 1983), respectively, from a mitochondrial lysate of in vivo-labeled cells, and they should both precipitate the polyprotein, should it exist.

References:

1 Undergraduate, California Institute of Technology.

26. Mode of Synthesis of the ATPase 6 and ATPase 8 Subunits in Human Mitochondria

Ivan Tarle 1, Anne Chomyn

Two cases of two reading frames overlapping out of frame occur in human mitochondrial DNA. In both cases, a single messenger RNA corresponds to the two overlapping reading frames.

We are investigating the possibility that the primary translation product from a pair of overlapping reading frames is a polyprotein precursor destined to be processed to yield one or two mature proteins. We are investigating in particular the ATPase 8/ATPase 6 genes, which overlap by 45 nucleotides out of frame. Thus, the synthesis of a polyprotein precursor from these genes would require a frameshifting on the ribosomes near the 3' end of the ATPase 8 reading frame, to put the ribosomes into the ATPase 6 reading frame. This phenomenon is not without precedent (see Jacks and Varmus, 1983).

We are using mitochondria from HeLa cells labeled in vitro. Permeabilized HeLa cells are labeled with 35S-methionine in the presence of cycloheximide (an inhibitor of cytoplasmic protein synthesis). The mitochondria containing 35S-methionine-labeled polypeptides are lysed and the lysate is incubated with anti-peptide antibodies directed against an amino-terminal peptide of ATPase 8 or antibodies against a carboxy-terminal peptide of ATPase 6. These antibodies are known to be able to precipitate ATPase 8 and ATPase 6 (Chomyn et al., 1983; Mariottini et al., 1983), respectively, from a mitochondrial lysate of in vivo-labeled cells, and they should both precipitate the polyprotein, should it exist.

References:

1 Undergraduate, California Institute of Technology.

27. DNA-mediated Transformation of Mitochondria

Michael P. King

In an effort to understand better the molecular genetics of various human mitochondrial diseases, we
are developing a procedure to introduce stably foreign DNA molecules into mitochondria. Initial experiments have focused on the transfer of mitochondria from one cell line to another. CAP23 is a VA2B cell line variant that is resistant to high levels of chloramphenicol. We have determined that this resistance results from a single T to C transition near the 3' end of the mitochondrial large ribosomal DNA. We are microinjecting mitochondria isolated from CAP23 cells into various chloramphenicol-sensitive cell lines. Following micro-injection, transformed cells are selected for in the presence of chloramphenicol. Transformation can then be confirmed by various restriction fragment length polymorphisms which exist between the CAP23 mtDNA and mtDNA from the recipient cell lines. At present, we have developed several lines that are resistant to high levels of chloramphenicol and are now analyzing their mtDNA to confirm their transformation.

28. Cloning of the Human Nuclear Gene(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 a number of mitochondrial proteins, the carrier protein is encoded by a nuclear gene(s). This project is involved with identifying the respective gene in human cells.

A human liver cDNA library in the plasmid vector pEX1 is being screened by colony hybridization for recombinants containing inserts homologous to a mixture of 15-mer oligonucleotides coding for the sequence ArgArgMetMetMet. This sequence is totally conserved in the beef heart enzyme and in the translated sequences of the homologous Neurospora crassa, Zea mays L. and Saccharomyces cerevisiae ADP-ATP carrier genes. RNA transfer hybridizations of human poly(A)+ RNA with the oligonucleotide probes have revealed the presence of hybridizing mRNA species. Secondary colony hybridization screening will be performed using cDNA probes of the homologous gene product in Neurospora crassa and Zea mays L. It was previously proposed to screen the library by hybridization selection of the cDNA to mRNA that directs the in vitro translation of the ADP-ATP carrier. However, it has been found that antisera directed against the homologous protein isolated from bovine heart or Zea mays L. do not immunoprecipitate a specific protein(s) of HeLa cell mitochondria under varying lysis conditions.

29. Isolation of Extrachromosomally Located Dihydrofolate Reductase Genes from Methotrexate-resistant Human Cell Lines Using Reverse-field Gel Electrophoresis

Barry J. Maurer, Eric H. C. Lai

Dihydrofolate reductase (DHFR) is the target enzyme of the folate antagonist methotrexate (MTX). Stepwise selection of human VA2-B and HeLa S3 cells in the presence of MTX has resulted in the amplification of the DHFR gene by formation of double minute chromosomes and homogeneously staining regions. One line, HeLa 10B3, contains very small double minute chromosomes. Reverse-field gel electrophoresis has been used to isolate 10B3 double minute chromosomes. The DNA of these chromosomes (~700 kb) is being investigated for complexity and structure. Polysomal RNA from HeLa 10B3 and other MTX-resistant cell lines is being examined to determine if other genes are co-amplified and expressed in concert with the DHFR gene or the double minute chromosomes.

PUBLICATIONS


Associate Professor of Chemistry & Biology: Judith L. Campbell
Visiting Associate: L. Elizabeth Bertani
Research Fellows: Martin Budd, Colin Gordon, Ambrose Y.-S. Jong, Friedrich Srienc
Graduate Students: Doreen Ma*, Kevin Sweder1, Stephen Mayo1
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Summary: In our laboratory we take a combined genetic and biochemical approach to the study of DNA replication. We work primarily with yeast because it is a good model for higher eukaryotic cells and because of the versatility of the experimental system. Our overall goal is to understand how initiation of DNA synthesis is controlled in the eukaryotic S phase and what proteins are involved in DNA replication. In order to understand these processes, it is essential to know what gene products are involved and how they are regulated.

In the past, we have used in vitro replication systems to identify the proteins and DNA sequences involved in replication (Celniker and Campbell, 1982). Recently, we have purified several proteins by bio-chemical 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 for DNA polymerase I. Gene disruption experiments indicate it is essential for replication. This is the first eukaryotic polymerase ever cloned.

DNA sequencing should prove invaluable for structure/function studies. The second gene isolated is for a single-stranded DNA binding protein that may interact with polymerase during replication. Mutants in this gene have not yet been constructed. This reverse-genetics approach will be extended in the near future to the remaining primase, RNase H and helicase activities important for replication.

As to the initiation problem, we have further refined our functional map of sequences likely to be chromosomal origins of replication, namely, autonomously replicating sequences (ARS). Furthermore, we have identified a protein that binds to the 11 base pair consensus sequence within all yeast ARS. Application of reverse genetics to this protein should tell us if these sequences participate in replication or transcription, another role sometimes ascribed to them. A by-product of these studies was the development of a flow cytometric assay for yeast cells, that is, the ability to measure yeast cells in the cell sorter. This has been used to assess plasmid stability and cell cycle regulation of expression of the single-stranded binding proteins.

Another project has focused on the prokaryotic model of replication. The dnaA protein of E. coli is
analogous to the yeast ARS-specific protein 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. Amplification within the 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:**


30. Are Autonomously Replicating Sequences Origins of DNA Replication?

**Kevin Sweder, Judith L. Campbell**

While it is generally believed that yeast DNA replication occurs bidirectionally from multiple, specific origins of replication, no direct evidence of a specific DNA sequence requirement exists. However, several lines of circumstantial evidence tend to support the idea of specific origins of replication. Sequences that enable high frequency transformation and extrachromosomal replication of any colinear DNA in yeast are called autonomously replicating sequences (ARS) and are good candidates for chromosomal replication origins (Stinchcomb *et al.*, 1979; Struhl *et al.*, 1979). In addition to the above properties, microscopy and fiber autoradiography indicate origin-to-origin distances of 10 µm to 60 µm, corresponding to approximately 400 origins per haploid genome, which is in good agreement with the estimate of the number determined by cloning of chromosomal sequences that mediate high frequency transformation.

The functional domains of ARS1 have been dissected using Bal31 and SI mapping (Celniker *et al.*, 1984; Kearsley, 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 present in ARS activity on centromeric plasmids (Srienc *et al.*, 1985). Domain B immediately flanks domain A and is between 46-109 bp. 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. 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 may contain the 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.

Our laboratory is currently involved in isolating proteins that interact directly with the ARS region. Since the function of such proteins is not known *a priori*, we have chosen to isolate them by their ability to bind the ARS and protect it from DNase I digestion (Galas and Schmitz, 1978). By using DNase I footprinting, we avoid assays that require either a highly purified protein or large amounts of the protein. Having completed purifying the protein that binds to the consensus sequence, we can now biochemically characterize it. We have produced antibodies to the protein and are isolating the gene from a yeast genomic DNA library in λgt11. Isolation of the gene will enable us to mutagenize the gene and by gene replacement to determine the role of the protein in the yeast cell cycle.

**References:**


31. Does dnaA Protein Participate in the DNA Replication of ColEI-type Plasmids?

**Doreen Ma**

The dnaA protein was demonstrated to be an essential component in the initiation of replication from oriC (origin of *E. coli* chromosome) (Wechsler,
This protein has been purified and used in an in vitro replication system to provide specific initiation at oriC (Fuller and Kornberg, 1983). Fuller et al. (1984) have proposed a consensus dnaA protein sequence, 5'-TTATC(A)CAC(A)A-3', which is repeated four times within the minimal oriC sequence. The consensus sequence is also present in a few other promoters and origin sites of different plasmids. A DNase I footprinting study by Fuller et al. (1984) has definitively reported dnaA protein binding to the oriC region of pBR322. This result, together with the mutational and biochemical studies, strongly suggests that the dnaA protein participates in ColEI replication (Abe, 1980; Polaczek and Ciesla, 1984).

Initiation of replication of ColEI-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 become 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 ColEI plasmid replication (Conrad and Campbell, 1979). Preliminary results indicated that dnaA protein inhibits ColEI replication in vitro. We have also developed an in vitro transcription system that faithfully mimics the in vivo process. This system synthesizes both the 108 nucleotide RNA I and the preprimer RNA II. Addition of RNase H cleaves the preprimer to a 555 nucleotide RNA that appears as a new band on high resolution polyacrylamide sequencing gel. At present, we are using the in vitro transcription system to help unravel the mechanisms involved. We have also acquired polyclonal antibodies against the dnaA protein which serve as an invaluable tool in our in vitro systems.

References:

32. Temperature-sensitive DNA Polymerase I Mutants of Yeast

Martin Budd, Judith L. Campbell

The gene encoding DNA polymerase I was isolated in the lab by screening a Ygt11 yeast library (Johnson et al., 1983). A gene disruption experiment using an internal fragment of the cloned gene has shown that POLI is a single copy essential gene. To further understand the role of DNA polymerase I in replication, repair, and recombination, temperature-sensitive mutants were isolated by site-directed mutagenesis. The strain used in the isolation, MB3-8C-ura3-52, trpl, leu2, has the polymerase gene disrupted with a fragment containing the LEU2 gene. The polymerase gene disruption is complemented by the plasmid 2cB255 which contains 2 μ, URA3, and POLI DNA. The plasmid pPOLI-8 has POLI, TRP1, trpl, and CEN4 DNA and is the target for DNA polymerase mutations. pPOLI-8 was treated with hydroxylamine and then used to transform MB3-8C. Transformants were replica plated onto media containing 5-fluoro-orotic acid and screened for growth at 23°C and cell death at 36°C. Growth on 5-fluoro-orotic acid eliminates the plasmid 2cB55 leaving the plasmid pPOLI-8 as the only source for a functional polymerase. Twelve temperature-sensitive mutants of DNA polymerase I were isolated on plates containing 5-fluoro-orotic acid. DNA coding for the mutant polymerase was cloned into Ylp5, integrated into the strain SS111, and subsequently removed, resulting in six temperature-sensitive DNA polymerase I strains isogenic to SS111 and a seventh that exhibited defective growth at 36°C. The subsequent strains with integratants were complemented for growth at 36°C by plasmids containing the polymerase gene.

When poll strains are incubated at 36°C for four hours, they form a uniform population of dumbbell-shaped cells similar to that seen after germination of spores with a disrupted polymerase. DAPI staining shows that the nucleus has migrated to but has not entered the isthmus between mother and bud. This
terminal phenotype is similar to that of cdc2 cells but different from cdc8 cells. Genetic mapping using the temperature-sensitive mutants shows that the DNA polymerase I gene is located on chromosome 14 near met4 and top2 in agreement with results from OFAGE gels. The DNA fragments coding for the mutant DNA polymerase I proteins have been subcloned into high copy number vectors and are being used to study the proteins. Overproduction of mutant polymerase I proteins in poll strains does not result in viable cells at 36°C. The poll-5 protein is defective at both 23°C and 36°C. In vivo DNA synthesis is defective in all the poll mutants at 36°C.

The poll mutants will be used to study the role of DNA polymerase I in repair. Studying revertants of poll strains will be an attempt to find possible genes whose products interact with polymerase.

Reference:

33. Yeast Single-stranded Nucleic Acid Binding Proteins (SSBs)

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 kilodaltons (kd); SSB2 = 50 kd; SSBm = a mitochondrial species of 20 kd. Also abundant are a 31 kd species shown to be homologous to higher cell hnRNPs (M. Cusick and J. Abelson, unpublished results) and a 23 kd species that has not been further characterized. SSB1 stimulates DNA 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 kd 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. First, in the internal portion of the gene, there is a 6- to 7-fold 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 aspartate (total acidic amino acids >55%). Such an acidic structure may be characteristic of all nucleic acid binding proteins.

References:

34. Biochemical Approach to Yeast cdc7 Mutant

Ambrose Jong

A yeast cell division cycle mutant, cdc7, arrests in the late G1/S phase at the restrictive temperature. Earlier genetic work established that it is required for the initiation of mitotic DNA synthesis. The mutant cells fail to form a synaptonemal complex, to show commitment to genetic recombination, to form ascospores, and to show almost no mutagenic repair. Recent attention has focused on the fact that the sequence of CDC7 protein shares homology with a number of vertebrate protein kinases, including those of the src oncogene family. Presumably, it plays important roles in the control of cell proliferation. We are interested in the identification and study of the protein product of CDC7. To do this, we isolated the CDC7 gene by screening with a recombinant plasmid pool (YCpl50 library) to complement the mutants at the restrictive temperature. Of 2 x 105 colonies screened, two positive colonies were obtained. The gene is located on a 2.1 kb MvuI-Sphl DNA fragment. We also cloned the gene into a vector of T7 RNA polymerase/
promoter expression system. Presumably, *E. coli* will express the cloned gene exclusively in this system under induction conditions and in the presence of rifampicin. I have used this system to express a yeast SSBl protein successfully, although the preliminary experiments indicated that the *CDC7* protein could not be detected using this system. By DNA sequence analysis, the *CDC7* gene does not contain an efficient *E. coli* 16S ribosomal binding site, resulting in absence of initiation of protein synthesis. In order to overcome this problem, a new plasmid is used which contains the T7 phage Gene 10. This DNA fragment includes a strong 16S ribosomal binding site in front of the poly linker. It is expected that a large quantity of *CDC7* protein will be produced for further characterization.

35. Affinity Purification of *S. cerevisiae* Proteins Required for DNA Replication

Colin Gordon

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 (Alberts et al., 1983). One promising approach that has been used to isolate proteins required for DNA replication in the phage T4 is affinity chromatography (Formosa et al., 1983). Affinity chromatography is able to detect even relatively weak protein-protein interactions when the concentration of protein immobilized on a column matrix 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 gene was subcloned into the galactose-inducible expression vector pBM150 (Johnston and Davies, 1984).

References:

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

Stephen 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-l cytochrome c (Montgomery et al., 1973; Smith et al., 1979) for studying intramolecular electron transfer mechanisms via the ruthenium modification method (Margalit et al., 1983). 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 Evens and Sutherland PS300 (an interactive computer graphics system) using the protein engineering simulation capabilities of program BIOGRAF/1 (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-l cytochrome c. The first two mutations, Cys102/Ser and Ser102/Thr, were designed to circumvent complications in the physical characterizations caused by the cysteine sulphydryl. 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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37. E. coli DNA Replication Promoted by Integrated pBR322

L. Elizabeth Bertani, Giuseppe Bertani1, Judith L. Campbell

The replication requirements of the small ColEl-type plasmid, pBR322, are quite distinct from those of its host, E. coli: pBR322 replication is unidirectional and requires the product of host gene polA (DNA polymerase I) (Kingsbury and Helsinki, 1970) but not of dnaA. DNA replication in E. coli, on the other hand, is bidirectional (Bird et al., 1972) and requires the dnaA gene product for initiation (Hirota et al., 1968) but is independent of DNA polymerase I.

Plasmid pBR322 exists extrachromosomally in high copy number but has no mechanism of its own for integration into the host chromosome. In fact, there has been some discussion about the consequences if such an integration should occur in the absence of extrachromosomal copies of the plasmid. One view is that such an event would be lethal for the host because the integrated pBR322 replicon would attempt to maintain a high copy number of initiating multiple rounds of replication, thereby disrupting normal bacterial replication.

Some support for this view came from Yamaguchi and Tomizawa (1980) who obtained integration of pBR322 by first cloning a piece of chromosomal DNA into the plasmid and then selecting for recombination between the cloned DNA and its homologous sequence on the chromosome. Integration was detected by the "suppression" of a temperature-sensitive dnaA- mutation: i.e., bacteria carrying an integrated pBR322 replicon could replicate at 40°C. It was found, however, that the presence of extrachromosomal copies of the plasmid were necessary for viability, and it was concluded that plasmid integration would otherwise be lethal.

We have prepared a derivative of pBR322 carrying the site-specific integration system of the temperate phage P2 (Ljungquist and Bertani, 1983). Using a temperature-sensitive polA- host, we have obtained strains carrying a single copy of integrated plasmid with no detectable extrachromosomal DNA present. Such strains are quite stable at all temperatures, in contradiction to Yamaguchi and Tomizawa's original prediction, although in this case, the plasmid is not required to replicate the host chromosome.

To test its ability to mediate chromosomal replication, the plasmid was introduced into a temperature-sensitive dnaA- host and selection made for temperature independence. One of the few strains obtained was then cured of extrachromosomal plasmid by treatment with novobiocin (Wolfson et al., 1982) at 30°C. According to genetic tests and Southern blot analysis, the strain carried a single integrated copy of the plasmid and it could be shown that its ability to grow at 40°C was due to the presence of the pBR322 replicon. The replication of pBR322 can be repressed by a plasmid-encoded species of RNA, RNA I, that interferes with the processing of pBR322 primer RNA. When a plasmid carrying several copies of the gene for RNA I was introduced into the strain, its ability to grow at 40°C was lost, although the integrated plasmid was retained.

References:

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III


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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 model systems in much of our work. A number of sea urchin genes active at particular times and in particular cell lineages have been cloned and analyzed. 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.

Methods for gene transfer in the sea urchin are being exploited in a number of studies of gene regulation during embryogenesis. In particular, we are beginning to analyze the regulatory regions of various sea urchin actin genes, which are expressed in a spatially-defined manner in young embryos. The gene transfer system is expected to be useful as well in elucidating recently cloned genes that are active in embryogenesis. Other studies concern sea urchin genes active in adult tissues. In all, our aim is to better understand the mechanism of gene control in development and the functional relationships among DNA sequences in higher animals.

38. The Sea Urchin Bindin Gene

**Boning Gao**

Bindin, a major protein of the sea urchin sperm acrosome granule, mediates the species-specific adhesion and binding of the sperm to the egg. In earlier work, a bindin cDNA clone containing a 1873 base pair (bp) insert was isolated and sequenced. It was found to code for a bindin precursor (prebindin) which is twice as large as the mature bindin protein. Recent studies have concerned the expression of the bindin gene and its organization in the genome. The bindin cDNA clone...
was used to study tissue specificity of expression. The results showed that bindin is a sperm-specific protein—its messenger RNA was not detected in the sea urchin eggs, ovaries, early embryos, coelomocytes, tube feet, lantern tissue or intestine that were tested.

Sperm DNA isolated from several individuals was probed with the bindin cDNA clone and revealed that there is one bindin gene per haploid genome. Haploid female coelomocyte DNA also possesses one bindin gene.

Bindin cDNA was used to screen cloned genomic libraries of sea urchin DNA. A clone containing a 14 kilobase (kb) insert hybridized to, and was found to have overlapping restriction enzyme recognition sites with, the 3′ end of the bindin cDNA. There is an intron in the genomic clone upstream of the overlapping region, which did not hybridize. A 1.3 kb genomic DNA clone that also hybridized with bindin cDNA has been characterized and partially sequenced. It contains a 219 bp exon in which the 5′ end lies 2 bp upstream of the AUG translation initiation codon. This exon is flanked by introns on either side. Thus, there are at least three introns in the bindin gene.

39. Bindin Gene Evolution

Joseph Minor

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 Strongylocentrotus 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. 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 isolated testis poly(A)+ RNA from Lytechinus 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.

40. Characterization of a Major Matrix Protein of the Sea Urchin Spicule

Henry M. Sucov, Steve Benson1, Fred Wilt1

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 embryos, 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 at the time of their formation are endowed with the determinants necessary and unique for their ontogeny.

We have isolated and characterized a single-copy gene active specifically in the micromere lineage that encodes one of the four predominant matrix proteins of the spicule. Typically, matrix proteins serve as a lattice-like microenvironment into which ions are introduced and caused to crystallize. Sequence analysis of this gene, termed SP50, revealed that 4596 of the encoded protein consists of a tandemly repeated 13-amino-acid motif which by virtue of its composition, repeated nature, and the function of matrix proteins in general, may interact directly with the mineral phase of the spicule.

Our major interest in this gene lies in its tissue-specific expression. Northern gel analysis reveals that the SP50 message prevalence increases over 100 fold with only a doubling of cell number; therefore, the message is regulated almost certainly at the transcriptional level. We are in the process of subcloning the SP50 promoter into CAT-containing vectors for introduction into embryos as a means of determining
the control regions of this gene. The SP50 promoter does not contain the CAAT or TATA boxes found in most eukaryotic genes, possibly indicative of a utilization of a particular set of tissue-specific transcription factors. It is conceivable that one or several such factors that interact with the SP50 gene are localized in the egg at the vegetal pole and have a determinative influence on the ontogeny of the micromere cells that inherit them.

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41. Sea Urchin Immunology

L. Courtney Smith, Michael F. Graham

Immune systems function in vertebrates to allow differentiation between "self" and "non-self" molecules. Sea urchins, in addition to all other invertebrates, also display this basic immune characteristic. However, no molecular and almost no cellular mechanisms of immune function in echinoderms (or any other invertebrate) are understood. Therefore, we have undertaken some initial studies that will lead to a characterization of the immune responses in urchins.

Sea urchin coelomic fluid contains a mixture of cells with an overall concentration of about ten million cells per milliliter. The urchin "immunocytes," which are responsible for immune recognition and effector functions, have been assumed to be found within these cell populations. Preliminary studies characterizing the coelomocytes have shown that challenge by xenogeneic contact (injection of coelomocytes from a closely related species of urchin) does not result in any gross changes in coelomocyte population sizes. In addition, when the coelomic fluid is flushed out and replaced with "urchin medium," the cells repopulate to equal or higher numbers in one to seven days. Whether the cells are recruited from other spaces, originate from coelomic stem cells, or both, is unknown.

In Northern blot experiments, poly(A)⁺ RNA isolated from sea urchin coelomocytes and 24-hour embryos were probed with eight immunologically relevant mammalian genes. A faint banding pattern was seen in both coelomocyte and embryo lanes when the beta and gamma chains of the T-cell receptor and a major histocompatibility complex (MHC) class I probes were used. Distinct bands were noted only in the coelomocyte lanes when the filters were probed with Thy 1 cDNA. Structural similarities of the single Thy 1 domain to both the constant and variable domains of immunoglobulin (lg) has led to speculations that Thy 1 might be descended from the "ancestral" lg-family gene. Its identification in the sea urchin in addition to the urchin MHC-like gene(s) may offer insights into the evolution of immunity and the origins of the Ig gene superfamily.

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42. The Cloning and Expression of the Sea Urchin Myosin Heavy Chain Gene

Samuel J. Rose III

A study of genes coding for proteins normally expressed in sea urchin muscle tissue continues. As reported last year, a portion of the gene encoding the sea urchin myosin heavy chain (MHC) has been cloned and sequenced. The goal of these studies is to compare the coordinate expression of muscle-specific genes during early development in the sea urchin embryo.

An 800 base pair (bp) genomic fragment containing approximately 130 bp of coding sequence for sea urchin MHC has been subcloned into an Sp6 transcription vector. Labeled antisense transcripts from this vector have been hybridized in solution to total RNA from sea urchin embryos of varying stages of development. The quantitative titration of MHC transcripts has demonstrated that prior to 38 hours postfertilization, no MHC transcripts can be detected. Within (at most) six hours of this time, the muscle-specific "M" act in gene of the sea urchin also begins transcription. Thus, coordinate induction of these two muscle-specific genes appears tightly regulated. However, quantitative levels of steady state transcripts between the two genes quickly diverge soon after the onset of transcription.

A cDNA library derived from sea urchin muscle has been synthesized and cloned into λgt10. A clone from this library hybridizing to sea urchin MHC probes has been mapped and is being sequenced by Sanger dideoxy-M13 methods. It is 3.7 kb in length and thus it is likely that a little more than half of the sea urchin MHC transcript (6.5 kb long) has been cloned.
43. Activation of Sea Urchin Actin Genes during Embryogenesis

James J. Lee, Frank J. Calzone

Previous studies have provided a wealth of information about the actin gene family of the sea urchin *Strongylocentrotus purpuratus*. The haploid genome of this species contains eight different actin genes, of which six are known to be functional. These genes display distinct patterns of expression in adult sea urchin tissues, and while they are all transcribed to some extent during embryogenesis, they function in different cell lineages or sets of cell lineages at diverse stages of development. Moreover, molecular titration assays of embryonic total RNA have established the absolute steady-state prevalence of transcripts from each of five different actin genes. In recent work, we carried out quantitative measurements of the rate of nuclear synthesis and decay of these actin genes during embryogenesis.

Actin gene-specific DNA clones were hybridized with metabolically-labeled nuclear RNA preparations and the rates of nuclear synthesis and of decay for each transcript species were determined at a number of stages of development. The embryos were labeled continuously for 2 hours with \(^3\)H-guanosine, beginning at 21, 36, and 65 hours postfertilization. The amount of nuclear \(^3\)H-poly(A) RNA that hybridized to each cloned sequence was determined and the specific activity of the \(^3\)H-GTP pool was measured in the same embryos. Rate constants for the nuclear synthesis of each transcript species and for its decay were extracted from these data. The actin transcripts identified by the cloned probes displayed a range of synthesis rates; yet, for all but one gene of this family, the kinetic parameters of synthesis and decay can account for the accumulation of actin transcripts during early development. Thus, the measurements show that the accumulation of actin gene transcripts is a direct consequence of gene activation and continued transcription during embryogenesis.

44. Sea Urchin Actin Gene Linkage

Joseph Minor, James J. Lee, Patrick S. Leahy

Earlier work done in this lab has shown that the *Strongylocentrotus purpuratus* genome contains six transcriptionally active genes and two pseudogenes. Overlapping clones place the six actin genes in three linkage groups. In order to further understand the evolutionary relationship and developmentally-controlled transcription of these genes, we determined the relative positioning of the three linkage groups in the genome. The approach taken was to determine the genetic linkage between the genes by studying the segregation of restriction fragment length polymorphisms (RFLPs).

Several male sea urchins were screened for heterozygous RFLPs by isolating their genomic DNA from sperm, digesting it with a restriction endonuclease, and probing the resulting fragments on Southern blots with gene-specific probes. Several female urchins were then screened for heterozygous RFLPs by probing genomic DNA isolated from pools of larvae resulting from the cross of a female to a male whose RFLPs had been determined from sperm DNA. The female's RFLPs were then inferred by subtracting the male's contribution to the larval DNA pool. From this information, particular males and females were selected such that crosses could be scored for the segregation of RFLPs. These crosses were made and the resulting offspring (F_1) were cultured through metamorphosis until the juvenile urchins were approximately 5 mm in diameter. Genomic DNA was then isolated from individual juveniles and scored for the segregation of RFLPs.

Specific RFLPs of actin genes Cyl and CyIIa always cosegregated, demonstrating the genetic linkage of these two genes. Genetic linkage was also shown for actin genes CyIIla and CyIlb. These results confirm the linkage groups Cyl-CyIIa-CyIlb and CyIIla-CyIlb previously determined by overlapping clones. In contrast, RFLPs of Cyl segregated at random with respect to the RFLPs of CyIIla, demonstrating that these genes are genetically unlinked. The sum of all the segregation data shows that the actin gene groups Cyl-CyIIa-CyIlb, CyIIla-CyIlb, and M are unlinked in the genome. This result precludes regulation of all the actin genes as part of one "chromosomal domain."

45. Control of Expression of the CyIIla Actin Gene in Sea Urchin Embryos

Constantin N. Flytzanis, Roberta R. Franks, Lisa S. Gobar

Sea urchin actin genes have been studied in this laboratory for several years. One of our cloned actin
constructs (Cyllla-CAT) consists of about 10.5 kb of 5' sequence from a cytoplasmic actin gene (Cyllla) attached to the bacterial chloramphenicol acetyltransferase (CAT) "marker" gene. Cyllla cytoplasmic actin is expressed in embryos and larvae but not in adult tissues. When Cyllla-CAT DNA is injected into sea urchin eggs, the embryos that develop produce CAT enzyme at appropriate stages of development, i.e., when the Cyllla gene is transcribed in control embryos. This indicates that the cis-regulatory elements that control the temporal expression of the Cyllla actin gene are included in the 5' flanking sequences and/or the part of the transcribed Cyllla sequence present on Cyllla-CAT.

Continuing experiments include measurements of CAT DNA, RNA, and enzyme levels in Cyllla-CAT-transformed embryos. These results have allowed us to quantitate the expression of the exogenous gene during embryonic development and have confirmed our earlier qualitative observations. We also have extended these studies to include quantitation of the putative trans-activator(s) of the Cyllla actin gene in embryos. In these studies, regulatory molecules are titrated by transforming embryos with increasing amounts of Cyllla-CAT DNA.

Other efforts are focused on the identification of regulatory elements on the Cyllla gene. Partial deletion of 5' flanking sequences on the Cyllla-CAT construct prior to microinjection have located probable regions of control, including a possible enhancer-like sequence positioned further than 2 kb upstream of the CAP site. Regions of the Cyllla gene recognized by trans-acting regulatory factors are also being located in competition assays. In these experiments, embryos are transformed by coinjecting Cyllla-CAT and a given upstream Cyllla DNA fragment. By this method we have begun to identify DNA sites that act as competitors for the binding of Cyllla transcription factors.

46. Tissue-specific Expression of a Gene Injected Into Sea Urchin Eggs

Barbara R. Hough-Evans

Cyllla cytoplasmic actin is expressed in aboral ectoderm cells of sea urchin embryos. Embryos that develop from eggs injected with the fusion gene Cyllla-CAT synthesize CAT enzyme at detectable levels after 20 hours of development (see Abstract No. 45). We have fixed and sectioned Cyllla-CAT embryos for cytological study. Radioactively-labeled anti-CAT RNA is being used for in situ hybridization with RNA of the embryo sections. In preliminary experiments, we detect CAT mRNA in aboral ectoderm cells of pluteus stage embryos, and in no other tissue of these embryos. Not all cells of the aboral ectoderm contain CAT message, indicating that the embryos are a mosaic of CAT-containing and normal cells. The in situ hybridizations show that the Cyllla 5' sequences included in the injected DNA are sufficient to specify the tissue in which the gene will be expressed, as well as the correct timing of gene expression.

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

Pierre Thiebaud

We are interested in the possibility of introducing cloned genes into sea urchin tissues, including the germline, with increased efficiency. We will use the fusion gene construct Cyllla-CAT and P element-mediated transformation. The P elements have been used as vectors to achieve germline reintegation of cloned genes in Drosophila (Spradling and Rubin, 1982). Recently, it has been shown that P element derivatives are able to transpose somatically (Laski et al., 1986). We will use these new derivatives combined with the nuclear injection system (see Biology 1985, Abstract No. 45) to achieve specific genetically-transformed animals with the Cyllla-CAT gene.

References:

48. Transcriptional Regulation of the Sea Urchin Spec I Gene

Donna L. Livant

Two families of genes are specifically transcribed in the aboral ectoderm of sea urchin embryos and larvae: Spec genes encoding calcium-binding proteins (Houck et al., 1986) and the cytoskeletal actin genes Cyllla and Cylllb. Spec mRNAs encode small, acidic proteins related to Troponin C by sequence and can be divided into two subfamilies. 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. A second group of transcripts, the Spec 2 mRNAs, are 2.2 kb long and begin to accumulate at about 30 hours postfertilization. Spec 2 mRNAs are about tenfold less abundant than Spec 1 mRNAs. The CyIIla gene is activated at about the same time as are the Spec 1 genes and in the same cells. Because expression of the CyIIla and Spec 1 genes begins only in the presumptive aboral ectoderm of the blastula stage, and because the kinetics of the accumulation of the two types of transcripts are similar, Spec 1 and CyIIla genes are candidates for coordinately regulated genes whose functions are distinct but must be present simultaneously.

We are currently doing experiments to test the hypothesis that CyIIla and Spec 1b are regulated by the same transcriptional activators. It has been shown that CyIIla trans activators are present in limiting amounts (see Abstract No. 45). If CyIIla and Spec 1b are regulated by the same trans activators, it should be possible to use DNA fragments containing the Spec 1b regulatory elements in excess to compete for trans activators binding the regulatory elements of the CyIIla-CA T construct during embryogenesis. Similar competitive behavior, assayed as decreased CAT activity in 20-hour embryos, by the same amounts of Spec 1b and CyIIla regulatory elements would be evidence that these two genes are regulated by the same trans activators. We would, therefore, predict from this experiment that Spec 1b and CyIIla would also have the same regulatory elements in the sequences surrounding them.

Reference:

49. DNA Binding Proteins of Cell Lineage-specific Sea Urchin Genes
Frank J. Calzone, David R. Fromson, Henry M. Sucov

Methods have been developed to extract DNA binding proteins from nuclei of sea urchin embryos. Specific gene fragments have been used to assay crude protein extracts for DNA binding activities. DNase I and exonuclease III protection assays have been most extensively employed. Four, or possibly five, DNA binding protein "footprints" have been detected in the 5' flanking sequences of the ectoderm-specific actin gene CyIIla. The locations of the footprints extend as far as 600 nucleotides from the start of transcription. Evidence also has been obtained for DNA binding proteins reacting with the 5' flanking sequences of a primary mesenchyme-specific gene. Future experiments will identify any DNA binding proteins showing cell lineage-specific localization or temporal developmental regulation. Strategies for cloning developmentally interesting DNA binding proteins are being investigated.

50. Larval Gene Expression
R. Andrew Cameron

The larval stage intervenes between the embryo and the adult in sea urchin development. Mechanistically speaking, the larva functions to disperse the genotype and precociously develop structures needed to form the adult. The sea urchin larva is adapted to swim and feed in the water column while the adult lives on hard sea bottoms. Metamorphosis marks the transition from the larva to the adult and the larva may delay metamorphosis until a suitable substrate is encountered.

The adaptations for feeding required to develop further and for a planktonic existence may include unique patterns of gene expression. For example, at the onset of feeding the synthesis of ribosomal RNA in sea urchin larvae greatly increases. Furthermore, specific mRNAs are synthesized exclusively in the larval epidermis (derived from the aboral ectoderm of the embryo). These include one member of the actin gene family, termed CyIIla, and the Spec mRNAs which code for acidic, low molecular weight proteins belonging to the Troponin C superfamily (see Abstract No. 48). These genes have been proposed to function in metamorphosis by facilitating the collapse of the larval epithelium.

The expression of these genes may correlate with particular events in larval development such as growth of larval arms, formation of the adult primordia or onset of metamorphic competence. Using well defined larval culture techniques and titration techniques for actin transcripts developed in our laboratory, we will measure the changes in gene expression that occur during larval development. From these data we can ask the following. Does the regulation of the actin gene implicated in metamorphosis parallel the onset of
competence to undergo metamorphosis? And, more importantly, we will have taken the first step to define a larval system for the study of molecular developmental biology.

51. Regulation of CyI, A Sea Urchin Cytoplasmic Actin Gene
Karen S. Katula

The CyI cytoplasmic actin gene of the sea urchin Strongylocentrotus purpuratus is expressed in all adult tissues, in oocytes, and at certain stages of embryogenesis in a developmentally-regulated manner. CyI is the second most abundant actin transcript throughout most of embryogenesis. In order to identify and study the DNA sequences responsible for the control of CyI transcription in embryos, a fusion gene was constructed in which the 5' flanking region of the CyI gene was joined to coding sequences of the bacterial enzyme chloramphenicol transferase (CAT). The cloned DNA was injected into sea urchin eggs, and embryos of several stages were assayed for the presence of CAT enzyme. Temporal regulation of the exogenous CAT gene by the CyI sequences was normal, with enzyme activity detected between 10 and 14 hours, although the level of CAT production was not very high. The 5' region was sequenced by the Sanger method and sequences around the "CAAT" box showed some homology with those of CyIIIa actin, another cytoplasmic actin represented in maternal mRNA. The mRNA cap site was identified by primer extension. In further gene transfer experiments, five fusion gene constructs with CAT were used, four involving deletions to leave from 250 to 2500 bp of CyI sequence 5' from the cap site, and one in which most of the 5' "leader" intron was removed. Preliminary results show that temporal regulation was normal in all cases and presumably resides in the 250 bp segment of sequence 5' to the mRNA cap site.

52. Transcriptional Regulation of the Sea Urchin Muscle Actin Gene
Donna L. Livant

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 (Lee et al., 1986). Muscle actin transcripts are found about 60 hours postfertilization only in 20-40 cells associated with the coelomic sacs of gastrulating embryos (Cox et al., 1986). In these cells, the M actin transcripts accumulate to about 1000 molecules per cell by late pluteus (Lee et al., 1986).

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 CyIIIa and CyI, 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 are currently making an expression construct with the bacterial CAT gene in order to assay this sequence for M actin regulatory elements. We also are determining the location of the M actin transcriptional start site by S1 analysis and primer extension. By analogy with the CyIIIa and CyI actin genes, we expect it to be 1.5-2 kb upstream of the translational start site.

References:

53. Repetitive Sequence Transcripts in Maternal RNA
Frank J. Calzone, James J. Lee, Nga Le

As much as 70% of the mass of poly(A) transcripts in the cytoplasm of the unfertilized sea urchin egg are interspersed with a relatively high concentration of repetitive sequences in a pattern uncharacteristic of translatable mRNA. We have cloned the complete genomic sequences of an interspersed maternal RNA called 2109A-10. Detailed RNA mapping experiments using genomic fragments as probes indicated that the 2109A-10 gene transcribes a single 3.7 kb RNA which is not spliced in the unfertilized egg or embryo. The 2109A-10 transcript does not appear to contain an open reading frame that is likely to be translated. Repetitive sequences occupy the 5' half of the 2109A-10 transcript. The sequences of an unusual interspersed repeat, repeat C, initiate the 2109A-10 gene transcript and approximately 300 other diverse transcripts. Unlike most interspersed repeats, the
sequences of repeat C appear to be highly conserved and are asymmetrically represented in RNA. A small set of transcripts containing only repeat C are unique to the egg and early embryo. RNA titration and kinetic-labeling experiments have shown that unlike most interspersed repeats, the majority of repeat C transcripts do not turn over in the nucleus. The majority of repeat C transcripts in the cell are relatively stable transcripts which accumulate in the cytoplasm. Genomic titrations indicate that the number of repeat C genes is very close to the estimated number of diverse repeat C transcripts. Taken together, these observations strongly suggest a function for repeat C in the expression of a diverse set of gene transcripts. Repeat C appears to be associated with frequent rearrangements that have occurred during the evolution of the sea urchin genome.

54. Oral/Aboral Axis Specification in Sea Urchin Embryos

R. Andrew Cameron

Gene expression by developing sea urchins is specific in both time and tissue. The analysis of these tissue-specific patterns requires a more complete understanding of the fate of individual embryonic cells through development into the adult organism. While aspects of early embryonic cell lineage are well known (Horstadius, 1939), the complete details of all cell origins and cell fates are not.

As a first step, we have addressed the specification of the embryonic axes. The first embryonic axis, the animal-vegetal axis, is established before fertilization in sea urchin embryos. However, the second or oral-aboral axis is first revealed at the gastrula stage. Even earlier, at the hatching blastula stage, several genes are expressed specifically in the aboral ectoderm (reviewed in Angerer and Davidson, 1984). Using published information on cell fates and determinations, we have constructed a cell lineage diagram from fertilization to pluteus. The lineage diagram makes specific predictions of cell fate which we set out to test by dye injection. We randomly injected dextran linked to fluorescein into individual blastomers at the eight-cell stage and followed their fate to the early pluteus stage. These injections produced five mutually exclusive patterns of dyed pluteus cells. When compared to the sea urchin cell lineage chart, the five staining patterns can be explained if two cells are specified at the eight-cell stage to form the oral side, i.e., if the oral/aboral axis is specified at the eight-cell stage. Furthermore, these studies demonstrate a clear clonal quality to the normal development of the highly regulative sea urchin embryo.

References:

55. More on the Long, Interspersed Repeat Family cs2108

Lanny M. Lake, Deborah Klein, Roy J. Britten

The cs2108 repeat family of Strongylocentrotus purpuratus consists of many long, interspersed repeats that occur in subfamilies with 5-20 closely related members. A member of one subfamily and surrounding DNA has been cloned from S. purpuratus nuclear DNA and a 3 kb region of the repeat has been subcloned. For most of this 3 kb length, there is relatively precise homology with the genomic DNA of the distant sea urchin species Lytechinus pictus, which shares with S. purpuratus a last common ancestor that existed 100-200 million years ago. The observed 8% divergence is only 3% greater than the 5% divergence observed among family members in S. purpuratus. Thus, over this long region the sequence divergence has been less than for the histone-coding sequences of the same pair of species. (The histone amino acid sequences are typically totally conserved, but silent substitutions in the coding regions are nearly saturated at this evolutionary distance.) There is no simple explanation for the conservation of this repeated sequence and it may be the result of an interspecies transfer of a viral-like sequence element.

56. The Region Surrounding an Occurrence of the 2108 Long Repeat

Deborah Klein, Roy J. Britten

The subfamily structure of the cs2108 repeat sequences of the sea urchin genome can be readily demonstrated by hybridizing a labeled fragment of cs2108 to genomic Southern blots at very high stringency. Under these conditions, 10 to 20 bands are seen with each of several restriction enzymes, reflecting the restriction sites in the regions of the
members of the subfamily. This supplies a method for investigation of the length and location of the termini of a cloned example of the long repeat, since different probes from within the repeat should give very similar patterns. A probe that is outside the cloned repeat will give a different pattern corresponding to the nature of the adjacent region of the clone being examined. The expectation for an interspersed repeat is that the adjacent regions will be single copy, but in this case the adjacent regions are actually from another low frequency repeat that gives quite a different band pattern.

When the DNA from several individual sea urchins is blotted, most of the bands are the same size. Only a few variant sizes are seen for each individual, indicating that the regions in which the subfamily members occur have a low degree of interindividual sequence variation. For typical DNA, owing to the 4% sequence variation, the expectation is that only a quarter of such bands would have the same size in any two individuals, as has been observed with several single copy probes.

57. *"New" Interspersed Human Repeat Families*

Huey Ru Hwu, Roy J. Britten

In earlier work, a clone was selected from the Maniatis human genomic library because it contained repeats that did not cross-hybridize with the Alu family at low stringency. Three repeat regions in this 15 kb clone apparently do not hybridize with each other nor with the interspersed human repeated DNA families that have been investigated (Alu, Kpnl-L1, LTR of THE1, polyAC). One of these repeats has been shown to occur on 1.5% of the inserts in the Maniatis library. The second repeat hybridizes with 3.5% of the inserts in the Maniatis library, while a single test shows that the third hybridizes with a large fraction of the library. Thus, each of the three is probably a member of a different family of interspersed repeats that has not been previously investigated.

58. *Insertion and Deletion in Primate Genomic Evolution*

Roy J. Britten

The beta globin pseudogene sequences (Koop et al., 1986) were compared for human, chimp, gorilla, orangutan, old world monkey and new world monkey, and the number of substitutions and rearrangements were summed for all of the branches of the cladogram. There were 461 base substitutions and it could be determined that at least 37 were double hits at particular locations in the sequence. This is about as expected by chance, indicating a lack of "hot spots" for base substitution in this region.

In comparison, 189 nucleotides were affected by insertion/deletion events, of which the largest affected 24 nucleotide pairs. In contrast to the case for substitutions, there are "hot spots" for rearrangement. Of the 18 insertion/deletion events (greater than one in length), it can be shown that three have occurred more than once during the evolution of these primates, since their pattern of occurrence is directly inconsistent with known cladogram. The presence of local sequence duplication for several of the insertion/deletion events may have implications for the mechanism involved.

Reference:

59. *Average Single Copy DNA Diverges at the Rate for Silent Substitutions*

Roy J. Britten

Interspecies DNA comparisons are available for a number of gene-coding regions for pairs of species for which the total single copy DNA divergence has been measured. Examination of these measurements shows that the average extent of divergence for silent substitutions in gene coding regions equals that for total single copy DNA. For example, between mouse and rat the average degree of silent substitutions for six genes is 22%, while single copy DNA measurements show 20% divergence of total single copy DNA. Comparisons among higher primates of beta globin pseudogene sequences (Koop et al., 1986) show almost exact equality between the divergence of this 2000 nucleotide region and the total single copy DNA divergence for each of about a dozen species pairs.

The simplest explanation is that most silent substitutions in coding regions and substitutions in the total single copy DNA do not affect the phenotype with the result that the sequences may drift freely with little selective pressure. Obviously, in each case a small fraction of the substitutions will be under
selection, but their effects will be significant only at great evolutionary distance.

**References:**


60. Changes in mRNA Abundance in Early Mouse Embryos

Lajos Pikó, K. D. Taylor

Previously, we have shown that the mouse egg inherits a large supply of maternal poly(A)+ mRNA but that the bulk of this RNA is degraded at the two-cell stage; the poly(A)+ RNA content increases sharply during cleavage through the preimplantation blastocyst due to a high rate of embryonic RNA synthesis (Pikó and Clegg, 1982; Clegg and Pikó, 1983). In order to shed light on the behavior of some individual mRNAs during this period, we have prepared a cDNA-plasmid library to poly(A)+ RNA from two-cell embryos. Out of several hundred clones obtained, 76 were selected at random and screened in dot hybridization experiments with 32P-labeled cDNA from unfertilized eggs, two- and eight-cell embryos, and early blastocysts.

About one-half of the cDNA clones gave a positive reaction with the labeled cDNA probes (indicating transcript abundances of about 0.1% or higher) at least at some stages of development. Three patterns of mRNA abundance emerged. One sequence was highly abundant in the egg, decreased sharply in abundance in the two-cell embryo and continued to decline during cleavage. Nine clones varied little in abundance at the one-cell and later stages. However, the great majority of clones were of relatively low abundance, or undetectable, in the egg but became much more prevalent during cleavage beginning with the two-cell stage. These results suggest that the mRNA population derived from the embryonic genome is quantitatively and qualitatively different from the maternally-derived mRNA population stored in the egg. These findings are in agreement with previous observations in several laboratories that a major shift in the qualitative pattern of protein synthesis occurs between the two-cell and eight-cell stages of development.

**References:**


1Veterans Administration Medical Center, Sepulveda, CA.

**PUBLICATIONS**


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Staff Associate: Janet M. Roman
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Summary: We (W.D., J.R., and J.F.K.) have spent most of the past year at the Max Planck Institute for Developmental Biology in Tübingen, Germany. While there we initiated a new collaborative research project aimed at studying the proteins and genes responsible for the addressing of neural growth cones in the developing embryo. During the early 1960s, Roger Sperry's work here at Caltech stimulated our interest in his proposed chemoaffinity model for neural specificity (Sperry, 1963). Based on our knowledge about generation of specificity in the immune system and principles of protein evolution, we proposed the existence of families of highly specific cell recognition molecules (Dreyer and Bennett, 1965; Dreyer et al., 1967). It seemed reasonable that such families of evolutionarily-related molecules be involved in the development of the entire organism, not just the nervous system or the immune system, and we termed these molecules area code molecules (Hood et al., 1977).

The technologies for detection and analysis of these rare cell-surface receptors were not available in the sixties, or even in the seventies, and work in this lab focused on analyses of receptor molecules present in much greater quantities (for example, rhodopsin). Much of our energy during this time also was focused on the development of highly sensitive instruments that would be capable of analyzing very rare molecules (see, for example, Hunkapiller et al., 1984). These efforts and those of our collaborators in the Hood group led to the first of the advanced, highly sensitive instruments known collectively as the Caltech Microchemical Facility.

Now, for the first time, the sensitivity afforded by the Microchemical Facility puts us close to the range of detection we feel will be necessary to study the "Sperry molecules" or "area code molecules" and we have focused our new research on that goal. As a means of determining the feasibility of isolating and analyzing rare cell-surface molecules using the Microchemical Facility, we have carried out studies of members of the MAC-1/LFA-1 family of cell-cell recognition proteins. In this system we have been able to isolate the proteins, determine their N-terminal amino acid sequences, synthesize oligonucleotide probes and isolate the gene directly from a genomic library (Springer et al., 1985; Sastre et al., 1986; see Abstract No. 61). Based on the success of this prototype project, we initiated the new project with our collaborators at the Max Planck Institute in Tübingen.

The project aimed at detecting and analyzing area code molecules is based on the assumption that such molecules will be scarce cell-surface molecules involved in specific cell interactions. We plan to detect such molecules by means of monoclonal antibodies and have already generated monoclonal antibodies against cell-surface molecules of the chick embryo retinotectal system. The retinotectal system of chick embryos was chosen for several reasons. First, it is a system in which large quantities of material may be collected due to the ready availability of large numbers of fertile eggs. This is a critical factor when studying scarce molecules. This system is also ideal for detecting the specificity of cellular interactions, and our collaborators in Tübingen have already established several in vitro and in vivo assays for such specificity. We have limited ourselves to
analysis of cell-surface molecules by use of a biotin-avidin isolation procedure (see Abstract No. 62). These cell-surface molecules have been isolated, further purified by size separation and used as immunogens for generation of monoclonal antibodies. These antibodies are now being screened for their specificity and pattern of reactivity on tissue sections and in biological assays.

Since very little is known concerning the nature of the surface molecules of the nervous system, our project will certainly raise monoclonals to previously undescribed and uncharacterized proteins of importance. We feel that in addition there is a very good chance that some of these molecules will play a role in the addressing of cellular and neuronal assembly during embryogenesis.

References:

61. Isolation of a Partial Genomic DNA Clone for the Cellular Adhesion Molecule MAC-1

Leandro Sastre1 Janet M. Roman, David B. Teplow, William J. Dreyer, Connie E. Gea1, Richard S. Larson1, Thomas M. Roberts1, Timothy A. Springer1

Our long-term aim is to detect and analyze cell-surface molecules involved in the generation of specificity during development. As a feasibility study before attempting to detect such hypothetical cell-surface receptors on nervous tissue, we analyzed the MAC-1/LFA-1 family of proteins found on blood cells. MAC-1 and LFA-1 mediate different types of adhesion reactions on macrophages and T cells, respectively.

We have isolated a genomic clone coding for the a subunit of MAC-1 directly from a genomic library using synthetic oligonucleotide probes based on the N-terminal amino acid sequence of the protein. The success of this isolation depended upon: 1) preparation of monoclonal antibodies, 2) isolation of the scarce cell surface proteins, 3) determination of the amino-terminal sequence of minute amounts of protein, and 4) sequential use of overlapping synthetic probes at carefully selected stringencies. The identity of the clone has been established by DNA sequencing and translation in vitro of hybrid-selected mRNA. This is one of the first examples of the isolation of a gene directly from a mammalian genomic library.

The tissue-specific regulation of this family of proteins is most interesting. We have determined that MAC-1 a-subunit mRNA is present in macrophages but not T lymphoma or L cells. During y-interferon-stimulated maturation of the premyelocytic cell line M1, Mac-1 a-subunit mRNA is induced. This corresponds with the tissue distribution of the MAC-1 a subunit, showing that expression is regulated at least partially at the message level.

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

Jon Faiz Kayyem, Janet M. Roman, Enrique de la Rosa1, William J. Dreyer, Uli Schwartz1

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 selectively biotinylated cell-surface proteins on intact retinotectal tissue using an hydroxysuccinimide ester which
derivatizes proteins via amino groups on lysine residues. After cell lysis, the proteins were purified on avidin columns and eluted with free biotin. The cell-surface proteins were further purified by separation 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.

Monoclonal antibodies have been produced against proteins isolated in this manner and are currently being screened against fixed tissue sections of chick retina and tectum. Patterns of reactivity on tissue sections differ. Further screening procedures 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.

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PUBLICATIONS


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Summary: Our laboratory has a broad spectrum of interests including the molecular biology and evolution of the immune system, the biology and chemistry of hepatitis B virus, the molecular biology of myelin basic protein, structure-function analyses of hormones and DNA binding proteins, and the development of new instrumentation and chemistries for the synthesis and analysis of genes and proteins.

The immune system employs three major categories of antigen-binding receptors: antibodies, T-cell receptors, and class I and II proteins encoded by the major histocompatibility complex (MHC). Antibodies and T-cell receptors are encoded by a multiplicity of variable (V), diversity (D), and joining (J) gene segments that rearrange during the differentiation of the respective B and T lymphocytes to generate complete \( V_H \) and \( V_H \) as well as \( V_\alpha \) and \( V_\beta \) genes, respectively. We are analyzing the mechanisms for diversification, rearrangement, expression and evolution of the \( \alpha \) and \( \beta \) T-cell receptor genes (Kronenberg et al., 1986) and the diversification of immunoglobulin heavy chain (H) genes. We are also studying the organization, patterns of developmental expression and regulation of class I MHC genes. We are interested in studying the evolution of the immunoglobulin gene superfamly which currently contains eight multigene families and 12 single-copy members (Hunkapiller and Hood, 1986).

The hepatitis B virus infects perhaps 200,000,000 individuals worldwide. Dr. Kent has been interested in raising antibodies, using synthetic peptides, against this virus, which may have diagnostic and therapeutic potential. In addition, he has been interested in identifying the liver receptor for this virus.

Myelin basic protein (MBP) is a major component of the myelin in the central nervous system. We have cloned the mouse and rat MBP genes and established that each of the four MBP forms is derived from a single gene by alternative patterns of RNA splicing. We also have identified a mutant mouse, shiverer, that has deleted the 3' half of the MBP gene. We have constructed transgenic shiverer mice with a functional MBP gene and find that shiverer mice homozygous for the MBP transgene no longer have the shiverer phenotype. This is an ideal model system for studying the factors regulating the expression of a CNS-specific gene.

Dr. Kent and his coworkers have developed techniques that allow us to synthesize large peptides (>50 residues). These techniques have been employed to study the structure-function relationships of TGF\( \alpha \) (50 residues) and IL-3 (140 amino acids). In addition, we are characterizing the DNA binding portions of several interesting eukaryotic proteins.

In the Microchemical Facility, we are developing new chemistries and instrumentation for femtomole protein sequencing, for automation of the Maxam-Gilbert DNA sequencing chemistry, and for more rapid analyses of protein and nucleic acid data banks using newly developed computer superchips and software. We have spun off from the developmental microchemical facility an applied microchemical facility that has two DNA synthesizers, a peptide synthesizer and will soon have a protein sequenator (see Facilities section, page 218). Thus, the Microchemical Facility has provided powerful tools for approaching the biological problems of interest to our laboratory.

References:


63. Mouse T-cell Receptors Specific For Cytochrome c: Predominant Usage of a \( V_\alpha \) Gene Segment

Astar Winoto, James L. Urban, Nancy C. Lan, Joan Goverman, Daniel Hunsburg

The antigen-specific cell-surface receptors of B and T lymphocytes are structurally similar in that they are both disulfide-linked heterodimeric glycoproteins (heavy/light and \( \alpha/\beta \) chains) encoded by somatically rearranged variable (V), joining (J) and diversity (D) gene segments and constant (C) genes. Nonetheless, several important functional differences exist for the two types of receptor, including the fact that B cells exhibit direct antigen binding whereas T cells possess a dual requirement for antigen and certain polymorphic
cell-surface determinants encoded by the major histocompatibility complex (MHC). To analyze the molecular interactions involved in T-cell antigen/MHC recognition, we have characterized the \( \alpha \) and \( \beta \) gene segments of 15 T hybridomas specific for the C-terminal peptide of pigeon cytochrome c. Cytochrome c was chosen for study because the primary amino acid sequence of both the foreign antigen and the restricting MHC molecule is known. Using DNA sequence and Northern blot analyses, we determined that a single \( \text{Va} \) gene segment is predominantly used in this cytochrome c T-cell response. In addition, we found that \( \beta \)-junctional sequences seem to be important in determining antigen fine specificity. However, since no absolute correlation of V/D/J gene segments with antigen or MHC reactivity was found, we conclude that both \( \alpha \) and \( \beta \) chains contribute to the overall specificity of this immune response.

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

James L. Urban, Raúl Saavedra

Experimental allergic encephalomyelitis (EAE) is an autoimmune disease of the central nervous system induced in genetically susceptible animals by challenge with myelin basic protein (MBP). Chronic and relapsing forms of the disease have some similarities to human demyelinating disorders, particularly multiple sclerosis. We are currently studying the T cell immune response of EAE mice at the molecular level. Such T lymphocytes are thought to play a major etiological role in EAE because: 1) T cell-depleted animals do not develop the disease, 2) the disease can be reproduced by passive transfer of T-cell lines or clones obtained from MBP-injected animals, and 3) vaccination with attenuated MBP-reactive T cells confers resistance to further induction of the disease. We have generated a number of T-cell lines reactive with MBP in the EAE-susceptible SJL/J strain of mouse and have cloned them by limiting dilution. We are currently analyzing their immune receptors for MBP at the molecular level. Such T cells are thought to play a major role in EAE because: 1) T cell-depleted animals do not develop the disease, 2) the disease can be reproduced by passive transfer of T-cell lines or clones obtained from MBP-injected animals, and 3) vaccination with attenuated MBP-reactive T cells confers resistance to further induction of the disease. We have generated a number of T-cell lines reactive with MBP in the EAE-susceptible SJL/J strain of mouse and have cloned them by limiting dilution. We are currently analyzing their immune receptors for MBP at the molecular level in an effort to determine the size of the repertoire recognizing MBP and producing EAE in these mice. If the total number of variable (V) gene segments of either the \( \alpha \)- or \( \beta \)-chain gene of the T-cell receptor is relatively restricted, as is the case for \( \text{Va} \) in the response to pigeon cytochrome c (Winoto et al., 1986), it may be possible to direct monoclonal antibodies against this particular V gene segment and thus prevent the development of EAE.

Reference:

65. Analysis of T-cell Receptor \( \alpha \) and \( \beta \)-Chain Genes from Lysozyme-specific T-cell Hybridomas

Joan A. Kobori, Nilabh Shastri

We have analyzed \( \beta \)-chain gene rearrangements in eight T-cell hybridomas specific for the T11 peptide (residues 74-96) of hen egg lysozyme. Three of these T-cell hybridomas recognize the peptide antigen in the context of the MHC-encoded I-A\(^k\) molecule, whereas the other five T-cell hybridomas are I-E\(^k\) restricted. The I-A\(^k\)-restricted cells recognize determinants contained within the amino-terminal residues 74-86, while the I-E\(^k\)-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.

Using specific hybridization probes for the C\( \beta \)1 gene and the J\( \beta \)2, D\( \beta \)1, and D\( \beta \)2 gene segments, we identified and isolated the productive lysozyme-specific \( \beta \)-chain rearrangement in each T-cell hybridoma. Size-selected limited genomic DNA libraries were constructed using \( \lambda \) phage vectors to isolate the productive \( \beta \)-chain rearrangements. We identified five distinct V\( \beta \) and six distinct J\( \beta \) gene segments 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. We are currently sequencing the V-D-J gene segments to analyze the detailed structure of these T11 peptide-specific \( \beta \) chains. Our results indicate that T-cell responses to single determinants such as peptide antigens are made up of many independent clonotypes.

We have isolated several genomic rearranged \( \alpha \) clones using specific J\( \alpha \) probes. In the cells in which we cannot identify a rearrangement, we are constructing cDNA libraries.
Both the a- and a-chain gene analyses have been extended to lysozyme-specific T-cell hybridomas derived from C57BL/6 and B6-C-H-2bm12 mice. These studies are in progress.

66. Genomic Organization of Genes Encoding T-cell Receptor a-chain Variable Region

Bernhard Arden

From T-cell receptor a-chain transcripts in a cDNA library constructed from thymus messenger RNA, 19 a cDNA clones were chosen for nucleotide sequence analysis (Arden et al., 1985). Eight full-length Vα gene segments, each representing a different Vα subfamily, were subcloned into pUC8. A subfamily is a group of related V gene segments with greater than 75-80% sequence similarity. Usually a Vα gene subfamily contains from 5-8 members. Among the 12 Vα subfamilies that have been found by now, there are only two that contain a single member. In contrast, most Vβ gene segments exist as single-member subfamilies. The Vα subfamily organization therefore resembles those of the immunoglobulin Vκ and Vλ gene segments. I am using the Vα subfamily-specific probes to study the genomic organization of Vα gene segments.

A cosmid library constructed from B10.D2(dml) liver DNA was screened with a V gene segment-specific cDNA probe hybridizing to the 8-10 members of the Vα1 subfamily. I isolated and mapped four partially overlapping cosmid clones, each containing one Vα1 gene. One of these clones also hybridized with a cDNA probe specific for the Vα4 subfamily that consists of eight members (Arden et al., 1985). The gene from the Vα4 subfamily maps at a distance of only 15 kb from that of the Vα1 subfamily. Therefore, genes from different subfamilies may be interspersed.

The organization of the Vα1 subfamily was also studied using the Schwartz-Cantor gel electrophoresis system (see Abstract No. 68). This agarose gel system employs two alternately pulsed, inhomogeneous orthogonal electric fields that allow the separation of DNA molecules of sizes from 30-2000 kb. DNA digested with infrequent-cutting enzymes was hybridized with a Vα1 subfamily-specific probe. Two bands (50-100 kb) were obtained with Xhol and only one band (~150 kb) was observed with ClaI. Therefore, genes of the Vα1 subfamily appear to be clustered. I have started chromosomal walking to link the Vα1-containing cosmid clones. In addition, in Southern blots from pulsed field gels hybridized with different Vα probes, linkage relationships for the other Vα subfamilies will be determined.

Reference:

67. Characterization of the T-cell Response against Very Closely Related Alloantigens

Nancy A. Costlow

I am examining the genes encoding the T-cell antigen receptors that are reactive with a number of very closely related molecules. These antigens are mutations of the Kβ class I molecules which have been described and characterized (Nairn et al., 1980). These "bm" mutations of Kβ differ from Kβ and from each other by only 1-3 amino acids. Dr. Jeffrey Bluestone (NIH, Transplantation Biology Section, Immunology Branch, Bethesda, MD) has provided me with cytotoxic T cells cloned from mice carrying a bm mutation that react with the Kβ molecule. Each cloned T-cell line has been tested for its ability to recognize the parental Kβ molecule as well as a number of the different bm mutants. I am presently examining T cells with different reactivity profiles based on the results of this panel analysis.

I have made cDNA libraries from these cells and am in the process of cloning and sequencing their T-cell receptor genes. This sequence data will indicate if a limited number of variable genes is involved in these responses to such similar antigens or if a large number of variable genes is involved. This study will show by what mechanisms T cells are able to distinguish between such closely related antigens.

Reference:

68. Chromosomal Mapping of Human T-cell Receptor Vβ8 Subfamily by Field-inversion Gel Electrophoresis

Eric H. C. Lai

The human T-cell receptor Vβ8 subfamily has been found to have five germline members by Southern blot analysis. All five germline gene segments have been isolated and sequenced. Two of the five members are linked and separated by only 3 kb. However, the
genomic organization of the other members of the V\textsubscript{\beta}8 subfamily is not known. Thus, a field-inversion gel electrophoresis apparatus was used to characterize the genomic organization of the V\textsubscript{\beta}8 subfamily. Field-inversion gel electrophoresis, which is capable of separating DNA fragments ranging in length from 20 to 3,000 kb, has been constructed in our laboratory and all the methods required for study of the mammalian genome (i.e., preparation of large size mammalian DNA, restriction enzyme digestion and efficient transfer of large size DNA onto filters) have been worked out.

When a cDNA probe corresponding to the V\textsubscript{\beta}8 subfamily was used to hybridize human DNA that were digested with infrequently cutting enzymes (XhoI, Clal and SmaI), multiple bands were observed. However, a single band of 110 kb was observed with restriction enzyme SfiI. Hybridization probes from each member of the V\textsubscript{\beta}8 subfamily were then isolated and they all hybridized to the same SfiI fragment. A single SfiI restriction fragment length polymorphism shifts the size of the whole V\textsubscript{\beta}8 subfamily to 115 kb, suggesting that the entire subfamily is present on a single Sfi fragment. I am now in the process of isolating cosmid clones to link together all the members of the V\textsubscript{\beta}8 subfamily.

69. A Provisional Genetic Map of Murine V\textsubscript{\beta} Gene Segment Subfamilies

Richard K. Barth

DNA rearrangements of the T-cell receptor \(\beta\)-chain genes are associated with the process of generating a large repertoire of antigen specificities in the T-lymphocyte population of an individual. The \(\beta\)-chain gene locus, located on chromosome 6 of the mouse, frequently undergoes rearrangement on both chromosome homologs and usually results in the deletion of DNA intervening the rearranged gene segments. In a chromosome that has experienced a V\textsubscript{\beta}-D\textsubscript{\beta}-J\textsubscript{\beta} deletional rearrangement, all V\textsubscript{\beta} gene segments located between the rearranging V\textsubscript{\beta} and D\textsubscript{\beta} gene segments are lost. The identity of the absent V\textsubscript{\beta} gene segments can be determined by Southern blot analysis of the T-cell genomic DNA, if there is extensive deletion on the homologous chromosome 6 of the T cell. By analyzing a large number of cloned T cells for those V\textsubscript{\beta} gene segments that are rearranged, those that are deleted and those that remain in germline configuration, it is possible to establish the chromosomal order of different V\textsubscript{\beta} gene segments relative to the D\textsubscript{\beta}-J\textsubscript{\beta}-C\textsubscript{\beta} locus. This deletional mapping approach was tested on 15 independent, cloned T lymphomas using six different V\textsubscript{\beta} gene segment DNA probes. An analysis of the results yielded the following provisional map of subfamilies:

\[
(V_{\beta}^{2}, V_{\beta}^{4}) - V_{\beta}^{1} - V_{\beta}^{5} - V_{\beta}^{8} - V_{\beta}^{3} - D_{\beta}^{1} - J_{\beta}^{1} - C_{\beta}^{1} - D_{\beta}^{2} - J_{\beta}^{2} - C_{\beta}^{2}
\]

Additional T-lymphoma lines and V\textsubscript{\beta} DNA probes are being used to confirm and expand this map.

70. Characterization of Partially Rearranged (D-J) \(\beta\)-chain Genes

Erich Strauss, Richard K. Barth

The gene segment components (V, D and J) of immunoglobulin heavy-chain and T-cell receptor \(\beta\)-chain genes are assembled into a complete variable gene (V-D-J) through an ordered developmental program. The initial step in this program is the juxtaposition of a D gene segment to a J gene segment by a specific DNA rearrangement event. The product of D to J rearrangement, a so-called partially rearranged or D-J gene, may undergo a subsequent rearrangement event with a V gene segment to form a completely rearranged or V-D-J gene. Recently, it has been shown that both heavy-chain and \(\beta\)-chain D-J genes are transcribed in B and T lymphocytes, respectively. In addition, there is evidence that specific polypeptides are translated from heavy chain D-J transcripts. These observations led to the interesting possibility that partially rearranged heavy-chain and \(\beta\)-chain transcripts or proteins play an active role in lymphoid development, either by regulating subsequent immunoglobulin/T-cell receptor gene rearrangements or by participating in other processes crucial for lymphocyte differentiation. We have begun to further characterize \(\beta\)-chain D-J RNAs and to investigate the potential of these transcripts to direct the synthesis of specific, partial \(\beta\)-chain polypeptides. Using an oligonucleotide-directed primer extension technique with poly(A)\textsuperscript{+} RNA from several murine T lymphomas that transcribe D-J genes, we have localized the transcriptional start site of D\textsubscript{\beta}1-J and D\textsubscript{\beta}2-J transcripts to 178 nucleotides and -275 nucleotides upstream from the D\textsubscript{\beta}1 and D\textsubscript{\beta}2 gene segments,
respectively. We are in the process of confirming these results using indirect RNA sequencing and nuclease protection assays. Both D_{l1} and D_{l2} transcripts contain a single open translational reading frame extending through the D segment. Each open frame begins with a methionine codon located 137 nucleotides upstream of D_{l1} and 233 nucleotides upstream of D_{l2}. Thus, one of every three D-J genes generated by rearrangement has the potential for encoding a partial B-chain polypeptide. We are in the process of selecting "in frame" D-J transcripts and testing for their ability to direct the synthesis of partial B-chain polypeptides in an in vitro translation system.

71. Ontogeny of T-cell Antigen Receptor Gene Rearrangement

Mitchell Kronenberg, Joan L. Klotz, Eric H. C. Lai, Richard K. Barth

Rearrangement of immunoglobulin genes in developing B cells is an ordered process. Rearrangement in the heavy chain gene family always precedes light chain gene rearrangements. In addition, those V_H gene segments that are closer to both the J_H gene segments and constant region genes tend to be expressed preferentially early in the ontogeny of the immune system. This ordered expression of V_H gene segments suggests that the rearrangement mechanism may somehow scan or move along the chromosome.

We are currently testing whether the same two phenomena, ordered rearrangement with respect to unlinked gene families and ordered rearrangements among the V gene segments in a family, also pertain to the unlinked T-cell receptors α, β and γ genes. Our previous experiments showed that γ transcripts are detected first on day 14 of gestation in murine fetal thymocytes, followed by β transcripts on day 16 and α transcripts on day 17 (see Biology 1985, Abstract No. 73). This result suggests but does not prove the existence of an ordered rearrangement process for these gene families.

To study the gene rearrangement process directly, we have prepared over 130 T-cell hybridomas made by fusing fetal thymocytes from days 16, 17 and 18 gestation with the T lymphoma BW5147. DNA has been prepared from these cells and a preliminary analysis demonstrates that chromosome loss in these cells is minimal and that these hybrid cells directly reflect the cell population found in fetal thymus at each developmental stage. For example, day 17 hybridomas with germline β genes presumably derived from very immature cells, hybridomas with partially rearranged genes (D_{β-}J_{β}), and hybridomas with V_{β-D_{β-}}J_{β} rearrangements have been found in a set of 16 cells tested. We have begun to successfully analyze α gene rearrangements in these cells using reverse-field gel electrophoresis to separate restriction fragments greater than 50 kb in length. Although the data are too preliminary to prove that the α, β and γ genes rearrange in an ordered fashion, we have been able to demonstrate that very immature day 16 fetal thymocytes express most of the V_β gene segment repertoire. This argues against an ordered expression of V gene segments in this gene family.

72. Analysis of a Human V_β Gene Subfamily

Gerald J.-M. Siu, Errich Strauss, Eric H. C. Lai

We have isolated and sequenced five germline V_β gene segments that are homologous to the V region of the YT35 cDNA encoding the β chain of the T-cell antigen receptor from the tumor MOLT-3. Cosmid and lambda clones containing the five members of this V_β gene subfamily, called the V_β8 subfamily (previously defined as the V_β BM3 subfamily; Siu et al., 1984), were isolated by using the YT35 V region-specific probe. One of these gene segments, V_{β 8.1}, is identical to the YT35 V segment and therefore is the corresponding germline V_β gene segment encoding the YT35 cDNA. The remaining four members, V_{β 8.2}, V_{β 8.3}, V_{β 8.4} and V_{β 8.5}, exhibit 98%, 82%, 79%, and 76% homology to the YT35 V gene segment, respectively. Analysis of the coding regions reveals that the members of this family are mutating at a rate comparable to that of most eukaryotic genes. Two of the five members, V_{β 8.4} and V_{β 8.5}, have in-frame stop codons and therefore are pseudogenes. Characterization of the genomic clones containing the five members of the V_β8 family indicate that two of the five members, V_{β 8.1} and V_{β 8.2}, are only 3 kb apart; these two gene segments are the most homologous to each other and are probably the result of a recent gene duplication event. The other members of the family do not appear to be closely linked. To further characterize the genomic organization of the V_β8 subfamily, field-inversion gradient gel electrophoresis was utilized.
This novel technique permits the separation and analysis of large fragments of DNA for gene linkage. A single 110 kb restriction fragment was obtained when genomic DNA was digested with SfiI and hybridized to the YT35 V region probe. Probes derived from each subfamily member also hybridized to a single 110 kb SfiI fragment. Mapping of the lambda and cosmid clones containing the members of this subfamily have identified only one SfiI site, located 6.6 kb 5' to the V_8.1-V_8.2 cluster. These data suggest that the entire V_8 subfamily is present on a single 110 kb SfiI restriction fragment, although it is also possible that the members of the V_8 subfamily hybridize to similar-sized but different SfiI restriction fragments. The resolution of these two differing models will require the actual linkage of the members of the V_8 family using genomic clones.

Reference:

73. Analysis of the 5' End Control Element for T-cell Receptor Gene Expression

Nancy C. Lan

Recent studies of the regulation of immunoglobulin (lg) gene expression show that in addition to the enhancers of both heavy and light chains exhibiting cell type specificity, the promoters located at the 5' end of these genes also confer cell type specificity. Sequence analysis reveals an octomer sequence ATGCAAAT present in nearly all heavy chain gene promoter regions, its complement being found at the same position in light chain promoters. Furthermore, a nuclear factor from a human B cell has been found recently to bind to this conserved sequence motif. However, the role of this factor in the specific expression of Ig is not clear.

The promoter regions of rearranged T-cell receptor V_α and V_β genes will be examined to determine whether these promoters act in a tissue-specific manner and further to compare their specificity with that of immunoglobulin gene promoters.

We have transiently and stably transfected mouse Tk^-L cells and T cells with a complete V_β gene with and without a viral enhancer. The results of these experiments are currently being analyzed. Once the tissue-specific expression of the entire T-cell receptor gene is established, the promoter regions with deletion mutants will be constructed onto the bacterial gpt gene for the transfection assay to define the region conferring specificity. In addition, 5' end regions from available V_α and V_β genes are currently being sequenced to locate possible consensus sequences. DNA footprinting experiments will be performed using nuclear extracts from lymphoid and non-lymphoid cells to establish binding specificity. An affinity column containing multicopies of the recognition sequence will be used to purify the protein(s). Comparison of the specificity with that of immunoglobulin gene promoters then can be performed.

74. Expression of H-2 Class II Genes and T-cell Receptor Genes in Murine T Cells

Chia-lam Kuo

By DNA-mediated gene transfer techniques, I was able to introduce cloned mouse A^k and A^k genes, which encode the class II I-A molecules, into mouse EL4, a thymoma of helper lineage. Monoclonal alloantibodies defining distinct polymorphic regions of I-A^k molecules were used and showed that the I-A^k molecules are expressed on the transfected cell surface. These T-cell transfectants serve as a good substance for studying the regulation of MHC gene expression since I-A driven off its own promoter is not expressed in EL4 cell. One of the interesting questions we would like to ask first is whether the expression of I-A genes can induce the expression of the invariant chain (ii) in the transfected T cell. Il chain is a membrane protein associated intracellularly with class II molecules and expression of Il chains has been shown to be closely linked to that of class II molecules. The chain may play an as yet undefined physiological role in intracellular transport of class II molecules and in the functioning of class II molecules in the immune response. From the result of Northern blot analysis, we were able to show that expression of the Il chain message is induced while the introduced I-A genes are expressed in the transfected T cells. Whether the Il chain gene is expressed as a protein, and how and why the expression of class II molecules induces the expression of Il chain in these transfected cells will be further investigated to understand the regulation of MHC class II gene expression and the function of the Il chain.
A genomic clone of β chain and a cDNA clone of α chain of T-cell receptor genes were isolated from a cytochrome c-specific T-helper hybridoma by Winoto et al. in this laboratory. I have subcloned these genes into expression vectors and introduced both genes into EL4 to determine if the expression of the transfected α and β genes will endow the recipient cell with the specificity of the donor cell. Since antibody directed against this T-cell receptor molecule is not available at present, I could only collect transfected cells which, based on Northern blot analysis, express the message of the introduced T-cell receptor gens as candidates for the functional assay. The functional assay, which is to test the ability of transfected cells to produce IL-2 in the presence of antigen and antigen-presenting cells, is in progress in collaboration with N. Shastri to answer the above questions.

75. Secretion of a Murine Alloreactive T-cell Receptor Produced in Insect Cells by a Baculovirus Expression Vector

James L. Urban, Nancy A. Costlow, Joan A. Kobori

Recent molecular characterization of the T-cell receptor for antigen has demonstrated that it is similar in many respects to the B-cell antigen receptor, the immunoglobulin: 1) both are heterodimers of individual chains (α/β and heavy/light, respectively) divided into variable (V) and constant (C) regions, 2) each V region is encoded by two or three analogous gene segments, 3) both employ similar mechanisms for generating diversity, and 4) both apparently exhibit the same general primary and secondary structure. A detailed three-dimensional analysis of the T-cell receptor has been hampered by the fact that unlike its immunoglobulin counterpart, it is not produced in a soluble secreted form and therefore cannot be easily isolated and purified in the large amounts needed for binding studies and X-ray crystallography. We are currently attempting to produce large amounts of soluble T-cell receptor molecules for such studies by transfecting with truncated α- and β-chain genes lacking their transmembrane anchor regions. The transfection utilizes the expression vector Autographa californica nuclear polyhedrosis virus (AcNPV) introduced into cultured Sf9 insect cells. A similar system has been successfully employed for the generation of high levels of secreted human interferon-β (Smith et al., 1983) and interleukin-2 (Smith et al., 1986). The particular T-cell receptor we are attempting to express recognizes a mutated form of the MHC class I protein K^b, which we also hope to produce in a soluble form for use in future binding studies.

References:

76. Chimeric T-cell Receptor Genes as Tools in Analyzing T Cell/Target Cell Interactions

Joan M. Goverman, Tim Hunkapiller

We have recently proposed a model of T-cell antigen recognition in which the T-cell receptor and immunoglobulin molecules have structural and functional similarities. Other molecules are implicated in functioning as additional receptors mediating T cell/target cell interaction. In addition, we have suggested specific target sequences that may be recognized by T-cell accessory molecules. Our current efforts are directed toward testing the tenets of this model. We are constructing hybrid T-cell receptors in which the variable regions of the T-cell receptor are replaced by the variable regions of an immunoglobulin molecule of known specificity. Using this approach we hope to generate T cells that will be specific for a soluble antigen instead of cellularly presented antigen. This strategy should enable us to examine independently the interactions of T cell cell-surface molecules and target cell molecules and the influence of these interactions on T-cell activation and effector function. In addition, we are synthesizing peptide analogues of putative recognition sequences for T-cell accessory molecules and investigating the ability of these peptides to affect T-cell binding to target cells.

Reference:

77. Organization, Diversity and Polymorphism of Genes of the Human T-cell Antigen Receptor α Family

Michel Klein

The antigen-specific receptors on human T cells
can recognize and bind an almost unlimited number of antigenic determinants provided they are presented in association with cell-surface proteins encoded by the major histocompatibility complex (MHC). The T-cell antigen receptor is a disulfide-linked heterodimeric integral membrane protein composed of α and β chains, each divided into variable (V) and constant (C) regions.

The structural analysis of the β-chain gene locus revealed that its organization is similar to that of the immunoglobulin genes and is composed of non-contiguous Vβ, Dβ, and Jβ gene segments and two Cβ region genes. In contrast to the VH and VL repertoires, the repertoire of expressed Vβ genes is limited (Concannon et al., 1986a, 1986b).

The genomic organization of the human T-cell antigen receptor α-chain gene locus differs from that of its β-chain counterpart. It consists of a number of Va and Ja gene segments and a unique Ca region gene. To gain structural information on the Va gene family, we have isolated 140 α cDNA clones from a human peripheral blood lymphocyte library. Analysis of their nucleotide sequences will allow us to: 1) estimate the size of the Va gene repertoire, and 2) characterize the combinatorial and somatic mechanisms responsible for the antigen receptor diversification. Preliminary sequence data obtained from 12 α cDNA clones have already indicated that the Ja gene segment repertoire is significantly larger than the Jβ repertoire. Va gene-specific probes will be used to probe large germline DNA fragments by field inversion gel electrophoresis and analyze cosmid clones to map the entire Va gene family. The study of Va gene polymorphism should facilitate the search of interesting associations with autoimmune diseases and lymphoproliferative disorders.

References:

78. Diversity and Structure of Human T-cell Receptor Vβ Genes

Patrick J. Concannon, Lulu A. Pickering1, Patrick Kung1

The nucleotide sequences of 27 T-cell receptor β cDNA clones isolated from a human peripheral lymphocyte library were determined and compared to five additional published sequences. These cDNA clones represent 22 distinct Vβ gene segment sequences, which fall into 15 different human Vβ gene subfamilies, each containing six or fewer members. From this analysis we estimate that the repertoire of expressed human Vβ genes is less than 59, apparently much smaller than the immunoglobulin VH or VL repertoires. Variability plots comparing these human Vβ regions with each other and with published mouse Vβ regions provide evidence for only four hyper-variable regions homologous to those seen in comparisons of immunoglobulin V regions. Somatic hypermutation appears to be used infrequently, if at all, in these Vβ genes.

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79. Isolation and Characterization of T Suppressor-specific Genes

Hilde Cheroutre

Many experiments support the hypothesis that a T-cell subpopulation can suppress the immune response of other lymphocytes, responding to particular antigens. The molecular bases of these phenomena are poorly understood. Some unresolved questions include the following. 1) Do suppressor T cells express a cell-surface T-cell receptor, consisting of an α and a β subunit, or do they have a different type of antigen receptor? 2) Are there genes that are expressed only in suppressor T cells, which define these cells as a separate subpopulation?

In our approach to these problems, we are attempting to identify T suppressor-specific genes from a T-suppressor hybridoma (372D6.5) that reacts with the synthetic polymer GAT. This T-suppressor hybridoma was created by cell fusion with BW5147 thymoma cells. We have synthesized a cDNA library from 372D6.5 poly(A)+ RNA. To isolate suppressor-specific genes, we have screened this library by both subtractive and differential screening techniques. One hundred and twenty-five clones were isolated by this method, although none of the selected clones were completely specific for the T-suppressor hybridoma RNA. Some clones hybridized very strongly with the T-suppressor cDNA and only weakly with BW5147 cDNA. Clones that are enriched could reflect the up-regulation of some transcripts in the suppressor
hybridoma cells. Alternatively, these clones could be
coded by genes that cross-hybridize with but are not
identical to genes expressed by BW5147. Such genes
could include the T-cell receptor and MHC genes
which have some conserved and some highly variable
sequences. We hybridized the 125 selected clones with
\( \alpha \) and \( \beta \) constant region probes. \( \alpha \)-Positive clones were
rehybridized with the \( V_\alpha \) probe specific for BW5147.
Several full-length \( \alpha \) clones did not hybridize with this
V probe, suggesting that another V region, derived
from the suppressor-cell fusion partner, is expressed in
the hybridoma. Further, a characterization of these \( \alpha \)
clones and suppressor \( \alpha \) clones is in progress. We also
identified several class I genes among the 125 selected
clones. Further characterization of those class I clones led to the identification of several transcripts
encoding MHC differentiation antigens, such as the
T9/T17 gene and the \(^{37}\) gene. Preliminary evidence
indicates that these class I transcripts may be specific
for the suppressor cells opposed to other lymphocytic
lineages. The analysis of class I genes has been done in
collaboration with Dr. S. Hunt and K. Brorson and is
still in progress.

80. Influence of Ia Polymorphism on the
    Specificity of T Cells Specific for
    Short Peptides of Lysozyme

Nilabh Shastri, Cheryl A. Davis, Suzanna J. Horvath

Studies on the specificity repertoire of T cells have
repeatedly demonstrated a major influence of the
polymorphic Ia molecules encoded by the major histo-
compatibility complex (MHC). Thus, pairs of MHC
congenic mouse strains show dramatic differences in
responsiveness to the same antigen, a phenomenon
referred to as an immune response (Ir) gene defect.
Conclusive distinctions have not been possible between
the two major hypotheses accounting for these dif-
ferences. Response status could be a reflection of
either the available T-cell repertoire or the ability of
antigen-presenting cells to stimulate specific T cells.
Both the intrathymic development of the T-cell
repertoire and the antigen-specific activation of T
cells by antigen-presenting cells are known to be
intimately linked to the Ia molecules encoded within
the MHC.

Our studies using synthetic peptides corresponding
to a 23 amino acid region of lysozyme (residues 74-96)
have revealed that the choice of minimal antigenic
determinant is strictly dependent upon the Ia molecule
required for recognition by T-cell clones. For example,
all T-cell clones requiring the I-\( \mathbf{A}^k \) or the I-\( \mathbf{E}^k \)
molecules for recognition are specific for antigenic
determinants found within residues 74-86 or 85-96,
respectively. Furthermore, I-\( \mathbf{A}^b \)-requiring T-cell
clones recognize either residues 74-90 or 81-96
(Shastri et al., 1986a, 1986b). These clear distinctions
in minimal antigenic specificity are indicative of the
structural differences in the polymorphic Ia molecules
known to be co-recognized by the T-cell receptor.

Interestingly, T-cell clones requiring the I-\( \mathbf{A}^{bm-12} \)
molecule were found to recognize only residues 74-90.
Thus, the antigenic specificity of these T cells is
apparently identical to that of a subset of I-\( \mathbf{A}^b \)-
requiring T cells. This apparent exception to the
distinctions found in the minimal antigenic
specificities associated with structurally distinct Ia
molecules (e.g., I-\( \mathbf{A}^k \) and I-\( \mathbf{E}^k \) molecules) is perhaps
related to the fact that the I-\( \mathbf{A}^b \) and I-\( \mathbf{A}^{bm-12} \)
molecules differ only in three amino acid substitutions.
Studies are in progress using Ia-expressing L-cell
transfectants as antigen-presenting cells to precisely
determine the structural features of the Ia molecules
that are responsible for these reactivity differences
and to relate these to those of the T-cell antigen
receptor.

References:
Shastri, N., Gammon, G., Horvath, S., Miller, A. and
Shastri, N., Gammon, G., Miller, A. and Sercarz, E.

81. Genes Encoding the \( V_\mu \) Region of
    Anti-\( \alpha(1\rightarrow3) \) Dextran Antibodies

Carol Readhead, Joan L. Klotz, Teresa Geffroy,
Roger Perlmutter

The response of mice to the polysaccharide \( \alpha(1\rightarrow3) \)
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 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 \( V_\mu \) segment. This implies that the \( V_\mu \) gene
segment encoding J558 plays a major role in the \( \alpha(1\rightarrow3) \)
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 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 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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82. Isolation of Brain cDNAs that Cross React with L3T4

Bernard Venooy, Hilde Cheroutre, Jane Parnes

L3T4 is a molecule that is present on the surface of a subset of T cells in the mouse. In general, these L3T4-bearing T cells are helper T cells, which facilitate the immune response. The helper cells recognize antigen together with class II H-2 molecules on the surface of other cells. Based on antibody inhibition studies, it has been proposed that the L3T4 molecule plays a role in the interaction between the T cell and the antigen-class II complex. More specifically, it has been hypothesized that L3T4 interacts with nonpolymorphic regions of class II molecules.

An L3T4 cDNA hybridizes with two RNA species from the brain. S1 protection experiments show that the 5' part of these brain RNAs is different from the 5' part of the T cell L3T4. The RNA level in the brain is very low. This is reflected in the fact that two cDNA libraries from the brain (a total of 1.3 million plaques) contained no recombinant phages that hybridized with the L3T4 cDNA.

Presently, we are making and screening a larger cDNA library in order to isolate the brain cDNAs. We want to determine the sequence and the genomic organization of the gene that encodes these brain RNAs. This will show us whether or not the brain RNA is made from the same gene as the L3T4 RNA in T cells through alternative splicing. Furthermore, we want to study the anatomical distribution in the brain by antibody staining and by in situ hybridization. By studying this in fetal development of normal mice and in mice with neurological disorders, we hope to get insight into the function of this molecule.

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83. Expression of Major Histocompatibility Complex Class I Genes in Normal Murine Tissues

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

Class I antigens are a family of related glycoproteins that include the transplantation antigens K, D, and L and the nonclassical antigens Qa-1, Qa-2 and TL (thymus leukemia). The transplantation antigens are expressed on most somatic cells and are involved in the recognition of foreign antigen by cytotoxic T cells. The nonclassical class I antigens are expressed in a tissue-specific manner, usually on hematopoietic cells; hence, they have been called hematopoietic differentiation antigens. The function of these antigens is unknown.

Class I antigens are encoded by genes in the major histocompatibility complex. In the BALB/c mouse
there are at least 35 class I genes. Three of these genes encode the transplantation antigens. The remaining 32 class I genes encode the nonclassical class I antigens. We are interested in establishing which of these antigens are expressed, where they are expressed and when they are expressed. This information may provide insights into the function of this large group of very interesting genes.

We have developed a very sensitive system for studying class I gene expression in mouse tissues. cDNA libraries have been made from various BALB/c tissues including thymus, brain, spleen, liver and bone marrow. Clones containing class I genes were isolated from the libraries using a probe from a highly conserved region of all class I genes. The individual clones were identified using a set of oligonucleotide probes capable of distinguishing among the 35 BALB/c class I genes (Hunt et al., submitted for publication).

Using this system, we have shown that at least three class I genes are expressed in the mouse brain, a tissue thought to be devoid of class I gene expression. In the thymus, we have shown that at least 14 different class I genes are transcribed whereas only five had been detected using serological reagents. Two of these clones represent new class I genes that were not identified in earlier analyses of genomic cosmid clones. Further characterization of the class I genes expressed in these and other tissues is currently under way.

84. Characterization of a Highly Divergent Murine Class I Gene Encoded in the Tla Region of the Major Histocompatibility Complex

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 14 different class I genes are expressed. Two of these genes (designated T9 and T17) reside in the Tla region of the major histocompatibility complex. These genes show greater than 95% homology at the DNA sequence level and have probably resulted from a gene duplication event in the recent past.

As a first step in the characterization of these genes, we have completely sequenced a full-length T9 cDNA clone and have partial sequence data from a T17 cDNA clone, both of which were isolated from a thymus cDNA library. In addition, we have partial sequence information from a T17 cDNA clone isolated from a T-suppressor hybridoma cDNA library. These genes are the most divergent of the class I genes studied to date. Virtually the only region of sequence conservation, when compared to other class I genes, is in the highly conserved fourth exon which encodes the \( \beta_2 \)-microglobulin-binding region. Stretches of homology can be found in the exon coding for the a1 region but the leader, a2, transmembrane and cytoplasmic regions are unique. The cDNA sequence predicts that this gene encodes a membrane-bound protein product. The T17 cDNA clone possesses a very interesting 5' untranslated region which may be involved in the regulation of the expression of this gene. The T9/T17 gene pair is expressed in the thymus, spleen and lymph node and at much lower levels in the liver. Future studies will include characterization of the T9/T17 protein product and identification of the subpopulations of cells in the mouse that express these genes.

85. Structural Studies on Murine Tla Genes

Douglas A. Fisher

Thymus leukemia (TL) antigens are cell-surface glycoproteins that are expressed only on thymocytes and on some leukemia cells of thymic origin. TL antigens are biochemically similar to transplantation antigens and are encoded at a genetic locus, Tla, that is closely linked to the H-2 complex. The H-2, Qa and TL antigens are encoded by members of the class I gene family.

We have determined the DNA sequence of a BALB/c Tla gene encoding the thymocyte TL antigen (Fisher et al., 1985). This gene, the T13C gene, is homologous in both sequence and in most intron-exon boundaries with H-2 genes, with the major exception being the cytoplasmic region where gene T13C utilizes one cytoplasmic exon, whereas H-2 genes generally use three. As expected, the DNA sequence of gene T13C has diverged considerably (25%) from H-2 genes.

Low copy number DNA probes from gene T13C have allowed us to analyze the 18 BALB/c Tla region class I genes and compare them with those cloned in the TL+ C57BL mouse and to study the expression of TL antigen. The T13C gene is deleted from the C57BL genome, explaining the TL+ phenotype of this mouse. In addition, both BALB/c and C57BL mice contain a
second Tla gene, the T3 gene, which is highly homologous (>95%) to the T13C gene and encodes a TL antigen detected only on leukemia cells. The T3 gene has been sequenced both in C57BL (Pontarotti et al., 1986) and in BALB/c mice (S. Nathenson, unpublished). It is not yet understood why these genes are leukemia specific, or what, if any, role they play in leukemia.

Another Tla gene, the T1c gene, has been analyzed in detail. Originally thought to encode a TL antigen, the T1c gene was shown to be a pseudogene (Fisher et al., 1986). Comparison of the T1c gene with the T13c gene showed a point of genetic recombination. Interestingly, a B2 Alu-like repetitive element forms the boundary of the recombination, raising the possibility that these transposon-like elements may catalyze large transpositions or recombinations.

References:

86. MHC Class I Antigen Expression during Cellular Differentiation

Suzanne Ostrand-Rosenberg

Murine teratocarcinoma tumors consist of undifferentiated, malignant stem cells (embryonal carcinoma) and differentiated, benign cells. Many of these tumors can be cultured in vitro as undifferentiated cell populations and induced to differentiate into derivatives to the three primary germ layers. Teratocarcinomas are therefore considered an excellent experimental system in which to study and regulate differentiation. Numerous studies have suggested that MHC class I antigens, as well as playing a critical role in immunological cell-cell interactions, may also play an important role in cellular differentiation and/or development. The present studies were therefore undertaken to identify and characterize MHC class I antigen expression as a function of the state of differentiation. Igf10 cDNA expression libraries have been constructed from poly(A)+ RNA from undifferentiated teratocarcinoma PCC4 and 402AX cells and from retinoic acid- and cyclic AMP-induced, differentiated PCC4 cells. These libraries are being screened with H-2b-specific transmembrane probes to identify MHC class I clones. Once identified, the class I clones will be further characterized and sequenced. These studies should precisely define which MHC class I molecules are expressed during cellular differentiation and should provide us with a panel of genes whose role in cellular differentiation can be further tested.

87. Functional and Serological Analysis of Secondary Mutants Derived from dm1 Hybrid H-2 Antigen

Duane W. Sears, Iwona Stroynowski

Secondary mutants of the hybrid dm1 H-2D/L gene, cloned from the B10.D2 (dm1) mutant mouse strain, were constructed for functional analysis of the T-cell recognition sites on H-2 antigens. The dm1 H-2D/L gene is a hybrid of the parental H-2Dd and H-2Ld genes in that the leader exon, the first structural domain exon, and a large part of the second structural domain exon derive from the H-2Dd gene, whereas the remaining 3' portion of the gene derives from the H-2Ld gene.

By exchanging exons between the mutant dm1 gene and the parental H-2Dd or H-2Ld genes, two secondary mutant genes were created. One of these genes encodes an H-2 antigen that differs from the parental H-2Ld amino acid sequence at position 92 (Thr+Ser), 99 (Tyr+Ala), 104 (Gly+Glu) and 114 (Glu+Trp). The other secondary mutant gene encodes an antigen that differs from the parental H-2Dd amino acid sequence at positions 155 (Arg+Tyr), 156 (Asp+Tyr) and 169 (Arg+His). The mutant genes were introduced into mouse L cells and are now being analyzed with monoclonal antibodies to map serologically-defined determinants. Transfectants also will be infected with reovirus and analyzed in terms of T-cell recognition with allospecific lymphocytes and with virally-restricted T-cell killers. Reovirus, a double-stranded, naked RNA virus, elicits potent cytotoxic T-cell response in strains encoding parental molecules (B10.D2-H-2Ld) as well as in mice carrying mutant dm1 antigens (B10.D2-H-2+dm1). The cells expressing the two secondary mutant genes will allow us to analyze how the localized amino acid substitutions affect T-cell recognition during the reovirus cytotoxic response.

Iwona Stroynowski, Bette Korber, Kathleen N. Blackburn, Suzanne Ostrand-Rosenberg

Cell-mediated immune response to pathogens requires participation of many different components in concert. The identity of some of these components is known, while many others remain to be discovered. We study murine transplantation antigens, or major histocompatibility complex (MHC) class I molecules, whose role in recognition and elimination of virally-infected and neoplastically-transformed cells is well documented. These cell-surface polymorphic glycoproteins function as target/guidance molecules for cytotoxic T lymphocytes (CTL) during recognition of "self" from "nonself." Our studies are directed towards a quantitative analysis of different factors involved in regulation and effector functions of class I-dependent immune responses.

1. A Single MHC Gene Encodes Tissue-specific Secreted and Membrane-bound Forms of Qa-2 Antigen

Iwona Stroynowski, Kathleen N. Blackburn, Mark Soloski1, Ulf Landegren

Murine BALB/c major histocompatibility complex (MHC) contains at least 33 structurally-related class I genes. Three of them, H-2Kd, Dd and Ld, were shown to encode classical transplantation antigens expressed on most somatic cells of the body and known to function in presenting antigens to CTL. The remaining 30 genes could potentially encode protein products related to the transplantation antigens. Serological evidence suggests that, in fact, two regions of the MHC, Qa-2 and Tla, encode transplantation-like antigens whose function is presently unknown. Interestingly, these Qa and TL proteins have limited tissue distribution. Their expression is usually restricted to the cells of the lymphoid origin, although some can also be found in liver. We are interested in identification of different Qa and TL protein products, their structural characterization and in assigning function(s) to these molecules. Because of their similarity to the transplantation antigens, it has often been assumed that they play a role in cell-cell interactions during an immune response. On the other hand, Qa and TL proteins can be regarded as naturally occurring mutants of transplantation antigens that lost the ability to express ubiquitously and to function in presenting viral antigens to CTL. By studying them, we hope to learn more about transplantation antigens themselves. What features of class I molecules make them function in immune responses? Does the tissue-specific expression of class I molecules affect their ability to present antigens and does it play a role in the establishment of self tolerance?

We have identified BALB/c (H-2d) and C57BL/10 (H-2b) genes encoding serologically and biochemically-defined murine Qa-2 antigen. This nonpolymorphic molecule is expressed on selected subpopulations of bone marrow-derived cells and on liver. Class I genes subcloned from cosmid libraries (constructed in L. Hood's and R. Flavell's laboratories) were introduced into fibroblastic L cells and epithelial hepatoma cells by DNA-mediated transfer. The expression of Qa serological determinants was monitored by radioimmunoassay with different monoclonal antibodies. None of the genes transfected into L cells expressed cell-surface products reactive with anti-Qa antibodies. In contrast, hepatoma cells transfected with Q6d, Q7b and Q9b genes were positive in the same assay. To show that Qa cell-surface expression is tissue specific, we introduced Qa genes into a number of other mouse/rat cell lines: embryonal fibroblast line, another derivative of L cells, pituitary gland tumor line and primary cloned liver cells. Only liver-derived cells supported cell-surface expression of Qa antigen. However, when L-cell transfectants were radio labeled biosynthetically and Qa-reactive proteins were characterized by two-dimensional gel electrophoresis, we found that a 40 kd protein associated with β2-microglobulin is present in the cytoplasm of L cells and is secreted into the growth medium. The secreted form of Qa protein synthesized in L cells is indistinguishable in molecular weight from the membrane-bound molecule found normally on resting T cells or in hepatoma transfectants. It is also identical in size by the two-dimensional gel criteria with the soluble Qa molecules secreted from activated T cells (Soloski et al., 1986). These results indicate that the same gene encodes both forms of Qa antigen and that their expression is dependent on tissue-specific factors. We have mapped the trait responsible for cell-surface expression to the 3' half of the Qa gene. We are presently investigating the possibility that the two
Qa forms are products of differential mRNA splicing. Alternatively, cell-surface display of Qa molecules may require stabilizing proteins/factors which are present in some cell types only. The results of these studies will lead to the understanding of molecular mechanisms involved in tissue-specific expression of class I proteins and will be useful in designing experiments testing biological functions of transplantation antigens and their variants.

2. Regulation of Class I Antigens Expression: Cell Surface Levels of Qa-2 Antigen are Inducible by Interferons

Iwona Stroynowski, Donald Mann, Bette Korber, James Forman

It has been reported previously that various class I molecules (H-2, Qa and Tla) are under complex regulatory control of interferons, growth factors, glucocorticoids and prostaglandins. Of particular interest are lymphokines produced by activated T cells and secreted into the medium, where they locally modulate expression of MHC antigens. The level of expression of these antigens regulates, in turn, the magnitude of immune response. It has been shown for example that interferon-induced increase of transplantation antigens on target cells correlates with enhanced susceptibility to CTL lysis. We studied response of Qa gene expression to treatment with α and γ interferons. We showed that cloned Q6d and Q9b, both of which encode Qa-2 antigen (see above), are enhanced by interferons in murine hepatoma cells and rat liver transfectants to a similar degree as classical H-2 transplantation antigens. This finding has to be reconciled with previous reports (Soloski et al., 1986) in which it was found that the levels of Qa cell-surface expression remain constant in activated T cells producing γ interferon. It is possible that other lymphokines present in activated T cells antagonize interferon, or that T-cell activation results in change of Qa expression from cell surface to soluble form, thus accounting for the observed results. We are presently testing these possibilities by introducing cloned H-2 and Qa genes into T cells in which these processes can be studied and by testing existing T-cell hybridomas expressing Qa antigens. We plan to correlate these analyses with studies on molecular mechanisms involved in interferon action (see Abstract No. 89).

3. Expression of Transplantation Antigens in Malignant Cells and their Role in Tumor Rejection

Iwona Stroynowski, Suzanne Ostrand-Rosenberg

Several independent studies have established previously that tumor-derived cell lines often express very low or undetectable levels of class I MHC antigens. Because these molecules play important roles in immune responses, the correlation between decrease in class I membrane expression and the cell malignancy is viewed with great interest for its potential implications in tumor growth and metastasis. Of special interest are also molecular mechanisms used to regulate the expression of MHC antigens in transformed cells. For example, it has been reported that lymphoma cell lines often express the products of the H-2D but not H-2K locus, suggesting that H-2K and H-2D gene expression may be regulated independently of each other.

We have characterized expression of transplantation antigens on murine hepatoma cell lines derived from spontaneous tumor BW7756 of C57L/J mice (H-2b haplotype). Three independently established cell lines were tested by radioimmunoassay for the cell surface display of H-2Kb and H-2Db antigens. All three were found to express high levels of H-2Db but undetectable quantities of H-2Kb proteins. This finding indicates that either the parental tumor is an H-2Kb loss variant or the in vitro cloning of the BW7756 tumor leads to a reproducible "dedifferentiation" accompanied by attenuation of H-2Kb expression. We are presently trying to distinguish between these possibilities and to find out if the attenuation of H-2Kb expression has occurred at the DNA, RNA or protein level. We have shown that introduction of H-2Kb gene into hepatoma cells by DNA-mediated transfer allows expression of the H-2Kb protein—thus the mechanism involved in the repression of the endogenous gene does not operate in trans on exogenously introduced class I DNA.

We also are testing the role of MHC class I expression in the in vivo proliferation of the BW7756 cells. The parental hepatoma grows as subcutaneous tumor in syngeneic hosts while the in vitro established cell lines fail to do so even if inoculated at high concentration. Interestingly, these cell lines are insensitive to cytopotoxic T (CTL) cells (J. Forman, personal communication) but are very sensitive to lysis by natural killer (NK) cells (A. Orn, personal
communication). Since it has been proposed that NK cells are specifically active against cell targets expressing low levels of class I antigens (Piontek et al., 1985), we are testing 1) whether NK cells are in fact responsible for the rejection of hepatoma cell lines, and 2) if H-2K\textsuperscript{b}-positive transfectants having enhanced class I cell-surface expression will be more tumorigenic in vivo than the H-2K\textsuperscript{b}-negative cells. The results of these studies should increase our understanding of the role of MHC class I antigens, CTL and NK cells in tumor rejection.

References:

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89. Molecular Analysis of Interferon Regulation of Murine Transplantation Antigens Indicates that Two Regions of the Genes are Responsive to Interferon

Bette Korber, Iwona Stroynowski

Interferons (IFNs) regulate expression of a wide variety of molecules including several components of the immune system. We have studied IFN-mediated enhancement of expression of H-2 class I genes that encode murine transplantation antigens. Transplantation antigens play a critical role in the destruction of virally infected and malignantly transformed cells. The pathogenic antigens are recognized by cytotoxic T cells in the restricted context of a self-H-2K, D or L molecule.

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 H-2D\textsuperscript{d} and H-2L\textsuperscript{d} were linked to the structural gene of chloramphenicol acetyl transferase (CAT) and the constructs were introduced into L cells. Measurements of CAT activity in γ-IFN-treated versus untreated cells revealed that the 5' flanking region of class I genes contains a sequence sensitive to γ-IFN. Deletion analysis of this region indicates that the critical sequence for a response is a 30 bp sequence which was previously proposed to be a consensus for α-IFN-regulated genes (Friedman and Stark, 1985). Riboprobe RNase protection assays show the CAT transcript is initiating correctly and that the CAT RNA parallels the increase in protein activity after treatment with γ-IFN. We are presently beginning footprinting studies to look for nuclear DNA binding proteins that may be activated in IFN-treated cells.

Class I constructs carrying a nonregulated feline leukemia virus (FeLV) promoter inserted in place of the parental 5' flanking region of the H-2L\textsuperscript{d} gene were also analyzed (Wong et al., 1985). The expression of the H-2L\textsuperscript{d} antigens in cells transfected with such hybrid genes was still under γ-IFN control. FeLVL\textsuperscript{d} antigen levels increased upon γ-IFN treatment less than the parental H-2L\textsuperscript{d} transfectants when assayed by radioimmunoassay. We are currently attempting to determine if the regulation of the FeLVL\textsuperscript{d} construct is at the RNA level, and if it is, if this regulation is due to an increased transcriptional rate or due to RNA stabilization.

Similar effects on gene regulation were observed using α-IFN. Thus, the IFN-mediated induction of class I genes depends on two independently operating mechanisms: one is controlled by the 5' flanking region and is encoded in the IFN consensus sequence proposed by Friedman and Stark (1985) and the other by sequences downstream from the transcription initiation site.

References:

90. Transgenic Mice Carrying Novel Transplantation Antigen Genes

Carol Readhead, Iwona Stroynowski

The murine major histocompatibility complex contains 2-3 classical transplantation antigen genes and in addition 27 structurally-related sequences that could potentially encode proteins. A few of these, Qa-2 and TL, were identified serologically. In contrast to the true transplantation antigens, which are expressed on most somatic cells of the body, Qa-2 and TL proteins are found only on some subsets of lymphoid cells. Their functions are unknown.
To assess the ability of tissue-specific class I proteins to perform functions similar to H-2K, D and L transplantation antigens, we injected mouse embryos with a Qa-2 gene modified in such a way as to allow its expression on all cells of the body. This gene, Qa-2/Dd (Stroynowski et al., 1985), is composed of the 5' half of the Qa-2 gene and the 3' half of the H-2Dd gene. When introduced into fibroblasts, it is expressed as a hybrid molecule carrying the α1-α2 domain of the Qa-2 protein (region important for the functional interactions with T cells) and the α3, transmembrane and cytoplasmic region of H-2Dd transplantation antigen (region required for non-tissue-specific expression).

We microinjected the Qa hybrid gene into the nuclei of C57BL/6J and C3H/HeJ embryos and transferred the embryos to B6D2F1 pseudopregnant foster mothers. The transgenic mice are presently being analyzed. The parental C57BL/6J mice have a Qa-2 gene but it is only expressed on a subset of lymphoid cells, whereas C3H/HeJ mice have no Qa-2 gene. We anticipate that the Qα hybrid gene will be expressed universally on all tissue types as are the transplantation antigens. If this is the case, we will test if the Qα hybrid molecule can present antigens to T cells and participate in the graft rejection processes.

Reference:

91. Integration and Expression of Microinjected Myelin Basic Protein Gene in the Transgenic Shiverer Mutant Mouse

Carol Readhead, Brian J. Popko, Jessica A. Dausman

Myelin is a multilayered membrane structure that surrounds most axons of the central and peripheral nervous systems. The high electrical resistance of the myelin sheath permits the rapid conduction velocities of nerve impulses. Approximately 70% of the dry weight of myelin is lipid and the remaining 30% is protein. The lipid is believed to convey the electrical resistance of myelin and the protein is thought to have a structural role. The myelin basic protein (MBP) constitutes about 30% of CNS myelin proteins.

The shiverer mutation is one of several that disrupts myelination in the mouse. Shiverer homozygotes develop a generalized tremor at about 12 days that becomes progressively more severe. The level of CNS myelination in shiverers is greatly reduced and the myelin that is present lacks MBP protein. The majority of the protein coding region of the MBP gene is deleted in shiverer mice and no MBP RNA can be detected in shiverer brains. The shiverer mutation as well as the MBP gene have been mapped to chromosome 18. These data suggest that the shiverer mutation is the MBP gene deletion.

In order to show directly that the MBP gene deletion is the shiverer mutation and to examine the factors that regulate the MBP gene's strict tissue and temporal specific expression, we have begun to make transgenic shiverer mice with the wild-type MBP gene. The mouse MBP gene spans approximately 35 kb of DNA, and we have microinjected it as a cosmid clone. We have produced a transgenic line that is expressing the transgenic MBP gene tissue specifically in shiverer mice at approximately 10% of the level of the wild-type gene. These mice still shiver, possibly because the level of transcription is inadequate. We are in the process of examining these mice for MBP protein production by polyacrylamide gel electrophoresis, electron microscopy, and immunocytochemistry. Transgenic mice with a single copy of the MBP gene are being mated to each other to produce a homozygous line to determine whether two expressing copies of the transgenic MBP gene per cell will have a phenotypic effect. Additionally, we are continuing to microinject the cosmid with the hope that at another integration site the transgenic gene will be expressed at a level closer to that of the normal gene.

We are also microinjecting a series of additional constructs that have a cDNA clone of the MBP gene under the transcriptional control of various regulatory regions. One construct has the cDNA promoted by the control region for the early proteins of SV40. The other constructs have various amounts (from 0.5 to 6.0) of the 5' region of the genomic MBP gene promoting the cDNA's transcription. The SV40-promoted cDNA should tell us what effect promiscuous transcription of the gene will have on the mouse, and the cDNA constructs promoted by genomic MBP sequences should give us an indication of what regions are necessary for appropriate MBP transcription. We have made transgenics with several of these constructs and are beginning to examine their transcriptional activity.
92. Examination of the Myelin Basic Protein Gene in the Dysmyelinating Murine Mutant MLD
Brian J. Popka, Carol Readhead, Eric H. C. Lai

The myelin-deficient mutation (MLD) is allelic to the shiverer mutation and in homozygotes results in central nervous system (CNS) hypomyelination and dysmyelination. MLD mice are characterized by very low but detectable levels of myelin basic protein (MBP) in their CNS myelin. The MBP gene has been implicated as the genetic defect of shiverers; therefore, we have examined the MBP gene of MLD mice for similar genetic abnormalities.

Whereas there is no detectable MBP RNA in shiverer mice, a relatively high level (-10% of controls) of MBP RNA can be detected in the brains of adult MLD mice. Genomic blots indicate that all of the exons of the MBP gene are present in MLD DNA. We have isolated genomic clones of the MBP exons from MLD DNA. Five of the seven exons have been sequenced and show no alterations relative to the normal MBP gene. Nevertheless, the restriction map of the clone that contains the first exon of the MBP gene reveals a rearrangement approximately 5 kb 5' to the transcriptional start site. Genomic blots of MLD DNA, hybridized with the first exon of the MBP gene, reveal the normal 5' region as well as the rearranged version detected in our clone. Therefore, it appears that the MBP gene, or a portion of it, has been duplicated in MLD mice.

We are taking two approaches to gain a better understanding of the MBP gene duplication in MLD mice. We are using the field inversion gel electrophoresis systems, in which very large segments of DNA can be mapped, to determine if the duplicated regions of the MBP gene are closely linked. It is possible that if the duplicated 5' region of the MBP gene is close to the normal MBP gene, transcription from the abnormal gene may adversely affect normal MBP gene expression. Additionally, we have made a cosmid library from MLD DNA. We are in the process of characterizing several MBP clones isolated from the library. Cosmid clones of the MLD MBP gene should help determine if the duplication is responsible for the reduction of MBP gene transcription in MLD mice.

If we are able to show directly in the transgenic studies that the shiverer mutation is the MBP gene deletion (by correcting their dysmyelinating phenotype), we will also attempt to show directly that the MLD mutation is the result of abnormal MBP gene expression. This will be accomplished by crossing the transgenic gene of the phenotypically normal shiverer into the MLD background. If these mice are phenotypically normal as well, it will indicate that the reduction of MBP gene expression in MLD mice is responsible for their abnormal phenotype.

93. Alternative Usage of Exons in the Myelin Basic Protein Gene during Mouse Development
Raul A. Saavedra

Myelin is the electrically insulating sheet that surrounds nerve fibers. One of its major proteins is myelin basic protein (MBP), and in the mouse central nervous system there are four different forms of MBP: 21.5 kd, 18.5 kd, 17 kd and 14 kd. The stoichiometric proportions of these proteins change from 1:5:2:10, respectively, during the age of maximum myelination (18 days) to 1:10:3.5:35, respectively, in the adult. Early in mouse development, larger proteins can be isolated that are precipitated by antibodies specific to mouse MBP. However, the exact relationship of these larger proteins to MBP is presently unknown. The mouse MBP gene contains seven small exons, and the second and sixth exons are used alternatively to produce the four forms of MBP.

We have designed experiments to study the alternative usage of exons during mouse development. The experimental approach includes 1) the isolation of poly(A)* messenger RNA at various ages after birth, 2) S1 nuclease protection experiments, and 3) primer extension experiments using DNA probes specific for particular regions of the MBP gene. The results of these experiments will provide information on the relationship between the various MBP forms during the process of formation and maintenance of myelin.

94. Myelin Protein Genes from Primitive Vertebrates
Lance Fors, Raul A. Saavedra

Myelin is a membrane complex that surrounds nerve fibers and serves as an electric insulator to increase the velocity of the nerve impulse. Myelin basic protein (MBP) and proteolipid protein (PLP) are the major components of the central nervous system (CNS) myelin. The comparison of the shark MBP and
PLP genes with their mammalian counterparts will provide valuable information on the evolution of myelin and insights into the structure-function relationships of these genes.

In order to study the evolution of the MPB and PLP genes, we have undertaken the cloning and characterization of these genes from the shark Heterodontus using mammalian MBP and PLP cDNA as probes. Once these genes are cloned and sequenced, their genomic structure will be compared to that of their mammalian counterparts. By making use of the transgenic mouse system available in our laboratory, we plan to determine whether the shark MBP gene can functionally substitute for the mouse gene and thus reestablish normal neural function in the mutant mouse "shiverer."

95. Structural Analysis of Scrapie-associated Proteins

David B. Teplow, Stanley B. Prusiner

Scrapie is a disease of sheep characterized by progressive neurological degeneration. Human counterparts include Kuru and Creutzfeldt-Jakob disease (CJD). All three diseases exhibit a common neuropathology characterized by extensive intracellular and extracellular deposition of protein. Purified protein isolated from brains of hamsters infected with the scrapie agent is infectious. This protein, termed PrP33-35Sc, is not associated with nucleic acid and may thus be a novel infectious agent. A structural variant, termed PrP33-35C, exists in normal brains.

The central question we are addressing is what the chemical difference is between PrP33-35Sc and PrP33-35C. We are using protein chemical methods to study the structure of the polypeptide backbone of each protein and to identify and contrast posttranslational modifications of each polypeptide chain. These studies are a first step toward understanding the etiology of scrapie infection and of Kuru and CJD.

96. Approaches to Subpicomole Protein Sequencing

Ruedi Aebersold, David B. Teplow, Stephen B. H. Kent

Many biologically important proteins occur in very low abundance. Current sequencing levels (5-20 picomoles) must be improved about 1000 fold in order to give information about these rare proteins. To achieve this 10-100 femtomole sensitivity, all aspects of protein sequencing methodology must be addressed. We have undertaken a systematic research program with this goal in mind. This includes: 1) separation of proteins by SDS-PAGE, Immobiline IEF, or two-dimensional gels, followed by electroblotting onto activated glass fiber sheets for high yield isolation of subpicomole amounts of proteins in covalently-immobilized form directly suitable for sequencing; 2) microscale chemical deglycosylation techniques compatible with these new isolation techniques; 3) design, construction, and performance of an improved protein microsequenator; 4) use of fluorescent and cryptic-functionality Edman reagents; 5) modified reaction conditions for accelerated Edman degradation; 6) alternative procedures for the analysis of the resulting amino acid derivatives; 7) enhanced visible and fluorescence detection of these derivatives; and 8) digital acquisition and manipulation (identification and quantitation) of the analytical data sets and deduction of the amino acid sequence. These techniques have proven useful in sequencing of a variety of novel proteins.

97. Micropurification of Proteins for Sequencing

Ruedi Aebersold, David B. Teplow, Stephen B. H. Kent

We have developed a series of procedures for the high-yield purification of picomole amounts of proteins in a form suitable for automated Edman degradation. All of these procedures take advantage of the chemically-modified glass fiber supports that we recently introduced (Methods in Protein Sequence Analysis Meeting, Cambridge, U.K., August 1984). One-dimensional separation methods for isolating proteins include: 1) SDS-polyacrylamide minigels, followed by electroblotting at high pH onto quaternary-ammonium (QA) derivatized glass; 2) iso-electric focusing (IEF) in Immobiline polyacrylamide gels, followed by electroblotting in dilute acetic acid onto TFA-treated glass; 3) reverse phase HPLC, followed by direct application of the protein- or peptide-containing fraction onto a QA-glass disc. Two-dimensional separation methods include: 1) IEF-
SDS-polyacrylamide (O'Farrell) gels; 2) SDS-PAGE separation of glycoproteins before and after microscale HF deglycosylation; and 3) separation of proteins by SDS-PAGE, followed by in-gel digestion and separation of the fragments on SDS-PAGE gradient gels. In these three applications, the separation is followed by electroblotting at high pH onto QA-glass. Proteins and peptides prepared by all of the above methods are directly suitable for sequence determination on the gas phase sequenator. These approaches have been used for picomole sequencing unknown proteins from a variety of sources.

98. Electroblotting onto Activated Glass: High Efficiency Preparation of Proteins from Analytical SDS-Polyacrylamide Gels for Direct Sequence Analysis

Ruedi Aebersold, David B. Teplow, Stephen B. H. Kent

We have developed a new method for the isolation of proteins for microsequencing. It consists of electrophoretic transfer ("electroblotting") of proteins or their cleavage fragments onto activated glass filter paper sheets immediately after separation by SDS-polyacrylamide gel electrophoresis. The proteins are immobilized on the glass fiber sheets by ionic interactions or by covalent attachment. A wide range of proteins can be prepared in this fashion with no apparent restriction due to solubility, size, charge, or other intrinsic properties of the proteins. As little as 50 ng of the transferred proteins can be detected using Coomassie blue or fluorescent dye staining procedures and even smaller amounts of radiolabeled proteins by autoradiography. After detection, the protein-containing bands or spots are cut out and inserted directly into the gas phase sequenator. The piece of glass fiber sheet acts as a support for the protein during the sequencing. Amounts of protein in the 2-150 picomole range can be sequenced, and extended runs can be obtained from the blotted samples because of improved stepwise yields and lower backgrounds. This method has been successfully applied to the sequencing of a variety of proteins and peptides isolated from one-dimensional and two-dimensional polyacrylamide gels.

99. Electroblotting from Immobiline Isoelectric Focusing Gels For Direct Protein Sequence Determination

Ruedi Aebersold, David B. Teplow, Stephen B. H. Kent

Isoelectric focusing is the most highly resolving analytical technique available to the protein chemist. However, in the past its usefulness in protein purification for sequencing has been limited because of problems related to the use of carrier ampholytes and recovery of the focused proteins in a sequenceable form. The electroblotting technique can be applied not only to SDS gels but also to proteins/peptides focused in Immobiline isoelectric focusing gels. The electroblotted proteins are cut out and directly inserted in the cartridge of commercially available gas phase sequenators. A variety of proteins ranging in isoelectric points from 4.5 to 8.5 have been successfully isolated from gels containing up to 6 M urea and subjected to picomole-level amino terminal sequence determination. This method provides for the first time a general method for isolation of proteins for microsequencing based on ionic charge, rather than size or hydrophilicity.

100. An Improved Automated Protein Sequenator

Wade M. Hines, Paul K. Cartier, Vincent R. Farnsworth, Stephen B. H. Kent

We have designed and constructed an improved instrument for automated Edman degradation with the goal of developing a flexible research tool for exploring alternative chemistries. The instrument has a fully-programmable controller, based on the VIC20 microcomputer, and any desired chemistry can be entered by means of light pen input to a menu-driven operating system. Different reaction temperatures are available under computer control. The Wittman-Liebold/Caltech zero dead volume valve design was modified to allow solenoid operation without vacuum assist. The same valve design was used for the reagent bottles, with the threaded bottle cap incorporated directly into the KelF valve block. Narrow range, low pressure regulators with flow restrictors were used for greater precision in the metering of small amounts of reagents and solvents. The instrument was designed for easy access to sub-assemblies. For example, the reaction cartridge/conversion flask assembly lifts out of the machine as a single unit. Several sequenators
have been constructed and have been used for: routine quantitative Edman degradation of synthetic peptides and proteins; picomole-level microsequencing of unknown peptides and proteins; solid phase Edman degradation of proteins covalently attached to DITC-glass fiber discs; and development and testing of new chemistries allowing fluorescent detection of the amino acid derivatives from sequencing.

101. Fluorescence Detection in Solid-Phase Sequence Analysis

Ruedi Aebersold, Lloyd M. Smith, Robert J. Kaiser, Stephen B. H. Kent

The current sensitivity of protein sequence determination is still not adequate for the analysis of many proteins whose biological functions are of great interest and importance. We have therefore undertaken the development of a more sensitive method for protein sequence analysis, in which the amino acid derivative is detected by fluorescence methods and which is inherently about $10^4$-fold more sensitive than UV detection. Our approach consists of three parts: 1) the protein sample is covalently attached to the support, in order to allow the use of a wide range of chemical and solvent conditions; 2) a new instrument using a versatile microprocessor-based controller has been constructed, which allows flexible control of the chemistry; and 3) novel Edman sequencing reagents for use with fluorescence detection have been synthesized. Three different chemistries for fluorescent protein sequencing have been developed and evaluated. We are now focusing on one of these and optimizing it for sequencing of proteins in the femtomole range.

102. Off-Line Digital Data Analysis For High Sensitivity Protein Sequencing

Stephen B. H. Kent, Lloyd M. Smith, Peter Hughes, Wade M. Hines, Christopher T. Dodd

We have developed a system for the acquisition, enhancement, and processing of PTH amino acid analytical data. The voltage signal from the HPLC detector is digitized using a 12-bit analog-to-digital convertor, acquired using an IBM PC-XT microcomputer, stored onto hard disk, and copied onto a 5-1/4" floppy disk (about 40 complete PTH chromatograms per disk). Subsequent processing of the data is carried out after the chromatography experiment has been completed. Each PTH chromatogram is analyzed as follows: high frequency noise is removed by digital filtering; peaks are located, and identified by reference to a standard pattern of PTH derivatives; baseline anomalies are removed by a general routine that is independent of reproducibility of the baseline between analyses; PTH peaks are then accurately integrated by non-linear least squares Gaussian fitting. At each stage, the operator can adjust parameters to optimize the analysis. The software package we have developed includes a variety of graphics routines for presenting the processed data to the operator in forms that aid interpretation. For example: baseline-corrected, high frequency-filtered chromatograms can be displayed six at a time; cycles can be normalized and subtracted from one another to highlight changes; histograms of the quantitative PTH data can be printed out. This data analysis system has proven useful for the sequence determination of unknown proteins.

1 Undergraduate, California Institute of Technology.

103. Microscale Structure Analysis of a High-Molecular-Weight, Hydrophobic Membrane Glycoprotein Fraction With PDGF-dependent Kinase Activity

Paul Tempst, David D.-L. Woo, David B. Teplow, Ruedi Aebersold, Stephen B. H. Kent

General methods have been developed for the study of the primary structure of picomole quantities of large, hydrophobic membrane glycoproteins with blocked amino termini. Three techniques have been designed to be used in concert with each other: 1) modified protein preparation and fragmentation techniques; 2) a simple but very selective two-dimensional reverse phase-HPLC system for the resolution of complex mixtures of small to medium size tryptic peptides using Vydac C4, C18, and diphenyl chemistries; and 3) a two-dimensional separation method for large, denatured polypeptide fragments using size exclusion-HPLC combined with either reverse phase HPLC (C4) or SDS-PAGE in conjunction with electroblotting and autoradiography. These methods have been applied to studies of the platelet-derived growth factor receptor.
A Novel Method for Determination of Structural Domains in Proteins By Electroblotting and Direct Sequence Analysis of Peptide Fragments Generated by Limited Enzymatic Cleavage

David B. Teplow, Chikao Nakayama
Rasika M. Harshey, Stephen B. H. Kent

We have developed a general approach to isolate structural domains of proteins and to determine their N-terminal amino acid sequences. The isolated domains can be used in functional studies to determine structure/function relationships. The technique was applied to two proteins of the temperate bacteriophage Mu. These two polypeptides, termed transposase and B, function to efficiently integrate and transpose the Mu DNA within the genome of the susceptible host. These proteins were purified to homogeneity and then subjected to limited proteolysis with chymotrypsin, trypsin, or V8 protease. SDS-PAGE analysis showed that, although of different specificity, all three enzymes produced similar families of peptides, suggesting that susceptible peptide bonds were clustered in, and defined, nonglobular interdomain boundaries. By directly electroblotting from polyacrylamide gels onto chemically derivatized glass fiber filters, we were able to simultaneously prepare the multiple peptide fragments for sequence analysis. The N-terminal amino acid sequence data, together with predictions of hydrophobicity and secondary structure, confirmed our interpretation of the proteolysis experiments. In conjunction with Western blot and DNA-binding analyses, the multi-faceted analytical approach we have taken has allowed us to elucidate the organization and function of the structural domains in these proteins in a simple, direct fashion.

Identification of a Peptide Fragment from the Carboxyl-terminal Extension Region (E-domain) of Rat Proinsulin-like Growth Factor II

Vincent W. Hylka, David B. Teplow, Stephen B. H. Kent, Daniel S. Strauss

A fragment of the carboxyl-terminal extension region (E-peptide) of rat proinsulin-like growth factor II has been purified from medium conditioned by cultured BRL-3A rat liver cells. The fragment, identified by microsequence analysis, was discovered in a biologically active fraction of insulin-like growth factor II (IGF-II). The fragment begins at position 117 in pro-IGF-II, two amino acids downstream from an Arg-Arg potential prohormone processing site. A synthetic analogue of the E-peptide at high concentrations stimulates $^3$H-thymidine incorporation in NIH3T3 hamster cells, raising the possibility that the E-peptide might bind with low affinity to a mitogen receptor. Peptides from the E-regions of pro-IGF-I and pro-IGF-II should be useful for development of radioimmunoassays for measurement of the somatic production of IGF-I and IGF-II, analogous to the radioimmunoassay for the insulin C-peptide.

Difficult Sequences in Stepwise Peptide Synthesis: Common Molecular Origins in Solution and Solid Phase

Stephen B. H. Kent

In both stepwise and segment solution synthesis, the most common difficulty is poor solubility of some protected peptide intermediates. In stepwise solid phase synthesis, the phenomenon of "difficult" sequences has been reported for more than a decade. We have shown that peptides covalently attached to swollen polymeric supports are effectively in solution at a molecular level, and that the interpenetrating polymer network and the covalently attached peptide chains mutually enhance each others solvation. Thus, interference by the resin support is not the explanation for the difficulty in assembling some peptide chains on solid supports. Some protected peptides show a strong tendency to form intermolecular beta-sheet aggregates (i.e., precipitate) rather than interact with the solvent. This tendency is dramatically reduced for peptides attached to swollen polymeric supports but can still affect the course of a synthesis. Observations consistent with this explanation include: the problem is sequence rather than residue specific; it is maximal at chain lengths of about 15 residues and rare at greater than 20 residues, consistent with the known maximal tendencies to form beta-sheet aggregates or adopt helix/random coil conformations; higher loadings of peptide on the resin exaggerate the problem; and the use of better solvents for protected peptides helps minimize the effect. This understanding has been used to design a practical chemistry of considerable generality for the assembly in high yields of long,
protected peptide chains in the chemical synthesis of proteins such as interleukin-3 and transforming growth factor α.

107. Automated Chemical Synthesis of a Protein Growth Factor for Hemopoietic Cells, Interleukin-3
Ian Clark-Lewis, Ruedi Aebersold, Herman J. Ziltener, John W. Schrader, Stephen B. H. Kent

Interleukin-3 (IL-3), a protein of 140 amino acids, was chemically synthesized by means of an automated peptide synthesizer and was shown to have the biological activities attributed to native IL-3. Assays of synthetic analogues established that: 1) an amino-terminal fragment has detectable IL-3 activity, but that the stable tertiary structure of the complete molecule was required for full activity; 2) the cysteine at position 17 was required for activity; and 3) the two different isolated forms of IL-3 have equivalent activity. The results demonstrate that automated peptide synthesis can be applied to the study of the structure and function of proteins.

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108. In vivo Activity of Chemically Synthesized Interleukin-3
John W. Schrader, Ian Clark-Lewis, Herman J. Ziltener, Stephen B. H. Kent

Interleukin-3 (IL-3) prepared by automated chemical synthesis was administered to mice by 11 subcutaneous injections at 8-hour intervals. Spleens of treated mice were increased in weight and contained increased numbers of mast-cell precursors, myeloid colony-forming cells, pluri-potential hemopoietic stem cells, and histologically identifiable mast cells and megakaryocytes. Mast cells were increased in number in the gut wall and in the skin at the injection site. Biologically active synthetic IL-3 was cleared from the serum with rapid kinetics that were similar to those observed with the natural molecule. Chemical synthesis offers a convenient source of large amounts of material uncontaminated by other biological products and should aid in the definition of possible therapeutic applications, the clarification of structure-function relationships and the development of useful structural analogues.

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109. Antibodies of Predetermined Specificity Raised Against Synthetic Peptides Recognize and Neutralize the Bioactivity of Interleukin-3
Herman J. Ziltener, Ian Clark-Lewis, Stephen B. H. Kent, John W. Schrader

Ten peptides that correspond to portions of the T-cell-derived lymphokine interleukin-3 (IL-3) were synthesized, coupled to keyhole-limpet hemocyanin and used to raise antipeptide antibodies in rabbits. These antisera cross-reacted to greatly varying degrees with native, biologically active IL-3. Antibodies directed against peptides corresponding to residues 1-29 at the N terminus and 123-140 at the C terminus of IL-3 and to two selected mid portions of the molecule corresponding to residues 64-82 and 91-112 were affinity-purified using Sepharose beads conjugated with the corresponding peptides. Affinity columns produced from affinity-purified antibodies to the 1-29, 91-112 and 123-140 peptides although not the 64-82 peptide were effective at depleting biologically active IL-3 from conditioned medium. However, only in the case of the column coupled with anti-1-29 antibody was biologically active IL-3 recovered from the column in good yield (98%). Affinity-purified antibodies were also tested for their ability to neutralize the biological activity of IL-3. Antibodies directed to the peptides 1-29 and 91-112 inhibited bioactivity significantly, although with different dose-response characteristics. These were interpreted as indicating that the anti-91-112 antibodies, although of lower affinity for native IL-3 than the anti-1-29 antibody, bound closer to the active site. These experiments demonstrated that antibodies to defined peptides can be used to generate antibodies to native IL-3 and should form useful tools in analyzing structure of native molecules.

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110. Use of Antipeptide Antibodies to Define the Occurrence of Two Naturally Occurring Forms of Interleukin-3 (IL-3)
Herman J. Ziltener, Ian Clark-Lewis, Barbara Fazekas de St. Groth, Stephen B. H. Kent, John W. Schrader

Two forms of murine IL-3, differing by a six amino acid extension at the N-terminal of one form, have been isolated. In order to investigate the natural occurrence of these two forms of this important
molecule, antibodies of predetermined specificity were generated against chemically synthesized peptides corresponding to the unique N-terminal six amino acid extension or to the next 22 amino acids of the IL-3 sequence. Virtually all of the PSH secreted by T cells is recognized by antisera to the six N-terminal amino acid extension, suggesting that the long (1-140) form of the IL-3 molecule is the primary secretion product. In contrast, the IL-3 secreted by the myelomonocytic leukemia WEHI-3B contained a significant amount (25%) of material that failed to bind to anti-1-6 antibodies. The simplest interpretation of these findings is that all of the IL-3 initially secreted has residues 1-6, and that proteolytic cleavage at the Arg₆-Asp₇ bond generates the shorter form.

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111. Synthesis and Characterization of Human Transforming Growth Factor α

David D.-L. Woo, Ian Clark-Lewis, Stephen B. H. Kent

We have synthesized human transforming growth factor α (TGFα), a mitogen for cells of epithelial and fibroblast origin. It is a 50 amino acid protein with three disulfide bonds. The overall yield of chain assembly determined by sequencing was 82% (99.6% per step, average). After refolding by air oxidation, the correctly refolded, biologically active TGFα was purified from the incorrectly refolded molecules and minor by-products of the synthesis by reverse-phase HPLC. The purified TGFα molecule gave a single peak on reverse-phase HPLC and a single band on analytical isoelectric focusing. Furthermore, the synthetic TGFα had the same qualitative and quantitative activity as purified natural epidermal growth factor, a biologically and structurally related hormone. Analysis of proteolytic fragments of human TGFα showed that disulfides are formed in a similar manner to those in epidermal growth factor.

112. Antibodies of a Synthetic Peptide from the Pre-S 120-145 Region of the Hepatitis B Virus Envelope Are Virus-Neutralizing

A. Robert Neurath¹, Stephen B. H. Kent, Karen F. Parker, Alfred M. Prince¹, Nathan Strick¹, Betsy Brotman¹, Phyllis Sproul¹

Our studies with synthetic peptides have provided evidence for the presence of pre-S-coded sequences in the envelope (env) proteins of hepatitis B virus (HBV) and indicated that these sequences are involved in the specific attachment of HBV to liver cells. Scanning of the pre-S sequence for immunodominant continuous epitopes identifies the sequence within residues pre-S (120-145) as one of the two most immunogenic in eliciting antibodies recognizing HBV and as the most efficient for binding antibodies from sera of rabbits and humans immunized with HBV env proteins. To assess the potential of pre-S (120-145) as a synthetic vaccine against hepatitis B, in vitro neutralization of the virus by rabbit antiserum to the peptide was assayed in chimpanzees. The animals, subsequently proven to be susceptible to HBV infection, did not develop hepatitis B as judged by negative serological tests for HBV-associated antigens and antibodies and by normal serum alanine aminotransferase levels and normal liver biopsies. These results establish the role of a pre-S domain in the process of virus neutralization and enhance the potential of synthetic pre-S analogues for hepatitis B vaccination.

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113. Epitope Scanning: Strategies for Determining the Precise Location of Continuous Epitopes Applied to the Pre-S Region of Hepatitis B Virus

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

Denaturation-resistant epitopes coded for by the pre-S region of the env gene of HBV have been implicated in the immune response to hepatitis B infection. We have systematically scanned the open reading frame of the pre-S region to precisely locate these epitopes using a combination of strategies. Synthesis and immunochemical screening of overlapping large peptides covering the entire pre-S region showed that the N-terminal regions (open reading frame residues 12-32, 120-145) of the large and middle envelope proteins contained the two immunodominant continuous epitopes of the virus. Two sets of peptides corresponding to the halves (N terminal, C terminal, and middle) of each region were synthesized. Two families of free peptides of increasing length from the C terminal of each region were generated by automatic sampling of machine syntheses. A novel method for the parallel synthesis of large numbers of free peptides was applied to the preparation of two families
of peptides covering the regions in a moving register of eight residues. These sets of free peptides were screened for antigenic cross-reactivity with anti-(full length peptide) and anti-virion antisera. Microtiter plate and solution immunoassays were used as well as competitive inhibition assays. In addition, a novel method for the parallel synthesis of peptides covalently attached to glass fiber filter discs in the wells of microtiter plates was used to scan the two regions for epitopes, using a moving register of six- and eight-residue peptides. The results obtained with these different epitope scanning methods were compared. The precise locations of the two continuous immunoglobulin-binding epitopes in the pre-S region of the HBV envelope proteins were amino acids 26-32 and 132-137. The antigenic and immunogenic properties of peptides designed from these data have been investigated as an aid to the design of a synthetic vaccine and diagnostics for hepatitis B.

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114. Identification and Chemical Synthesis of a Host Cell Receptor Binding Site on Hepatitis B Virus

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

Hepatitis B virus (HBV) has not yet been propagated in vitro and knowledge concerning its reaction with receptors on target cells is scant. We have located within the HBV envelope proteins a sequence mediating the attachment of HBV to human hepatoma HepG2 cells. A synthetic peptide analog (PLGFFPDHQDLPAFGANSNPDWDFNP) is recognized by cell receptors and also by anti-HBV antibodies and elicits antibodies reacting with native HBV. Antisera raised against the synthetic peptide prevent virus attachment. More significantly, the synthetic peptide itself inhibits HBV attachment to liver cells. This represents the first time that the precise host cell receptor binding site has been located on a virus and mimicked by chemical synthesis. The synthetic peptide is a promising immunogen expected to elicit protective antibodies based on the concept of blockade of the attachment site as a mechanism of virus neutralization. The approach used here, based on anti-peptide antisera and the binding of peptide analogs to cell receptors, is generally applicable for the delineation of cell receptor binding sites on viruses whose gene sequences are known.

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115. Synthetic Peptide Vaccines and Diagnostics based on the Production of Chemically-activated Peptides by Genetic Engineering Techniques

Thomas Kempe1 Stephen B. H. Kent, Flora Chow1, Susan M. Peterson1, Wesley I. Sundquist1, James J. L'Italien1, Douglas Harbrecht1, David Plunkett1, William J. DeLorbe1, Chin Sook Kim1

A vector system has been designed for obtaining high yields of small peptides synthesized in Escherichia coli. Multiple copies of a synthetic gene encoding the neuropeptide Substance P Arg-Pro-Lys-Pro-Gln-Gln-Phe-Gly-Leu-Met-NH2 have been linked and fused to the lacZ gene. Each copy of the Substance P gene was flanked by codons for methionine to create sites for cleavage by cyanogen bromide. The isolated trimeric fusion protein was converted upon treatment with CNBr in formic acid to monomers of Substance P analog, each containing a carboxyl-terminal homoserine lactone (Hse-lactone) residue Arg-Pro-Lys-Pro-Gln-Gln-Phe-Gly-Leu-Hse-lactone. The Hse-lactone moiety was subjected to chemical modifications, including aminolysis to Substance P [11Hse amide]. This method permits synthesis of peptide amide analogs and other peptide derivatives by combining recombinant DNA techniques and chemical methods. In particular, we (SBHK, CSK) intend to use the method to genetically engineer the production of synthetic vaccines.

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116. Automated DNA Sequence Analysis Using Fluorescence Detection

Lloyd M. Smith, Jane Z. Sanders, Christopher T. Dodd1

A prototype DNA sequenator has been constructed (Smith et al., 1986). This instrument uses laser excitation of fluorescence to detect bands of DNA present in polyacrylamide gels. The DNA fragments generated in the enzymatic sequencing reaction are labeled with one of four fluorescent dyes, a different dye being used in each of the four different sequencing reactions. The four reactions are combined in appropriate ratios and co-electrophoresed in a poly
acrylamide tube gel. The fluorescence detector measures the fluorescence at each of four different wavelengths, and this information is stored in a microcomputer. The DNA sequence is inferred from the temporal sequence of DNA bands of different colors electrophoresing through the detector region of the gel. This instrument has been used to sequence known DNAs, and the principle of the method has thus been demonstrated.

Current work on this project is headed in three principal directions. First, the prototype instrument is being improved to increase the reproducibility of the data; this will allow the software for auto-analysis of the data to be developed and the accuracy of the method to be determined and improved. Second, chemistry for the use of the Maxam-Gilbert chemistry in conjunction with the instrument is being developed; this will increase the versatility of the instrument, facilitate automation of the sequencing reactions, and possibly improve the accuracy of the auto-analysis. Third, chemistry for the generation of more highly fluorescent DNA fragments is being developed to increase the sensitivity and resolution of the method.

Reference:

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117. The Chemical Synthesis of Fluorescent Dye-Oligonucleotide Conjugates on a Solid Support

Robert J. Kaiser Jr., Lloyd M. Smith

We have improved the chemistry for preparation of synthetic oligonucleotides conjugated to fluorescent dyes for use as primers in connection with our recently developed automated DNA sequencer (see Abstract No. 116). Overall, the strategy involves the chemical synthesis of an oligonucleotide via the standard phosphoramidite approach which contains the modified nucleoside 5'-amino-5'-deoxynucleoside at its 5' end. After cleavage and deprotection, the product oligonucleotide containing a free amino group at the 5' terminus is reacted with an amino-reactive derivative of the desired fluorescent dye. Initially, these dye-conjugation reactions were carried out in basic, buffered aqueous or aqueous/organic solution and required multiple chromatographic steps to achieve purification. Under these conditions, many dyes are either poorly soluble or rapidly degraded in aqueous solution, resulting in low yields of the desired dye-oligonucleotide conjugates.

We have therefore devised a new synthetic method that utilizes the advantages of solid support-based chemistry. The key feature of this method is the use of the acid-labile monomethoxytrityl (MMT) group for protection of the 5' amino group during the chemical synthesis of the amino oligonucleotide as described above. At the end of the synthesis, instead of cleaving the 5'-MMT-protected amino-oligonucleotide from the solid support, the MMT group is removed with dilute acid to yield the amino-oligonucleotide still attached to the support and ready to react with dye. The dye conjugations are done under basic, anhydrous conditions (20% [v/v] triethylamine in N,N-dimethylformamide [DMF]) using a 20- to 50-fold excess of dye. The use of DMF as solvent allows for much-improved dye solubility and stability, and strictly anhydrous conditions ensure that little if any oligonucleotide is cleaved from the support during the conjugation reaction. Excess dye is then washed from the support using anhydrous DMF and methanol, the dye-oligonucleotide is cleaved and deprotected as usual, and the desired pure product is easily and cleanly obtained after a single reverse-phase HPLC run (Smith et al., 1986).

The new procedure is quite rapid and the yields of pure product are much improved over the solution-phase method. Furthermore, it is easily amenable to automation. Utilized in conjunction with a primer-directed sequencing strategy (Strauss et al., 1986) and the automated fluorescent DNA sequencer, extremely rapid sequencing of large numbers of bases will be possible. The method also can be used to covalently couple other moieties, such as biotin, metal-chelating agents, or intercalating molecules, to an amino-oligonucleotide via an appropriately activated derivative. We are currently pursuing work along these lines. The only disadvantage of the method is that molecules that are incompatible with the oligonucleotide cleavage and deprotection conditions cannot be utilized in this manner. We are currently investigating new cleavage and deprotection conditions in order to increase the general utility of the procedure.
References:

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

Robert J. Kaiser Jr., Lloyd M. Smith

In order to enhance the sensitivity of fluorescence-based DNA sequencing approach (see Abstract No. 115), we are investigating the incorporation of more than one dye molecule into the synthetic DNA sequencing primer in such a manner as to achieve a nearly linear relationship between number of dye molecules per primer and fluorescence intensity. Our strategy is to incorporate multiple reactive amino groups into internal positions of an oligonucleotide to serve as points of attachment for dye molecules.

We have synthesized the 3'-O-phosphoramidite derivative of 5'-O-dimethoxytrityl-2'-N-(N-trifluoroacetylglucyl)-2'-amino-2'-deoxyuridine. This molecule can be incorporated into internal positions of a synthetic oligonucleotide via the standard phosphoramidite chemistry, since the amino group at the 2'-position of the modified nucleoside does not interfere with the construction of the 3',5'-phosphodiester backbone. After normal cleavage and deprotection, the resultant product is an oligonucleotide containing one or more internal amino groups ready to be conjugated with an activated dye derivative. Currently, we are able to routinely achieve 65-85% coupling yields with this phosphoramidite; we are pursuing ways to improve this yield to the 98+% level necessary for the successful incorporation of more than a single 2'-N-glycyl-2'-amino-2'-deoxyuridine (2'-gly-dU) moiety.

We have initially concentrated on oligonucleotides containing a single 2'-gly-dU. These oligonucleotides give good yields of dye conjugates when prepared by solution phase methods. However, the presence of even a single internal 2'-gly-dU severely reduces the hybridization strength of the oligonucleotide with its complementary strand (in which 2'-gly-dU has been base paired with dA), and this reduction is exacerbated in the corresponding dye conjugate. In order to obviate this problem, which is fatal to successful DNA sequencing, we have synthesized a sequencing primer containing a single 2'-gly-dU in a nonhybridizing "tail" 5' to the hybridizing portion of the primer. Such an oligonucleotide conjugated to fluorescein has been shown to be highly effective in Sanger (dideoxy) sequencing. This strategy is now being expanded to oligonucleotides containing more than one dye molecule.

We also are investigating other amino-derivatized nucleosides as alternatives in the above strategy.

119. Automated Fluorescence Maxam/Gilbert Sequencing

Jane Z. Sanders, Robert J. Kaiser Jr., Lloyd M. Smith

The automated fluorescence DNA sequencer (see Abstract No. 116) 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 fluorescent sequence analysis.

The Maxam/Gilbert sequencing method relies on base-specific chemical degradation and strand cleavage to produce a nested set of DNA fragments. These reactions can be carried out while the DNA to be sequenced resides on a solid support, attached either by ionic attraction or by covalent bonding. The use of a solid support eliminates much of the time-consuming ethanol precipitation and lyophilization involved in the usual procedure and offers a route to automation of the process.

We are investigating a solid phase approach to fluorescence-based Maxam/Gilbert sequencing. In this approach, an oligonucleotide containing a fluorescent dye molecule conjugated to its 5' terminus and an internal reactive free amino group will be covalently attached through the amino group to a solid support via a long molecular "tether." This tether will incorporate a cleavage link somewhere in its length that is stable to the chemicals used in the sequencing procedure but cleavable subsequent to fragment generation. The oligonucleotide will then be hybridized to a template containing the DNA to be sequenced and employed as a primer to enzymatically synthesize the complementary strand of the unknown sequence. After thorough washing of the support to remove template, nucleotide triphosphates and enzyme, the support-
bound DNA will be subjected to chemical degradation and cleavage, leaving a nested set of fluorescent fragments bound to the support. The fragments can then be cleaved from the support and electrophoresed to obtain sequence information.

We are currently synthesizing the requisite oligonucleotides using the phosphoramidite derivatives 5'-monomethoxytrityl-5'-amino-5'-deoxythymidine and 5'-O-dimethoxytrityl-2'-N-(N-trifluoroacetylglucyl)-2'-amino-2'-deoxyuridine to incorporate two amino groups distinguishable by the acid/base lability of their protecting groups. This allows the introduction of a fluorescent dye on the 5' end of the synthetic oligonucleotide via solid phase dye conjugation (see Abstract No. 117) and will produce a fluorescent dye-oligonucleotide conjugate containing a free amino group subsequent to cleavage and deprotection (see Abstract No. 118). We are also derivatizing a variety of solid supports with tethers containing a cleavage linkage and an amino reactive end for covalent attachment of the fluorescent primer. Work has also been undertaken to determine the effects of the chemicals used in sequencing on the fluorescent dyes utilized in the fluorescence sequencing technique.

120. Model Substrate for Leader Peptidase: A Synthetic 73 Amino Acid Residue Protein, M13 Procoat

Suzanna J. Horvath, John M. Tomich 1, Michael R. Emerling 1, Ron H. Sweet

The "membrane trigger hypothesis" (Wickner, 1979) suggests that for certain pre-proteins the presence of a leader sequence confers a water-soluble conformation upon secretory or membrane proteins during and after synthesis in the cytosol. The bulk of the information needed to bring about secretion through or insertion into membranes is believed to be encoded in the tertiary conformation induced by the leader sequence. Another feature of this hypothesis is the requirement that the leader sequence be removed enzymatically by leader peptidase to make the process irreversible. While several of the steps of this hypothesis have been identified, the precise nature of the events remains to be elucidated. M13 procoat produced in a cell-free system has been used as a substrate in leader peptidase assay (Zwizinski and Wickner, 1982) yet it can only be isolated in minute quantities. Utilizing an improved manual stepwise solid phase chemical synthetic method, we have successfully synthesized the entire procoat protein (73 amino acids in length) in gram quantities. There are several problems associated with the synthesis of large peptides. These problems generally interfere not with our capacity to build the correct sequence, but rather with our ability to isolate the product in the correctly folded conformation. The procoat sequence contains no cysteine residues and therefore no disulfide bonds which might complicate refolding. Simply denaturing and renaturing (through use of heat or chaotropic reagents) in the presence of various detergents failed to produce an active substrate for leader peptidase. However, refolding under conditions that permit isomerization of proline residues resulted in a biologically active product. The refolded-isomerized peptide is cleaved by E. coli leader peptidase at the amide linkage between ala23-ala24 forming the mature coat protein (50 residues in length) and the leader peptide (23 residues in length). The hydrolysis of procoat protein can be followed kinetically due to the introduction of a radioactive leucine residue of high specific activity at position 16.

Our ability to produce large quantities of this biologically active synthetic model substrate should greatly facilitate studies in membrane-protein interactions and crystallization of a pre-protein.

References:

1 This research project was carried out in collaboration with Prof. John H. Richards' group from the Division of Chemistry and Chemical Engineering, California Institute of Technology.

121. Application of Chemically Synthesized Deoxyoligonucleotides in Biological Research

Christine A. Hill, Suzanna J. Horvath

We have continued our effort to provide hundreds of synthetic deoxyoligonucleotides for a large variety of research projects at Caltech. In order to further encourage new, creative utilization of synthetic DNA in biological research, we have introduced the synthesis of DNA probes containing deoxyinosine residues. Deoxyinosine base pairs with A, T and C bases (Ohtsuka et al., 1985; Takashi et al., 1983). Thus, deoxyoligonucleotide probes with
deoxyinosine at ambiguous codon positions can be the best choice as hybridization probes for cloning genes for proteins containing amino acids with highly degenerate codons.

We have improved the synthesis of long DNA fragments (up to 90 bases) by using β-cyanoethyl phosphoramidites instead of methyl phosphoramidites. It is known that certain side reactions such as base modification of G residues, which leads to chain branching and methylation of T residues, can occur and become more significant as the oligonucleotide grows in length (Pon et al., 1985; Urdea et al., 1985). Recent studies showed that using the traditional cyanoethyl protecting group, these side reactions are less significant. We have studied reaction conditions of β-cyanoethyl phosphoramidite couplings and we have been working to optimize synthetic cycles for long deoxyoligonucleotide synthesis. Long deoxyoligonucleotides are mainly used for synthetic gene assembly. Changes also have been made regarding the scale of synthesis: short DNA primers for rapid primer-directed DNA sequencing are routinely made on 0.2 µM scale; and long DNA fragment synthesis is usually carried out on 1 µM scale; and 10 µM, large scale synthesis has been tested for affinity column preparation.

References:

PUBLICATIONS


Associate Professor: Elliot M. Meyerowitz

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Summary: The goal of our research is to understand the molecular mechanisms of development in multicellular organisms. Two developmental processes concern us: the process by which individual cells recognize their physical and temporal location in a developing organism, and the process by which the cells use positional and temporal information in the regulation of gene expression. We use two organisms in our experiments, the fly Drosophila and the flowering plant Arabidopsis.

Previous work in this laboratory has shown that Arabidopsis, a diminutive member of the mustard family, is uniquely suited for certain types of recombinant DNA experiments. Among the properties of this flowering plant are an extremely small nuclear genome (70,000 kilobase pairs, less than 20 times the size of the genome of the bacterium E. coli) and the near absence of dispersed repetitive DNA. These
properties allow gene cloning and chromosome walking experiments to be performed with great facility and speed. This year we report further progress in the characterization of developmentally-regulated genes and genes important in pattern formation in floral development, as well as the development of new techniques for the study of these genes.

Our *Drosophila* studies are primarily concerned with the hormonally-regulated 68C gene cluster. This cluster contains three genes expressed as a result of steroid hormone induction; in addition to the steroid regulation, the genes are regulated in their tissue and time of developmental expression. The work of the last year has included characterization of the DNA sequences required in *cis* for appropriate expression of all three genes, analysis of genes whose products are required in *trans* for gene cluster regulation, and an analysis of the relation between expression of these genes and the chromatin state change known as puffing. In addition to these studies, work has continued on the structure and regulation of a rhodopsin gene expressed in the R7 photoreceptor cell of the *Drosophila* eye.

122. Characterization of Genes Abundantly Expressed in Seeds of *Arabidopsis thaliana*

Patty P.-Y. Pang

The cloning and preliminary characterization of genes that are abundantly expressed in developing seeds in *Arabidopsis thaliana* have been reported previously (see Biology 1985, Abstract No. 132). These genes can be classified into four groups by their restriction map and their ability to cross-hybridize to one another under normal hybridization stringency conditions. Three of the classes hybridize to abundant seed RNA species of approximately 1700 nucleotides and the final class to an RNA species approximately 650 nucleotides in length.

The expression of these four genes during seed development has been analyzed on RNA gel blots. The developmental profiles of the messages of the three large genes are very similar. RNA can be detected about day 9 after pollination and its level peaks at about day 12. The smaller message appears about day 6, peaks about day 8, and is undetectable at day 11. Seeds usually take about 14-15 days to mature.

Of the three *Arabidopsis* large seed-specific genes, only one cross-hybridizes to the *Brassica napus* 12S storage protein gene. This *Arabidopsis* gene has been sequenced and it shares extensive homology with the *Brassica* gene (R. E. Pruitt thesis, 1985). Determination of the DNA sequence of a second large seed-specific gene is underway and preliminary results suggest that this second gene is about 70% homologous to the 12S storage protein on the DNA level and about 75% on the amino acid level. In order to determine whether the third large seed-specific gene belongs to this distantly-related gene family, a 32P-labeled 12S RNA probe made from a SP6 vector was used to hybridize to DNA fragments of the three large seed-specific clones which had been blotted onto a nitrocellulose filter. Both the hybridization and the wash were done at extremely low stringency. Under these conditions, weak homology was detected with the partially sequenced clone and even weaker homology with the third gene, while no cross-hybridization was detected with the *Arabidopsis Adh* gene. The initial observation that the 12S storage protein gene is present only in single or two copies per haploid genome in *Arabidopsis* is difficult to reconcile with the high heterogeneity of the 12S storage proteins seen in two-dimensional gels (M. Crouch, Indiana University, personal communication). Our recent results suggest that there are at least three genes that code for 12S-like seed storage proteins in *Arabidopsis* that cross-react with each other only under very low stringency hybridization conditions. This can explain the high complexity of the 12S seed storage proteins and implies that similar distantly-related 12S protein genes may be found in other plants.

123. Mutations Affecting Floral Development in *Arabidopsis*

Elliot M. Meyerowitz

A number of laboratories have reported *Arabidopsis* mutations that cause the flowers to appear abnormal. We have obtained a number of lines bearing these mutations and have found that several of them have very specific developmental effects on floral morphogenesis. For example, the mutant *pistillata*, obtained from M. Koornneef of the University of Wageningen (Netherlands), has antherless filaments in place of normal stamens and petals converted to sepals. *apetala-2*, also from Koornneef, shows a conversion of
petals to stamens and a conversion of sepals to something like leaves. As a preliminary characterization of these mutants, which can properly be termed homeotic variants (as first defined by Bateson in 1894), I have made a number of double mutant combinations. An example of a result is the strain homozygous for both of the mutations described above, which has flowers with two whorls of leaves and no petals or stamens surrounding a normal ovary. This result indicates that pistillata is epistatic to apetala-2 in the determination of the fate of those cells that would, in wild-type plants, become petals. A simple hypothesis is thus that the wild-type product of the pistillata gene, which prevents petal precursor cells from differentiating into sepals, is required before the product of the apetala-2 gene, which prevents petal precursor cells from becoming stamens, in normal development. Molecular cloning of these genes may help clarify their wild-type function.


124. Restriction Fragment Length Polymorphism Mapping of the Arabidopsis Genome
Caren Chang, Arthur W. DeJohn
We are continuing the construction of a genetic map of restriction fragment length polymorphisms (RFLPs) in Arabidopsis thaliana as described by Robert E. Pruitt (Biology 1984, Abstract No. 92). The work involves two stages. One is to identify clones that are polymorphic in their restriction fragment lengths between the two parental strains of A. thaliana. The other is to analyze the linkage of the RFLPs by hybridizing radiolabeled polymorphic clones to filters containing restriction digests of seven of the genomic F3 DNA preparations.


125. Genetic Transformation of Arabidopsis thaliana
Caren Chang
We are interested in being able to genetically transform A. thaliana and to demonstrate with this technique that the cloned Arabidopsis alcohol dehydrogenase gene (Chang and Meyerowitz, 1986) can restore activity to an Arabidopsis alcohol dehydrogenase null mutant. The approach makes use of the natural Ti plasmid transformation system of the soil bacterium Agrobacterium tumefaciens (Caplan et al., 1983). In this system, a particular portion of an engineered Ti plasmid becomes transferred and stably integrated into the plant genome. One protocol of transformation that has been successful for several dicot plant species is the leaf disc transformation procedure (Rogers et al., 1986) in which wounded leaves are infected with agrobacteria containing the Ti transformation vector. With in vitro culturing, the infected leaf explants produce callus tissue which frequently gives rise to shoots. The shoots can be excised from the callus and regenerated into mature, whole plants.

The Ti-based transformation vector we are using contains a resistance gene for hygromycin B, which is an antibiotic lethal to plants. We have inserted in this vector the Arabidopsis alcohol dehydrogenase gene, including the DNA approximately 4.5 kb upstream and 3 kb downstream of the gene. Using the leaf disc transformation procedure, we have obtained hygromycin B-resistant calli and shoots. The next step is to regenerate stably transformed plants from these shoots and screen their progeny for alcohol dehydrogenase activity.
References:

126. Regulation of Glue Gene Expression: What Are the Factors Involved and Where Do They Act?

Peter H. Mathers

The third chromosome of Drosophila melanogaster contains a cluster of three glue genes at position 68C that are expressed in a tissue- and stage-specific manner. The developmental expression of these three genes is known to be coordinately regulated by at least two factors: the steroid hormone ecdysterone and the product of the 2B5 locus on the X chromosome (see Biology 1985, Abstract No. 121). Ecdysterone has been shown to be required for both the induction of glue RNA accumulation and its repression later in development. Mutations at the 2B5 locus, such as the l(1)npr-1 mutation, appear to be blocked in development at a stage just prior to induction of glue RNA synthesis and therefore accumulate no detectable levels of Sgs-3, Sgs-7 or Sgs-8 RNAs. Because the 2B5 locus is ecdysterone inducible and both ecdysterone and the 2B5 product are required for RNA accumulation, it is possible that the requirement for hormone is manifested through its inductive effect at 2B5.

In an effort to elucidate the action of these factors on gene expression at 68C and to determine their site(s) of action, studies have looked at expression of altered glue genes, which have been reintroduced into the fly by P element-mediated transformation, and how they are affected by mutations that eliminate the activating factors. Using the l(1)npr-1 mutations, we have shown that the product of the 2B5 locus or a factor induced by it is interacting within a region from -130 base pairs upstream of Sgs-3 to a site approximately 1 kilobase downstream of the RNA start site and within the Sgs-3 coding region. Experiments in progress are designed to distinguish whether the 2B5 product is acting upstream of the RNA initiation site or downstream and whether the mutation affects RNA synthesis or stability. In addition, a mutation in ecdysterone biosynthesis, l(1)su(f)²₉⁷g, has been used to delimit the site of hormone action to a region from 2.3 kilobases upstream of Sgs-3 to within the coding region. By further delimiting the sequences necessary for proper glue gene expression, we hope to determine the cis-acting sequences required for interaction with known trans-activating factors and to understand how these factors regulate developmental gene expression.

127. Studies on Sgs-3 Gene Regulation

K. Vijay Raghavan, Madeline A. Crosby, Peter H. Mathers

In continuation of work reported in the last year's report, we have established in three different ways that 130 base pairs of 5' flanking sequence is sufficient for the correct tissue- and stage-specific expression of Sgs-3. First, when an Sgs-3 gene with 130 bp upstream of the mRNA cap site is cloned into a P-element vector with alcohol dehydrogenase as the selectable marker and germline transformant flies obtained, the introduced gene is expressed in the salivary glands of third instar larvae as detected by RNA blot hybridization at levels of about 10% of the endogenous. Second, the same insert in a vector with the rosy gene product as the selectable marker expresses at the correct stage and tissue at similar levels. Finally, an Sgs-3 lacZ fusion gene with 130 bp of 5' flanking Sgs-3 sequence in a rosy vector is expressed in the salivary glands of third instar larvae as seen by both histochemical staining for ß-galactosidase activity and by RNA blot procedures. Animals that carry the Sgs-3 gene with 130 bp of 5' flanking sequence and are hemizygous for the X-linked mutation l(1)npr-1 do not express RNA from either the endogenous or the introduced gene as examined on RNA blots.

128. Sequences Required for Regulated Expression of the Sgs-8 Gene

Elliot M. Meyerowitz

In last year's annual report we reported experiments in which an E. coli ß-galactosidase gene was fused in-frame to the Drosophila Sgs-8 gene, with both 5' upstream and 3' downstream sequences from Sgs-8 surrounding the fusion gene. These surrounding sequences caused normal tissue and developmental time of expression of the fusion construct after its transformation into the Drosophila genome. In the past year, further transformants of related constructs have been obtained, including constructs in which the
3' downstream Sgs-8 sequences have been removed entirely. After introduction to the fly genome, these sequences direct proper expression of the fusion gene (and, consequently, production of a bacterial enzyme in the fly larval salivary glands). From this it can be concluded that sequences downstream of Sgs-8 are not necessary for its normal regulated expression, and that all of the required sequences are either just upstream of, or within, the gene.

129. Fusion Gene Manipulations of the Drosophila melanogaster Sgs-8 and Sgs-7 Genes

Mark D. Garfinkel

The two small glue protein genes at 68C, Sgs-8 and Sgs-7, are oriented oppositely with their 5' ends separated by 475 base pairs. Analysis of the functional content of this region is proceeding with a series of fusion gene constructions that modify both of these genes. The first modification is described in the preceding abstract: the 5' flanking sequences of Sgs-8 and an amino-terminal segment of this gene were attached in-frame to the E. coli β-galactosidase enzyme-coding region (lacZ). The second modification was the fusion of the 5' flanking sequences of Sgs-7 and its 5' untranslated region to the 5' untranslated region of the D. melanogaster alcohol dehydrogenase gene (Adh). The glue protein intergenic region was reassembled by restriction and ligation of appropriate DNA molecules; the 475-base-pair region is now attached to two different structural genes, each of which encodes a readily-assayed enzyme.

Germline transformants of the Sgs-8-lacZ, Sgs-7-Adh fusion gene pair were obtained in a D. melanogaster host strain that is homozygous for null mutations at the Adh locus and at the ry locus. A minimum of three independent germline transformation events were obtained. Histochemical staining of third instar larvae descended from three different primary transformants revealed β-galactosidase activity, derived from the Sgs-8 promoter, in the salivary glands only, as seen previously (Biology 1985, Abstract No. 129). Two of these transformant lines were tested for and showed Adh activity derived from the Sgs-7 promoter, in third instar larval salivary glands.

These results form the initial observations for a dissection by in vitro mutagenesis of the 475 bp region to determine the extent of cis-acting DNA sequences required for regulated expression of both the Sgs-7 and the Sgs-8 genes. Convenient restriction sites enable the intergenic region to be manipulated easily, and the histochemical enzyme assays permit each in vitro-synthesized mutation to be tested for both genes' function. Use will be made of both germline transformation and transient expression procedures.

The existing germline transformants will be tested for their response to chemicals that have been used to select either for or against Adh enzyme activity. Following mutagen treatment, the selective agents should permit the recovery in vivo of mutations, either in cis or in trans, that perturb glue gene regulation.

130. Evidence for the Cell Autonomous Regulation of Sgs-3 by l(1) npr-1

K. Vijay Raghavan, Carol A. Mayeda, Elliot M. Meyerowitz

The product of the 2B5 locus as defined by the loss-of-function mutation l(1)npr-1 is required for either the synthesis or stabilization of freshly synthesized RNA from the 68C locus (Crowley et al., 1984). We had shown earlier that an Sgs-3-lacZ fusion gene with 2270 bp of sequence 5' of the Sgs-3 mRNA start site, when transformed into the germline of flies, expresses the fusion protein in the salivary glands of third instar larvae (as detected in a histochemical assay for β-galactosidase activity), and that when transformant animals are also hemizygous for the mutation l(1)npr-1, there is no detectable activity for β-galactosidase activity in the salivary glands of third instar larvae.

We have now examined, using the above Sgs-3-lacZ gene in a histochemical assay, the phenotype of l(1)npr-1 in clones of cells. Female flies that were homozygous for an Sgs-3-lacZ transgene on the third chromosome and carried an unstable Ring-X chromosome were crossed to males that were l(1)npr-1. Such males survived since they carried a wild-type copy of the npr-1 region as a duplication on the Y chromosome. The l(1)npr-1 chromosome also carried the additional markers y (yellow), w (white) and mal (maroon like). Fertilized eggs from the above cross sometimes lose the Ring-X chromosome at the first nuclear division; the animals into which such embryos develop are part XO (phenotypically male, and expose
the recessive markers on the $l(1)npr-1$ chromosome) and part XX (phenotypically female). We examined the salivary glands of third instar larvae that were gynandromorphs to see if we could detect mosaicism in the pattern of staining for $\beta$-galactosidase activity. In half body mosaics for $l(1)npr-1$, we see cases in which one lobe stains for $\beta$-galactosidase activity and the other does not. These results indicate that it is the genotype of the lobes, whether or not they are $l(1)npr-1^+$, that decides whether they stain, and that half the animal being wild type is not sufficient for expression of the Sgs-3-\textit{lacZ} gene product in all the cells of the salivary glands. Evidence for cell autonomy comes from the examination of another category of mosaics in which it is inferred that the male-female dividing line has passed through a lobe. Such lobes, in which some cells stain and the others do not and in which clearly unstained cells border strongly stained cells, argue for the cell autonomous regulation of Sgs-3 by the $l(1)npr-1^+$ product.

Reference:

131. A Transient Assay for Regulated Expression of Sgs-3

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

Studies of the Sgs-3 gene utilizing germline transformation have defined the sequences required in \textit{cis} which are sufficient to allow correct stage- and tissue-specific expression of Sgs-3. Further analysis of the specific nucleotides required will necessitate testing of many potentially informative constructs generated in \textit{vitro} for regulated expression. To do this using the technique of germline transformation could be a very lengthy process. Recent reports have indicated that copies of the \textit{Drosophila} alcohol dehydrogenase gene (Martin \textit{et al.}, 1986) and Sgs-5 glue gene (G. Guild, personal communication) injected into embryos persist as supercoiled and nicked circles and are expressed later in the development of the injected animal with the correct stage and tissue specificity. Such a "transient" assay could greatly facilitate the testing of genes with altered sequence for alterations in expression pattern.

A preliminary series of injections to test the feasibility of using such an assay with the Sgs-3/\textit{lacZ} gene has been carried out. DNA of a plasmid containing an Sgs-3/\textit{lacZ} fusion gene with 2.3 kb of 5' flanking sequence was injected into preblastoderm-stage embryos. Those animals that developed to the third instar larval stage were dissected and their salivary glands stained histochemically for $\beta$-galactosidase activity. Control glands of this strain never show staining in the conditions of the assay, yet 80% of the glands of injected animals showed at least some cells that stained blue. Staining was not seen in the cells of other tissues or at other stages tested. This result indicates that the plasmid DNA persists and is expressed correctly. This technique should therefore allow us to quickly test many sequence alterations, including deletions, inversion, and point mutations, for their effects on expression.

Reference:

132. Evolution of the Sgs-3 Glue Gene in Species Related to \textit{D. melanogaster}

Christopher H. Martin, Elliot M. Meyerowitz

The three glue genes at 68C are members of a related gene family. The largest of the three, Sgs-3, differs from the other two by the presence of a central region consisting of 37 tandem repeats of five amino acids. Sequences sufficient for proper tissue and time of expression have been found to lie within 130 base pairs of the transcription start site. The Sgs-3 homologous genes from three other closely related \textit{Drosophila} species (\textit{D. erecta, D. yakuba} and \textit{D. simulans}) were used to approach the following questions. How does the repeat region evolve? What regions of the protein are highly conserved (and thus likely to be necessary for proper function)? Are any regulatory sequences sufficiently conserved to make their positions obvious among a background of less conserved, non-essential, sequences? Will the genes be expressed properly in a different species?

Sequencing of the three Sgs-3-like genes has shown that the general structure of the gene is conserved: a conserved hydrophobic leader, interrupted by an intron, followed by a repeat region and then by a highly conserved carboxy-terminal region. The repeat region itself seems to be evolving rapidly. It differs greatly among the species in length, protein sequence,
and DNA sequence. The region upstream of the gene known to be involved in regulation is highly conserved; sequences further upstream are often found to be much more variable among the species. In several instances, deletions have removed hundreds of base pairs of sequence upstream of the Sgs-3-like gene.

The Sgs-3-like genes from *D. erecta* and *D. yakuba* have been stably integrated into the genome of *D. melanogaster* by P factor-mediated transformation. These introduced genes are expressed in the proper tissue and time at moderate-to-high levels. The ability of these genes to be properly regulated in the background of another species allows the use of sequence comparisons to help locate possible sites of action of trans-acting factors.

133. Characterization of a Boundary between Conserved and Non-conserved Sequences

Christopher H. Martin

The site of a dramatic change in the rate of DNA sequence evolution exists near the 68C glue gene clusters of several *Drosophila* species. The presence and approximate location of the transition site was determined by comparison of the restriction maps of the regions surrounding the 68C-like glue gene cluster of five members of the *melanogaster* species subgroup (Meyerowitz and Martin, 1984). In order to learn more about the boundary between the two regions and about any possible function of the conserved region, the DNA sequences of the boundary regions from three members of the *melanogaster* species subgroup were determined. The relatively non-conserved region contains the glue gene cluster and appears to be evolving by a number of mechanisms: point mutations, insertions and deletions, inversions, duplications and the gain or loss of repetitive sequences. In contrast, the conserved region appears to evolve through relatively infrequent point mutations and small insertions and deletions. Using a best fit alignment of these sequences, the site of the transition from slowly to rapidly evolving sequences has been found to occur abruptly within a region fewer than 50 nucleotides in length. While the frequency of base substitution undergoes a five to tenfold change across this boundary, the frequency of insertion/deletion events remains approximately the same.

The slowly evolving sequences are of unknown function; no transcripts are known to originate from the conserved region. Furthermore, no large open reading frames were found in the sequenced portions of the conserved regions. These sequences also are not likely to be essential to the regulation of the nearby glue gene cluster: P factor-mediated transformation experiments of the glue gene cluster using constructs not containing any sequences from the conserved region have shown normal tissue, time, and level of expression. Thus, the basis for the high level of sequence conservation seen in the conserved region is unknown.

References:

134. The Opsin Genes of *Drosophila*

Karl J. Fryxell

In the previous year, I reported the isolation of seven "eye-specific" (or optic lobe-specific) genomic clones (Biology 1985, Abstract No. 130). In this year, I have shown that these seven clones encode six different genes, and one of these six genes (nDm6030) hybridizes with oligonucleotides representing conserved regions of the rhodopsin gene. nDm6030 is not labeled by a 32P-cDNA probe derived from adult heads of the *Drosophila* mutant sevenless (this mutant was discovered in Prof. Seymour Benzer's lab), but five other eye-specific genes are labeled by this probe. Thus, nDm6030 encodes an opsin that is expressed only in the R7 photoreceptor cell. By methods similar to those described above, I have also cloned *ninaE*, an opsin gene that is expressed specifically in R1–6 photoreceptor cells, as well as additional putative opsin genes.

A 2.6 kb region of nDm6030 has been sequenced, including the entire transcribed region, 5' and 3' flanking sequences. The transcript appears not to have any introns, since there are no consensus splice donor sites, no interruptions of the open reading frame, and no obvious insertions with respect to the coding sequence of other opsin genes. Conceptual translation of the coding sequence predicts a protein of 41,124 daltons, with seven transmembrane-spanning segments, a lysine residue in the middle of the seventh transmembrane segment, a serine- and threonine-rich carboxy-terminus, and other characteristics of the
opsin gene family. The overall homology to ninaE, the major rhodopsin gene of *Drosophila*, is rather low (37% amino acid identity). Since amino acid substitutions in mammalian rhodopsin evolve infrequently, this suggests that at least two opsin genes (and possibly color vision) were present early in the arthropod lineage. The conserved amino acids are clustered near the cytoplasmic surface of the rhabdomere membrane, a fact that was not evident in previous sequence comparisons.

Dipterans have two kinds of R7 photoreceptor cells (R7γ and R7p) with different action spectra, and it will be interesting to see if one or both of these cell types expresses nDm6030. Similarly, Dennis Ballinger has isolated mutations affecting *Drosophila* color choice behavior that map close to nDm6030 (on the third chromosome), and it will be interesting to see if these mutations reside within nDm6030. Finally, P factor-mediated transformation will allow me to find the differences in regulation between nDm6030 and ninaE, a problem of fundamental importance.

135. **Subtractive Hybridization of Tiny Tissues**

*Michael J. Palazzolo*

The isolation of tissue-specific genes in *Drosophila*, where subsequent genetic analysis is possible, would allow experiments aimed at understanding the physiology of differentiated cells. Subtractive hybridization is the most sensitive technique for identifying mRNA molecules expressed in one tissue and not another. However, this procedure requires large amounts of poly(A)* mRNA. Since most dissectable tissues in the fruit fly are quite small, this requirement effectively limits the use of standard subtractive procedures.

We have recently developed a new methodology that allows the amplification of mRNA sequences. Use of this technology should greatly facilitate attempts to clone tissue-specific genes in tissues that are not available in large amounts. Plasmid vectors containing specific bacterial RNA polymerase promoters have been shown to be useful for the in vitro synthesis of large amounts of single-stranded RNA sequences. A family of λ cDNA cloning vectors (SWAJ) has been constructed using these same promoters. In these vectors, two asymmetric and unique restriction sites have been positioned between two different promoters. A simple cloning scheme, based on an oligonucleotide primer-adapter, allows high efficiency, directional cDNA cloning into these vectors (see Figure 1). DNA prepared from these libraries can be used as a template for the selective amplification of sense or antisense sequences. These single-stranded cRNA molecules, which can be prepared in large amounts, should be useful in subtractive hybridization procedures.

The first advantage of this SWAJ system is that not as much dissected tissue is required for the use of subtractive hybridization procedures. For example, a representative cDNA library (more than a million individual clones) can be constructed from the poly(A)* mRNA from a thousand imaginal discs. cRNA from this library can then be used in an essentially unlimited number of subtractive hybridizations. In contrast, using standard procedures, more than 200,000 discs would be necessary for a single subtraction.

Another advantage of this system is that both ends of the cRNA molecules contain defined nucleotide sequences so that cDNA copies can be generated from the cRNA sequences. Thus, it should be possible to simply construct a cDNA library enriched in subtracted, and therefore, tissue-specific clones. In standard subtractive hybridization procedures, typically only a small fraction (less than 5%) of the radioactivity is incorporated in the tissue-specific sequences. With the family of SWAJ vectors, it is simple to synthesize single-stranded, high specific activity probes directly from a subtracted library in which essentially all of the radioactivity is introduced into the tissue-specific clones. This should make it easier to identify rare tissue-specific sequences.

*In situ* hybridization to tissue sections requires high specific activity antisense sequences from individual clones. A third advantage of SWAJ is the simple preparation of such probes. Since all clones are directionally inserted behind a polymerase promoter, there is no need to determine orientation or subclone. The cDNA clones can be used to directly screen for temporally- and positionally-specific transcription.

The final advantage is the ease of DNA sequence analysis available in SWAJ cDNA clones. The sequence at each end of the cRNA molecule is defined and both sense and antisense sequences can be prepared from each clone. Using appropriate oligonucleotide primers, dideoxynucleotides and reverse transcriptase, it should
be possible to Sanger-sequence both strands without restriction mapping or subcloning.

**FIRST STRAND SYNTHESIS USING PRIMER ADAPTER**

**SECOND STRAND SYNTHESIS**

**SACI LINKERS**

**XBAI DIGESTION**

**SACI DIGESTION**

**Figure 1.** A SWAJ directional cDNA cloning scheme. The left side of the diagram describes the synthesis of double-stranded cDNA. The Amersham cloning kit was used for the initial steps, with the exception that a chemically synthesized oligonucleotide primer-adapter was used instead of the oligo(dT) provided in the kit. This primer is a single-stranded sequence containing the recognition site of the enzyme XbaI upstream of an oligo(dT) sequence (CCAGTCACTCTAGATTITTTTITTTT). It is represented in the diagram by the boxed X. The oval S symbol represents SacI linkers. The right side of the diagram illustrates restriction of the vector and ligation to double stranded, asymmetric cDNA.

**PUBLICATIONS**


**Summary:** I continue with various aspects of the program in developmental biochemistry that has been in progress for the past several years. The primary system makes use of the morphogenesis of wings during pupal stages of Drosophila, a system that was developed here in collaboration with Dr. Nancy S. Petersen. This collaboration continues and I am concerned primarily with the purification and properties of the major proteins that enter into the construction of cell epicuticle and cell hairs. The biochemistry of the heat shock protection phenomenon is under extensive consideration also.

136. Autoproteolysis of Some Eukaryote Proteins

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

Several RNA viruses transcribe and then translate the entire genome in one piece. The large protein product is then cut into useful polypeptides by protease action upon itself. We presented evidence recently that the 70 kd heat shock protein of Drosophila and of a mouse cell line also undergo autoproteolysis. We have now extended the study to proteins in general which are abundant and concerned with processes of differentiation in Drosophila. At least 15 proteins have apparent autoproteolytic properties. One is actin and another appears to be a precursor of epicuticle. The latter, which is the impervious layer in the arthropod exoskeleton, is formed by secretion outside the epithelial cells followed by processing to produce two or three layered sheets. Self-processing by autoproteolysis may be involved. These results have been submitted for publication in Science.

¹Department of Microbiology and Biochemistry, University of Wyoming, Laramie.

137. Phenocopies and Morphogenesis

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

We have continued work using a combination of mutants and heat shock phenocopies of Drosophila to evaluate time sequences in morphogenesis. Studies on the mutants forked, singed and multiple wing hair as well as wild type have shown that wing hairs are extruded to about full size at 32 hr after pupariation whether they are single (normal) or multiple (mwh mutant). Branching occurs in all at 38 hr and waviness is induced at 43 hr in wild type. Waviness occurs in the forked and singed mutants. Mutant gene expression (of the recessive mutants) can be induced in several situations in heterozygotes by heat shock when they do not show by the same treatments of wild types. Data of forked have been published and those on multiple wing hair have been submitted for publication.

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**PUBLICATIONS**


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- National Science Foundation
- Weizmann Fellowship
Summary: Our laboratory has focused on two primary areas of research. We are interested in understanding the mechanisms that generate heritable patterns of gene expression and in the mechanisms that cells use to process information and signals provided by their environment.

One mechanism for regulating gene expression in a heritable fashion involves gene rearrangement. A variety of organisms, including pathogens, use gene rearrangement to vary the nature of their surface antigens. We have been studying the details of the site-specific recombination system that controls flagellar phase variation in *Salmonella*. This system has turned out to be relatively complex. In addition to the recombinase that mediates DNA inversion, other proteins that bind at sites distant from the recombination site are required to enhance the rate of recombination. The interaction among enhancer, recombinase and the HU, histone-like protein provides us with an interesting model system in which to study the role of DNA binding proteins in regulating site-specific recombination and by analogy their role in other reactions that involve specific DNA substrates.

We are continuing our efforts to develop a system that would allow us to study the events that regulate differentiation in meiotic cells in the mouse and eventually the mechanisms involved in recombination during meiosis. We are also continuing our mapping studies correlating the genetic maps in mouse and man and mapping brain-specific functions in the murine system.

Our work on the mechanisms involved in signal transduction has been carried out in two systems. We have concentrated on defining the functions of the transmembrane chemoreceptors in the bacterial chemotaxis system. We are using extensive characterization of mutants in specific portions of the aspartate transmembrane receptor to characterize the functions of subdomains of the receptor. Two approaches have thus far been very useful. One involves introducing specific mutations and then isolating intragenic suppressors of these mutations or using specific extragenic suppressors to modify the protein. Our data thus far suggest that the signal sequence region of the protein plays an important role in maintaining the structure of the C-terminal end of the receptor and perhaps in transmembrane transmission of signals. Another approach involves the isolation of dominant mutants that behave as if they were "locked" in a specific signaling mode and the use of the mutant gene products to develop biochemical assays for signaling.

Another system that mediates signal transduction in eukaryotic cells is the G-protein system. In the past year most of our effort has gone into characterizing the various α, β and γ components of the system in terms of their primary structure, the number of different homologous genes that encode members of this family and the nature of the genomic correlates of the cDNA clones that we have isolated. We are trying to develop an in vitro reconstituted system using the components of the visual signal transducing system in order to better understand the function of specific components of the system.

138. Requirements for Hin-mediated DNA Inversion in vitro

Reid C. Johnson, Michael F. Bruist, Carol C. Lee, Melvin I. Simon

The expression of certain genes is controlled by the rearrangement of specific DNA sequences. One such system involves the alternate expression of different flagellin genes in *Salmonella typhimurium*. This alternate expression is the result of inversion of a 995 bp segment of chromosomal DNA.

The site-specific recombination reaction can be performed in vitro using extracts of *E. coli* in which the Hin protein has been overproduced. The in vitro system has been fractionated to determine the components necessary for the reaction. Maximum rates of recombination in the purified reconstituted system require 1) a supercoiled DNA substrate, 2) the Hin protein, 3) the *E. coli* histone-like protein HU, and 4) a 12,000 molecular weight (MW) unidentified protein from *E. coli* (Johnson et al., 1986). We have previously shown that the DNA substrate for the inversion reaction must contain two 26-bp recombination sites in the proper configuration (inverted) plus an additional cis-acting sequence—a recombinational enhancer (Johnson and Simon, 1989). The 60-bp recombinational enhancer increases the rate of recombination over 150 fold. It can be located on either side of the recombination site, in either orientation, and at variable distances (less than 100 bp to over 4000 bp) from the recombination site.
The 20,000 MW Hin protein binds to the recombination sites and is the enzyme that performs the DNA strand exchange reaction. The 12,000 MW host protein interacts with the DNA at the enhancer and thus is involved in the enhancer-mediated stimulation of the inversion reaction. In addition to the 12,000 MW protein, the HU protein appears to be indirectly involved in enhancer function since the dependence on HU varies with respect to the location of the enhancer relative to the recombination sites. The results suggest that the association of HU with the DNA between the enhancer and the proximal recombination site may facilitate bending of the DNA, allowing for a protein-mediated interaction between the enhancer and the two recombination sites. It is presumed that the recombinational enhancer is involved in aligning the recombination sites into the proper synaptic complex to allow for DNA strand exchange.

References:

139. The DNA Binding Properties of the Proteins of the Hin Site-specific Recombination System

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

The interactions of the proteins of the Hin site-specific recombination system with their substrate DNA have been investigated using DNA footprinting. In footprinting, proteins bound to DNA interfere with DNase I cleavage or dimethyl sulfate methylation at the binding site. It was demonstrated that the Hin protein binds to the recombination sites (hi:rL and hi:rR) at each end of the inverting DNA segment. Through the use of several altered hi:r sites and using a synthetic peptide corresponding to the last 52 amino acids of Hin, we have demonstrated that the C-terminal domain of Hin interacts with the outer 10 bp of each half of a hi:r site. This leaves the center of the site free for the actual recombination reactions.

An efficient DNA substrate for the inversion reaction contains a recombinational enhancer. Footprints show that Factor II binds to the enhancer at two separate and independent sites. Binding also was shown using the DNA fragment retardation assay which separates discrete protein-DNA complexes by electrophoresis. Two complexes, corresponding to one and two sites on the enhancer being occupied, were seen. Related experiments, which use the fact that proteins cannot bind to DNA methylated at the binding site, showed that the site in the enhancer that is farthest from the nearby crossover site binds Factor II first.

The HU protein did not show any site-specific binding to DNA, in agreement with the idea that HU interacts nonspecifically with all DNA. HU may help bend DNA to allow the interaction of the Factor II and Hin DNA complexes on a supercoiled substrate. By using the technique of E. Richet and H. Nash (National Institutes of Health, personal communication), we have determined the footprint of all of the proteins required for Hin-mediated recombination on a supercoiled substrate. This technique showed that the binding pattern on supercoiled DNA was the simple sum of the Hin and Factor II binding patterns on linear DNA.

140. Synthesis of a Site-specific DNA Cleaving Reagent

James P. Sluka', Michael F. Bruist, Suzanna J. Horvath, Melvin I. Simon, Peter B. Dervan

We have demonstrated that an artificial peptide whose sequence is the same as the last 52 amino acids of the Hin protein binds to the Hin recombination site. The binding protects the exact same bases from dimethyl sulfate as the intact Hin protein, indicating that the same DNA-protein interactions occur with both molecules (Bruist et al., 1986).

An amino acid analog containing EDTA was synthesized and coupled to the amino terminus of the 52 amino acid Hin peptide. This modified peptide can be used to bring Fe(II) to a hi:x site and cleave the DNA. Fe(II) is chelated to the EDTA and in the presence of a reducing agent such as dithiothreitol or ascorbate and dissolved oxygen, generates free hydroxyl radicals. These radicals cause the oxidative decomposition of the sugar in the DNA, cleaving the backbone. The cleavage pattern produced by the modified peptide is two clusters of cuts, about four bases each, near the center of the hi:x site. This confirms our previous observation that at least two peptides are bound to a hi:x site and shows that the N termini of two of the peptides are near the center of the site. At present, this reagent still gives some nonspecific cleavage of the DNA. Modifications of the
peptide are being planned that will increase the specific binding constant so that this can be used as a reagent for cleaving specific complex DNA sites.

Reference:

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

141. Function of the Transmembrane Region of the Aspartate Receptor in E. coli
Kenji Oosawa, Melvin I. Simon

In E. coli, the nucleotide sequences of four genes encoding the chemosensory transducers, tar, tsr, trg, and tap, have been determined, and amino acid sequences of gene products were deduced from the nucleotide sequences. From these data and from the results of biochemical and behavioral experiments, we conclude that the chemosensory transducers can be divided into four domains: 1) first transmembrane region responsible for insertion of the protein into the membrane; 2) periplasmic region for ligand binding; 3) second transmembrane region; and 4) cytoplasmic region for signaling the flagellar motor and for regulation of methylation of the receptor correlated with sensory adaptation.

We reported last year that mutations in the first transmembrane region of tar have some effect on the activity of the Tar protein. These mutant proteins, one with a replacement of alanine 19 by lysine and another with a deletion of the amino acids from valine 7 to methionine 13, were used for further analyses. These mutants showed no swarms on tryptone motility plates, and the protein was localized in the membrane. Furthermore, binding of aspartate was similar to wild type. However, they were not modified by CheR and CheB, i.e., there was no apparent methylation or demethylation of the mutant proteins. In behavioral experiments, the lys 19 mutant responded to the addition of repellent and removal of aspartate but not to the addition of aspartate. These results suggest that the first transmembrane region plays a role not only for protein insertion into the membrane but also for maintaining the functional configuration of the protein in the membrane.

We studied the pattern of suppression of the lys 19 mutant. Amino acid changes in three regions of the protein suppressed the lys 19 mutation: 1) adjacent insertion of a glutamic acid residue; 2) changes, including the insertion of glutamate residues, in the second transmembrane region; and 3) a variety of amino acid changes (23) all localized in a stretch of amino acids from residue 264 to 303.

On the basis of these results, we suggest that the lysine mutation can be suppressed by interaction with negatively-charged glutamate within the membrane; thus, the second transmembrane region is probably quite close to the first transmembrane region. In addition, small modifications in structure in a specific domain (amino acids 264 to 303) can compensate for the lys 19 insertion into the membrane. The domain of the molecule may be involved in the transmembrane transmission of signals from the periplasmic to the cytoplasmic side of the membrane.

142. Isolation of the Mutants of the Aspartate Receptor in E. coli
Norihiro Mutoh, Kenji Oosawa, Melvin I. Simon

For mapping of the functional domains in the protein, isolation of mutants provides a first step. For mutagenesis of the tar gene encoding the aspartate receptor, we treated tar plasmid DNA with hydroxylamine. Transformants with treated plasmid DNA were tested on tryptone motility medium for swarms. After incubation, tight colonies were picked as mutants and plasmid DNA was purified for further characterization. Mutations in the tar gene were mapped by a series of restriction digests and eventually by DNA sequencing.

Two classes of mutants were isolated, nonsense mutants and missense mutants. The nonsense mutations mapped all over the gene, but most of the missense mutations clustered in the portion of the gene encoding the cytoplasmic portion of the Tar protein. In these experiments, we believe that we have isolated mutants defective in the general process of signaling.

From the results of behavioral and methylation experiments, the missense mutants were classified into three groups: 1) mutant cells that showed no tumbles and the receptor protein was overmethylated; 2) cells that tumbled all the time and the protein was undermethylated; and 3) the methylation pattern was normal and responded to stimulus but the cells showed no behavioral response.
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Prof. John Abelson's group has been constructing suppressor tRNA genes to replace different amino acids at amber codons. These are very powerful tools to analyze the phenotypes of the Tar proteins that have different amino acid residues at the same position of the protein. We are now using the suppressors and amber mutants in the tar gene to further analyze the correlation between structural domains and transmembrane receptor function.

143. The Transducin \(\gamma\) Subunit: Characterization of the Gene and its Expression

**Manfred Baetscher, Henry K. W. Fong, Melvin I. Simon**

There are two major prerequisites in order to study the factors controlling tissue-specific gene expression: a highly differentiated type of cell and a number of genes known to be specifically expressed in that cell. One suitable system might involve the photoreceptor cells of the retina which contain the major light absorbing pigment, rhodopsin, and the phototransducing G proteins. The gene for rhodopsin was previously isolated and the transcriptional regulatory sequences characterized by Nathans and Hogness (1983). We have used the cDNA for the retina-specific \(\gamma\) subunit of transducin to screen bovine genomic DNA cloned into the \(\lambda\EMBL4\) vector. Five clones were isolated of which three hybridized to the entire cDNA, one to the 5' end and one to the 3' fragment. Restriction site mapping revealed that all five clones contain overlapping sequences from the same gene which spans a region of approximately 8 kb. Of this region, 95% is covered by two introns: one of approximately 7 kb interrupts the coding sequence and the other one, 93 bp long, is located in the 5' untranslated region. The putative promoter region was sequenced. We are planning to study the specificity of the promoter for the transducin \(\gamma\) subunit in transgenic mice and in retinoblastoma-derived cell lines.

**Reference:**

144. Expression of the \(\beta\) Subunit of Transducin in *E. coli*

**Narasimhan Gautam, Melvin I. Simon**

Retinal transducin is composed of three subunits, \(\alpha\), \(\beta\) and \(\gamma\), as in other G proteins. The \(\alpha\) subunit of transducin activates cGMP phosphodiesterase when rhodopsin is photolyzed. The known role for the \(\beta\gamma\) subunits that remain bound together is to facilitate the binding of \(\alpha\) to rhodopsin. The \(\beta\) subunit has a repetitive segmental structure (Fong et al., 1986) and its structure is conserved across different G proteins. The \(\gamma\) subunit is small (8000 daltons). It seems different in various G proteins.

An approach towards understanding the relationship between the structure and function of these subunits is to synthesize large amounts of native as well as mutagenized forms of these proteins in *E. coli* and to purify these and assay for function after reconstituting with other components of the transducin system.

The cDNA for transducin \(\beta\) (T\(\beta\)) (Fong et al., 1986) was transcribed using the SP6 *in vitro* transcription system and this mRNA translated *in vitro*. The polypeptide product was indistinguishable based on molecular weight and isoelectric point from T\(\beta\) purified from bovine retina. After introducing an appropriate restriction site close to the initiator codon by oligonucleotide site-directed mutagenesis, this cDNA was cloned into a vector containing the tac hybrid promoter. When induced with isopropyl \(\beta\)-D-thiogalactoside (IPTG), this directs the synthesis of low amounts of T\(\beta\) in *E. coli*. We are now attempting to increase the amount of protein synthesized by different methods. One of these would be to introduce the cDNA for the T\(\gamma\) subunit into the same vector since T\(\gamma\) might protect T\(\beta\) from proteolytic degradation.

Peptides corresponding to different regions of the T\(\beta\) and T\(\gamma\) subunits have been synthesized by Dr. S. Horvath. We have tried to raise antibodies to some of these and so far have succeeded in obtaining antisera directed against "native" but not denatured T\(\beta\) and T\(\gamma\). We plan to use some of these peptides to determine if T\(\beta\) and T\(\gamma\) undergo posttranslational modifications, specifically acylation.

**Reference:**

145. Isolation of Genes Encoding \(\alpha\) Subunits of G Proteins

**Ryn Miake-Lye, Michael Strathmann, Melvin I. Simon**

Guanine nucleotide-binding regulatory proteins (G
proteins) are \( \alpha-\beta-\gamma \) heterotrimers that transduce a wide variety of extracellular signals across the plasma membrane by stimulating or inhibiting the production of second messengers within the cell (e.g., cAMP, cGMP, inositol triphosphate). The \( \alpha \) subunits of G proteins are different for each type of G protein. They interact with both the signal receptor and the enzyme responsible for changing the level of second messenger. Thus, the \( \alpha \) subunit appears to be the subunit responsible for conferring specificity of each G protein's function. Several different G proteins have been identified biochemically, and there are signal transduction systems in which G proteins have been implicated but not yet isolated. Thus, it is reasonable to expect that there may be a family of related but distinct \( \alpha \) subunits of G proteins.

In order to ask about the diversity of the family of \( \alpha \) subunits, we have isolated cDNAs encoding proteins homologous to known \( \alpha \) subunits. We have constructed \( \lambda gt10 \) cDNA libraries from bovine brain and bovine heart mRNA and screened them with oligonucleotides to sequences that are conserved in known \( \alpha \) subunits. From several screens of the brain library with a variety of oligonucleotides, 56 clones were isolated, each of which hybridized reproducibly to one oligonucleotide. As a second, stronger criterion for calling these clones \( \alpha \) subunit-like, we asked if any of them would hybridize to the coding regions of known \( \alpha \) subunits at reduced stringency. Each of nine clones hybridize to all the coding regions of the \( \alpha \) subunits of \( G_s, G_i \), rod transducin and cone transducin. None appear to encode these known \( \alpha \) subunits, since they do not hybridize at higher stringencies. Furthermore, probes prepared from each of three of these clones hybridize only to themselves, implying that these clones represent independent cDNAs. Currently, we are determining the nucleotide sequence of these clones to ascertain the extent of homology to known \( \alpha \) subunits.

146. 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 function of myeloid cells is 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 \textit{in vitro} by pharmacological agents such as phorbol esters and dibutyryl CAMP which interact with G protein-regulated systems.

We have constructed a cDNA library with RNA extracted from a human myeloid leukemic cell line, HL-60. We plan to screen this library with oligonucleotide and cDNA probes and to identify G-protein genes that are specifically expressed in myeloid cells. We will then study the pattern of expression of the G-protein genes and their protein products in normal and neoplastic myeloid cells.

In collaboration with Drs. Bruce Birren and Henry Fong, we have screened the HL-60 cDNA library and identified two distinct cDNA clones that are homologous to the \( \beta \) subunit of bovine transducin. One cDNA clone hybridizes predominantly with a message of 1.7 kb in HL-60 cells and in a number of bovine tissues. This cDNA is strongly homologous with the \( \beta \) subunit of transducin at the amino acid level but diverges in nucleotide sequence, particularly in the 3' region of the transcript. The other cDNA clone hybridizes with messages of 1.7 and 3 kb and is closely homologous with bovine transducin in both 5' and 3' regions. We plan to characterize these cDNAs further and determine the pattern of expression of the distinct \( \alpha \)-subunit genes in human myeloid cells and other tissues.

In order to look for changes in the pattern of expression of \( G_\alpha \)-subunit proteins, we have examined the pattern of ADP-ribosylation of myeloid proteins after membrane preparations were incubated with cholera and pertussis toxins. After HL-60 cells were induced to differentiate \textit{in vitro}, the pattern of ADP ribosylation changed. We plan to use nucleotide and antibody probes to determine whether this change in labeling reflects a change in transcription or translation of \( G_\alpha \) subunits.
nucleotide-binding proteins, or G proteins. In response to activation of cell membrane receptors by light, hormones, neurotransmitters, odorants or other chemical signals, specific G proteins stimulate or inhibit target biochemical processes, such as the activity of adenylate cyclase, phospholipase C, ion channels and retinal cGMP phosphodiesterase. We are interested first in the molecular structure of G proteins and how the functions of the specific molecular components are achieved.

Retinal transducin, which stimulates a cGMP phosphodiesterase in photoreceptor cells, and other well-characterized G proteins are comprised of three subunits. We have identified and analyzed cDNA clones of the bovine transducin a subunit that may be highly conserved or identical to that in other G proteins. The encoded a subunit has a molecular weight of 37,375 and is comprised of repetitive homologous segments arranged in tandem. Furthermore, significant homology in primary structure and segmental sequence exists between the a subunit and the yeast CDC4 gene product. The transducin a subunit polypeptide is encoded by a 2.9-kilobase (kb) mRNA. However, there exists in retina other a-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 a-related mRNAs, usually 1.8 and 2.9 kb in length.

These results indicate that there is a diversity of a subunit-related mRNAs that could encode different proteins. It seems likely that there are a number of highly homologous a-related genes on the basis of hybridization with genomic DNA. One other bovine a-related cDNA clone has been identified from a brain cDNA library. A partial amino acid sequence derived from this clone is about 90% homologous with the transducin a subunit. We are interested in the extent of diversity of a-related gene products and are investigating their specific roles in the mechanism of signal transduction.

Together the a and y subunits of G proteins act as a tightly bound entity that reversibly associates with the a subunit. The expression of the transducin y subunit appears to be 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 y transducin peptide and the carboxyl termini of ras oncogene products. Preliminary results suggest that homologous cDNAs can be isolated using the Ty cDNA probe, and we also are investigating the diversity and homology of the gene family for y subunits.

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

Vivian H. Cohn, 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—a, β, and γ. Multiple genes have been identified coding for each subunit. In an attempt to study the organization of these genes in the genome and to understand the relationships between the various subunits, we have begun to map them in the mouse.

The gene mapping has been carried out using recombinant inbred strains of mice as well as somatic cell hybrids. We have so far determined the map position of two of the retinal transducin a genes, Tal and Ta2, on chromosomes 9 and 17, respectively. The fact that these two genes lie on different chromosomes may suggest that they are evolutionarily quite distant or that they each evolved independently. Indeed, they are active in different cell populations. Tal encodes the transducin subunit found in rod outer segments and Ta2 encodes the homologous protein expressed in cone cells. We are also studying two retinal β genes and have preliminary data suggesting that one of them lies on mouse chromosome 3, near the Adh-3 gene. Localization of a-subunit genes coding for Gs, G, and G is ongoing; initial data suggest that G may also lie on chromosome 9, near Tal (adjacent to the β-galactosidase gene).

Although mapping genes with these techniques 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. The mapping studies may also be useful in identifying the number of a, β, and γ genes. Eventually, we plan to clone regions containing the
murine G-protein genes in order to examine at the molecular level the structure and organization of this multigene family.

149. Molecular Aspects of Meiosis in Mouse Testis

Kelwyn H. Thomas, Melvin I. Simon

The specific aim of this project is to utilize recombinant DNA techniques to isolate nucleic acid probes that can be used as markers to characterize events in meiotic prophase. In mouse testis there exists a series of well-defined sequential steps that are reflected in very distinctive cytological changes as the germ cells progress through meiotic prophase. Autoradiographic, biochemical and physiological studies done to date have implicated early meiotic-specific transcriptional events as an absolute requirement for progression of meiosis and also for events involved in later meiotic stages (e.g., recombinational events). However, none of these studies could give any precise information concerning the nature of these meiotic-specific transcripts. We have been able to utilize high efficiency cloning vectors to isolate and identify a series of meiotic-specific gene transcripts. We are in the process of characterizing the stage and cell specificity of these transcripts by Northern analysis of RNA from prepubertal testis and in situ cytohybridization on frozen sections of testis. The long-term goals of this project include isolation and characterization of meiotic-specific genes to study gene regulatory signals, which are important in controlling the precision and specificity of cell differentiation during meiosis. Preliminary studies on this aspect of gene regulation are now in progress utilizing transgenic mouse techniques. Subcloning of these cDNA sequences into expression vectors such as λgt11 will also enable sufficient overproduction of meiotic-specific proteins to justify their use in functional studies. Through utilization of a combination of these techniques, we hope to be able to correlate specific transcripts with structural and functional features observed in the cells undergoing differentiation during spermatogenesis. From these studies we should gain some insight into the presently unknown molecular mechanisms regulating the precision and specificity of meiotic pairing and genetic recombination.

PUBLICATIONS


Summary: Our work concerns two families of human pathogens, the flaviviruses and togaviruses. 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. The virus remains present in sylvan cycles, however, and periodically causes epidemics in human populations. In addition, every year there are also a number of deaths of people who intrude into the jungle habitat in which the virus is present. Last year we reported the complete sequence of the 17D strain of yellow fever virus which allowed us to deduce the genome organization and many details of the replication strategy of the flaviviruses. It also showed that flaviviruses are distinct from other families of RNA containing animal viruses and probably separated from the other viruses at a distant time in the past.

The alphaviruses also contain a number of important human pathogens. Much of our work is with the type alphavirus, Sindbis virus, which is avirulent in man but for which model systems of neurovirulence exist in the mouse. We are studying the location of the neutralization epitopes on glycoprotein E2 of Sindbis virus and the possible relationships of these to neurovirulent markers. We also have initiated a number of studies on nonstructural protein genes of alphaviruses which form the viral replicase. These include sequencing studies of nonstructural proteins, attempts to suppress the opal termination codon in the nonstructural region of Sindbis virus, and studies using monovalent antibodies to the nonstructural proteins in biochemical approaches to the synthesis and processing of nonstructural protein precursors. These latter studies also involve attempts to identify or localize the protease believed to be virus encoded and respon-
sible for processing the nonstructural polyprotein. These studies of nonstructural proteins also involve mapping of RNA mutant strains of Sindbis, temperature-sensitive mutants that have defects in the various nonstructural proteins.

We also report two projects whose goal is to understand functions of the structural proteins of the virus. The first includes mutant ts103, in which the interaction between the RNA and the nucleocapsid protein appears to be defective. The second involves studies of the autoprotease in Sindbis virus present in the capsid protein which makes the first cleavage in the processing of the structural polyprotein. Last year we reported that this capsid protein might be a serine autoprotease and studies designed to test this hypothesis are under way.

Finally, we are studying a promoter in vaccinia virus that has been useful in expressing foreign genes such as alphavirus or flavivirus proteins in vaccinia virus.

150. Nucleotide Sequence of Virulent Yellow Fever Virus (Asibi Strain); Comparison with the Vaccine Strain, 17D

Chang S. Hahn, Joel M. Dalrymple, Charles M. Rice

We have sequenced the virulent parental strain (Asibi) of the yellow fever virus and compared the sequence with that of the 17D vaccine strain. There is a 0.67% difference at the nucleotide level and 1.03% difference in amino acid sequence between Asibi and 17D at the nucleotide level and 1.03% difference in amino acid sequence. The nucleotide changes between the two strains are almost randomly distributed throughout the coding region with the exception that the regions encoding ns2a and the E protein have almost twice the overall mutation frequency. However, the particular amino acid changes that occur are dramatically different among these polypeptides. The ns2a protein has the highest frequency of amino acid changes (3%) followed by ns2b (2.3%) and E (2.4%). The changes occurring in the ns2 region are conservative and do not change the hydrophobicity of these membrane-associated proteins. In the E protein (the major antigenic determinant of the virus), more than one-third of the substitutions are nonconservative changes and we believe the changes in the E protein lead to different tissue tropisms in the two strains and thus differing virulence. Furthermore, nonstructural proteins NS1, NS3 and NS5 show the least divergence at the nucleotide level as well as the fewest amino acid changes between these two strains of yellow fever virus. More than half of the nucleotide substitutions occur in the third codon position and thus are silent. These data imply that virus passage places different selective pressures on structural and nonstructural proteins.

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151. Structure of Dengue 2 Virus Genome

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

The virus family Flaviviride contains many human and veterinary pathogens, most of which are transmitted in nature by insect vectors, either mosquitoes or ticks. An important member of this family is dengue virus, a mosquito-borne flavivirus which is currently epidemic in Asia, the Caribbean and South America. Dengue exists in four serotypes (types 1, 2, 3 and 4) of which type 2 has been implicated in some of the more serious manifestations in human infection.

Using RNA from a potential vaccine strain (SI) of dengue 2, which was supplied to us by Dr. Joel Dalrymple, we have constructed a cDNA library by reverse transcription, using a random oligonucleotide primer for first strand synthesis. The clones contain viral sequences between 2 and 7 kb in length inserted into the commercial vector pGem 1 (Promega Bioec). These were mapped by homologies in their deduced amino acid sequences to the known amino acid sequence of the I7D strain of yellow fever virus (Rice et al., 1985) and sequenced by the chemical methods of Maxam and Gilbert (1980).

The sequence is nearing completion and shows that dengue contains a single, long open reading frame at least 10,149 nt long. The genome organization of dengue 2 appears to be identical to yellow fever virus [5' C-prM(M)-E-NS1-ns2a-ns2b-NS3-ns4a-ns4b-NS5 3'], and the sizes of the predicted virus-coded proteins are similar between the two viruses.

References:
152. Expression of Dengue 2 Virus Structural Proteins in Recombinant Vaccinia Viruses

Young S. Hahn, Edith M. Lenches

Dengue virus (types 1, 2, 3, and 4) is the causative agent of millions of cases of human illness worldwide every year. Although uncomplicated 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.

As a first step towards the development of a safe and efficacious dengue vaccine, we are attempting to insert regions of the dengue RNA genome encoding the structural proteins as cDNA sequences into recombinant vaccinia viruses. Vaccinia is a DNA-containing virus with a large (108 kd) genome which was used for decades in routine vaccination against smallpox. This virus has recently become important as an expression vector. The cDNA copy of the gene to be expressed is inserted within the sequences encoding the vaccinia thymidine kinase (tk) gene adjacent to a vaccinia promoter in a bacterial plasmid. Cells infected with wild-type vaccinia (tk<sup>+</sup>) are transfected with the plasmid DNA. Virus containing the heterologous DNA insert can be obtained after homologous recombination at the tk sequences. Such viruses are phenotypically tk<sup>-</sup> and can be selected by growth in bromodeoxyuridine. In this way a number of viral antigens have been expressed in vaccinia such as the hepatitis B antigen, the influenza hemagglutinin, and the rabies glycoprotein. Using our clones of dengue 2 (described in the preceding abstract), we hope to construct recombinant vaccinia viruses that express dengue antigens in mammalian cells.

Stec et al. (1986) have isolated a series of Sindbis variants that are resistant to neutralization by a number of different Sindbis anti-E2 monoclonal antibodies (MAbs), as well as some variants sequentially selected to be simultaneously resistant to up to three MAbs. They have also isolated neutralizable revertants of many of these resistant variants. We have sequenced the E2 encoding region of a number of these variants by chain termination methods, using intracellular virus-specific RNA, specific oligonucleotide primers and reverse transcriptase.

We have found that variants resistant to neutralization have altered amino acids in a highly hydrophilic region of E2 between residues 170 and 220. Most of these changes involve charged amino acids, often basic residues (lysine or arginine) in the parental virus that are replaced by acidic or neutral amino acids. For one MAb (MAb 23), changes at position 216 lead to altered reactivity; for another (MAb 50), changes at residue 190 lead to escape from neutralization. These studies have shown that single amino acid alterations can have profound effects on the antigenic structure of the Sindbis virus glycoproteins.

Reference:

153. Sequence Analysis of Antigenic Variants of Sindbis Virus: Localization of Neutralization Epitopes on Glycoprotein E2

Ellen G. Strauss

Sindbis virus contains three species of structural protein: the capsid protein (C), which complexes with the genomic RNA to form the viral core, and two species of glycoproteins (E2 and E1) which are integral membrane proteins present in the viral envelope. Studies with monoclonal antibodies have shown that E1 is the viral hemagglutinin while E2 contains at least three neutralization epitopes of the virus.

Ellen G. Strauss, James H. Strauss, Chang S. Hahn, Charles M. Rice

We had previously determined the genomic RNA sequence of the type alphavirus, Sindbis virus. This virus encodes four nonstructural proteins that are translated from the genomic RNA. The long open reading frame encoding (N terminal to C terminal) nsP1, nsP2, nsP3, and nsP4 is punctuated after nsP3 with an opal termination codon (UGA). Comparative studies with one of the most distantly related alphaviruses, Middelburg virus, revealed an identical stop codon at this location (Strauss et al., 1983). Upstream, the sequences diverged, but downstream of the opal codon there was a very highly conserved region.

These facts suggested to us that readthrough translation to produce nsP4 was an intrinsic feature of the alphavirus genome. However, it has been reported (K. Takkinen, personal communication) that Semliki Forest virus lacks this opal codon.
We have constructed cDNA libraries of two additional alphaviruses, Ross River virus and O'Nyong-nyong virus (RNA from the latter virus was furnished by Dr. Joel Dalrymple) and obtained the nucleotide sequence of each in the vicinity of the nsP3-nsP4 junction. Ross River contains an opal codon (UGA) but O'Nyong-nyong does not; in this case a single nucleotide change to CGA changes the termination codon to one encoding an arginine residue. In both cases the downstream sequences are highly homologous (in nsP4) while the carboxyl termini of nsP3 are quite different.

During Sindbis infection, nsP4 is made in very small amounts relative to nsP1, nsP2, and nsP3. How such a modulation is achieved in viruses lacking the termination codon is currently under investigation. We are attempting to change the opal codon to either ochre or amber termination codons, or to Ser, Leu or Trp codons, by site-specific mutagenesis. RNA transcribed from such mutagenized clones can then be examined in vivo for the effects of these changes on the replication and growth of the virus.

Reference:

155. Synthesis and Processing of the Nonstructural Proteins of Sindbis Virus

W. Reef Hardy

Sindbis virus encodes four nonstructural proteins with which it transcribes first a minus-strand RNA copy of its genome and second, using this copy as a template, virus-specific mRNA and full-length genomic RNA. The three N-terminal nonstructural proteins are synthesized as one large polyprotein precursor that is subsequently processed to yield the three mature proteins. At a much lower frequency, a precursor is translated and processed that includes all four nonstructural proteins as the result of suppression of an in-frame opal stop codon.

Although we now know the complete sequence of Sindbis virus and many specific details concerning the expression and processing of the structural proteins, relatively little is known about the nonstructural proteins. This is because the nonstructural proteins are produced in such small amounts relative to host cell proteins and virus structural proteins during the infection.

The approach we have undertaken to surmount this problem utilizes polyclonal antisera to immunoprecipitate each of the four nonstructural proteins from infected cell lysates. Using these antibodies, we have examined the kinetics with which the four mature nonstructural proteins are produced from their precursors for both wild-type virus and for mutants, which are temperature sensitive in RNA synthesis and accumulate large polyprotein precursors at the non-permissive temperature.

At this time we are trying to locate the putative viral-encoded protease believed to perform the cleavages that generate the mature nonstructural proteins from their respective precursors. We have been using an Sp6 system to transcribe copies of full-length viral RNA from an infectious DNA clone and then translating these transcripts in vitro. The antisera is then used to immunoprecipitate the cleavage products and so identify them. By making large deletions in each of the genes encoding the nonstructural proteins and then translating these tailored transcripts, it is hoped that we can determine in which gene the viral protease resides.

156. Mapping of RNA− Mutants of Sindbis Virus

Young S. Hahn, Charles M. Rice

Several temperature-sensitive (ts) RNA− mutants of Sindbis virus have been isolated, characterized and grouped into four complementation groups (Strauss and Strauss, 1980). They are presumably defective in one of the nonstructural proteins (nsP1, nsP2, nsP3 and nsP4) which function as the viral replicase. The presence of four complementation groups and four nonstructural proteins suggests that each complementation group defines mutations in one of the nonstructural proteins, but no definitive evidence exists to support this hypothesis. 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 complementation groups (A: ts21; B: ts11; F: ts6, 110, 118; G: ts7, 18) and their revertants was synthesized with reverse transcriptase using mutant virion RNA as template. It has been analyzed by the Fisher and Lerman (1983) gel system (which localizes
single base substitutions), but it was difficult to unambiguously identify the nucleotide changes responsible for the ts phenotype in each RNA^ mutant. Now that an infectious clone of Sindbis virus has been developed, we are attempting to map the mutations using this infectious clone.

References:


Chang S. Hahn, 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 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 multicored particles. Originally, we had postulated that the defect in ts103 was probably in the nucleocapsid protein, such that the interaction of the capsid with the cytoplasmic domains of the glycoproteins during budding was weakened. Sequencing of the ts103 genome has shown that the sequences of ts103 and of revertants of ts103 are unchanged from the parental HRSP sequence in the coding region for the structural proteins. It therefore became necessary to examine the nonstructural region of the genome. Neither the 5'-terminal structure nor a highly conserved 51 nucleotide region about 150 nucleotides from the 5' end of ts103 contained alterations. However, within the 5' terminal 1.5 kb (a domain thought to be essential for replication from studies of Sindbis defective interfering RNAs), some changes have been localized. Currently, we are trying to construct hybrids between the infectious clone of the parental HRSP genome and that of ts103 on the cDNA level to determine which region and/or nucleotide(s) is responsible for the ts103 maturation-defective phenotype.

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

Chang S. Hahn

There is evidence suggesting that the precursor of the structural proteins of the alphaviruses is processed by an autoprotease activity that exists in the C-terminal domain of the capsid protein (Simmons and Strauss, 1974; Scupham et al., 1977; Aliperti and Schlesinger, 1978; Hahn et al., 1985).

We have postulated previously that His-141, Asp-147, and Ser-215 of the alphavirus capsid protein form a catalytic triad similar to that found in serine proteases, in part because of the extensive amino acid homology between the capsid protein and mammalian serine proteases near these residues (Hahn et al., 1985). 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, Thr or Asn. These mutagenized plasmids are being used as templates for in vitro transcription using T7 or Sp6 polymerases.

During in vitro translation of wild-type Sindbis 26S mRNA, the capsid protein is cleaved quantitatively from the nascent polypeptide chain (Simmons and Strauss, 1974). When transcripts from the mutagenized clones are translated and the products examined by gel electrophoresis, we predict that some of the amino acid substitutions will destroy the protease activity and only the polyprotein precursor will be seen. If some of these mutants or combination of mutants retain the autoprotease activity, they will be tested in vivo using the infectious clone.

References:

159. 3'-9 Deletion Analysis of the Vaccinia Virus 7.5 kd Early Promoter Region: Viral Transcription Can Initiate within Foreign Sequences

Young S. Hahn, Charles M. Rice, Walter M. Hodges, Chris A. Franke, Dennis E. Hruby

A series of 3' to 5' deletions extending up to and
through the transcription initiation site of the early promoter for the vaccinia virus 7.5 kilodalton gene (7.5 kd) were constructed and fused to the bacterial chloramphenicol acetyl transferase (CAT) gene. The effects of these deletions on early and late viral transcription were studied by assaying CAT activity and 5' mapping of CAT-specific transcripts in cells infected with vaccinia recombinants containing the chimeric genes. A deletion to +12 nucleotides (relative to the major vaccinia recombinants containing the chimeric genes. A deletion to +12 nucleotides (relative to the major 5' terminus of these transcripts were mapped using both S1 nuclease and primer extension and the results indicate that in the -7 deletion, proper initiation of early transcription can occur, although inefficiently, within this foreign insert.

PUBLICATIONS


Assistant Professor: Barbara J. Wold
Research Fellow: V. Craig Bond
Graduate Students: Paul A. Garrity, Paul R. Mueller, Frank Preugschat, David W. Ruff, Sean Tavtigian, Roger A. Wagner
Laboratory Staff: Daniel E. Barth, Jessie Mei Huei Jong

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Summary: We are studying the events that lead to selective gene expression, emphasizing several early steps that occur within the cell nucleus. Three lines of research in the lab explore different aspects of RNA synthesis and RNA transport from nuclear to cytoplasmic compartments. Frank Preugschat and Dave Ruff are working on RNA transport and its linkage to RNA maturation. Recent developments include the
isolation and characterization of complete cDNA clones of a major component of Xenopus nuclear RNPs called C protein. In addition, monoclonal antibodies specific for several other components of Xenopus nuclear RNPs have been isolated. In the coming year, we look forward to combining these probes for RNP structure with the microinjection assay for RNA processing and transport developed previously. The objectives are to further define the cellular machinery involved in RNA transport as well as the RNA sequence requirements for export to the cytoplasm.

Another nuclear protein, cMyc, appears to influence the expression of genes that regulate cell growth and differentiation, but its mode of action remains a mystery. cMyc is one of a family of phosphoproteins localized in the cell nucleus. They were first identified years ago by their capacity to influence cell growth patterns when they are expressed at high levels in tumor cells. Indirect evidence has suggested that myc may increase the activity of existing transcription factors, thereby altering the expression of other genes that require these factors, although alternative functions are also possible. We have previously found that the pattern of myc expression in normal cells is not varied within each cell cycle, but is up-regulated as cells emerge from the $G_0$ resting state into active cell division. To further define the function of myc in normal cells, several related approaches have been taken. Sean Tavtigian recently joined the lab and has constructed genes that provide for regulated over-expression of cMyc. cMyc protein coding sequence was placed under glucocorticoid and heavy metal regulation. This puts us in a position to measure directly the effect of excess myc on genes whose expression is presently thought to be myc regulated. Feedback regulation of newly induced myc on endogenous cMyc expression can also be measured. The first issue to be settled is whether the effects on other genes are transcriptional, posttranscriptional or some combination of both. The long-term goal is to use this information along with conditional myc expression to further define myc function at the biochemical level. Roger Wagner and Paul Garrity are also studying myc function. Wagner is focusing on its expression in Xenopus oogenesis and embryogenesis, while Garrity has set out to define myc's posttranscriptional regulation.

Paul Mueller, Steve Salser and Jesse Jong have asked the following question: What are the consequences of varying the gene dosage of a regulated promoter? They have observed the titration of key regulatory molecules governing the expression of the mouse metallothionein gene, which is regulated in response to heavy metals and glucocorticoids. Their approach also has provided us with a favorable target cell population for further study of the interaction between regulatory molecules and the promoter.

160. hnRNPs and RNA Transport

Frank Preugschat

One of the most basic processes that eukaryotic cells must carry out is that of transporting the products of transcription out of the nucleus to the cytoplasm. My main interest lies in defining some of the molecular components involved in the transport process. It has been recognized for a long time that precursor hnRNA is found complexed with proteins in the nucleus. Many of these proteins are confined to the nucleus and are not found complexed with mature mRNA in the cytoplasm. Functional roles in processing and transport have been postulated for these proteins. In collaboration with Dr. Gideon Dreyfuss and his colleagues at Northwestern University, cDNA clones corresponding to several of these proteins have been isolated from our human $\lambda gt11$ library. One of these cDNAs codes for an isoform of the $C$ or "core" protein, which is tightly associated with precursor hnRNA in the nucleus.

Using the human C-protein cDNA as a probe, I have cloned and sequenced a Xenopus laevis C-protein cDNA. The nucleic acid and peptide sequence is highly conserved between man and frog (90% at the amino acid level) over a stretch of 36 residues near the amino terminus of the molecule. One sequence feature of interest encodes the peptide HKGFAFVQFSNERTAR-TA. Closely related sequences are found in all nuclear RNA binding proteins for which sequence information is presently available ranging from yeast poly(A)-binding protein (Sachs and Kornberg, 1986) and SSBI (A. Jong and J. Campbell, personal communication) to mammalian nucleolin and helix destabilizing protein. It seems possible that this sequence may be of importance for RNA binding and/or subcellular localization.

Using the Xenopus cDNA sequence, I constructed a
C-terminal β-galactosidase fusion protein of approximately 145 kd molecular weight. Oligo-peptides were also synthesized using the cDNA-derived peptide sequence. The fusion protein and synthetic peptides were then used for monoclonal antibody production. I will use these antibodies to define whether or not the C proteins are involved in nucleocytoplasmic transport of RNA. I have set up a model system to study transport by using microinjected mRNA and the *Xenopus laevis* oocyte. Large amounts of pure RNA are synthesized *in vitro* with the SP6 transcription system, and I then inject the RNA into the large germinal vesicle of the oocyte. I am able to monitor RNA efflux from the nucleus through manual micro-dissection and I can measure turnover through RNA excess hybridization/RNase protection experiments. I will now use RNA substrates with various modifications in sequence content as well as Cap and poly(A) content to discover which features are critical for transport. I also would like to monitor the association of microinjected RNA with *Xenopus* nuclear proteins through the use of antibodies to *Xenopus* C protein. By injecting anti-C protein antibody into the germinal vesicle, I hope to ask the question, "Does C protein have a functional role in RNA transport?" An alternative experimental approach to this question will involve injection of antisense C RNA into oocytes and embryos to see what steps in RNA biogenesis, if any, are impaired as C-protein stocks are depleted.

Reference:

161. *Xenopus laevis* hnRNPs
David W. Ruff

We are characterizing the protein components of *Xenopus laevis* oocyte hnRNPs (heterogeneous nuclear RibonucleoProteins). Newly synthesized RNA is found associated with these proteins in nuclei, and they are of interest to us because they undoubtedly play a significant role in RNA maturation and transport. The hnRNPs are found associated with nuclear RNA as a 40S monomer particle in a sucrose gradient. These monomers consist of proteins and 500-800 nucleotides of RNA. The 40S monomers are breakdown products generated by endogenous ribonuclease nicking of longer RNA molecules. *Xenopus* is an attractive system in which to study the linkage of transcription, RNA maturation and subsequent transport because the nuclei can be easily micro-manipulated. SDS-polyacrylamide gel experiments find three predominant proteins along with several minor ones associated with the *Xenopus* 40S particles. We are currently studying these hnRNP complexes using UV crosslinking and monoclonal antibodies. Irradiating nuclei containing the hnRNPs results in covalent crosslinking of RNA to closely-associated proteins. After sucrose gradients are used to purify the 40S monomers, oligo(dT) cellulose columns can be used to affinity purify those proteins bound to poly(A) RNA. This will help to determine which of the proteins are most closely associated with RNA. We have generated monoclonal antibodies that recognize several proteins in the 40S monomer. We are currently characterizing these immune reagents. These antibodies provide the means to clone additional genes that code for the hnRNP proteins by screening a λgt11 library (see also the above abstract from Frank Preugschat). Perhaps more importantly, they can be used to explore the steps in RNA maturation and transport. In one set of experiments, the monoclonal reagents will be used to follow the maturation and transport of newly injected RNA precursors. Gradient analysis coupled with Western blotting should reveal how processing and transport are related to 20S and 40S RNP structures. A second set of experiments will entail microinjection of the antibodies to see whether and how they might perturb normal RNA biogenesis.

162. Regulation of Mouse Metallothionein
Paul R. Mueller, Stephen Salser¹, Jessie Mei Huei Jong

We are interested in understanding how certain inducible genes are regulated. Our efforts have focused on the metallothionein-I gene of mouse, which can be induced by heavy metals and dexamethosone. This induction is dependent on the interaction of cis-controlling elements (5' flanking regions of the metallothionen gene) and trans-controlling elements (some yet unknown transcription regulatory factor(s)).

A portion of our effort has focused on determining the optimal induction conditions for this gene using zinc and cadmium alone or together as inducers. Metallothionein induction is quantitated by measuring the metallothionein RNA level of cells exposed to various conditions. Zinc and cadmium require different
times of exposure and different concentrations to yield the highest degree of induction. Our objective is to understand the differences in response in terms of cis-acting sequences within the gene and their interaction with transcription factors.

In another series of experiments, cell lines have been created in which there are 30 to 1000 extra copies of the metallothionein promoter. In some cases these extra copies are fused to the protein coding region of dihydrofolate reductase (DHFR). These lines have been tested to see if the large number of metallothionein regulatory DNA sequences have titrated trans-acting regulatory factor(s). The titration effect is monitored by evaluating the state of expression of endogenous metallothionein genes as the number of functional metallothionein promoters is increased. We have found that in the presence of several hundred copies of the metallothionein promoter, the endogenous metallothionein gene is inducible to only 20% of the level obtained in the parental cell line. Basal level expression (in the absence of inducers) is unaffected by the increased gene dosage at gene numbers that do suppress the metal response of endogenous MetI and MetII. Lower numbers of Met promoters also reduce induction, but to a lesser degree as would be expected for in vivo titration of one or more regulatory molecules acting on the Met promoter. The reduction in inducibility of endogenous Met genes shows that the dominant mechanism of induction is the action of one or more limiting positive regulatory molecules. This is by contrast with release from a repressed state maintained by one or more negative regulatory molecules. If the latter were true, we would have expected to see induction of the endogenous gene as the copy number of Met promoters increased. Preliminary data hold open the possibility that at the highest promoter copy numbers, the basal level of expression from the endogenous gene is beginning to increase. If this is the case, it would suggest that both positive and negative trans-acting factors govern expression of this gene.

The cell lines with multiple copies of the metallothionein promoter are also being used for in vivo footprinting experiments. In vivo footprinting in mammalian cells is inherently difficult due to the large genome size which leads to a low signal to noise ratio. This can be significantly improved by increasing the copy number of the gene, as in our cell lines. The objectives of the in vivo footprint experiments are to ascertain which elements are responsible for induction, whether identical patterns of protection are seen for different inducing agents and which elements are titrated as gene dosage is increased.

1Undergraduate, California Institute of Technology.

163. Conditional Expression of Cellular Myc (c-myc)
Sean Tavtigian

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; furthermore, its overexpression in transgenic mice vastly increases the incidence of malignancies in a number of tissues. In tissue culture, c-myc overexpression relaxes the growth factor requirements of primary cell lines and increases the frequency with which they become established. Yet 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 HSP 70 and $\beta$-tubulin. 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 70 genes. Furthermore, c-myc proteins are primarily nuclear in localization and bind to DNA cellulose in vitro. These data suggest that c-myc may be involved, directly or indirectly, in the regulation of gene expression.

Our approach is to study the effect of regulated c-myc overproduction on expression of genes including HSP 70 and $\beta$-tubulin. In order to do this we have introduced into cells a gene in which the MMTV glucocorticoid enhancer and MT-1 metallothionein promoter have been fused to a minimal c-myc coding sequence into both mouse 3T3 cells and mice. Our myc message is induced 10-15 fold in 3T3 cells, has a half-life of about 2 hours, and reaches much higher steady state levels than the nonrecombinant myc message.

By inducing our myc construct with heavy metals and/or dexamethasone and then monitoring the levels
of HSP 70, β-tubulin message by solution hybridization/RNase protection or Northern blot, we will determine which, if any, of these genes is actually myc inducible. 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 these genes.

164. Xenopus Myc
Roger A. Wagner

The nuclear phosphoproteins called Myc are thought to influence normal and aberrant cell proliferation and perhaps play a role in commitment and differentiation as well. It is presently thought that they may 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. As an initial step, we have isolated cDNA clones for Xenopus cMyc and confirmed their identity by determining their DNA sequence and comparing it with mammalian and avian myc sequences. We are presently using the cloned gene along with myc immune reagents to characterize the expression and activity of the gene and its product in oogenesis and early embryogenesis. Tests of the role myc may play in oocyte maturation and subsequent fertilization and cell division are being probed, assaying the effects of altered myc RNA and protein levels. The diversity of the myc family and the pattern of expression in early embryogenesis will be further explored in the coming year.

165. Homologous Recombination in Animal Cells
V. Craig Bond

The cloned herpes thymidine kinase (TK) gene and the hamster adenine phosphoribosyltransferase (APRT) gene are being used to examine the physical events leading to mitotic recombination in mammalian somatic cells. Fragments of these genes were used as substrate. Upon transformation of these fragments into mouse L cells in the appropriate combinations, the cell's recombination machinery can rearrange them into intact, functional genes. Using this system, we have examined a number of parameters that affect the frequency with which the cell is able to recombine these substrate molecules. Variables such as linearization versus supercoiling, location of the site of linearization, and the type of cut were tested and their effect quantitated.

From the data of a number of experiments, we began to define a possible mechanism for these mitotic recombination events. It invokes double strand nicking, or two close single strand nicks of a supercoiled substrate, exposing ends. These ends are opened by breathing or chewback, exposing single-stranded regions of homology. These regions can anneal, and gaps are filled in and ligated. Key features of this proposed mechanism suggest that the recombination frequency will become inversely proportional to the length of the region of homology when the length of homologous sequence exceeds an optimal value. This aspect was tested using a nested set of truncated overlaps of the hamster APRT gene. The results, in large part, support the exonuclease repair mechanism.

The system developed to monitor recombination also can be used to screen mutant cell lines for those that cannot carry out recombination correctly. Cells are available from patients with several genetic diseases, including Bloom's syndrome and ATT, that are likely to affect steps in mitotic recombination. Because most of these human cell lines are primary lines, colony formation is not a practical way of monitoring recombination. However, dominant markers, such as pNEO, cannot be monitored transiently. A transient assay that will use herpes TK gene as a dominant marker is currently being developed. This dominant assay and the recombination assay will allow us to screen many human mutant cell lines.

PUBLICATIONS

CELLULAR BIOLOGY AND BIOPHYSICS

Howard C. Berg
Charles J. Brokaw
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Elias Lazarides
Jean-Paul Revel
Ellen Rothenberg
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Summary: Flagellated bacteria possess a remarkable motility system based on a reversible rotary motor linked by a flexible coupling (the proximal hook) to a thin helical propeller (the flagellar filament). The motor derives its energy from protons driven into the cell by chemical gradients or electrical fields. The direction of rotation of the motor depends, in part, on signals generated by sensory systems, the best studied of which analyzes chemical stimuli.

The rotation of the motor can be observed directly by fixing the filament to a glass slide; the cell body spins alternately clockwise (CW) and counterclockwise (CCW). These cells are called tethered cells. If chemical attractants are added to the medium surrounding tethered cells, chemotactic responses can be elicited. When the several motors of a free-swimming, peritrichously-flagellated cell such as *Escherichia coli* or *Streptococcus* strain V4051 turn CCW, the filaments work together in a bundle that drives the cell steadily forward (the cell "runs"); when the motors turn CW, the bundle flies apart, and the motion is highly erratic (the cell "tumbles"). Runs and tumbles occur in an alternating sequence, each run constituting a step in a three-dimensional random walk. When the cell swims in a spatial gradient of a chemical attractant, runs up the gradient are extended; this imposes a bias on the random walk that carries the cell in a favorable direction. Changes in concentration are sensed by specific chemoreceptors; CCW rotation is favored as more attractant is bound. We would like to understand how the motor works, what the signal is that controls its direction of rotation, and how this signal is processed by the chemical sensory system.

The spore-bearing stalk of the fungus *Phycomyces* grows away from solid objects. It is thought to have some kind of chemical radar. We are trying to learn how this system works.

166. Nature of the Signal Coupling Receptors to Flagella

Alan J. Wolfe, M. Patricia Conley, Tina Kramer

Bacteria respond to rising concentrations of attractant by increasing the extent of CCW rotation of their flagella. This response is mediated by receptors on the cell surface that bind attractant; it does not require transport or metabolism of this attractant. We are investigating the link between receptors and flagella by constructing strains of *E. coli* that are deleted for all the genes required for chemotaxis toward amino acids, except those for which the null phenotype is non-flagellate. Such "gutted" strains rotate their flagella CCW. Addition of the cheY gene product, a cytoplasmic protein of low molecular weight, switches the direction to CW. The extent of CCW rotation can be increased in this strain by the addition of the tar gene product, a receptor for aspartate that spans the cytoplasmic membrane. Thus, the cheY gene product could be a CW-generating substance inactivated by the receptor that links the receptors to the flagella by diffusion, as suggested by the work of Ravid and Eisenbach (1984) and Segall et al. (1985).

References:

167. Incorporation of Gene Products Required for Flagellar Rotation

David F. Blair

It was shown earlier that different force-generating units in the bacterial rotary motor became active in sequence when defective MotB proteins were displaced by newly synthesized wild-type copies (Block and Berg, 1984). Construction of the necessary plasmids and transducing phages is under way that will allow such experiments to be done with motA and motB in null backgrounds. More stringent means of
controlling the synthesis of wild-type MotA and MotB proteins are being devised; these should make possible more detailed characterization of flagellar motors that contain only one or a few functional force-generating units.

References:

168. Behavior of the Flagellar Rotary Motor at Low Speeds

Markus Meister

If a tethered bacterial cell is energized and then subjected to rapid transverse flow, it stops spinning: its body is forced downstream. If the flow rate is gradually decreased, the cell turns upstream until the long axis of its body becomes perpendicular to the direction of the flow. At this point, the cell starts spinning, slowly during the part of the cycle in which it turns against the flow, rapidly during the part of the cycle in which it turns with the flow. The value of this critical flow rate is a measure of the motor's stall torque. Running torques were deduced from the rotation rate at zero flow. As nearly as could be judged from estimates of viscous drag, the two were the same, even as cells were subjected to extremes of pH or protonmotive force. It appears that the rate of flagellar rotation does not limit the generation of torque under any of these conditions.

169. Behavior of the Flagellar Rotary Motor at High Speeds

Graeme Lowe

A fully-energized tethered cell is driven by a flagellar motor at a frequency in the range of 5 to 15 Hz. In this regime, the angular velocity is inversely proportional to viscosity: the torque is constant. We have found that motors of fully energized swimming cells run at much higher frequencies, about 100 Hz. These measurements were made by repeatedly computing and averaging estimates of the power spectrum of light sensed by a photomultiplier at the top of an inverse phase-contrast microscope. This spectrum contains one peak due to body roll and a second peak due to vibrations in the body generated by rotation of the flagellar bundle. Cells in this regime appear to run their motors at constant angular velocity: the bundle vibration frequencies did not change much as the external viscosity was raised by the addition of Ficoll. In addition, the cells showed both temperature and deuterium solvent isotope effects, both of which are absent in tethered cells (Khan and Berg, 1983).

Reference:

170. Response of the Flagellar Rotary Motor to Abrupt Changes in External pH

Katsuhiko Shimada, Howard C. Berg

Cells of Streptococcus strain SM197 were starved, tethered to a quartz coverslip, energized with a potassium diffusion potential, and exposed to sudden decrements in external pH generated by flash photolysis of 2-hydroxyphenyl-1-(2-nitro)phenyl phosphate. The rotation rate of the cells increased following the flash but only after a brief time lag. Lags of order 0.1 sec were observed in a dilute buffer (0.05 mM), confirming results obtained earlier by Khan et al. (1985). These lags were longer when the buffer was prepared in D2O. However, lags as short as 0.1 sec were found in more concentrated buffers (1 and 3 mM). In this case, there was no deuterium solvent isotope effect. These differences arise from the extra time required for diffusion of protons from a dilute medium into the cell wall, which has a large buffering capacity. The short lags observed in concentrated media probably are inherent to the flagellar motor, but the possibility that they are due to buffered diffusion through the wall has not been ruled out.

Reference:

171. Off-Line Analysis of the Motion of Tethered Bacteria

Andrew Nathan

Tethered bacteria are being used extensively in studies of energetics and behavior. Relevant data include the speed, duration and timing of the periods of clockwise (CW) and counterclockwise (CCW) rotation. The sheer volume of these data is noteworthy: cells of E. coli spin 10 Hz, reversing on average once per second. An experiment in which a microscopic field of 10 cells is recorded on video tape for one hour yields records of some 360,000 revolutions
and 36,000 CW/CCW rotation intervals. Work continues on an instrument capable of digitizing the angular positions of video images of tethered cells in real time. The hardware, built by John Power in the Biology Division Electronics Shop, is working well, and the software is nearly complete.

172. The Avoidance Response in Phycomyces

Paul W. Meyer, Ivan J. Matus

The sporangiophore of the fungus Phycomyces bends away from nearby objects without ever touching them. It has been thought that these objects act as aerodynamic obstacles that damp random winds, thereby generating asymmetric distributions of a growth-promoting gas emitted by the growing zone. In the interest of testing this hypothesis, we studied avoidance in an environmental chamber in which convection was suppressed by a shallow thermal gradient. We also controlled pressure, temperature and relative humidity of the air, electrostatic charge, and ambient light. A protocol was established that yielded avoidance rates constant from sporangiophore to sporangiophore to within ±10%.

We found that avoidance occurred at normal rates in the complete absence of random winds. The rates were smaller at 100% than at lower values of relative humidity, but not by much. Remarkably, at a distance of 0.5 mm, avoidance from a 30 µm-diameter glass fiber (aligned parallel to the sporangiophore) was about the same as that from a planar glass sheet. The rate for the fiber fell rapidly with distance, while the rate for the sheet remained nearly constant out to about 4 mm. We conclude that avoidance depends either on adsorption by the barrier of a growth-inhibiting substance or emission by the barrier of a growth-promoting substance; it cannot occur by passive reflection.

PUBLICATIONS


173. Dynein Arm Mutants of Chlamydomonas

Charles J. Brokaw, Ritsu Kamiya¹, David J. L. Luck²

Dynein is the flagellar ATPase, located in two rows of arms along the doublet microtubules, that is responsible for the generation of active sliding. Mutants deficient in arms of either the outer row or the inner row have different phenotypes. In the outer dynein arm mutants, there is always a major reduction in flagellar beat frequency. At least three distinct motility phenotypes of outer dynein arm mutants have been identified; these have different degrees of reduced amplitude of beating in addition to the reduced frequency. A mutant with a partial deficiency of outer arms shows a similar reduction in amplitude without the reduced beat frequency. A mutant with a
complete inner arm deficiency is paralyzed, but complete outer arm deficiency does not prevent motility. These observations indicate that inner arms and outer arms have different functions.

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174. The Relationship between Flagellar Asymmetry and Calcium Concentration

Charles J. Brokaw, James Trimmer

Work reported last year suggested involvement of calmodulin in mediating the increased asymmetry obtained when demembranated sea urchin spermatozoa are reactivated at increased calcium concentrations. However, the relationship between asymmetry and calcium concentration extends over four decades of calcium concentration and does not resemble calcium-binding by calmodulin. We have reinvestigated this relationship using our new capabilities for obtaining extensive data by computer-assisted image analysis. The extended response curve appears to be the result of two distinct calcium receptors—one with affinity in the micromolar range, and another with affinity in the 0.01 to 0.1 micromolar range. The first receptor could well be calmodulin, but the second one is unknown.

It has been assumed that this in vitro relationship between calcium concentration and the asymmetry of flagellar bending is indicative of what happens in vivo. An attempt to confirm this has given negative results. This experiment made use of a monoclonal antibody to a sperm membrane protein; this antibody has been shown to cause increased intracellular calcium in live sea urchin spermatozoa. However, we found no changes in flagellar bending. This could be explained by assuming that the flagellum is not the compartment that is the source of the signal indicating an increase in intracellular calcium, but as yet there is no confirmation of this assumption.

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175. New Evidence for a "Biased Baseline" Mechanism for Calcium-regulated Asymmetry of Flagellar Bending

Dan Eshel, Charles J. Brokaw

New analysis methods for flagellar bending waves were applied to data from photographed images of ATP-reactivated spermatozoa of a sea urchin and Ciona, and from flagella on a uniflagellate mutant of Chlamydomonas. Curves of shear angle vs. time were constructed for each point along the flagellum. For each of these time series, four parameters were fitted with a sinusoidal function, using the simplex method for least-squares parameter estimation. The sinusoidal functions determined by these parameters can then be used to reconstruct shear curves that represent data averaged over several beat cycles. This process reveals the major features of the bending patterns with a much lower noise level and makes it easy to identify and subtract out the mean angle at each point along the flagellum. The results show that the asymmetric beat patterns can be interpreted in terms of completely symmetric bending waves which develop and propagate on a curved flagellum. Our analysis eliminates previous objections to a "biased baseline" mechanism for the regulation of bending wave asymmetry by calcium and supports other evidence favoring this mechanism over a "biased switching" mechanism, such as experiments showing calcium-dependent changes in curvature in non-beating flagella.

176. Regulation of Sea Urchin Flagellar Bending by Phosphorylation

Charles J. Brokaw, Sandra N. Nagayama

Spermatozoa from the sea urchin, Lytechinus pictus, are not fully active after demembranation and suspension in ATP solutions unless they are first incubated with cAMP and ATP at a reduced salt concentration. This activation can be reversed by subsequent incubation with a protein phosphatase preparation from rabbit skeletal muscle and then restored by incubation with the catalytic subunit of the cAMP-dependent protein kinase and ATP. When the results of activation are assayed in reactivation solutions containing 1 mM ATP, activation is accompanied by increases in frequency, bend angle, and symmetry. However, when the results of activation are assayed at lower ATP concentration (0.1 mM), there is a decrease in bend angle after phosphorylation, so that there is then no phosphorylation-dependent change in the velocity of sliding between microtubules. Activation by phosphorylation must be more complicated than simply a turning on of the active sliding process. It may be involved in "fine-
over a wide range of substrate concentrations.

177. Computer Simulation of Bend Propagation by Axoplasmic Microtubules

Charles J. Brokaw

The generation of bending waves by microtubules in squid nerve axoplasm has been modeled using appropriately modified versions of computer programs developed previously for simulation of flagellar bending waves. The results confirm that a constant longitudinal force directed along the axis of the microtubule is sufficient to cause the generation of regular oscillations and propagated bending waves when the forward gliding movement of the microtubule is obstructed. No control mechanism is required to modulate the active force-generating system. In order to obtain bending waves similar to those observed experimentally, it was necessary to use a model for the force-generating system in which the active force decreases with increasing sliding velocity. If the elastic bending resistance of axoplasmic microtubules is similar to that of microtubules in sperm terminal filaments, the longitudinal force per unit length generated by the axoplasmic microtubules must be of the same order of magnitude as the force generated by dynein arms along the doublet microtubules of eukaryotic flagella.

PUBLICATIONS


Assistant Professor: Scott D. Emr
Research Fellows: Vytais A. Bankaitis, David M. Bedwell, Jinnie M. Garrett, Lianna M. Johnson, Daniel J. Kilionsky
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Summary: In all cells, different cellular functions are segregated into distinct membranes or membrane-enclosed organelles (e.g., mitochondria, nuclei, lysosomes, etc.). The unique biochemical characteristics exhibited by each of the different subcellular compartments are a function of the unique set of proteins that reside within each of them. Little presently is known about the mechanisms responsible for directing these proteins to their correct intracellular destinations. Our research goal is to understand how a few representative proteins in yeast are selectively and efficiently transported from their site of synthesis in the cell cytoplasm to their unique site of function in one of more than a dozen potential subcellular compartments. Such an understanding is likely to contribute to a more general appreciation of how protein traffic is regulated in eukaryotic cells.

We are employing the technique of gene fusion to map the sequence or conformational signals in a nuclear-encoded mitochondrial protein (the F1-ATPase a-subunit protein), two vacuolar proteins (carboxypeptidase Y and protease A) and a protein presumed to reside in the Golgi complex (Kex2) that allow these proteins to be properly sorted to their respective organelle destinations. Different segments of the
yeast genes that encode these proteins have been fused in frame with the yeast SUC2 gene. The SUC2 gene codes for the normally secreted enzyme invertase. This enzyme serves as a biochemical marker enabling us to follow the intracellular delivery of the hybrid proteins coded for by each of the gene fusions. Sequences identified in these organellar proteins that are capable of redirecting yeast invertase to a new subcellular destination are being modified by site-directed mutagenesis. The effect these mutant sequences have on intracellular delivery of both the fusion proteins and the respective prototypic organellar proteins will be analyzed. These studies should help to define the information content of a few representative protein sorting signals in yeast.

Yeast mutants altered in their ability to properly recognize these protein sorting signals are being selected by exploiting certain phenotypes conferred to yeast by the gene fusion constructs. The genes affected in these mutants may code for components of the cellular machinery that functions in the recognition and delivery of the proteins being studied. By cloning these genes and characterizing the proteins they code for, we hope to obtain a better picture of how cells accomplish the assembly and maintenance of their highly compartmentalized structure.

178. Mitochondrial Protein Import in Yeast

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 NH$_2$-terminal leader sequence that presumably facilitates the binding and import of the protein 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. We have utilized site-directed mutagenesis to introduce a series of mutations into the leader sequence of an imported mitochondrial protein, the $\beta$ subunit of the F$_1$-ATPase complex (which is encoded by the ATP2 gene). This approach will allow us to determine the critical residues involved in the import process, as well as examine whether the importance of these residues is due to their net charge or some other factor, such as an involvement in secondary structure. From these studies, we hope to isolate several mutations that effectively abolish the import of the $\beta$-subunit protein. By introducing a plasmid encoding such an import-defective $\beta$ subunit into a $\Delta$atp2 yeast strain, conditional mutations that restore the import of the defective $\beta$ subunit can be selected by demanding growth on a non-fermentable carbon source such as glycerol. Second-site mutations that map in the ATP2 gene may define additional regions of the $\beta$ protein that interact with the import machinery, while unlinked mutations will presumably represent alterations in various components of the import machinery that allow the recognition of the defective $\beta$-leader sequence. The characterization of these mutations may allow us to identify certain of the cellular components involved in directing mitochondrial protein import.

179. Search for Mutations Affecting Mitochondrial Protein Import

Julia A. Yang, Jinnie M. Garrett

We are interested in how nuclear-coded mitochondrial proteins are imported into mitochondria. Previously, gene fusions between the ATP2 gene (nuclear gene coding for the $\beta$ subunit of yeast mitochondrial F$_1$-ATPase) and the E. coli lacZ gene (coding for $\beta$-galactosidase) have been used to study the process of mitochondrial protein import. $\beta$-subunit-$\beta$-galactosidase hybrid proteins are efficiently targeted to mitochondria and prevent normal functioning of the organelle (Emr et al., 1986). Cells expressing the ATP2-lacZ gene fusions cannot utilize a non-fermentable carbon source, e.g., glycerol. This respiration-defective phenotype (Gly$^+$) has been exploited to select Gly$^+$ revertants. Unfortunately, a significant number of nuclear mutations obtained are dominant, making genetic analysis of these mutants cumbersome. Another drawback to this approach is that separation of mutations that affect mitochondrial protein import from those that affect unrelated respiratory functions of the organelle has been
difficult. Therefore, we would like to isolate a new set of mutations that only influence the import process.

We have devised an alternative strategy for selecting mutants affecting mitochondrial protein import. We have placed both an ATP2-lacZ gene fusion and the wild-type ATP2 gene under transcriptional control of the GAL1-GAL10 promoter. Cells carrying the GALp ATP2-lacZ construct are able to utilize glycerol (repressing conditions) but grow very slowly on medium containing galactose as the sole carbon source (inducing conditions). The retarded-growth phenotype of these cells on galactose is caused by overexpression of the hybrid protein. We did not observe a similar phenotype in cells overproducing the wild-type a-subunit. We are in the process of biochemically analyzing the effects of hybrid protein and wild-type a-subunit overproduction on mitochondrial import of the a-subunit protein. Concurrently, we are further characterizing the retarded growth phenotype exhibited by induced cells carrying the GALp ATP2-lacZ construct and intend to use this phenotype to select mutants, hoping that such mutants will be affected in functions related to the import process.

Reference:

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

Lianna M. Johnson, John A. DeModena

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). Analysis of these hybrid proteins has shown that the first 20 amino acids of CPY direct secretion of the CPY-INV fusion, whereas 50 amino acids or more of CPY lead to efficient vacuole localization of the hybrid protein. Hence, information sufficient for vacuolar targeting is located within the first 50 amino acids of CPY. Deletion of amino acids 20 through 50 in a CPY-INV fusion containing 156 amino acids of CPY results in a significant secretion of the hybrid protein. In addition, when the coding sequence for amino acids 20 through 50 are deleted from the wild-type PRC1 gene (codes for CPY), a severe defect in the localization of CPY to the vacuole is observed.

All proteins that enter the ER have signal sequences located near their NH2 terminus. These signal sequences are involved in directing translocation into the ER lumen, where they may either be cleaved from the protein or serve as membrane anchors. It is unclear whether vacuolar proteins are sorted via a membrane intermediate or as soluble proteins. We have found by mutagenesis of the presumed signal peptide cleavage site in CPY that this signal is cleaved from the protein at the level of the ER. Thus, the CPY signal sequence is removed from the protein prior to vacuole sorting and is unlikely to play a role in the sorting event.

As proteins traverse the secretory pathway, they are modified. CPY normally is modified by the addition of GlcNAc2Man8-18P0-2 at four sites in the protein, while invertase is more extensively modified by the addition of GlcNAc2Man100-150P at 12 sites in the protein. CPY-INV hybrid proteins containing up to 95 amino acids of CPY become extensively modified in a manner that appears to be similar to that observed for invertase, whereas a hybrid protein containing 433 amino acids of CPY is modified to a much lesser extent, like CPY. Hence, there appears to be information within CPY that acts independent of the vacuole sorting signal and functions to regulate the degree to which this vacuolar enzyme is modified.

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

Daniel J. Klionsky

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 Nos. 180 and 182). We have begun additional studies utilizing a gene fusion approach to analyze delivery of proteinase A 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. A **PEP4-SUC2**-encoded hybrid protein containing 138 amino acids from the N terminus of proteinase A and lacking the invertase signal sequence is efficiently directed to the vacuole. The information necessary for directing proteinase A to the vacuole then is apparently contained within this N-terminal segment of the polypeptide. Additional gene fusion will allow a more precise identification of the sorting signal and will enable us to determine the important features of the signal. The sorting signal of proteinase A can then be compared to that of CPY to see if these two vacuolar proteins have targeting signals that share common features.

### 182. Isolation of Yeast Mutants Defective in Protein Targeting to the Vacuole

**Vytas A. Bankaitis, Jane S. Robinson**

We have constructed a **PRCl-SUC2** gene fusion that directs the synthesis in *Saccharomyces cerevisiae* of a hybrid polypeptide consisting of a 433-residue amino-terminal domain derived from the yeast vacuolar protease carboxypeptidase Y (CPY) and a 511-residue carboxy-terminal domain derived from the secreted yeast enzyme invertase. Fractionation data indicate that this amount of CPY primary sequence is sufficient to quantitatively divert invertase to the yeast vacuole. The phenotypic consequence of localizing active invertase to the vacuole has enabled us to select for mutants that "mislocalize" the hybrid protein to the cell surface. The corresponding mutations that lead to this effect are all **trans**-acting, recessive, and define at least 14 complementation groups. These vacuolar protein targeting (**vpt**) mutants also exhibit hybrid protein-independent defects in wild-type CPY delivery to the yeast vacuole. Precursor forms of CPY accumulate in the mutants and are secreted into the yeast periplasm and extracellular medium. This secretion appears to be dependent upon a functional **SEC1** gene product, a component of the secretory pathway that is normally required for protein secretion but not for vacuole protein delivery. The **vpt** mutants should provide useful information pertaining to the mechanisms by which yeast regulate vacuolar protein traffic.

### 183. Altered Vacuole Morphology in Certain Vacuolar Protein Targeting Mutants

**Lois M. Banta**

We are interested in the mechanism by which vacuolar proteins are sorted from secretory proteins and are targeted specifically to the vacuole. Previous work in this laboratory has identified a series of **trans**-acting mutations that affect the targeting of vacuolar proteins (see Abstract No. 182). We have examined the morphology of these vacuolar protein targeting (**vpt**) mutants using Nomarski optics and fluorescence microscopy. Makarow (1985) has demonstrated that the fluorescent compound FITC-dextran is taken up by yeast cells and localized to the vacuole in a manner that is concentration- and temperature-dependent. Analysis of the overall cell morphology and the FITC-dextran uptake and localization properties has revealed at least two distinct groups of **vpt** mutants.

The class A mutants, comprising nine complementation groups, resemble the wild-type parent strain in that they have a single distinct vacuole which is easily observed using Nomarski optics and which is stained by FITC-dextran. A second class of mutants, class B, consists of three complementation groups and is characterized by an altered vacuolar morphology. These cells exhibit a highly vesiculated appearance, and no discrete large vacuole is visible. In two of these mutants, the FITC-dextran is localized in the vesicular structures present throughout the cytoplasm. The mutant cells of one class B complementation group exhibit little intracellular accumulation of FITC-dextran.

The large number of complementation groups and the existence of at least two distinct morphologies associated with the **vpt** defect suggest that a number of gene products, acting at different stages in the targeting process, are involved in the localization of protein to the yeast vacuole. Efforts to characterize these mutants further and to clone the genes responsible for the altered morphologies are in progress. These studies should help elucidate the role played by these gene products in the mechanism of vacuolar protein targeting.

**Reference:**

184. Delivery and Retention of a Golgi Protein

Paul K. Herman, Keith A. Matthews, John A. DeModena

The study of protein secretion in eukaryotic cells has led to the postulation and subsequent partial characterization of the protein secretory pathway and its subcellular components. 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*. This protein is an endopeptidase (cleaves on the carboxy-terminal side of pairs of basic amino acids) that functions, in vivo, during the maturation of both α-factor and killer toxin. Recent work, including studies with yeast mutants defective at various stages of the secretory pathway, has indicated that the Kex2 protein resides within the Golgi (Julius et al., 1981f). Our initial experiments have involved the construction of a series of KEX2 fusions with the structural gene for the normally secreted invertase protein, SUC2. Preliminary results indicate that the KEX2 sequences in one of the fusion proteins are capable of retaining invertase activity within the cell. Experiments presently under way are aimed at characterizing the signals present within the KEX2 protein that are responsible for this specific intracellular localization. We are also currently employing the fusion proteins in attempts to generate yeast mutants that mislocalize the wild-type KEX2 protein 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.

Reference:

185. Characterization of the SEC18 Gene of *Saccharomyces cerevisiae*

Kurt Eakle

A series of temperature-sensitive (ts) yeast mutants that have defects in the process of protein secretion have been described (Novick et al., 1980). 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, at the nonpermissive growth temperature accumulates the yeast equivalent of endoplasmic reticulum and blocks protein transport to the Golgi apparatus. We are interested in characterizing the location and function of the product of this gene. The SEC18 gene was cloned by complementation of the temperature-sensitive sec18 defect and localized to a 3 kb BamHI-HindIII fragment. Complementation analysis using Bal 31 deletions from either end of the fragment cloned into a yeast centromere vector has shown that more than 2.5 kb of the sequence is necessary to complement the ts allele. Sequencing of this fragment reveals an open reading frame over 2300 bp in length, which would code for a protein of approximately 85 kd. In addition, disruption of the SEC18 gene has demonstrated that it is essential for yeast survival. A gene fusion between the open reading frame of SEC18 and the *E. coli* lacZ gene has been generated. The fusion is being used to overproduce a Sec18-a-galactosidase hybrid protein in bacteria that then will be used to raise specific antisera against the Sec18 protein. This antisera will be used to determine the subcellular site of residence for the SEC18 protein and to analyze the biogenesis of this protein. It is hoped that these studies will help to provide a better understanding of the role Sec18 plays in regulating protein traffic between the ER and the Golgi complex.

Reference:

186. Mapping an Internal Export Signal in LamB

Elliot Altman

An observation has been made in *E. coli* that the synthesis of a normally exported protein harboring a defective signal sequence interferes with the export of other secreted proteins (Bankaitis and Bassford, 1984).
This interference, which is measured by precursor accumulation, still occurs with the removal of the entire signal sequence and suggests the existence of a second, internal export signal (I.E.S.) in E. coli secretory proteins. We are trying both to map this I.E.S. in the LamB protein and to identify any component of the host secretory machinery that may interact with it.

Deletions of both amino- and carboxy-terminal coding sequences in the lamB gene are being used to map the I.E.S. Amino-terminal deletions of lamB have been fused to the first seven amino acids of lacZ to generate LacZ-LamB fusion proteins. The amino-terminal mapping has shown that the first half of the mature lamB protein can be deleted without affecting export interference. Carboxy-terminal deletions of lamB have been fused to lacZ to generate LamB-LacZ fusion proteins. LamB-LacZ fusion proteins that contain 320 amino acids of mature LamB (contains a total of 421 amino acids) do not interfere, while LamB-LacZ fusion proteins that contain 370 amino acids or more of mature lamB do interfere. We plan to delete the region of lamB corresponding to the I.E.S. in order to test if interference is alleviated and to analyze the effect of such a mutation on the export of an otherwise wild-type LamB protein.

Future efforts will be directed at trying to genetically define and then clone components of the host secretory machinery that interact with the I.E.S. in LamB.

Reference:

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 sub-systems such as taste learning, a categorization in Limax maximus.

187. 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 compression, 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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188. Electron Transfer Processes

David N. Beratan, Alvin D. Joran, Peter B. Dervan, John J. Hopfield, Jose Nelson Onuchic, Burton A. Leland

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).

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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| O, 1, or 2 Units |

PUBLICATIONS


Professor: Elias Lazarides
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Research Fellows: Brigitte Boyer, Peter M. Burger, Nigel R. Burns, Marios A. Cariolou, John V. Cox, Horst Hinssen, Frank Sangiorgi, Matthias Staufenbiel, Catherine M. Woods
Graduate Students: John J. Ngai, Jeffrey H. Stack
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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 ultimately these cells to 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.

189. Sequence Analysis of the Ankyrin Gene

Marios A. Cariolou, Peter M. Burger

One of the major polypeptide species in the membrane skeleton of erythroid cells is ankyrin. This extrinsic membrane protein provides the anchorage site of cytoskeletal proteins such as spectrin and the transmembrane anion transporter (Bennett, 1982). The exact nature of this interaction at the structural level is not known.

Results from this laboratory have demonstrated that ankyrin (molecular weight 210,000) is fatty acid acylated after its assembly onto the membrane skeleton of erythroid cells (Staufenbiel and Lazarides, 1986). Furthermore, ankyrin has been shown to be present in a variety of non-erythroid cells such as neurons and muscle (Nelson and Lazarides, 1984a, 1984b). These studies identified peptides that shared antigenic and structural features with the erythrocyte ankyrin but differed in terms of their estimated molecular weights. Thus, it is not clear yet whether ankyrin exists in cells as a single molecule or as a family of molecules with similar properties.

Monospecific antisera generated against erythroid ankyrin were used as immunological probes to screen a λgt11 expression vector library of cDNA prepared from chicken erythroid poly(A)+ RNA (Moon et al., 1985). The 2.8 kb cDNA obtained from this analysis hybridized to only one gene of chick genomic DNA. Northern blot analysis on erythroid and myotube RNAs showed that the cDNA probe hybridized to a majority RNA species of 8.8 kb in erythrocytes and a 3.4 kb in myotubes. The 3.4 kb transcript identified in myotubes could be translated, assuming a maximum coding capacity, to a peptide with molecular weight of 125,000. This differs from the observed molecular weight of myogenic ankyrin of 235,000.

We are presently rescreening the λgt11 cDNA library, prepared from chicken erythroid poly(A)+ RNA, with cDNA probes to identify and isolate more ankyrin cDNA clones. The complete nucleotide sequence of the 8.8 kb erythroid ankyrin transcript will be obtained from the sequence of these overlapping cDNA clones. We will also construct cDNA from myotubal ankyrin poly(A)+ RNA. Results from these studies will allow us to discuss features of: 1) the primary amino acid structure of ankyrin and propose a model for its possible spatial arrangement and functional properties in cells, and 2) the molecular mechanisms regulating the differential expression of ankyrin and ankyrin-like polypeptides in erythroid and non-erythroid cells.

References:
190. A Gelsolin-like Actin-modulating Protein is Part of the Membrane Skeleton in Erythrocytes

Horst Hinssen, Chantal M. Champagne

The membrane skeleton of erythrocytes is a complex structural network underlining the plasma membrane and consisting of various filamentous and globular proteins. Actin has a central function in this network. It is present in the form of short filaments and has already been shown to bind to several of the membrane skeleton components like spectrin, protein 4.1, protein 4.9, and tropomyosin. We have been able to identify a gelsolin-like actin-modulating protein as an additional constituent of the membrane skeleton of avian erythrocytes.

Actin-modulating proteins have been found so far in several non-muscle and muscle cell types. Their molecular weight varies from 85-92,000 in vertebrates to 42-45,000 in invertebrates (Hinssen et al., 1985, 1986). Despite these and other differences, all modulators investigated so far exhibit a characteristic and identical pattern of interaction with actin: they are able to sever actin filaments in a calcium-dependent reaction, requiring $\gt 10^6$ M Ca$^{++}$ for activation; they nucleate actin polymerization and they cap actin filaments at the fast polymerizing end.

We have generated and affinity-purified a polyclonal antibody against the gelsolin-like actin-modulator protein from the chicken gizzard smooth muscle and used it to identify a corresponding protein of 85,000 molecular weight in chicken erythrocytes and embryonic erythroid cells. The actin modulator binds in a Ca-dependent manner to the actin of the membrane skeleton, thus functionally corroborating the immunological finding. From the known characteristics of the actin modulators in vitro, it can be deduced that the protein is bound to the fast polymerizing end of the membrane-bound actin oligomers, thus preventing further polymerization and restricting filament length.

We found that a relatively large pool of actin modulator is also present in soluble form in the cytoplasm of the erythrocytes. Depending on whether or not Ca$^{++}$ is present in micromolar amounts during cell lysis, the proportions of membrane bound to soluble modulator vary, indicating a reversible interaction of the modulator with the membrane skeleton. It remains to be determined if the soluble form of the actin modulator is also bound to unpolymerized actin in cytoplasm, thus inhibiting the formation of high polymer forms of actin.

Screening of a chicken erythrocyte-cDNA-library in lg11 has so far led to the identification of a positive clone with a 3.2 kb insert. RNA blot analysis revealed a message of approximately 4 kb in erythrocytes as well as in chicken myotubes. Current investigations involve the rescreening of the library with the 3.2 kb insert for further clones to cover the full message and RNA blot analysis of various other cell types to study expression of the protein in different tissues and organisms.

References:

191. Spectrin Assembly in Avian Erythroid Development is Determined by Competing Reactions of Subunit Homo- and Hetero-Oligomerization

Catherine M. Woods

Erythroid development entails the biogenesis of a membrane skeleton, a network of proteins underlying and interacting with the plasma membrane. The major constituent of this skeleton is the heterodimeric protein spectrin, composed of two structurally similar but distinct subunits, $\alpha$- (molecular weight ~240,000) and $\beta$-spectrin (molecular weight ~220,000) which interact side-on with each other to form a long rod-like coiled-coil molecule. Spectrin binding to the membrane is mediated by the protein ankyrin, which in turn binds to the cytoplasmic domain of the transmembrane anion transporter (reviewed in Branton et al., 1981; Marchesi, 1985). Spectrin subunits have been purified from spectrin isolated from membranes of mature red blood cells but only after prior denaturation. These purified spectrin subunits will spontaneously heterodimerize, suggesting that assembly of the spectrin-actin skeleton during development is a simple self-assembly process (Marchesi, 1985). However, previous studies from this laboratory indicated that assembly in vivo was more complex than simple self-assembly, since there was evidence for two pools of both $\alpha$- and $\beta$-spectrin formed immediately after synthesis: one pool being
destined for rapid assembly, the other for specific pathways of degradation (Woods and Lazarides, 1985). We have analyzed the nature of the unassembled pools of α- and β-spectrin "in vivo" and compared them with the products of "in vitro" translation (Woods and Lazarides, 1986). These studies have shown that immediately upon (or possibly concomitant with) synthesis, the spectrin polypeptides adopt one of two configurations: heterodimers (the form characteristic of the mature membrane-skeleton) or homo-oligomers (in the case of α-spectrin, a homodimer, β-spectrin a homo-tetramer). The homo-oligomeric forms are stable and will not interact with each other either in the absence or presence of membranes under native conditions. However, if the homo-oligomers are denatured in approximately stoichiometric amounts, upon renaturation heterodimers are the predominant resultant form. Thus, it appears that the ability to homo-oligomerize is unique to newly-synthesized spectrin polypeptides and cannot be mimicked by previously denatured molecules. The ratio of heterodimers to homo-oligomers formed in vitro closely parallels the ratio of newly-synthesized spectrin that assembles versus the amounts being targeted for degradation. Membrane-binding studies confirm that it is the heterodimers that are the species to assemble stably, with the homo-oligomers being targeted for distinct pathways of degradation. Thus, the extent of spectrin assembly is limited by the extent of heterodimerization.

References:

192. AEV-Transformed Cells Express All the Major Peripheral Constituents of the Erythroid Membrane Skeleton but Do Not Express the Anion Transporter
Catherine M. Woods, Brigitte Boyer, Peter K. Vogt1, Elias Lazarides

The avian erythroblastosis virus (AEV) ES4 strain is a retrovirus that transforms early erythroid progenitor cells and blocks their differentiation at the CFU-E stage of development, i.e., well before the stage of terminal differentiation (reviewed in Graf and Beug, 1978). Temperature-sensitive mutants of this virus are available; hence, studies using this system were initiated to investigate whether this system would enable us to analyze the initial steps involved in the expression and assembly of the erythroid membrane skeleton. Contrary to other previous studies on this system, we have found that these cells do express major 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 αβ-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 form a rudimentary membrane skeletal array under the plasma membrane 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 mRNA is uniquely lacking in fully-transformed cells.

The AEV-transformed cells synthesize these proteins at rates equivalent to rates found in mitotic erythroid cells and severalfold greater than the rates found in postmitotic erythroid cells. Kinetic analysis coupled with the solubility properties of newly-synthesized components has revealed that these proteins do partly assemble into a skeletal form, but this skeletal pool is rapidly turned over with a $t_\frac{1}{2}$ of ~4.5 hr. This is considerably less than the doubling time for these cells. In contrast, in differentiating erythroid cells, no detectable turnover of these proteins occurs once assembled onto the membrane-skeleton. Hence, the low steady state levels in the AEV-transformed cells are a consequence of increased turnover and not of lower synthetic rates. As these cells lose their transformed character, they begin to express and accumulate anion transporter. Concomitantly, the steady state levels of the peripheral components on a per-cell basis increase. At the same time, the cells also begin to accumulate hemoglobin. Preliminary analysis of erythroid progenitor cells transformed with a different virus, S13, has revealed a similar situation, namely, all of the peripheral components of the membrane skeleton are
expressed but the anion transporter is not expressed. These studies suggest that the stable assembly of the erythroid membrane skeleton is dependent upon the presence of the anion transporter. Furthermore, the expression of all of the components typical of the erythroid membrane skeleton is not activated in a coordinate fashion during development. Rather, the expression of one key component, the anion transporter, occurs later in the sequence of steps leading to terminal differentiation. This expression of the anion transporter apparently coincides with the expression of hemoglobin. The alternative is that viral transformation of the progenitor cells is specifically inactivating the anion transporter, either via the site of integration of the provirus or by the action of their oncogenes. However, this interpretation seems less likely given that the AEV-ES4 virus and the Sl3 virus are quite different and that their major oncogenes, v-erbB and gp155, respectively, are unrelated with quite different activities (Benedict et al., 1985).

References:

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193. Cell Cycle Regulation of Membrane Cytoskeletal Polypeptide Expression and Assembly in Avian Erythroid Development

John V. Cox, Jeffrey H. Stack

Steady state protein and RNA levels have been determined for band 3 (anion transporter), ankyrin and protein 4.1 in erythroid cells of both the primitive and definitive lineage of 6-day chicken embryos. Cells of these lineages have been separated from each other on the basis of their size using centrifugal elutriation. Immunoblotting analysis of these elutriated cells demonstrated that total band 3 protein per cell in each fraction was constant. However, two of the fractions, one in the primitive and one in the definitive lineage, showed increased levels of cytoskeletal-associated band 3. These fractions also showed increased levels of the cytoskeletal polypeptides, ankyrin and protein 4.1. Further analysis of these elutriated cells through the use of the fluorescence-activated cell sorter revealed that those cells with increased levels of cytoskeletal band 3 are in G0, G1, and S, while those fractions containing lower levels of cytoskeletal band 3 are predominantly in S, G2 and M. In addition, immunofluorescent staining of these cell fractions with tubulin antibodies revealed that those cells in G0 through S possess a marginal band and those in S through M contain a normal microtubule array. These results indicate that as the cells of each lineage enter G2 and M, they disassemble their membrane cytoskeleton and reassemble it following mitosis. SI nuclease analysis showed that the band 3 and protein 4.1 RNA levels were similar in all cells of the definitive lineage. However, the abundance of these RNAs in primitive lineage cells was greatest in G0 and G1. The cells in G2 and M had reduced levels of both RNAs. These results suggest that there is cell-cycle regulation of these membrane cytoskeletal transcripts and polypeptides. Currently, attempts are under way to culture and synchronize these primitive erythroid cells to study this regulation in more detail.

194. Expression and Assembly of the Anion Transporter during Avian Erythroid Development

John V. Cox, Jeffrey H. Stack

Previous pulse-chase studies have established that the fraction of the newly synthesized avian erythrocyte band 3 (anion transporter) polypeptides which become associated with the cytoskeleton varies with the day of embryonic development examined. A greater percentage of cytoskeletal-associated band 3 is observed at days 10 and 15 than that found at day 5. To further understand how both the abundance of band 3 and the extent of its cytoskeletal association is regulated, steady state protein and RNA levels have been determined by immunoblotting and SI analyses. These studies have shown that the level of band 3 polypeptides per cell increases until day 10 of development. Furthermore, the fraction of cytoskeletal-associated band 3 increases from ~10% at day 5 to ~60% at day 10 of development. The cytoskeletal polypeptides ankyrin and protein 4.1, which have been shown to interact with band 3 in vitro, accumulate with the same kinetics as the cytoskeletal-associated band 3. This suggests that the availability of these polypeptides is the limiting step for the interaction of band 3 with the cytoskeleton.

While the band 3 and protein 4.1 polypeptide levels
increase from day 5 to day 10 of development, the band 3 RNA levels decrease -3 fold and the protein 4.1 RNA levels remain essentially constant. This result indicates that the abundance of these two polypeptides is regulated posttranscriptionally during avian erythroid development. Future experiments are aimed at determining the specific level at which this regulation occurs.

195. Identification and Analysis of Multiple Chicken Protein 4.1 mRNAs

Jeffrey H. Stack, John J. Ngai

Protein 4.1, an extrinsic membrane protein that facilitates the interaction of spectrin and actin, exists as a multiplet of structurally-related polypeptides in chickens (Granger and Lazarides, 1984). Interestingly, the ratio of protein 4.1 variants expressed is developmentally regulated during terminal differentiation of chicken lens and erythroid cells (Granger and Lazarides, 1985). In order to understand the mechanism(s) by which multiple chicken protein 4.1 variants are differentially expressed, we have begun by isolating cDNA clones for protein 4.1 from a chicken erythroid cDNA library (Biology 1985, Abstract No. 190). Blot analysis of both lens and erythroid RNA using protein 4.1 cDNA probes reveals only a single 6.5 kb mRNA. However, S1 nuclease protection of end-labeled cDNA probes by erythroid RNAs demonstrates the existence of at least four distinct protein 4.1 mRNA species whose sizes are indistinguishable by both formaldehyde- and methyl mercury hydroxide-agarose electrophoresis. Furthermore, the ratios of these mRNAs change during chick embryo erythropoiesis, indicating that the multiplicity of mRNAs in part is responsible for the heterogeneity of protein 4.1 polypeptides expressed. Genomic DNA blots, in conjunction with an analysis of protein 4.1 genomic clones, indicate that one gene encodes the multiple chicken protein 4.1 variants. Hence, developmentally-regulated differential processing of protein 4.1 transcripts determines the variants to be expressed. Protein 4.1 expression is further regulated at the translational level, as we observe a difference in variant ratios when protein 4.1 is synthesized in vivo or in a rabbit reticulocyte lysate in vitro.

To understand the pathways by which multiple protein 4.1 mRNA are generated, we are analyzing both genomic and cDNA clones encoding protein 4.1. However, since cDNA sequences corresponding to -50% of the mRNAs hybridize to -40 kb of genomic DNA, we expect the size of the chicken protein 4.1 gene to be on the order of ~30 kb. It would be impractical at present to attempt to sequence the entire gene. Our alternative strategy involves nucleotide sequence analysis of the cDNAs that we have already isolated and further isolation and sequencing of full-length protein 4.1 cDNAs. A comparison of full-length cDNAs will allow us to identify the sites at which individual mRNAs differ from each other. Heteroduplex analysis of genomic and cDNA sequences will be used to determine the structure of the protein 4.1 gene.

References:

196. Expression of the Chicken Vimentin Gene in Murine Erythroleukemia Cells

John J. Ngai

We have used Friend murine erythroleukemia (MEL) cells to study the regulation of vimentin gene expression during mammalian erythropoiesis. During DMSO- or HMBA-mediated differentiation of MEL cells, vimentin mRNA levels are rapidly repressed, suggesting that the regulation of this gene during erythroid differentiation is regulated at least in part at the transcriptional level (Ngai et al., 1984). To examine the regulation of vimentin gene expression in further detail, we have transfected MEL cells with a cloned chicken vimentin gene (Capetanaki et al., 1983). A 12 kb chicken genomic fragment containing the 8 kb vimentin gene was cloned into pUC18 together with the hamster APRT gene. The resultant 20 kb plasmid was then used to transfect an aprt MEL cell line by poly-L-ornithine-mediated DNA transfer (V. C. Bond and B. Wold, submitted for publication). The transformation frequencies of MEL cells using this method range from ~10^{-6} to 2 x 10^{-7}, which is ~5-10-fold higher than the frequencies observed using calcium phosphate-mediated transfection. Of an initial 24 aprt transformants analyzed, 16 possess intact chicken vimentin gene sequences. Of these 16 MEL cell clones, six expressed the chicken vimentin mRNA,
as detected by RNase protection of an SP6-polymerase-synthesized \(^{32}P\)-labeled anti-sense chicken vimentin probe. During DMSO-mediated differentiation, three of these lines expressed the exogenous gene at approximately constant levels. Surprisingly, two lines expressed a higher (5-10X) level of chicken vimentin mRNA after DMSO induction. Hamster aprt mRNA levels either are constant or increase only slightly during differentiation of these two cell lines. As the vimentin gene normally is induced during chicken erythroid development, these observations suggest that the exogenous vimentin gene contains sequences that can respond to positive trans-acting factors induced during differentiation of MEL cells. The sixth vimentin-positive cell line expressed the chicken mRNA at low levels, and after DMSO induction, the levels of chicken vimentin mRNA decreased. Hence, many more cell lines harboring chicken vimentin gene sequences need to be generated and analyzed to determine the behavior of the chicken vimentin gene in differentiating MEL cells. In addition to continuing transfection of MEL cells by poly-L-ornithine-facilitated transfer, we are presently also attempting transfection by electroporation and protoplast fusion, with the expectation that these techniques will yield higher transfection frequencies.

References:

197. Vimentin and Protein 4.1 Expression throughout the Development of Murine Primitive Erythrocytes
Frank Sangiorgi, Catherine M. Woods

During the embryonic and fetal development of the mouse, a sequential change in the site of erythropoiesis and in the appearance of two morphologically distinct erythrocyte cell lineages occur (Kovach et al., 1967). Erythropoiesis during embryogenesis begins with the formation of yolk sac blood islands on the seventh day of gestation. From the eighth to twelfth days, primitive erythroid cells develop from these blood islands and persist in the fetal circulation until the sixteenth day. Unlike the erythrocytes of the adult, these primitive cells remain nucleated throughout their development and synthesize predominantly embryonic hemoglobins. Subsequently, larger numbers of enucleated erythroid cells of the definitive lineage containing exclusively adult hemoglobin are formed in the fetal liver at 12 to 14 days of gestation.

The gross morphological characteristics of the nucleated erythroid cells derived from the yolk sac blood islands appear similar to those of avian erythrocytes. We have investigated this resemblance by examining these primitive murine erythrocytes with regard to vimentin and protein 4.1, proteins that are constituents of the intermediate filament and membrane cytoskeleton of chicken erythrocytes, respectively. Western blot analysis of primitive erythrocytes isolated at 8, 11, 12, and 15 days of gestation using chicken anti-vimentin antibody depicts a protein of apparent molecular weight 55,000, comigrating with chicken vimentin, in cells from eight-day embryos. This protein subsequently decreases in amount through the remaining stages. Immunofluorescence microscopy of these cells and cells from additional time points confirmed and extended this observation; vimentin filament networks are present in these erythrocytes from days 8 to 12, collapsing into a perinuclear bundle and subsequently being eliminated from the cells through the remainder of their development (days 13 to 16). The analysis of protein 4.1, utilizing a chicken anti-protein 4.1 antibody, detects a predominant protein of approximate molecular weight 115,000 and two additional polypeptides with molecular weights of 100,000 and 87,000 at eight days of gestation, a pattern qualitatively similar to that of definitive chicken erythrocytes (Granger and Lazarides, 1984). As the primitive murine erythroid cells continue to develop, this early arrangement of polypeptide variants shifts to one in which the predominant protein 4.1 variants are of molecular weights 80,000 and 78,000 by the twelfth day, a pattern identical to that observed for murine definitive erythrocytes (Whitfield et al., 1983).

These preliminary studies suggest that in this cohort of primitive erythroid cells, a vimentin filament network present early in their development is gradually lost so that by day 15 a large number of cells no longer possess these filaments. Concomitantly, the three high molecular variants of protein 4.1 are also replaced by the polypeptide variants characteristic of enucleated mammalian erythroid cells. Thus, during
development, mouse primitive erythrocytes appear to modify their cytoskeleton towards the phenotype of proteins seen in the definitive mammalian erythroid cell but without going through the final enucleation step. A more detailed analysis using probes for other components of the erythroid cytoskeleton is planned in order to test this hypothesis. Analysis of how the mitotic state of these cells bears on these changes is presently under way, along with the characterization of the high molecular weight variants of protein 4.1 and their relationship to the mature mammalian erythroid forms.

References:

198. Expression of an Exogenous Vimentin Gene in Erythroid Cells of Transgenic Mice
Frank Sangiorgi, John J. Ngai

The hematopoietic system offers a useful model to examine the change in intermediate filament organization during cellular differentiation. Vimentin, one of several classes of intermediate filaments, is a protein whose expression varies throughout the differentiation of the hematopoietic cell lineages.

A structural role for vimentin filaments has been inferred from morphological observations (Lazarides, 1982). Nucleated chicken erythrocytes possess a vimentin filament network that attaches to the plasma membrane, spans the cytoplasm, and appears to anchor the centrally located nucleus (Granger and Lazarides, 1982). During the differentiation of these erythrocytes the chicken vimentin gene is induced (Capetanaki et al., 1983), with a concomitant increase in vimentin synthesis. In contrast, vimentin filaments are not observed in the mature, anucleate mammalian erythrocyte. Dellagi and co-workers have shown by immuno-fluorescence microscopy that vimentin expression is lost during the erythroblastic stages of human erythropoiesis in vivo (Dellagi et al., 1983). Furthermore, our studies utilizing murine erythroleukemia (MEL) cells have demonstrated that the repression of 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 proceeded to microinject the entire chicken vimentin gene into fertilized mouse oocytes through the use of established procedures (Brinster et al., 1985). The generation of transgenic mice will allow us to study 1) whether or not the chicken vimentin gene is regulated in the same manner as the endogenous mouse gene in erythroid cells, and 2) the assembly and cytoplasmic localization of chicken vimentin should the gene be successfully expressed. Preliminary studies have indicated that in differentiating MEL cells a transfected chicken vimentin gene is positively-regulated while the endogenous gene is down-regulated (J. Ngai, unpublished results). We therefore anticipate that the chicken vimentin gene will also be positively regulated during murine erythroid development in vivo. In addition, these transgenic animals offer the opportunity to examine the function of this gene and of the protein in nonerythroid cells. The resultant mice from the microinjection and transplantation procedures are currently being analyzed for integration of the foreign DNA into the genome. Following the identification of several transgenic mice, these animals and their offspring will be examined for expression of the gene in erythroid and additional tissues.

Another approach is also currently being undertaken that involves the directed expression of chicken vimentin in erythroid cells. This objective is to be accomplished by the construction of a chimeric gene incorporating a full-length vimentin cDNA under the control of the mouse α- or β-major hemoglobin promoter. This arrangement of sequences will serve to target the expression of vimentin in a temporal and erythroid-specific manner.

References:
Summary: The laboratory's interest in gap junctions has continued during the year with significant advances on a number of fronts. Especially curious is the finding of a second protein (molecular weight 21,000) in gap junctions isolated from liver. This polypeptide is homologous but not identical to the major protein (28 kd) long recognized in this tissue. Immunocytochemistry carried out by Dermietzel and Traub in Essen (Germany) shows that the 21 kd entity co-localizes with the 28 kd. Thus, either connexons are mixtures of proteins as is the case for the acetylcholine receptor, or there are at least two types of connexons in a given junction plaque. What the role of these putative different connexons might be is not at all clear, but the finding has intriguing consequences for the control of assembly and permeability. The ratio of 21 to 28 kd seems to vary in different species, suggesting subtle differences in junction physiology even in the same organ.

Another finding of interest has been that the 47 kd protein described in heart gap junctions by Manjunath et al. (1985) has the same N-terminal sequence as the 27-30 kd polypeptides described previously in this tissue. It looks like the latter polypeptides are derived from the higher molecular weight protein by enzymatic degradation. The junction proteins do indeed turn out to be rather varied! We have begun to use synthetic peptides modeled on gap junction protein molecules and have raised antibodies against several of these. We have been fortunate to find some that react in Western blots and also in immunocytochemical staining of tissues. We hope these reagents will prove useful in the study of the role of different regions of the junctional polypeptide. We also have studied the organization of lens MIP in fiber cell membranes and are following the developmental expression of MIP mRNA. We have pursued our efforts to isolate a cDNA clone coding for the liver protein to compare with the lens clone we obtained a couple of years ago.

The year has also been epochal in that Jean Edens, long a mainstay of the laboratory, moved away! Thanks Jeannie for your help during the years; we'll miss you. We'll also miss Bruce Nicholson who left to take a position in Buffalo; the umbilical cord is not completely severed, however, since we are involved in joint projects even at this long distance.

199. Production and Characterization of Sequence-specific Antibody to the Heart Gap Junction Protein

Bruce J. Nicholson¹, S. Barbara Yancey, Brian J. Austin

Primary goals in our study of the heart gap junction protein are the production of both polyclonal and sequence-specific antibodies for use as probes for screening cDNA expression libraries and ultimately to determine the orientation of the protein in the membrane. The amino-terminal 20 or so residues of the protein have already been sequenced by Edman degradation (Nicholson et al., 1985). Based on this sequence, a peptide was synthesized by Dr. Stephen Kent in Prof. Lee Hood's laboratory. The peptide or peptide conjugated to hemocyanin was used to immunize rabbits. Initial assays of the peptide bound to microtiter wells show that the collected antisera from each of three rabbits react with the peptide at a dilution of 1/10,000. By good fortune, antiserum from one of the rabbits also reacts with isolated junctions by ELISA as well as with the protein on Western blots, making it suitable for experiments to determine the location of the amino terminus of the molecule as it is assembled in the gap junction. Initially, the antibody will be used for immunocytochemistry of isolated heart cell membranes and on tissue sections. Later, we will use the techniques of electrophysiology to determine whether antibody binding to gap junctions of cardiac myocytes in culture interferes with the ability of the cells to communicate.

Reference:

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200. The Heart Gap Junction Has a Tissue-specific Domain at the Carboxy Terminus

Bruce J. Nicholson¹, Chellakere K. Manjunath², David B. Teplow, Leroy E. Hood, Ernest Page², Jean-Paul Revel

The molecular mass of the heart gap junction protein was, until recently, believed to be about 28-30 kd, similar to that of other gap junction proteins characterized up to now. A larger polypeptide of molecular weight about 47 kd has been identified as a possible in vivo precursor, which undergoes rapid proteolysis during the isolation procedures. We have now shown that this 47 kd entity has the same amino-terminal sequence as the 28 kd polypeptide previously identified in heart junction fractions. Thus, the carboxy end of the heart junction protein is a peptide of molecular weight 17 kd, absent in liver. This finding further supports the idea that gap junction proteins are a highly diversified family.

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201. Identification of Two Homologous Proteins in Liver Gap Junctions

Bruce J. Nicholson¹, Rolf Dermietzel², David B. Teplow, Otto Traub², Klaus Willecke², Jean-Paul Revel

Biochemical and structural analyses have suggested that in each tissue, gap junctions are comprised of a single protein (molecular weight 28,000 in liver, 47,000 in heart and 26,000 in lens). While highly purified fractions of liver gap junctions display several bands when examined on SDS gels, these can virtually all be attributed to degradation and aggregation of the 27 kd protein. One exception, a molecule 21 kd, has now been partially sequenced. The amino-terminal 20 residues of this protein in both mouse and rat show a 45% identity with the previously sequenced 28 kd characteristic of liver.

Antibodies that bind specifically to either the 21 or the 28 kd protein bind to the same junctional plaques as determined by double labeling immunofluorescence. The colocalization is also supported by the co-precipitation of the two proteins from whole junction fractions by an antibody specific against the 28 kd component. It is not known whether a single channel (connexon) is a homo- or heteropolymer, but preliminary findings suggest the former. Thus, at least in liver, the gap junctions are comprised of two proteins, which, surprisingly, previous experiments had shown to be differentially regulated.

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202. Expression of the Gene for MIP during Development of the Rat

S. Barbara Yancey, Jan H. Hoh, Brian J. Austin

In sections of the eye lens even in its mature form
one is able to view the complete pathway of cell differentiation. The cells of the anterior epithelium near the equator of the lens continuously divide and differentiate into the elongated fiber cells which comprise the main body of the lens. MIP, the main intrinsic protein of the fiber cell membrane, is thought to be the major component of the numerous, large gap junctions that interconnect the fiber cells and is known as a marker for lens cell differentiation. To study the spatial and temporal pattern of expression of the gene for MIP, we have used the technique of in situ hybridization with antisense RNA probes transcribed from our cDNA template in the SP6 vector (Biology 1985, Abstract No. 192). Sections of rat embryos and eyes of neonatal rats are treated as described by Cox et al. (1984) and hybridized with the 35S-labeled probes. The message for MIP is detectable as early as day 12 of embryogenesis in the primary lens fiber cells formed as the cells in the posterior wall of the lens vesicle elongate to fill in the cavity of the vesicle. The cells of the anterior wall maintain their epithelial character and do not appear to express the gene for MIP at this or later stages, although we are currently devising procedures that will allow us to make a more accurate quantitative determination of the distribution of autoradiographic grains. With further development, as secondary lens fibers form by differentiation of the epithelial cells at the equator of the lens, RNA transcripts are predominantly localized in juxta-nuclear regions of the differentiating cells where membranous structures (rough endoplasmic reticulum and Golgi saccules) are seen by electron microscopy. We have not detected transcripts in the retina or any other non-lens tissues.

Reference:

203. Continuing Studies on the Orientation of MIP in Lens Membranes

S. Barbara Yancey, Brian J. Austin

From the complete amino acid sequence of MIP we obtained by cDNA cloning, we have proposed a hypothetial model for the arrangement of the molecule as it might be assembled in a gap junction. As described in Biology 1985, Abstract No. 193, we have begun to test this model with anti-peptide antibodies. One of the antibodies is to a region of the molecule (LP1) near the carboxy terminus which according to the model resides in the cytoplasm; the other is to a sequence (LP2) predicted to lie in the extracellular space, a possible site of cell-cell interaction in the junction not accessible to enzymes or antibody. Both antibodies recognize the intact protein on Western blots. We first tested the model by digesting lens junctions with either trypsin or chymotrypsin. According to the model, we would expect that either of these enzymes would remove the most C-terminal peptide but not the other. This was found to be the case, lending support to the model. Each of these antibodies also binds to sites in lens membranes bound to microtiter wells, but these preliminary ELISAs suggest that there is quantitatively less binding of the antibody to LP2. This result is also compatible with the model since crude membranes were used and MIP is believed to be present not only in junctional but also nonjunctional membrane. One would then predict that relatively more of this latter antibody should bind were the junctions to be split apart as is believed to happen when junctions are exposed to pH below 2.5 (Sas and Johnson, 1983). We are currently conducting experiments to determine whether this in fact occurs.

Reference:

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Assistant Professor: Ellen Rothenberg  
Senior Research Fellow: James P. Lugo  
Research Fellows: Christine Kinnon, Kathleen L. McGuire  
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Summary: Our laboratory is interested in lineage commitment and the sequential mechanisms of tissue-specific gene activation in the differentiation of T lymphocytes. T (thymus-derived) lymphocytes, which constitute an important branch of the vertebrate immune system, provide a particularly interesting subject for developmental study because of the variety of unique responses they exhibit that are beginning to be understood in molecular detail. All T cells recognize foreign antigens using cell-surface receptor structures that are related to immunoglobulins but unique to T cells. Different clones of T cells recognize different antigens because the genes encoding the receptors themselves, like immunoglobulin genes, are assembled from variable component sequences by DNA rearrangement during I-cell development. Contact with antigen activates the cells via rapid increases in cytoplasmic free calcium concentration and in membrane-bound protein kinase activity. These changes lead both to proliferation and to implementation of a "helper," "killer," or "suppressor" program of gene activity. The bases of these responses are probably best understood for helper T cells, which amplify immune responses by synthesizing and secreting a burst of polypeptide hormones. Many of these hormones are T cell-specific gene products. The one that concerns us here is interleukin-2 (IL2), the growth hormone that controls most if not all proliferation of the mature T cells themselves. Antigen contact also leads to proliferation because it stimulates expression of high-affinity receptors for IL2 on helper and all other classes of T cells. The binding of IL2 to its receptor drives resting lymphocytes into the cell cycle.

These phenomena involve at least three different expression patterns for T cell-specific genes. Genes encoding components of T-cell receptors are expressed in all T cells and appear to be transcribed constitutively. The gene encoding the IL2 receptor is also expressed in all T cells, but under tight activation-dependent control. Similarly, the IL2 gene is expressed only in transient response to induction, but appears to be further restricted to expression in a helper sublineage of T cells. Thus, while these genes all encode characteristic T-cell products, they are not coordinately regulated. We are interested in defining the processes whereby their separate expression patterns are established in T-cell ontogeny. To do this, we have looked in the thymus, the organ in which T-cell precursors differentiate.

Within the thymus, primitive precursor cells respond to the unique microenvironment by acquisition of phenotypic markers and functional properties of mature T cells. Peripheral lymphoid organs are seeded continuously by the emigration of these cells. Two classes of mature T cells, one set expressing the Lyt-2 glycoprotein (mainly killers) and one expressing the L3T4 glycoprotein (mainly helpers), are generated in the thymus from "double-negative" precursors that express neither marker. Tracking the steps in this conversion process is complicated, however, by the fact that most of the descendants of double-negative cells are not mature at all. They are cells that express both Lyt-2 and L3T4 ("double-positive" phenotype) and other unique markers, and they accumulate in the thymic cortex and die without emerging. We have analyzed this aberrant population in detail to find clues to the reason for their death. As described below, our results indicate that they are not, on the whole, T cells whose differentiation is aborted at an intermediate stage but rather cells embarked on a divergent ontogenic pathway, which we term the major cortical lineage. Thus, we are interested both in the gene expression changes that accompany T-cell maturation and in the basis for commitment to alternative lineages leading to maturation or to death.

In the past year, our results have elucidated profound differences between double-negative precursors, maturing T cells, and the major cortical population. Both "mature phenotype" thymocytes and the major cortical population are more mature than
double-negative cells by the criterion of expression of fully rearranged genes for the components of the T-cell receptor. The double-negative cells express only a subset of these genes and presumably have not completed the necessary gene rearrangements for others. On the other hand, the IL2 receptor gene is turned on early in the most primitive double-negative cells. While its expression is later turned off in mature T cells, it remains inducible. The major cortical cells, in striking contrast, apparently lose not only expression but also inducibility of the IL2 receptor. Yet a third pattern of expression is found for the IL2 structural gene. While it is not detectably expressed in the thymus in vivo, it becomes inducible in a subpopulation of double-negative cells and remains inducible in mature cells. Although RNA expression data are equivocal, major cortical cells cannot be induced to secrete IL2.

We have begun to study the regulation of IL2 gene expression in the most detail. Our recent results indicate that immature cells, even when induced, may only accumulate IL2 transcripts to substantially lower levels per cell than induced mature cells. This suggests that the cells first become inducible for IL2 transcription and subsequently undergo a further developmental change that increases the maximum transcription rate for fully-induced IL2 genes or increases the half-life of IL2 mRNA. We hope to test the relative importance of these effects by direct measurements of IL2 RNA synthesis and stability in the various populations. Our principal goal is to determine the molecular basis for the initial acquisition of IL2 inducibility. Few other genes have been studied that show both tissue specificity of expression and a reversible response to induction. An attractive hypothesis is that pre-T cell populations that become inducible for IL2 synthesis begin to express specific proteins that are conditional regulators of IL2 transcription. Using gene transfer of engineered IL2 genes, we have begun to study the sequence elements, within and flanking the gene, that are required for correct expression. This information will serve as a guide to direct assays for such regulatory proteins.

204. Two-color Fluorescence Analysis of Proliferating Thymocytes

James P. Lugo, Rochelle A. Diamond, Ellen Rothenberg

Unlike secondary lymphatic tissues, the thymus contains a significant pool (~10%) of dividing cells. Most likely, these cells represent the developmental step(s) during which developmental fates are fixed and committed daughter cells produced (Rothenberg and Lugo, 1985). By two-color fluorescence analysis of these cells (see the Cell Sorting Facility progress report, page 217, Abstract No. 2), using monoclonal antibodies (MAbs) against Lyt-2 and L3T4, we have shown that thymic lymphoblasts display heterogeneous amounts of these proteins on their surfaces, implying that it is during this period of rapid cell division that the Lyt-2 and L3T4 genes are first transcribed (Rothenberg et al., 1986). A striking aspect of these studies was the finding that the bulk of L3T4 surface heterogeneity was confined to cells that already expressed high levels of Lyt-2. This result provocatively suggests that cortical cells activate the Lyt-2 gene before activating the L3T4 gene, although the equally plausible interpretation that these cortical cells have repressed the L3T4 gene and are becoming single positive for Lyt-2 cannot be excluded. To distinguish between these two possibilities, direct in vitro tracing of lineage relationships will be required.

References:

205. In vivo Expression of IL2 Receptors in Thymocyte Populations of Different Maturational Stages

James P. Lugo, Rochelle A. Diamond, Ellen Rothenberg

Two years ago we demonstrated that very few proliferating thymocytes expressed IL2 receptors detectable by immunofluorescent staining (Lugo et al., 1985). This suggests that intrathymic proliferating is controlled by mitogenic signals that are quite different from those involved in peripheral T-cell responses to antigen. We have extended these studies by attempting
to characterize all those thymocytes that do express IL2 receptors, using two-color immunofluorescent staining analysis to type cells for expression of Lyt-2 and L3T4. Cells bearing low as well as high numbers of IL2 receptors have been quantitated by cytolytic elimination with an IgM antibody against the IL2 receptor and complement. These analyses have confirmed that the most numerous class of proliferating thymocytes, the Lyt-2\(^+\) L3T4\(^+\) cells, includes few if any IL2 receptor-bearing cells. IL2 receptor expression is largely confined to the double-negative cell population, where IL2 receptor-bearing cells constitute 50-70\% of the cells. There are two surprising aspects of this expression. First, the double-negative cells appear unable to respond to IL2 as a growth factor. Thus, the functional consequence of IL2 receptor expression is unclear. Second, although about half of double-negative cells are already in cycle as determined by acridine orange staining (see Cell Sorting Facility progress report, Abstract No. 2), both cycling and noncycling cells show equal frequencies of IL2 receptor expression. This result raises the possibility that immature cells do not even regulate expression of the IL2 receptor gene as do mature cells, but instead activate it independently of mitogenic stimuli per se. Thus, while the IL2 receptor gene clearly becomes available for expression early in ontogeny, both the signals that trigger its expression and the physiological significance of its expression remain unresolved.

Reference:

206. Inducibility of IL2 Receptor Expression by Cortical Thymocytes and Immature Pre-T Cells

James P. Lugo, Rochelle A. Diamond, Ellen Rothenberg

We are currently investigating the ability of various thymocyte subpopulations to express the IL2 receptor upon stimulation with the calcium ionophore A23187 and the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA). Our choice of A23187 and TPA as mitogens was based on the ability of these reagents to increase intracellular calcium and activate protein kinase C, respectively, two physiologic events that play important roles in normal T-cell activation and mitogenesis (Depper et al., 1984; Imboden and Stobo, 1985). As we reported last year (Lugo et al., 1986), these reagents can induce IL2 synthesis even in immature double-negative thymocytes that are unable to respond to receptor-mediated mitogenesis.

We have found that most (~90\%) immature (Lyt-2\(^+\), L3T4\(^+\)) thymocytes that do not express IL2 receptor can be induced to do so if stimulated with A23187 and TPA. This result indicates that like the IL2 gene (Lugo et al., 1986), the IL2 receptor gene is rendered inducible at an early stage of T-cell development, before expression of the mature (as) form of the antigen receptor has occurred. Furthermore, given the high proportion of immature precursors that can express IL2 receptor, it appears that most pre-T cells, regardless of their ultimate developmental fate, pass through a differentiative stage during which the IL2 receptor gene becomes transcriptionally inducible.

In contrast to our results with immature T cells, we have found that cortical lymphocytes, which simultaneously express both Lyt-2 and L3T4, cannot be induced to express IL2 receptor. Combined with our previous finding that A23187 and TPA fail to induce these cells to produce IL2 (Lugo et al., 1986), this result indicates that cortical cells comprise an inert T-cell population which has nonetheless undergone \(\alpha\)- and \(\beta\)-chain gene rearrangements at the genomic level and contains RNA transcripts from both of these loci (Kinnon, Diamond and Rothenberg, submitted for publication).

References:
if any α-chain transcripts (Kinnon, Diamond and Rothenberg, submitted for publication). These cells also express low levels of T3 δ-chain mRNA and preliminary studies suggest that they express even less T3 ε-chain mRNA, if any. Double-negative cells can be stimulated to proliferate by treatment with the calcium ionophore A23187 and the phorbol ester TPA (Lugo et al., 1986). This treatment also induces the transcription (see Abstract No. 212) and secretion of the protein product (Lugo et al., 1986) of IL2, a gene that is not transcribed in the resting cells. We are currently investigating the inducibility of T-cell receptor and T3 complex genes in response to these signals in this population of cells. Preliminary studies using a specific series of RNA dot blots hybridized with T-cell receptor and T3-specific probes have revealed that only the genes that are already active in double-negative cells are up-regulated in this system. Thus, there is a dramatic upsurge in the levels of the T-cell receptor γ (x10-12 fold), δ (x8 fold) and T3 δ (x7 fold) chain transcripts, while the levels for T-cell receptor α and T3 ε-chain transcripts appear to remain constant. We have estimated that there is roughly a threefold increase in the total RNA content of these cells, which is not sufficient to account for the observed increase in specific transcripts. These studies tentatively suggest that although neither the T-cell receptor α chain nor the IL2 genes are expressed in double-negative cells in vivo, they do not respond equally well to induction by these signals. It appears that the ease with which these genes can be induced is dependent on their state of specification prior to stimulation, and that the same signals do not up-regulate or down-regulate all chains of either complex to the same extent. It still holds true, however, that the T-cell receptor β chain and the T3 δ chain appear to be co-regulated (see Abstract No. 208).

References:

208. Transcription of the T3 δ Chain in Subpopulations of Adult Mouse Thymocytes
Christine Kinnon, Rochelle A. Diamond, Ellen Rothenberg

The T-cell receptor for antigen is a heterodimer composed of an α and β chain and is always found in close association on the surface of mature T cells with the T3 complex of polypeptide chains. Expression of either the T-cell receptor or T3 complex on the cell surface appears to be obligatory for expression of the reciprocal complex. We have shown previously that thymocytes of the major cortical lineage, which have a high probability of cell death, express high levels of mature mRNAs encoding both the α and β chains of the T-cell receptor (Kinnon, Diamond and Rothenberg, submitted for publication). However, it remains to be determined whether or not these cells express high levels of receptor proteins on their cell surfaces. Since there is such a close correlation between the expression of the T-cell receptor and the T3 complex on the surface of mature T cells, we explored the expression of the T3 complex in several populations of developing thymocytes. Using gel-blot analysis with a T3 δ-chain probe (kindly provided by P. van den Elsen and C. Terhorst), we were able to show that all populations of thymocytes that express high levels of T-cell receptor β-chain transcripts also express high levels of T3 δ-chain transcripts. In each case there was roughly a 1:1 correspondence of T-cell receptor β-chain mRNA to T3 δ-chain mRNA. Thus, it is possible that these genes may be co-regulated at the level of mRNA expression in developing thymocytes. Furthermore, this study has demonstrated that mRNA for at least one of the chains of the T3 complex also is expressed at high levels in thymocytes of the major cortical lineage, thus giving further credence to the idea that these cells are capable of expressing T-cell receptors for antigen on their cell surfaces.

209. Relation of Transcription to Surface Expression of T-cell Receptor Molecules in the Thymus
Christine Kinnon, I. Nicholas Crispe, Michael Bevan, Ellen Rothenberg

It is important to determine precisely at what level T-cell receptor molecules are being expressed on the surfaces of thymocytes of the major cortical lineage, since the expression of these molecules has important implications for the overproduction and elimination of this major class of thymocytes. Even assuming that the T-cell receptor α- and β-chain transcripts, which are present at high levels in these cells (see preceding abstract), are accessible for translation, we cannot rule out aberrant splicing patterns or translational
frameshifts due to out-of-frame rearrangements that would render either of these mRNAs inactive. Two monoclonal antibodies raised against mouse T cells, F23.1 from the Bevan lab and KJ16-133 from Kappler and Marrack's lab, both recognize the V_8 determinant on the β chain of the T-cell receptor when it is in combination with the α chain. About 10% of small thymocytes of the major cortical lineage, as compared to 20-25% of mature T cells, are recognized by both of these antibodies, demonstrating that at least some of these cells are capable of translating the α- and β-chain mRNA present in this cell population. We have been attempting to quantitate more precisely how much of the mRNA present at high levels in the cells of the major cortical lineage may be expressed as cell-surface receptor by eliminating the V_8-positive cells in this population. Depletion was achieved by treating the cells with F23.1 and then passing the cells over a protein A-Sepharose column. How much V_8-specific mRNA remains in the F23.1-negative cells is being determined by analyzing mRNA prepared from these cells by RNase protection experiments with a V_8-specific probe (provided by Dr. R. Barth). In this way we hope to determine more precisely how much of this mRNA is accessible for translation and expression within this population of cells and to extrapolate how many of these cells are expressing T-cell receptor proteins at their surfaces.

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210. Analyzing the Heterogeneity of T-cell Receptor Gene Expression in Thymocyte Populations by in situ Hybridization

Christine Kinnon, Kathleen McGuire, Ellen Rothenberg

Thymocytes are generally divided into different subpopulations and, thus, lineages on the basis of surface phenotype using the currently available markers. These subpopulations are not necessarily homogenous in terms of cell fate and may in fact represent a variety of cell types. In order to further study the role of T-cell receptor genes in determining the fate of developing thymocytes, we are currently attempting to examine the heterogeneity of T-cell receptor phenotypes expressed within different thymocyte populations. We wish to determine whether or not the T-cell receptor phenotypes expressed by populations isolated on the basis of surface marker phenotypes are indicative of the majority of the cells in a given population. We have prepared tritiated cRNA probes for the α, β and γ chains of the T-cell receptor which we are currently using for the in situ analysis of different thymocyte subpopulations. Preliminary studies using this technique have suggested that the high levels of α-chain mRNA seen in the cells of the major cortical lineage are representative of the majority of the cells in this lineage and not the result of a few cells expressing extremely high levels of these transcripts. We would like to expand these studies to investigate heterogeneity of expression in populations of both unstimulated and stimulated double-negative thymocytes.

211. Determination of the Sensitivity of IL2 mRNA Detection Using the Technique of in situ Hybridization

Kathleen McGuire, Ellen Rothenberg

We were interested in using the technique of in situ hybridization to cellular RNA to begin to measure the heterogeneity in levels of expression of the IL2 gene in normal T-cell populations. We have used the protocol of Cox et al. (1984) with a few modifications. To determine the sensitivity of the technique in our hands, we utilized the murine thymic lymphoma EL4.E1 (Farrar et al., 1980). These cells do not make IL2 RNA constitutively but transcribe the gene upon stimulation with the phorbol ester TPA (Bleackley et al., 1981). These cultures produce optimal amounts of IL2 RNA 8-12 hours poststimulation. In situ hybridization analysis of these cells revealed a progressive increase in the number of IL2-producing cells during the culture period and an increase in the quantity of IL2 RNA per cell, as evidenced by higher grain numbers per cell. Surprisingly, 18% of the EL4.E1 cells produced detectable IL2 by in situ hybridization even one hour poststimulation. This was unexpected because no IL2 RNA could be detected by RNA gel blot analysis at this time point. To verify the presence of IL2 RNA in these cells, RNA prepared from EL4.E1 cells stimulated for one hour with TPA was used to protect from RNase digestion the antisense strand of the HindIII-AccI fragment of the IL2 cDNA, pCD-IL-2 (Yokota
et al., 1985), transcribed using T7 RNA polymerase. This is the same probe as that used for in situ hybridization except that it was labeled with $^{32}$P-UTP instead of $^3$H-nucleotides. Using this technique, IL2 RNA (demonstrated by the 400 bp protected fragment) was not detectable in uninduced cells. However, IL2 RNA was detectable in cells stimulated for one hour. Measurement of protected probe by absorbance to DE81 filter paper and scintillation counting and by gel electrophoresis suggested that this population contained IL2 RNA at less than one copy per cell or an average of 2.5 copies per positive cell. While the grain count does not increase linearly with copy number in cells producing high levels of IL2 RNA, these results suggest that the in situ hybridization technique is an extremely sensitive method for detecting low levels of IL2 RNA.

References:

212. Heterogeneity in IL2 Gene Expression by Mature and Immature T-cell Populations

Kathleen McGuire, Ellen Rothenberg

The purpose of these studies was to begin to determine the regulation of IL2 gene expression in its endogenous position in normal T-cell populations. Therefore, spleen cells isolated from 6- to 8-week-old mice were cultured in the presence of the calcium ionophore A23187 and TPA which induces mature T cells to proliferate and produce IL2 (Albert et al., 1985; Truneh et al., 1985). In situ hybridization to stimulated spleen cells revealed that approximately 10% of total spleen cells have IL2 RNA at 24 hours poststimulation. RNase protection experiments suggested stimulated spleen has an average of 30 copies per cell overall and an average of 150 copies per positive cell. In contrast to spleen, only 5% of thymocytes produce IL2 when stimulated with ionophore and phorbol ester. In addition, RNase protection analysis showed that these cells contain only 30 copies per positive cell on average. In situ hybridization revealed that slightly more than half of the positive cells contain less than six grains (i.e., less than 25-30 copies) even though they are clearly above background. (Background levels were always less than one grain/cell in the 4-week exposures used for these determinations.) In the spleen, on the other hand, at least two-thirds of the positive cells contain more than six grains.

It would appear from these experiments, then, that T cells that are not fully mature, as evidenced by their continued presence in the thymus, are in general not capable of accumulating high levels of IL2 RNA as opposed to mature T cells. To further investigate this possibility, we analyzed IL2 RNA production by double-negative thymocytes (i.e., cells negative for the L3T4 and Lyt-2 T-cell surface markers) which have been demonstrated previously to secrete IL2 when stimulated with calcium ionophore and phorbol ester (Lugo et al., 1986). This competence is surprising in light of the fact that these cells are the most immature T cells demonstrable in the thymus and are thought to be the precursors of all other thymic lymphocytes (Mathieson and Fowlkes, 1984; Scollay and Shortman, 1985). In situ hybridization revealed that approximately 15% of these cells have low levels of IL2 RNA (1.6 grains/positive cell at four weeks) 24 hours poststimulation. RNase protection experiments suggested that these IL2-producing double negatives have approximately 5-10 copies per positive cell (i.e., approximately fivefold lower than total thymus). These results therefore substantiate the hypothesis that less mature T cells, while possessing an activatable IL2 gene, are not capable of accumulating high levels of IL2 mRNA. Further experiments are now in progress to determine where the difference lies and whether this effect is transcriptionally regulated or the result of posttranscriptional events.

References:
213. IL2 Gene Expression by PNA\(^+\) Thymocytes

Kathleen McGuire, Ellen Rothenberg

Cortical thymocytes can be distinguished from medullary thymocytes by their high levels of cell-surface receptors for the lectin peanut agglutinin. These so-called PNA\(^+\) cells do not appear to be capable of maturing into functional T cells and based on cell-surface markers, they are not related to a functional T-cell subclass. A subject of long debate has been the reason behind the failure of most of these cells to mature and what defect determines their fate in the thymus. It has been demonstrated that these cells do not secrete detectable IL2 when stimulated with either Con A (Caplan and Rothenberg, 1984) or calcium ionophore A23187 (Lugo et al., 1986) and TPA. We are interested in determining if the IL2 gene in these cells is inducible with these stimuli to begin to elucidate the level of control on IL2 production in this population.

Total thymocytes were isolated and separated into PNA\(^+\) and PNA\(^-\) populations by panning on PNA-coated petri dishes (Caplan and Rothenberg, 1984). Approximately 15% of total thymocytes do not adhere, 50-80% of the adherent cells can be eluted from the plates with galactose. Preliminary in situ hybridization results have demonstrated that when thymocytes are stimulated with ionophore and TPA, 60% of the cells containing IL2 mRNA can be recovered in these two fractions. However, only 22% of the positive cells recovered segregate with the PNA\(^+\) cells. The remaining 78% of cells transcribing the IL2 gene are in the PNA\(^-\) fraction. Although it is likely that the PNA\(^+\) fraction is contaminated with PNA\(^-\) cells, it seems unlikely that this totally accounts for the threefold excess of IL2-producing cells in this population. Therefore, we would like to more closely examine the IL2-producing capabilities of this population. It seems possible, in light of these results, that the defect in these cells may be at the level of RNA transport, translation and/or secretion rather than in the activation of the gene itself.

References:

214. Cloning the Mouse Gene for Interleukin-2 (IL2)

Thomas J. Novak, Ellen Rothenberg

Much of the work in this lab centers around the ability of different cells or thymocyte subpopulations to make the T cell-specific lymphokine IL2. We are interested in understanding at the molecular level the requirements for expressing this gene. This would help in the study of T-cell growth control; it would also be useful for following the progression of thymocytes from immaturity to functional competence.

We used a full-length IL2 cDNA (kindly provided by K. Arai and F. Lee, DNAX Corp.) to screen a BALB/c genomic library cloned into λ Charon 4A (kindly provided by Prof. L. Hood). Six clones were ultimately isolated that contain at least some IL2-coding sequence. The partial sequence of the murine IL2 gene has been reported (Fuse et al., 1984). Restriction enzyme mapping of our clones, however, has revealed several discrepancies between our clones and their reported sequence. We are now sequencing these regions ourselves.

The gene encodes 159 amino acids including a 20-amino-acid leader sequence. It spans four EcoRI restriction fragments of (5' - 3') 3.3 kb, 2.8 kb, 1.3 kb and 0.9 kb and it has four exons and three introns. Our six clones give a total of 10-15 kb of flanking DNA on either side of the gene so it is likely that all regulatory sequences are in hand.

Two clones appear identical and have ~1 kb deleted from the 5' end of the 3.3 kb EcoRI fragment leaving only ~1.8 kb of upstream flanking sequence. This truncated EcoRI fragment allows us to detect the cloned gene after transfection into other mouse cells. λIL2 DNA was mixed with pSV2-neo as a selectable marker and the DNA was introduced into mouse L cells by calcium phosphate coprecipitation. Several G-418-resistant colonies were expanded and analyzed by Southern blotting. Six of 15 had λ DNA and two had the 2.3 kb EcoRI fragment. These lines do not make IL2 mRNA or protein constitutively. Nor do they when stimulated with the phorbol ester TPA and/or the calcium ionophore A23187.

We must now determine if the L cells are deficient in some T cell-specific trans-acting protein involved in transcription, mRNA stability or both.

Reference:
Sequences Required for IL2 Gene Expression

Thomas J. Novak, Ellen Rothenberg

Having cloned the IL2 gene, we are now in a position to construct altered genes in an attempt to define sequences required for transcription and responsiveness to induction. Since at present we have no way of distinguishing between the IL2 mRNA of a transfected gene and that of its endogenous counterpart, we have instead made fusion genes containing the IL2 promoter and the bacterial gene encoding chloroamphenicol acetyltransferase (CAT). We have compared two constructs that differ in the amount of 5' upstream sequences that are present. Plasmid p42CAT48 contains 2.8 kb upstream of the IL2 promoter while p9CAT6 contains 1.8 kb. These two genes have been introduced by protoplast fusion into: mouse L cells; MTL2.8.2 cells, a mouse CTL line; and EL4.E1, a thymoma of helper lineage. One day postfusion, the cells are stimulated with 5 ng/ml TPA, 150 ng/ml A23187 or both. Twenty hours later, the cells are harvested and both CAT activity and transfected gene copy number are measured. The stimulated cells are compared to an unstimulated control group which was transfected in parallel. Initial results show that neither gene is expressed in L cells. Both constructs are constitutively active in EL4.E1 cells but do not appear to be inducible under the conditions used. It is noteworthy that only p42CAT48 appears to function in the MTL2.8.2 line, which being a cytotoxic line does not normally express IL2. Another interesting observation is that both IL2/CAT hybrids are expressed much less well than pRSV-CAT (see Biology 1985, Abstract No. 210), which contains the promoter/ enhancer present in the Rous sarcoma virus LTR. This is consistent with our previous results in T cells comparing the strength of the RSV LTR to T cell- tropic viral promoters and/or enhancers (Novak and Rothenberg, submitted for publication).

PUBLICATIONS


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Summary: Many brain functions such as vision, sensation, and motor control are subserved by different specialized brain regions. It has become clear that the molecular composition of neurons within these regions varies in ways that can affect their signaling properties. An important goal in neurobiology 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. Transient or permanent changes in the strength of synaptic connections are thought to be important for many mechanisms of information processing and storage in the brain. We are studying a calcium- and calmodulin-dependent protein kinase that is a major component of many central nervous system synapses. It is particularly highly expressed in the telencephalon where it is present throughout the neurons but appears to be concentrated in postsynaptic densities. The kinase is an oligomer composed of approximately 12 structurally-related 50,000 and 60,000 dalton subunits. We recently learned that calcium-dependent autophosphorylation of a small number of the subunits in an oligomer produces a new calcium-independent activity. This activity remains after the decay of the initial calcium signal. In a test tube, the calcium-independent activity can perpetuate itself 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.

We are attempting to learn more about the structure of the kinase and to develop a system in which we can examine its activity in intact cells. We are using recombinant DNA and biochemical methods to learn more about the structure and regulation of the important autophosphorylation sites. This will allow us to prepare reagents to measure occupation of the sites in homogenates and in tissue sections. We are studying possible effects of autophosphorylation on the subcellular location of the kinase. Finally, we are developing cellular preparations in which we can study the role of the kinase in synaptic regulation.

216. Sites of Autophosphorylation on the α and β Subunits of the Type II CaM Kinase: Identification and Characterization

Stephen G. Miller

The Type II CaM kinase undergoes autophosphorylation at multiple sites on both the α and β subunits. Brief autophosphorylation produces a significant Ca²⁺-independent kinase activity (Miller and Kennedy, 1986). The stoichiometry of phosphate incorporation suggests that the α subunit contains at least two and the β subunit at least three autophosphorylation sites. We are interested in characterizing the sites of autophosphorylation for several reasons including the following: 1) to determine whether the sites phosphorylated in vivo are the same as those used in vitro, 2) to locate the sites within the primary structure of the kinase subunits and determine their locations relative to putative functional domains, and 3) to determine which sites are responsible for the observed functional changes in kinase activity.

We have developed a method for isolating proteolytic peptides containing the autophosphorylation sites using reverse phase HPLC. The Type II CaM kinase is first labeled with 32P-phosphate under different autophosphorylation conditions (varying time, ATP concentrations, etc.). The kinase subunits are then separated by denaturing gel electrophoresis and gel
pieces containing the α and β subunits are isolated. The subunits are cleaved at arginine and lysine residues with trypsin and eluted from the gel by diffusion. The peptide mixtures are then fractionated by reverse phase HPLC, and $^{32}$P-phosphate-labeled peptides are located by measuring cerenkov radiation in each fraction. The phosphopeptide elution profiles demonstrate that the α subunit contains two and the β subunit three major phosphopeptides. Several minor phosphopeptides are also found, which may be due either to incomplete proteolysis or to limited incorporation of phosphate at other unique sites. The α and β subunits each incorporate phosphate on both serine and threonine residues. We are presently determining the amino acid sequence of each of these sites for comparison with the amino acid sequence determined from cDNA clones.

Reference:

217. Thiophosphorylation as a Tool in Studies of the Type II CaM Kinase

Sean S. Molloy

The adenosine triphosphate analog ATPγS has been useful in studies of biological systems involving regulation by phosphorylation/dephosphorylation. Although a broad range of kinases can use ATPγS as a substrate, thiophosphorylated proteins show an increased resistance to phosphatase activity when compared to corresponding phosphorylated proteins (Gratecos and Fischer, 1974).

This difference between the ability of kinases to catalyze thiophosphoryl transfer and phosphatases to catalyze thiophosphoryl hydrolysis has enabled investigators to dissociate the effects of phosphorylation and dephosphorylation in various biological systems. Thiophosphorylation has been used to study the role of phosphorylation in regulation of glycogen metabolism and smooth muscle contraction. We hope to apply strategies developed in such studies to gain an understanding of how autophosphorylation may play a role in determining the behavior of the Type II CaM kinase in vivo.

As an initial step, we established the ability of the purified Type II CaM kinase to use Mg-ATPγS as a substrate for its own autophosphorylation. We found that autothiophosphorylation proceeds nearly as rapidly as autophosphorylation with a parallel production of calcium-independent kinase activity. We have used the autothiophosphorylated kinase to aid in demonstrating the reversibility of the calcium independent state of the enzyme by phosphatases (see Abstract No. 218). Autothiophosphorylated kinase may also prove useful in physiological studies as a phosphatase-resistant form of the calcium-independent (state 2) kinase. The thiophosphokinase would be expected to produce the physiological effects mediated by the Type II CaM kinase in the absence of an increase in calcium concentration.

Reference:

218. Reversal by Dephosphorylation of the Activity Changes Associated with Autophosphorylation of the Type II CaM Kinase

Stephen G. Miller, Bruce L. Patton

Two changes in the activity of brain Type II CaM kinase are observed after Ca$^{2+}$/CaM-dependent autophosphorylation of the kinase subunits. Its activity is reduced to 30-50% of the control initial rate, and a substantial Ca$^{2+}$-independent activity is produced (Miller and Kennedy, 1986). In order to establish that kinase activity is regulated by the phosphorylation state of the enzyme and not by limited proteolysis or irreversible denaturation associated with the autophosphorylation procedures, we have shown that rat cerebellar homogenate, a crude source of protein phosphatase activity, both dephosphorylates the autophosphorylated kinase and reverses the activity changes associated with autophosphorylation.

Kinase was autophosphorylated with $^{32}$P-ATP in the presence (and absence) of Ca$^{2+}$/CaM. Reactions were quenched by addition of EDTA to deplete MgATP. Aliquots were removed for determination of both the extent of autophosphorylation and the catalytic activity in the presence and absence of Ca$^{2+}$. The autophosphorylated kinase was then incubated with crude phosphatase or heat-denatured crude phosphatase for five minutes. The extent of dephosphorylation and changes in kinase activity were determined as above.

Incubation with cerebellar homogenate returned
kinase activity in the presence of Ca\(^{+2}\) to approximately 80% of that of nonphosphorylated kinase controls, while the Ca\(^{+2}\)-independent activity was reduced to a barely detectable rate. At the same time, 85% of the phosphate was removed from the phosphokinase. Heat denatured cerebellar homogenate removed less than 10% of the phosphate and had little effect on the activity of the phosphokinase. As expected, the change in activity of the auto(thio)-phosphorylated kinase was not reversed by incubation with the cerebellar homogenate (see Abstract No. 217). Commercially available phosphatases did not dephosphorylate the phosphokinase at neutral pH.

These findings support the interpretation that autophosphorylation of the brain Type II CaM kinase produces a significant calcium-independent activity and a reduction in calcium-stimulated activity.

**References:**

219. Phosphorylation Increases the Association of Type II CaM Kinase with the Brain Microtubule Pellet

Ngozi E. Erondu

In a previous abstract (see Biology 1985, Abstract No. 215), we reported that the microtubule pellet (MT) isolated from rat brain cytosol after polymerization with taxol was enriched for Type II CaM kinase activity when the isolation was carried out in the presence of calcium and calmodulin. We suggested that the increased association could be a consequence of autophosphorylation of the enzyme.

To understand and characterize this phenomenon, we have studied the association between pure MTs and pure kinase. Microtubule proteins isolated from rat brain by two cycles of assembly/disassembly (2X MTs) were polymerized with taxol and then incubated with either autophosphorylated or nonphosphorylated kinase. The proportion of the enzyme recovered in the microtubule pellet was two to three times higher for phosphokinase than for dephosphokinase. This result supports the idea that phosphorylation increases the association of the Type II CaM kinase with brain microtubules. In view of recent findings in our lab (Miller and Kennedy, 1986) that submaximal incorporation of phosphate into the enzyme (3-12 out of a possible total of 30 phosphate groups per holoenzyme) is sufficient to produce changes in kinase activity, we have attempted to determine the threshold level of phosphorylation required to cause the increased association of kinase with microtubules. The results of our initial experiments indicate that the incorporation of as few as three moles of phosphate per mole holoenzyme is sufficient to cause maximal binding of kinase to microtubules. It therefore seems possible that both the changes in enzyme activity and the increased association of kinase with microtubules could result from the same autophosphorylation-induced conformational change.

**References:**

220. Type II CaM Kinase Activity in Hybrid Cell Lines

Sean S. Molloy

We have attempted to identify a neuronal hybrid cell line expressing Type II CaM kinase for use in biochemical and cell biological studies. We have examined three hybrid lines generated by the Nirenberg lab, N18-RE-105, NG108-15 and NCB-20, all of which express proteins associated with synaptic transmission (Nirenberg et al., 1983; Malouf et al., 1984).

Flasks of cells were harvested, sonicated in buffered medium, and then assayed for Ca\(^{+2}\)/calmodulin-stimulated phosphorylation of exogenous synapsin I. Low levels of such activity were detected as well as phosphorylation of several endogenous proteins. _Staph. aureus_ protease (SAP) mapping of the phosphorylated synapsin I indicated that the kinase present in the cell lines phosphorylates the same site as brain Type II CaM kinase.

An endogenous 60K protein, phosphorylated in a Ca\(^{+2}\)/calmodulin-dependent manner, was shown to be similar to the \(\beta\) subunit of the Type II CaM kinase by three criteria. 1) SAP mapping of the 60K protein produced an SDS-PAGE gel banding pattern nearly identical to that produced from the \(\beta\) subunit of brain Type II CaM kinase. 2) A polyclonal antisera that recognizes the \(\beta\) subunit on immunoblots reacts with a band in the 60K region of blots made from the cell line homogenates. A monoclonal antibody specific for the \(\alpha\) subunit of the brain enzyme did not react with the homogenates. The concentration of the \(\alpha\)-subunit-like
protein, measured by quantitative immunoblotting, was consistent with the observed low level of Ca\(^{++}\)/calmodulin-stimulated synapsin I kinase activity. 3) The use of monoclonal antibodies to inhibit cell line kinase activity indicated that only antibodies capable of inhibiting the \(\alpha\) subunit of the brain enzyme were effective. Antibodies that inhibit the \(\alpha\) subunit alone had no significant effect. The evidence indicates that all three of the hybrid cell lines studied express a Ca\(^{++}\)/calmodulin-dependent protein kinase that contains a subunit structurally and immunologically similar to the \(\beta\) subunit of the brain Type II CaM kinase but contains little \(\alpha\) subunit. The concentration of the \(\beta\) subunit in the hybrid lines is approximately 25% of the concentration of the \(\beta\) subunit in rat forebrain homogenates.

References:

221. Characterization of \(\beta\)-Subunit cDNA Clones

Mark K. Bennett

Some questions about the structure of the Type II CaM kinase holoenzyme and the relatedness of its two subunits can best be approached at the nucleic acid level. I previously isolated a 1.0 kilobase (kb) cDNA clone from a \(\lambda\)gt11 expression library that codes for the \(\beta\) subunit of the kinase (Biology 1985, Abstract No. 217). Since this cDNA cannot encode the entire 60,000 dalton \(\beta\) subunit, additional overlapping cDNA clones were isolated by screening a \(\lambda\)gt10 rat brain cDNA library with the 1.0 kb \(\lambda\)gt11 cDNA. A number of \(\beta\)-subunit cDNA clones covering a 1.84 kb region have been isolated and sequenced. The nucleotide sequence includes a single long open reading frame of 1626 nucleotides. This open reading frame encodes a protein made up of 542 amino acids and with a molecular weight of 60,333. The sequence also includes 62 nucleotides of 5' untranslated and 149 nucleotides of 3' untranslated sequence.

Two independent \(\beta\)-subunit cDNA clones contained identical 45-nucleotide deletions within the long open reading frame. The sequences on either side of the deletion were identical to all of the other cDNA clones. The deletion does not disrupt the open reading frame. A protein encoded by the open reading frame but lacking the deleted 45 nucleotides would have a molecular weight of 58,000. This is very close to the molecular weight of a minor protein present in purified kinase preparations, which is termed \(\beta'\) because it is closely related to the \(\beta\) subunit (Bennett et al., 1983). This raises the possibility that the \(\beta'\) protein is generated by a differential splicing mechanism.

In order to investigate the number of genomic sequences related to the Type II CaM kinase, I did a Southern blot analysis of rat genomic DNA. This analysis revealed that there are multiple genes or pseudogenes that are closely related to the \(\beta\)-subunit cDNA, although there may be only one or at most two genes coding for the \(\beta\) subunit itself. The other related sequences may include the \(\alpha\)-subunit gene or genes coding for related kinase expressed in other rat tissues.

References:

222. Amino Acid Sequence Homology Between the \(\beta\) Subunit and Other Protein Kinases

Mark K. Bennett

The amino acid sequence for the \(\beta\) subunit was deduced from the nucleotide sequence of \(\beta\)-subunit cDNA clones (see previous abstract). This sequence was compared to the protein sequences in the National Biomedical Research Foundation database. The top nine alignment scores were all protein kinases or protein kinase-related proteins. The three most homologous proteins were the catalytic subunit of phosphorylase b kinase, skeletal muscle myosin light chain kinase (both of which are calmodulin-dependent protein kinases), and the catalytic subunit of cAMP-dependent protein kinase. A sequence of about 300 amino acids near the amino terminal of the \(\beta\) subunit is identical at 38%, 31%, and 27% of its positions to the sequences of phosphorylase b kinase, myosin light chain kinase (both of which are calmodulin-dependent protein kinases), and the catalytic subunit of cAMP-dependent protein kinase. A sequence of about 300 amino acids near the amino terminal of the \(\beta\) subunit is identical at 38%, 31%, and 27% of its positions to the sequences of phosphorylase b kinase, myosin light chain kinase, and cAMP-dependent protein kinase, respectively. If conservative amino acid substitutions are included in the comparison, the homology increases to 58% for phosphorylase b kinase, 52% for myosin light chain kinase, and 48% for cAMP-dependent protein kinase. This region of homology is likely to be
involved in a catalytic function that is required of all protein kinases. A putative ATP binding site for the \( \beta \) subunit is within this region. It was identified by its homology to ATP binding sites chemically identified in other protein kinases.

The sequences outside the homologous region (about 250 amino acids for the \( \beta \) subunit) are likely to be involved in functions unique to each particular kinase, such as binding to regulatory components or interacting with other kinase subunits. A number of potential regulatory sites within the \( \beta \)-subunit sequence have been identified by homologies to functional domains in other proteins. These include a potential calmodulin-binding domain and a number of potential autophosphorylation sites. It remains to be experimentally determined which of these sites is actually involved in the regulation of the kinase.

223. Oligonucleotide Probe for the \( \alpha \) Subunit of the Type II CaM Kinase

Robert F. Bulleit, Alexander Gilman

Full-length cDNAs specifying the Type II CaM kinase subunits will be useful in asking experimental questions about the structure and expression of the kinase. cDNAs specifying the \( \beta \) subunit of the kinase have been isolated from both \( \lambda gt11 \) and \( \lambda gt10 \) libraries (see Abstract No. 221). The coding region has been sequenced and the \( \beta \)-subunit amino acid sequence deduced. In order to isolate \( \alpha \)-subunit-specific cDNAs, we wished to generate \( \alpha \)-subunit-specific probes. A 20,000 dalton peptide from the \( \alpha \) subunit has been isolated from a chymotryptic peptide digest of native kinase, isolated by HPLC and the sequence of its first 15 amino acids determined (see Biology 1985, Abstract No. 218). The amino acid sequence shows 60% homology to a corresponding portion of the \( \beta \)-subunit region. Based on this amino acid sequence and on the base sequence coding for the homologous \( \beta \)-subunit region, we have designed a "guessmer" of 45 nucleotides to use as an \( \alpha \)-subunit-specific probe. At sites where \( \beta \)-subunit and \( \alpha \)-subunit amino acids are identical, codons from the \( \beta \)-subunit cDNA were used. At sites where the amino acids are not identical, codons for the \( \alpha \)-subunit amino acid were used to define the first two bases. The third position was assigned based on either the most frequently used codon in a group of rat messages, or the corresponding nucleotides of the \( \beta \)-subunit message.

Under appropriate hybridization conditions, the guessmer hybridizes strongly to a 5.2 kb mRNA on Northern blots of brain poly(A)+ mRNA. This mRNA appears to be identical to the \( \alpha \)-subunit message detected previously with degenerate 17-base oligonucleotides (Biology 1985, Abstract No. 218). The guessmer hybridizes considerably more weakly to the \( \beta \)-subunit message. The hybridization conditions and the temperature at which half the duplexes dissociate suggest that the guessmer is 75–85% homologous to the \( \alpha \)-subunit message. The guessmer will be used to screen brain cDNA libraries carrying long cDNA inserts.

224. Phosphorylation of Smooth Muscle Myosin by Brain Type II CaM Kinase

Arthur M. Edelman¹, Mark K. Bennett, Mary B. Kennedy, Edwin G. Krebs¹

Brain Type II CaM kinase phosphorylates smooth muscle whole myosin, incorporating approximately 2 moles of phosphate per mole of myosin exclusively into the 20,000 dalton light chain. Tryptic phosphopeptide maps of myosin light chain after phosphorylation by myosin light chain kinase or by Type II CaM kinase suggest that these two distinct kinases phosphorylate a common site. This conclusion is supported by the observation that addition of Type II CaM kinase after maximal phosphorylation of myosin by the myosin light chain kinase produces no further incorporation of phosphate. Phosphorylation of whole myosin by brain Type II CaM kinase increases the actin-activated, Mg\(^{2+}\)-ATPase activity of myosin from 12.9 to 38.1 nmol/min/mg. The results suggest that Type II CaM kinase can regulate actin-myosin interactions. Thus, the kinase may be able to regulate cell shape, neurite motility or other acto-myosin functions.

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PUBLICATIONS


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**Summary:** The Lester laboratory is now almost completely devoted to the study of ion channels and drug receptors expressed in *Xenopus* oocytes after the injection of foreign RNA. The experiments, involving a combination of molecular biology and membrane biophysics, are performed in collaboration with the laboratory of Prof. N. Davidson (Divisions of Chemistry and Chemical Engineering and Biology).

We exploit three different aspects of this system. First, we study rather complex membrane properties that can be reconstituted in the oocyte, which provides a convenient system for both electrophysiology and biochemistry. In last year's annual report and in this year's, we report that voltage-dependent Ca channels can be both expressed and modulated in oocytes. We hope to begin studying the components necessary for this modulation—receptors, kinases, and other proteins involved in processing intracellular messengers.

Second, the oocyte provides a system for identifying cDNA clones that encode channels and receptors. Our pilot study for this program involves one of the receptors for serotonin; we now know that the RNA for this receptor is produced in the rat choroid plexus and is 5-6 kb in length.

Third, once one has full-length cDNA clones encoding a receptor or channel, one can ask questions about the role of various subunits and, more generally, about the relation between structure and function of the protein. The acetylcholine receptor continues to provide the most appropriate system for such studies, although progress is rapid on the electrically excitable Na channel as well.

This year witnessed a most interesting conclusion to our series of experiments probing the kinetic properties of membrane channels with flash-activated drugs. Intracellular concentration jumps of Ca$^{2+}$ activate a K$^+$ conductance within a few milliseconds. Detailed analysis is under way.

The Pine group continues to work towards experiments that explore the development and plasticity of synapses in cultured neuron networks. In addition, synapse elimination at cultured neuromuscular junctions is being studied. Non-invasive physiological techniques have been developed, which utilize voltage-sensitive dyes and culture dishes incorporating extracellular electrodes. In addition, this year a program exploiting novel silicon microdevices for long-term
communication with cultured neurons has begun. Finally, the group continues to work toward the application of soft x-ray microscopy for revealing ultrastructure in cultures.

225. Role of Acetylcholine Receptor Subunits in Competitive Antagonist Binding
Kiyonori Yoshii, Lei Yu, Katherine Mixter Mayne

The nicotinic acetylcholine receptor (AChR) consists of four homologous subunits assembled in a molar stoichiometry of α2βγδ. Roles of these subunits are still unclear except for the α subunits that contain binding sites for acetylcholine (ACh). Full sets of cDNA clones are now in hand for all four subunits from both Torpedo and mouse; SP6 transcripts from these clones produce functional ACh receptors when injected into Xenopus oocytes. Hybrid AChRs between different species of animals also have been expressed in the oocytes and used to investigate the role of subunits in the ACh response.

Competitive antagonists such as d-tubocurarine are thought to bind to the agonist site on the α subunit alone, without enabling the conformational change that opens the channel. This suggests that antagonist binding will provide a sensitive test for alterations in the binding process alone. We are therefore using interspecies hybrid receptors to investigate the role of the subunits in the binding of competitive antagonists such as d-tubocurarine. Dose-response relations for agonists are measured in the presence of various concentrations of the competitive antagonists under voltage clamp conditions. The parallel shifts produced by antagonists provide a direct measure of the $K_d$ for tubocurarine binding. We find that the α subunits are much more important than the δ subunits in controlling the binding affinity for tubocurarine.

We also intend to measure the inhibition of α-toxin binding by the competitive antagonists in oocytes injected with various combinations of subunit-specific mRNAs. The results will complement those obtained by the binding experiments and elucidate not only the binding mechanism of the competitive antagonists but also the interaction between AChR subunits.

226. Ca Channels Induced in Xenopus Oocytes by Rat Brain mRNA
John P. Leonard, Joel Nargeot, Terrance P. Snutch

RNA was isolated from fresh brains of 16-day-old rats and poly(A) samples were injected into stage V and VI oocytes. After allowing 2-5 days for expression, most oocytes were exposed to medium in which the K had been replaced by Cs for 24 hours prior to recording. Barium currents $I_{Ba}$ through calcium channels were measured in Cl-free Ba-methanesulfonate saline.

$I_{Ba}$ in non-injected oocytes was often undetectable but ranged up to 50 nA (22 ± 4 nA, $N = 21$). In contrast, injected oocytes showed peak $I_{Ba}$ of 339 ± 42 nA (N = 33). Threshold for activation of $I_{Ba}$ was -40 mV with peak currents at +10 to +20 mV. After a peak, currents decayed to a nearly steady level along a single exponential time course ($\tau = 630 ± 50$ ms at +20 mV). The maintained current was 67 ± 6% (N = 9) of the early peak amplitude. A prepulse duration of 5 sec was needed to examine inactivation of barium currents in injected oocytes. The inward $I_{Ba}$ could be observed in BaCl$_2$ and also in Na-free salines, indicating that neither Cl$^-$ nor Na$^+$ were carrying the inward current.

Although $I_{Ba}$ displayed voltage-independent blockade by Cd (50% inhibition at 6 µM), the organic Ca channel blocking agents (verapamil, compound W-7, and nifedipine) were uniformly ineffective. No effects were observed with the dihydropyridine antagonist, nifedipine (even at 10 µM and even when cells were held at -40 mV) or agonist Bay K-8644. However, $I_{Ba}$ was enhanced via activation of protein kinase C with 4-α-phorbol dibutyrate (PBT$_2$). In contrast to the effects of C-kinase activation by PBT$_2$, use of forskolin to activate protein kinase A did not alter $I_{Ba}$. However, experiments in the presence of Cd revealed that forskolin decreased $I_k$. Ca channels produced by rat brain mRNA thus contrast with the nifedipine-sensitive, Bay K-8644 enhanced, forskolin-enhanced Ca channels observed after injection of rat heart mRNA.

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227. Macroscopic Na Currents with Gigaohm Seals on mRNA-injected Xenopus Oocytes
John P. Leonard, Terrance P. Snutch, Hermann Lubbert

Oocytes were removed from ovaries, then exposed to collagenase for 3 hours, which (a) removed the
surrounding follicle cells, and (b) improved sealing. Some oocytes were injected with 70 nl (70 ng) poly(A) mRNA isolated from brains of 16-day-old rats, then incubated for 2-3 days. Oocytes then were exposed to hypertonic saline for 30 min; the plasma membrane shrank away from the vitelline membrane, which was then manually stripped away. Large diameter patch pipettes (20-30 µm tip, -250 kΩ) were fire-polished, coated with Sylgard, and used to form gigaohm seals enclosing up to 600 µm² of the surface. A standard virtual-ground circuit was used for voltage-clamp studies on the fast Na current induced by brain RNA. The capacitance was fully charged within <0.3 msec following a voltage jump. Maximal inward currents varied widely in amplitude among patches (0.1 to 3.4 pA/µm²). Na currents were maximal at -10 to 0 mV. hₐ₅ curves showed 50% inactivation after prepulses at -60 mV. The transient Na currents were followed at more depolarized voltages by a long lasting outward current of comparable amplitude. The outward currents are presumably carried by K⁺ but are not completely blocked by tetraethylammonium and 3,4-diaminopyridine. However, nearly complete elimination of these outward currents was obtained by treating the oocytes for 24 hours in a cesium-containing saline (K⁺ replaced) and adding 5 mM barium (with 100 µM cadmium to block Ca channels). This allowed examination of reversal of the Na current and analysis of kinetics in the absence of most contaminating currents. These rat brain mRNA-induced Na currents are remarkably similar to Na currents from a variety of nerve and muscle cells. The "big patch" method should allow analysis of currents that are too fast to be resolved using the standard 2-electrode voltage-clamp of these very large (1 mm) cells.

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228. The Function of the Various Subunits Comprising the Rat Brain Sodium Channel

Alan L. Goldin¹, Douglas S. Krafte

The sodium channel from rat brain is now known to consist of one large and two small subunits. At present the function of the smaller subunits is unknown. Our approach to this question involves extracting RNA encoding the sodium channel and injecting this message into Xenopus oocytes. By applying available electrophysiological techniques such as patch clamp and two-microelectrode methods, we can record currents generated by the opening of sodium channels and quantitatively characterize the properties of these proteins.

To specifically characterize the function of the specific subunits, two methods are currently being used to separate RNA encoding the large and small subunits. The first is to separate the RNA by size using density gradients. Because the molecular weight of the smaller subunits is about one-fifth that of the larger subunits, the RNA encoding these proteins is likely to differ significantly in size as well. Functional sodium channels are produced with only high molecular weight RNA. A second technique is to apply hybrid selection to specifically remove large subunit mRNA from the total mRNA pool. This approach is currently being attempted with previously identified cDNA clones for segments of the sodium channel message.

Results to date indicate that the high-molecular RNA by itself produces macroscopic currents with different kinetics from those produced by total mRNA. We are currently investigating whether this difference arises from the additional presence of β-subunit RNA or from another macromolecular component specified by total RNA.

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229. Expression of Calcium Channels from PC-12 Cells

Fedora Sutton

Diverse types of calcium channels have been described in various cells. These channels are designated as fast deactivating (FD), slow deactivating (SD) or L, T N channels, depending on the size of their conductance and their rate of deactivation. These channels are also distinguished from each other on the basis of their sensitivity to the organic Ca channel modulators, the dihydropyridines.

PC-12 cells have been shown to express both dihydropyridine-sensitive and insensitive calcium channels. PC-12 cells can be induced to differentiate into sympathetic neurons by the addition of nerve growth factor. The calcium channels expressed by these differentiated cells are predominantly
insensitive to both Bay K 8644 and to nitrendipine. It is therefore of interest to characterize the calcium channels expressed in both the undifferentiated and differentiated states of PC-12 cells in order to understand the mechanism of dihydropyridine sensitivity.

Xenopus laevis oocytes are injected with RNA isolated from both undifferentiated and differentiated PC-12 cells. These oocytes are monitored by the two-electrode voltage clamping procedure under conditions designed to reveal the various Ca channel types: dihydropyridines, inorganic Ca channel blockers, and variations in holding potential. Inactivation kinetics and permeation characteristics are also studied. The results will provide a survey of the type of calcium channel encoded by the different RNA samples.

230. The Rat Brain Serotonin Receptor Functionally Expressed in mRNA-injected Oocytes is of the 5-HT1C Type

Hermann Lübbert1, Terrance P. Snutch1, Nathan Dascal

Xenopus laevis oocytes injected with rat brain RNA synthesize functional serotonin receptors. These receptors appear to be coupled to a phosphoinositol pathway within the oocyte which acts on Cl channels. Since there are at least four pharmacologically distinct subtypes of 5-HT receptors in the rat brain, we characterized the receptor that is functional in the oocyte electrophysiological assay. Cl-conductance increases were half-maximal at a concentration of 5 ± 1 x 10^{-8} M serotonin, indicating that the receptor is of the 5-HT1 class. The Ki's of various antagonists were: spiperone, 4000 nM; mesulergine, 250 nM; mianserin, 40 nM; ketanserin, 35 nM; ritanserin, 30 nM; cyproheptadine, 300 nM; and yohimbine, >7000 nM. These affinities correlate with the expected values for a 5-HT1C receptor (Conn et al., 1986). Another way to confirm the receptor type was to isolate and compare RNA from various brain regions that are particularly enriched in a certain receptor type. Oocytes injected with RNA isolated from rat choroid plexus responded 8 to 16 times more strongly to serotonin than those injected with RNA from cortex, substantia nigra, or hippocampus. This confirms the identification of the receptor as of the 5-HT1C type. In addition, by fractionation of rat brain mRNA through agarose gels, we have identified a single RNA size class of about 5-6 kb, corresponding to an expected protein size of about 120-160 kd, which is sufficient for the generation of the response.

Reference:

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231. Activation of a K Current by Rapid Photochemically-generated Steps of Intracellular Ca

Alison M. Gurney, Joel Nargeot, Stuart Adams¹, John Kao¹, Roger Tsien¹

Although Ca^{2+} is a well-established intracellular messenger for a variety of processes, there are many questions concerning the kinetics and spatial localization of its effects. Some of these problems may now be approached using the photosensitive Ca^{2+} chelator, Nitr-5. The Ca affinity of this molecule decreases by roughly 40-fold after absorbing near-UV light; Ca is liberated with a time constant of ~300 µs. Nitr-5 or the related compounds Nitr-2 and Nitr-7, complexed with Ca^{2+}, were introduced into rat sympathetic ganglion cells by dialysis from a patch pipette electrode operating in the whole-cell voltage-clamp mode. Light flashes caused release of Ca^{2+} and activated an apamin-sensitive, but TEA- and charybdotoxin-insensitive, K⁺ current. Flash-induced current relaxations follow a simple exponential time course; time constants range from 4 to 40 msec, confirming directly that Ca can modulate membrane properties within a few msec after entering a cell. Half-maximal activation occurs with a Ca^{2+} concentration jump of about 300 nM to a final level of about 1000 nM. Comparison of the kinetics among the Nitr- derivatives, which release Ca at different rates, suggests that release of Ca from Nitr-5 is too fast to limit the rate of the relaxations. From the dependence of relaxation kinetics on the amplitude of the Ca^{2+} concentration jump, we conclude that a Ca^{2+} ion binds to a K⁺ channel with a forward rate of at least 10^8 s^{-1} M^{-1}.

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232. Synapse Elimination at Cultured Neuromuscular Junctions

John R. Gilbert, Becky G. Tanamachi, Jerome Pine

Microcircuit culture dishes that incorporate an array of 61 extracellular stimulating and recording electrodes have been developed, along with the necessary control electronics. The first experiments will be directed toward studies of synapse elimination at the neuromuscular junction, perhaps the most well-studied example of synaptic plasticity in the nervous system. In vivo, early in development, each muscle fiber is typically innervated by axons from several neurons. However, competition between the inputs ultimately leads to single innervation of the great majority of muscle fibers. This process of synapse elimination is known to be facilitated by neural activity, but the underlying rules that define the competitive interaction remain unknown.

We have developed a coculture system in which chick ciliary neurons innervate chick skeletal muscle fibers. The microcircuit dishes make possible controlled chronic stimulation of selected inputs to polyinnervated muscle fibers in these cultures. By observing the way in which synaptic inputs change strength in response to various patterns of stimulation, it is hoped that we will uncover the rules by which activity influences synapse elimination.

To successfully perform these experiments, it is necessary to observe the change with time of the synaptic input strengths to individual muscle fibers. This will be done by conventional intracellular recording from the muscle fibers while stimulating individual presynaptic neurons with appropriate microcircuit electrodes. Controlled concentrations of antagonists will be used to keep the postsynaptic potentials generated in the other cells of the culture. In this way, the electrical connectivity of the network can be mapped at a given time, without damaging the neurons. Studies made at later times will compare normal changes that occur during development with changes associated with chronic electrical stimulation. The microcircuit dish allows selective patterns of stimulation to be maintained chronically while the culture is in the incubator. It is hoped that this system will reveal some of the rules by which activity modifies synaptic connectivity.

To extend the initial studies to a culture system that has a wider variety of synaptic connections, including inhibition, we are also developing a microculture system for mouse spinal cord neurons.

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233. Development and Plasticity of Small Neural Networks

Chi-Bin Chien, Becky G. Tanamachi, Andrew Hsu, Jerome Pine

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 maintaining a microculture of a small number of neurons in isolation over this electrode array. The surrounding area is coated with silastic adhesive to confine the cell processes. A large surrounding area of the culture dish is used to grow a mass culture which biochemically conditions the medium. The microcircuit dishes are fabricated with transparent conductors to facilitate the use of optical recording techniques.

The initial experiments will study the development of connections between rat superior cervical ganglion neurons, which form a cholinergic network under appropriate culture conditions. The electrodes will be used to selectively stimulate each cell, while voltage-sensitive fluorescent dye will be used to record the postsynaptic potentials generated in the other cells of the culture. In this way, the electrical connectivity of the network can be mapped at a given time, without damaging the neurons. Studies made at later times will compare normal changes that occur during development with changes associated with chronic electrical stimulation. The microcircuit dish allows selective patterns of stimulation to be maintained chronically while the culture is in the incubator. It is hoped that this system will reveal some of the rules by which activity modifies synaptic connectivity.

To extend the initial studies to a culture system that has a wider variety of synaptic connections, including inhibition, we are also developing a microculture system for mouse spinal cord neurons.

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234. A Neuron-Silicon Connection

Wade Regehr, Heidi Langeberg, David Rutledge, Jerome Pine

A new technique for maintaining long-term two-way electrical communication with neurons is being explored. The basic idea is to emulate with a silicon microdevice the intimate contact which a patch electrode makes with a cell membrane. The device
that is being used for initial studies is called a "diving board" electrode, because it consists of a base that supports a long flexible arm. The base will be cemented to the bottom of a culture dish after the long arm has been positioned with its outer end touching the top of a neuron. The end of the arm incorporates a cup-shaped contact area containing a metal electrode with an insulated lead that connects to external electronics.

The diving board electrodes will be used in studies with cultured neurons to determine optimum conditions for establishing long-term contact and to study the electrical coupling that results. Experiments have been performed with glass micropipettes to simulate the contact that may be achieved, and it has been discovered that suitable applied voltage pulses can lead to reversible membrane poration which lowers the membrane resistance by an order of magnitude. This phenomenon remains to be studied in more detail with diving board electrodes, but it may greatly facilitate stimulation and recording.

A related effort has been the production of silicon "neurochips" which incorporate 16 wells appropriate for containing individual cultured cells. Each well has a conductor incorporated into the bottom to provide electrical contact as the diving board electrode does, with leads that carry signals to the periphery of the chip. These devices are designed for neural network studies, since the wells are spaced 100 microns apart and cells growing in them are expected to connect synaptically.

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235. Soft X-Ray Microscopy

Jerome Pine, Becky G. Tanamachi

A scanning soft X-ray microscope has been built by a group from SUNY Stony Brook and the IBM Watson Laboratories. The microscope is based on imaging in transmission at an X-ray wavelength of about 3 nm. At present the best resolution, limited by the size of the scanning spot, is about 150 nm, but as focussing devices are improved the resolution is expected to be about ten times better. The X-ray wavelength is chosen so that the X-rays are absorbed weakly by water and strongly by carbon. As a result, imaging of whole, wet live cells seems feasible, at an ultimate resolution intermediate between light and electron microscopy.

We are collaborating with the microscope builders to evaluate the usefulness of their device in studies of cultured neurons. During the past year, the first exposures were made of live cells, transported successfully from Pasadena to Brookhaven, Long Island. (The only appropriate X-ray source at present is the National Synchrotron Light Source.) Resolution comparable to that of phase contrast microscopy is the best that can be achieved at present, but it will improve steadily. Studies of live cells were hampered by the long exposure time—approximately one hour—during which time they were in saturated water vapor but otherwise unhydrated. Osmolarity problems made rehydration and survival studies very difficult.

An improved microscope is now being built, which will reduce the picture-taking time to about one minute. We are also building a wet cell at Caltech that will hold cultures in a hydrated state except during the short time of an X-ray exposure. We expect during the coming year to succeed in imaging live cells and evaluating their survival after various X-ray exposures. We also expect to achieve significantly better resolution than can be achieved with the light microscope.

PUBLICATIONS


Professor: Paul H. Patterson
Visiting Associate: Toshihiko Suzue
Senior Research Fellow: Keiko Fukada
Research Fellows: William V. Bleisch, Arlene Y.-C. Chiu, Sigrun Korsching, Ana I. Millaruelo, Irene V. Pech, Tetsuo Yamamori
Graduate Students: Nagesh K. Mahanthappa, Mahendra Rao
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Summary: The interests of this laboratory concern two aspects of the development of the nervous system: the factors controlling which type of phenotype a neuron acquires, and the identification of molecules involved in intercellular interactions leading to the formation of specific synaptic connections. Much of our effort in the 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. This protein has recently been purified and a partial N-terminal amino acid sequence has been determined. We have begun work...
on cloning the gene for this differentiation signal in order to facilitate further molecular studies of its function and mechanism of action. The role of hormones and growth factors has been extended to other phenotypic choices in this lineage, and interconversions of adrenal chromaffin cells, small intensely fluorescent (SIF) 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.

The extensive synaptic connections elaborated by neurons in culture provide an assay system for identifying neuronal macromolecules, surface-bound or secreted, which mediate intercellular interactions. A number of surface glycoproteins are highly enriched on neuronal surfaces and several of these are being studied in antibody perturbation experiments and with mutant cell lines. The NILE 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 in cultured neurons and in PC12 cells. Further work on the function of Thy 1 involves generating mutant cell lines deficient in this protein, characterizing their outgrowth and regeneration behavior, and transfection of the Thy 1 gene back into cells that lack the protein.

A novel method for producing monoclonal antibodies against rare antigens was used to generate an antibody that directly blocks the activity of an axon regeneration-promoting extracellular matrix complex. This antibody was used to localize the complex in situ. This experimental approach also is being used to investigate the molecular basis of the specificity of synaptic connections in the spinal cord innervation of sympathetic neurons. Purves, Sanes and colleagues demonstrated that this innervation displays a positional bias, with rostral cord neurons preferring to innervate more rostral sympathetic neurons as well as more 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. In order to test the effect of these and other antibodies on specific synapse formation, a culture system consisting of rostral and caudal spinal cord and sympathetic neurons is being developed. Thus, this second area of work involves the identification of molecules involved in intercellular interactions leading to axon outgrowth, regeneration and specific synapse formation.

236. The N-terminal Sequencing and Production of Antisera Against a Cholinergic Differentiation Factor

Keiko Fukada

A glycoprotein that controls transmitter choice in cultured sympathetic neurons by inducing cholinergic differentiation and reducing noradrenergic differentiation, without altering neuronal survival or growth, has recently been purified to homogeneity. The biological activity comigrates with a series of 125I-labeled protein spots of 45 kd in two-dimensional gel electrophoresis, and treatment of the 45 kd molecule with endo-α-N-acetylglucosaminidase F reduces the apparent molecular weight of both the labeled protein and the biological activity to 22 kd (Fukada, 1985). In collaboration with Drs. R. Aebersold, S. Kent and L. Hood, we sequenced 11 N-terminal amino acids of the intact and deglycosylated forms of this protein and found a single and identical sequence for both. In collaboration with Dr. S. Horvath, we produced a synthetic peptide of this sequence and coupled it to the proteins, keyhole-limpet hemocyanin and ovalbumin. Rabbit antisera were generated against each of the coupled molecules as well as the free peptide. These antisera specifically immunoprecipitate the 45 kd protein from a partially purified preparation, and the immunoprecipitation is completely blocked by either the peptide or the peptide coupled to the protein, but not by the proteins used for coupling. Antisera raised against the proteins used for coupling, and preimmune sera do not immunoprecipitate the 45 kd protein. In addition, the three antisera also are able to precipitate the biological activity of this
protein, as assayed by the conversion of noradrenergic sympathetic neurons to the cholinergic phenotype.

These results demonstrate that the sequenced protein contains the cholinergic differentiation activity and provide final proof that the purified protein is the cholinergic factor (Fukada, 1986). Moreover, these antisera will be useful in several ways: 1) for purifying large quantities of the factor, 2) for identifying the translation product of the appropriate mRNA as the cholinergic factor, and 3) for assessing the role of the factor in normal development.

References:

237. Cloning the Neuronal Differentiation Factor
Tetsuo Yamamori, Sigrun Korsching

The cholinergic differentiation factor described in the previous abstract by Fukada has interesting biological effects on neuronal development, but it is difficult to obtain in sufficient quantities. Further biochemical characterization, studies of the mechanism of its action, and analysis of its role in vivo will require considerably more material than is now available by biochemical purification methods. For these and other reasons, it would be desirable to clone the gene for this protein. We are using both in vitro translation assays and Northern analysis in this effort.

As described in the previous abstract, antisera were generated against a synthetic peptide made according to the sequence of the 11 N-terminal amino acids and coupled to the carrier protein KLH. These antisera recognize the native glycoprotein in immunoprecipitation assays. Poly(A)^+ RNA of different rat tissues, and of various developmental stages, was isolated and is being translated in a reticulocyte lysate system. Immune precipitation with anti-KLH-peptide antiserum reveals a 70 kd band which is not precipitated by the control anti-KLH antiserum. This band is found in RNA preparations from skeletal muscle and cultured heart cells, which are known to produce the cholinergic factor. A broad range of tissues will be analyzed in this way to compare the known distribution of the biological activity with the distribution of this band. At the same time we are evaluating the suitability of a 32 mer based on the amino acid sequence data in Northern analysis. At four and threefold code ambiguities, inosine was substituted. mRNA from several tissues and developmental stages is being examined. It is hoped that the two assay methods will agree on the best source of mRNA for further work.

238. Precursor and Mature Tyrosine Hydroxylase RNA Levels in Cholinergic Sympathetic Neurons
Dona Chikaraishi¹, Irene V. Pech, 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.
239. Control of Phenotypic Interconversions in vivo
Josette P. 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 or glucocorticoids. Injection of anti-NGF antisera for five days yielded the expected decrease in neuronal number, but preliminary results also demonstrate a significant increase in the number of SIF cells. This finding is consistent with a precursor role for SIF cells in that their number would depend in part on the availability of NGF for conversion to the neuronal phenotype.

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 injections. Membranes from neonatal chromaffin cells are then injected and hybridomas produced. Thus far, four antibodies have been obtained that bind to neonatal chromaffin cells and a subpopulation of small cells in the neonatal sympathetic ganglion but not to sympathetic neurons. Further work will be necessary to determine if the binding in sympathetic ganglia is actually to SIF cells.

240. The Function of Neuronal Thy 1
Nagesh K. Mahanthappa, Martha C. Zuniga, Paul H. Patterson

Thy 1 is a cell-surface glycoprotein that was first identified as two allotypes (1.1 and 1.2) 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. In an effort to elucidate Thy 1 function in T cells, poly- and monoclonal antibodies (MAbs) against Thy 1 were generated, and some induce mitosis and lymphokine secretion (Gunter et al., 1984). In the case of neurons, when dissociated rat retinal ganglion cells are cultured upon a surface on which anti-Thy 1 MAbs have been coated, a significant enhancement of neurite regeneration is observed (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 and the neuron-like, pheochromocytoma cell line, PC12. Initial studies with neonatal mouse sympathetic neurons in culture demonstrated that addition of anti-Thy 1.2 MAbs promoted neurite outgrowth and cell survival. Similarly, with dissociated neonatal rat sympathetic neurons, we find that anti-Thy 1.1 MAbs promote neurite outgrowth. This effect is not seen in experiments where the substrate is simply precoated with the MAbs, however.

These experiments suggest that the Thy 1 molecule may play an important role in neurite outgrowth and regeneration. In an effort to examine this idea from a different approach, we are using PC12 cells, which also exhibit an anti-Thy 1 MAb-inducible increase in neurite outgrowth. We are developing mutant PC12 cell lines deficient in Thy 1 surface expression by ethyl-methane sulfonate mutagenesis and selection with an anti-Thy 1 MAb-toxin conjugate. If deficiency in Thy 1 expression leads to a decrease in neurite
outgrowth, rescue of normal behavior will be attempted by transfection of the Thy 1 gene back into these cells. In pilot studies, we have successfully transfected the Thy 1.2 gene into wild-type PC12 cells, and these cells increase their neurite outgrowth in response to either anti-Thy 1.1 or 1.2 MAb's.

References:

241. Functions of a Neuronal Surface Protein
William V. Bleisch, Michael DeFreitas

We are continuing to study the function of the neuronal surface protein NILE. NILE is a 230 kd glycoprotein found on the surface of all neurons, and it is localized to the neurites of cultured neurons. Using a monoclonal antibody to this protein (ASCS4), we have found that NILE on cultured neurons is sulfated and is spontaneously converted from a 230 kd form to a 250 kd form by an unknown process. These and other similarities led us and others to suspect that NILE is similar to the cell adhesion molecule NgCAM/L1, which has recently been isolated from brain. Antibody studies have shown that this is the case. This implicates NILE in adhesion of neurons to glial cells and in the interaction of neurites to form 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. For example, we find that the ASCS4 antibody increases the proportion of neurons extending neurites in culture. As an alternative approach to the study of these molecules, we are using mutant cells of a neuron-like tumor line—PC12 cells. Using the anti-NILE ASCS4 monoclonal antibody, Bill Matthew selected PC12 mutant cells, which are deficient in the ASCS4 epitope. At least some of these cells have reduced or modified NILE protein by gel analysis. We are currently selecting clones of cells that are normal in morphology which contain other surface proteins for which we have antibodies but which do not stain with anti-NILE antibodies. Once we obtain several of these clones, we will examine them for deficits in their ability to perform those functions that have been attributed to NILE on the basis of antibody perturbation experiments, for example, the ability to extend neurites in response to NGF and ability to adhere to neurons and to glial cells. We will also examine other functions such as ability of cells to release neurotransmitter and recycle vesicles. Ultimately, we hope that this approach will yield a better understanding of the functions and mechanism of action of this neuron-specific surface protein.

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242. A Monoclonal Antibody that Blocks Neurite Outgrowth: Studies On the Binding Site and in situ Localization
Arlene Y.-C. Chiu, Jami Frost, William D. Matthew

A variety of nonneuronal cells secrete factors that can greatly enhance neurite outgrowth in culture; many of these are extracellular matrix (ECM) complexes containing primarily laminin and heparin sulfate proteoglycan (HeSPG). In order to understand how the organization of the ECM regulates neurite regeneration, a monoclonal antibody, INO (for Inhibitor of Neurite Outgrowth), was produced which blocks the activity of a secreted outgrowth-promoting factor (Matthew and Patterson, 1983). Biochemical analysis of this complex reveals that binding of the antibody requires an association of at least two matrix components, which appear to be laminin and HeSPG. In radioimmunoassays, INO binding is lost when the secreted complex is dissociated and fractionated. However, immunoreactivity could be recovered by selective reassociation of two of these fractions. Furthermore, monoclonal antibodies that recognize laminin or HeSPG could precipitate both the INO-bearing complex and neurite-promoting activity.

Immunofluorescence studies of INO binding sites in cryostat sections of unfixed rat tissues show the following. 1) INO binds to basement membranes that also bear laminin and HeSPG, which correlates well with the biochemical results. 2) The INO epitope is localized in the nervous system at sites known to support axonal regeneration; INO binding is restricted to the PNS and is not detectable in the CNS. In target tissues, staining is seen at sites which are normally contacted by innervating nerves. 3) INO immunoreactivity is dramatically increased in the basement membrane surrounding skeletal muscle fibers following denervation. However, no concomitant increase in
laminin binding is detected, suggesting that the rise in
INO staining intensity is not due to a general increase
in the amount of basement membrane. Thus,
denervated muscles may regulate neuronal sprouting
and reinnervation by a reorganization of the ECM
resulting in an increase in sites promoting neurite
outgrowth. These results also support the idea that
the INO determinant plays a role in axonal
regeneration in vivo.

References

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243. Rostro-caudal, Position-specific Monoclonal
Antibodies
Joann Imrich, 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 the rostral sympathetic
ganglia, while neurons in the caudal cord prefer to
connect with neurons in the caudal sympathetic
ganglia. This specificity continues to be displayed
even when adult ganglia are transplanted; 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 exper­
iment 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. These
phenomena suggest the possibility of a positional
information system for synaptogenesis that is shared
between neurons and muscle.

In an effort to investigate the molecular basis of
such a system, we would like to produce monoclonal
antibodies that display a rostro-caudal bias in their
binding to 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 (S1) root ganglia,
suppress the response to these antigens with cyclo­
phosphamide injections, and then inject membranes
from rostral (C3) ganglia. Dorsal root ganglia are used
because the segmental origin of the neurons in each
ganglion is restricted to that segment, unlike
sympathetic ganglia where the neurons in each
ganglion come from several adjacent segments during
development. This method produced many antibodies
that bind specifically to membranes from rostral but
not caudal ganglia. Some of these antibodies also bind
to membranes from sympathetic ganglia. The histo­
logical localization of antibody binding within the
ganglia is currently being determined, as is their
binding to rostral vs. caudal muscle membranes.

244. Selective Synapse Formation in Culture
Toshihiko Suzue

As discussed above in the Imrich and Patterson
abstract, spinal cord axons that innervate sympathetic
neurons display a positional bias in their innervation of
rostral vs. caudal ganglia as well as skeletal muscle.
Since the in vivo transplantation paradigm has a
number of limitations, it would be very useful and
informative to see if this positional bias in synapto­
genesis can also occur in culture. The experimental
system currently under development involves excising
fetal rat spinal cord from rostral (T1-2) and caudal
(T5-6) segments and placing them in small compart­
ments separated by Teflon partitions on a culture dish.
These compartments are adjacent to a third
compartment that contains dissociated neonatal rat
sympathetic neurons from rostral (superior cervical)
and caudal (T5) ganglia. The neurons from the two
ganglia are distinguishable because one set is pre­
incubated with a fluorescent dye which is internalized.
Within 2-4 weeks, axons from the spinal cord segments
grow into the third compartment through the space
between the Teflon partition and the dish surface.
When the cord segments are stimulated with extra­
cellular electrodes, synaptic potentials are
recorded from the sympathetic neurons with intracellular
electrodes. These potentials have characteristics
similar to those evoked by preganglionic nerve stimu-
lation in vivo, i.e., the excitatory postsynaptic potentials (EPSP) accompanied by conductance increases are sensitive to changes in extracellular [Ca++] and nicotinic antagonists. The relative strength of the innervation of a particular sympathetic neuron by each of the cord segments is estimated by examining the relation between the EPSP amplitude and the stimulus intensity.

If the rostral and caudal cord segments preferentially innervate rostral and caudal sympathetic neurons, respectively, the synaptic specificity observed in vivo is very likely due to pre- and postsynaptic markers on the neurons, ruling out extraneuronal factors. In addition, this co-culture system would be ideal for testing the ability of the position-specific antibodies discussed in the previous abstract to perturb the rostro-caudal bias in synaptogenesis.

**PUBLICATIONS**


Pittman, R. N. and Patterson, P. H. (1986) Characterization of an inhibitor of neuronal plasminogen activator released by heart cells. Submitted for publication.

**Assistant Professor:** Mark A. Tanouye

**Research Fellow:** Linda E. Iverson, Jonathan D. Pollock

**Graduate Students:** Alexander Kamb, Mani Ramaswami

**Laboratory Staff:** Ross J. McMahon

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**Summary:** We are concerned with basic molecular and genetic mechanisms underlying nervous system structure and function. The 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.

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 locus encodes the channel protein. The abstract by Mani Ramaswami describes our attempts to identify this structural gene.

Despite their importance, virtually nothing is known about K+ channel molecules. This is due to technical difficulties associated with biochemical purification. We have used a strategy that combines classical and molecular genetics to identify genes that encode or regulate the expression of K+ channels. Previous work has shown that *Shaker (Sh)* mutations in *Drosophila* eliminate or modify one class of K+ channel, the A channel. The Sh locus has been mapped cytologically using a collection of Sh translocation stocks to within a few bands at 16F on the X chromosome. Several EMS-induced Sh alleles have been mapped genetically with respect to each other and with respect to the translocation breakpoints. Sh appears to be a complex genetic locus. In the abstracts
by Linda Iverson and Alexander Kamb, we report the molecular cloning and partial characterization of the region containing Sh. In the report by Jonathan Pollock, we report electrophysiological analyses of a Drosophila cell line.

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 identifiable 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.

245. Genetics of Sh

Linda E. Iverson, Alexander Kamb

The Sh locus is defined by a collection of semi-dominant, EMS-induced alleles as well as several translocation mutations. All of these mutations cause leg-shaking behavior to various degrees. Gene dosage studies of EMS-induced alleles suggest a mutation of an antimorphic nature, that is, two doses of wild-type gene (Sh+) does not completely eliminate leg shaking (Sh/Sh+/Sh+). In addition, voltage clamp preparations of these mutant strains reveal defects in the fast potassium, or "A", current.

Several recessive lethal complementation groups flank the Sh locus. These lethals (t) map genetically to within a few hundredths of a centimorgan of Sh (A. Ferrus, unpublished data). t Heterozygotes (t/+ ) display mild action potential abnormalities such as multiple spiking (M. Tanouye, unpublished).

To investigate the relationship between the viable Sh alleles and the adjacent lethal alleles, we are constructing Sh-t heterozygotes. We will examine leg-shaking behavior, viability, and excitability properties of these heterozygotes.

To understand the molecular basis for the leg-shaking behavior and the potassium current abnormalities of Sh mutants, we have cloned a major portion of the chromosomal region known to contain Sh mutations (16F on X chromosome). We used a cDNA clone (adm135H4), obtained from Dr. M. Wolfner, to initiate a chromosomal walk in phage and cosmid libraries that has covered 300 kb of DNA. We have mapped three Sh translocation breakpoints within this region. These breakpoints are distributed over 65 kb of DNA.

Much of this 300 kb region has been used as hybridization probes to detect transcripts on Northern blots and to screen cDNA libraries (courtesy of Dr. L. Kauvar). We have identified some transcripts and isolated cDNA clones that originate from sequences near the Sh translocation breakpoints. Gene transfer studies with P-element constructs have demonstrated that one of these transcripts corresponds to one of the recessive lethal loci. We have not yet identified the gene(s) affected by the Sh viable mutations.

We have generated recombinants between Sh viable alleles and between Sh and adjacent lethal genes. Using restriction fragment length polymorphisms, we have mapped these crossover sites within the cloned region. This analysis delimits the area within which the Sh gene(s) lies.

Future efforts will focus on isolating cDNA clones that correspond to the Sh viable genes as well as on extending the chromosomal walk. In addition, we plan to compare the pattern of transcripts in Sh mutants relative to wild type.

246. Characterization of Ionic Currents in a Drosophila Cell Line

Jonathan D. Pollock

Experiments are currently under way to describe single channel and macroscopic currents in Drosophila Schneider Type 2 cells. Schneider Type 2 cells are an embryonic cell line derived from ectoderm (Schneider, 1972). Preliminary evidence suggests that the input resistances of these cells is between three to four gigohms and have a resting potential of -35 mV. It is
suspected that these cells lack the fast potassium current.

The ability to stably express cloned DNA in these cells (Rio and Rubin, 1985) and the putative absence of the fast potassium channel in these cells would help identify DNA sequences from the Shaker region that code for the fast potassium channel. Assuming that the gene for the fast potassium channel can be expressed in these cells, a number of analyses can be carried out. For example, studies on structure/function using site-directed mutagenesis can be performed. These analyses would help to answer the question of what amino acids in the primary structure of the potassium channel protein determine the ionic selectivity, open time, activation, and voltage sensitivity of the channel. For instance, Hille (1984) suggests that the selectivity filter for potassium is an amino acid sequence that forms a ring of five oxygens around the mouth of the channel. If such an amino acid sequence is found, it can be manipulated to determine whether ion permeation and selectivity for potassium is altered.

Another analysis that this system would permit is the purification and structural characterization of mutant and wild-type channel since the channel, stably expressed in these cells, could be over-expressed using an inducible promoter. Such studies would complement structure/function studies employing site-directed mutagenesis.

Last, this system may also be useful in studying the regulation of the fast potassium channel by other gene products. One distinct advantage of this system is that mutant gene products lethal in the fly but which regulate expression of the fast potassium channel could be studied.

References:

247. Cloning of the Na+ Channel Gene in Drosophila

Mani Ramaswami

The molecular structure of ion channels that underlie their complex functions is not well understood. Prototypic voltage- and ligand-gated channels have been cloned only recently (Mishina et al., 1985; Noda et al., 1986), and from their primary sequences much new information has been obtained. The powerful genetics of Drosophila offer the opportunity for the study of molecular interactions that are required for the proper synthesis, assembly, and function of these channel proteins. Mutants such as nap², para², and set² undergo rapid and reversible temperature-dependent paralysis above their "restrictive temperatures." They show abnormal neural excitability that appears to result from altered Na⁺ channel activity. The cloning of the Na⁺ channel in fruit flies should allow analysis of the participation of these genetic loci in ion-channel, and hence neural, function. It might also allow the dissection of functional domains in channels by the construction of fusions to other ion-channel sequences that should be available soon.

With rat cDNA clones for the Na⁺ channel from A. Goldin and A. Dowset of Prof. N. Davidson's lab as probes, we have used low stringency hybridizations designed to search the fly genome for homologous sequences, and have isolated several putative Na⁺ channel coding domains from a Drosophila genomic library (a kind gift from Alexander Kamb). We are in the process of analyzing these segments of DNA using synthetic oligonucleotides, in situ hybridizations to salivary gland chromosomes, and DNA sequencing.

References:

248. Genetic Analysis of bendless

Mark A. Tanouye, Alexander Kamb, Ross J. McMahon, Susannah Hannaford1, Anne Lewis1

The giant fiber pathway mediates the jump/flight escape response in Drosophila. Mutations that interrupt this pathway, such as bendless (ben), render mutant flies unable to jump. This mutation appears to affect one neural connection in the pathway. Thus, ben may encode a molecule that directs the formation of specific synapses. Only one ben mutant allele has been isolated (Thomas and Wyman, 1982).
We have mapped ben to a position 1.42 cM right of garnet on the X chromosome. ben must also lie to the left of narrow abdomen. Cytogenetically, ben lies between 12B6-7 and 12D3. We are mutagenizing flies with EMS, X rays, and hybrid dysgenesis (transposon insertion mutagenesis) to isolate additional ben alleles. Dysgenesis-induced mutant alleles may be used to obtain DNA clones of the ben locus in order to characterize the structure and function of the ben gene product.

References:

1Undergraduate, California Institute of Technology.

PUBLICATIONS
NEUROBIOLOGY AND BEHAVIORAL BIOLOGY

John M. Allman
James M. Bower
Bela Julesz
Masakazu Konishi
Marianne E. Olds
R. W. Sperry
David C. Van Essen
Summary: In collaboration with Marcus Raichle and Peter Fox of Washington University, and Francis Miezin and David Van Essen of Caltech, we have been mapping the functional organization of the visual cortex in human subjects using stimulus-evoked changes in local cerebral blood flow measured with positron emission tomography (PET scanning). In our mapping of primary visual cortex, we have been able to obtain a much higher resolution than has ever been achieved before using PET scanning. By comparing stimulus-evoked changes in cerebral blood in monkeys and humans, it will be possible to relate our findings in humans to the extensive body of data available for non-human primates on cortical function.

In another approach to the study of visual cortex, we have sought to determine the chemical basis of neural specificity of visual cortical areas and associated pathways. Colin McDonald, To Ha Thai and I have generated monoclonal antibodies that bind to particular pathways that connect the Middle Temporal visual area (MT) with other cortical visual areas. The discovery of the chemical nature of the substances bound by these monoclonals may reveal molecular and genetic basis of the circuitry connecting cortical areas. The MT pathway-specific monoclonals are also potentially a way of mapping this system in human brains obtained at autopsy.

We also have been using microelectrode recordings to map functional pathways in owl monkeys. Martin Sereno, Colin McDonald and I have been mapping the hitherto largely unexplored visual cortex on the ventral surface of the occipital and temporal lobes. Steve Petersen, Francis Miezin and I have found evidence for functionally distinct pathways connecting cortical visual areas. Fast conducting pathways with transient responses ascend to area MT and the Medial Visual area (M). These areas analyze visual motion and possibly serve to orient the animal to novel stimuli in the periphery of the visual field. A much slower pathway with sustained responses ascends to the dorsolateral visual area (DL), which is involved in the analysis of visual shape and form.

Finally, we have continued our comparative study of the organization and evolution of the visual system in primates. Evelynn McGuinness and I are collaborating with Patricia Wright, a Visiting Associate from Duke University, who has studied extensively the behavior of owl monkeys and prosimian primates.

249. Central Nervous System Electrophysiology in a Visually Predatory Arachnid

David W. Sivertsen

Jumping spiders (Salticidae) are visual predators. Brightly colored salticids wave their front legs like semaphores as a courtship display and stalk their prey with relentless efficiency, dropping into a cat-like crouch to stalk prey beyond the reach of their leap. The Caltech campus is home to several species, including Phidippus johnsonii, a large black, red, and iridescent green spider.

Previous work has quantified behavior (Land, 1972; Forster, 1982), receptor properties, and anatomy (Hill, 1975). Ethological studies relating brain and behavior have been hampered by technical difficulties in recording signals from the central nervous system. I have developed techniques that open this area to study.

Salticids have eight eyes. One pair is largely vestigial, two pair are primarily concerned with orienting toward visual stimuli, and the anterior medial (AM) pair perform complex visual analysis, similar to foveal vision in mammals. The AM eyes have 2° wide by 20° high retina. The receptors are ordered into four distinct layers, which are tiered along the 2° width. This array along the optical axis takes advantage of the chromatic aberration to focus green and UV wavelengths independently.

Although the lenses of the AM eyes are fixed in the chitinous exoskeleton, the retinæ are mobile. Land
(1969) has shown that salticids scan their world with a variety of stereotyped retinal movement patterns, similar to vertebrate eye movements. The use of a narrow, high resolution retina to raster a large visual field is an elegant solution to the compromises inherent in a miniature and compact system.

Using computerized stimulus presentation and data collection techniques adapted from primate research, I have characterized receptive fields and their properties. There are similarities to vertebrates, such as cells that are extremely velocity selective. In addition, I have found several new properties with single and multiple unit recording in the CNS. Last year's report describes space and size constancies found in the system. A new class of cells in the protocerebrum receives input from multiple eyes (usually the anterior lateral and AM pairs). These cells have very large receptive fields with response decreasing uniformly with eccentricity. They respond to movement of previously static stimuli. Such a cell is a good candidate for mediating visual attention. I also made assessments of spectral response characteristics of CNS neurons and found broader response curves in cells receiving input from AM eyes.

References:

250. Owl Monkey Extrastriate Cortex

Martin I. Sereno, Colin T. McDonald, John M. Allman

We have continued our chronic mapping experiments in owl monkey extrastriate cortex. Our most densely mapped hemisphere yielded about 1,200 different receptive fields from over 200 penetrations. Several previously unexplored regions in the rostral part of visually responsive cortex apparently contain topographically organized areas.

The new areas will be described in a medial to lateral sequence. First, medial and rostral to MT and DL, there is a topographically organized representation of most of the visual field in the temporo-parietal region (TP). Second, there is a superior temporal area (ST) rostral to MT with large receptive fields and at least local topographic order. Third, there is a strip of cortex directly lateral to MT representing the upper visual field; but unlike medial DL, which contains a representation of the entire lower visual field, this area only represents azimuths out to 15°. Fourth, in posterior inferotemporal cortex, there is a topographically organized area containing an upper visual field and a partial lower visual field representation. Finally, rostral to VA on the ventral surface, there is an area containing an orderly representation of at least the central 15° of the visual field.

These results suggest that there are several topographically organized visual areas in extrastriate cortex of the owl monkey beyond those already described. Thus, most of the visually responsive cortex in this animal shows some degree of retinotopic order.

251. Segregation of Information Flow in Four Extrastriate Areas of the Owl Monkey

Francis M. Miezin, Steven E. Petersen, John M. Allman

There is considerable evidence that two parallel processing channels for visual information exist in macaques between the retina and striate cortex. P-beta retinal ganglion cells produce sustained responses to prolonged stimulus presentation. Their relatively slow conducting axons project to the parvocellular layers of the dLGN which in turn project to layer 4C-beta of V-1. The cells in the parvocellular layer have long response latencies and sustained discharges. P-alpha retinal ganglion cells, however, produce transient responses, have short conduction latencies and project to the magnocellular layers of the dLGN. The cells of those layers have short response latencies and transient responses and their axons terminate in layer 4C-alpha of V-1.

These results raise the question whether this separation of response characteristics continues in areas beyond striate cortex. Cells were recorded from four extrastriate areas in the owl monkey: the middle temporal area, MT; the dorsal lateral area, DL; the dorsal medial area, DM; and the medial area. A histogram for each cell's response was constructed and normalized, and then the histograms for each area were summed. The combined histograms were used to distinguish characteristics of each area.

The response of cells in MT have the shortest latency and are the least sustained of the areas. DL on the other hand has a response latency 22 msec
longer than MT and the most sustained discharge rate. These results agree with those reported by Maunsell (1984) in macaques, in which he demonstrates that MT cells have a transient response with a latency of 38 msec while cells in V4, homologous to DL in the owl monkey, have a sustained response with a 50 msec latency.

The medial area responses are very similar to MT with the latency being essentially identical and the discharge just as transient. The remaining area, DM, combines aspects of both channels. As in MT, an early activation peak is present but the response is of the more sustained DL type.

References

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252. Retinotopic Organization of Human Visual Cortex

Peter T. Fox†, Francis M. Miezin, John M. Allman, David C. Van Essen

As a first step to relating the large body of data on non-human visual cortex directly to the human visual system, we have begun mapping human visual cortex using positron emission tomography (PET). PET was used to image focal cerebral blood flow (CBF) increases induced by a red/black checkerboard annulus that was placed at various eccentricities in the subject's visual field while the subject fixated a small crosshair in the center of the annulus. From previous work it is known that blood flow is modulated by changes in neural activity; hence, our PET measurements provide a valid indicator of visually evoked neural activity patterns. Oxygen-15-labeled water was used as the blood flow tracer; its very short half-life (2 min) allows multiple stimulated state and control state measurements to be acquired during a single scanning session for each subject. In each of six normal volunteers, cortical responses were mapped for three retinal eccentricities: macular stimuli using an annulus with an inner radius of 0.1° and an outer radius of 1.5°; perimacular stimuli with a 1.5-5.5° extent; and peripheral stimuli with a 5.5-15.5° extent. Response foci were imaged by subtracting a control response (fixation point only) from each stimulated state response.

Cortical response locations vary systematically with the position of the stimulus in the visual field (Figure 1). For all subjects, each restricted-field stimulus induced an intense, focal increase in regional CBF on the medial surface of occipital cortex. Based on stereotactic coordinates, the maximum responses were determined to be at the level of the calcarine fissure. Responses to the macular stimulus were slightly anterior to the occipital pole. Perimacular responses were 6 mm anterior and 4 mm superior to the macular response. Peripheral responses were 3 mm anterior and 5 mm superior to the perimacular response. All subjects showed the same general pattern of organization. After scaling the stereotactic locations of all of the responses to a standard size brain, the differences in location of the three responses are all statistically significant (p < 0.01).

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Figure 1. Stereotactic plot of the retinotopic organization of human striate cortex. The plot is a sagittal view of the location of the responses to visual field stimulation. The horizontal axis corresponds to distance in cm posterior to the center of the anterior commissure-posterior commissure (AC-PC) line. The vertical axis is the distance in cm dorsal (+) or ventral (-) to the AC-PC line. The mean coordinates (+/- one standard deviation along each axis) are shown for each stimulus. The points labeled Lower Field and Upper Field are responses for hemi-annuli stimuli restricted to the lower and upper visual field, respectively.
253. The Nocturnal Primate Niche in the New World

Patricia C. Wright

The niche of the only nocturnal monkey is discussed in light of our knowledge of the nocturnal prosimians from Asia, continental Africa and Madagascar. *Aotus trivirgatus* differs from diurnal monkeys by having a low basal metabolic rate and specializations in the eye and brain that better equips it for night vision. Although the nocturnal prosimians studied to date also have a low basal metabolic rate, they also have a *tapetum lucidum* for increased night vision. Olfaction is less important to the night monkey than for the prosimians and the olfactory lobes are one-third the size. These morphological and physiological differences are then reviewed in light of recent field work on the behavior and ecology of the night monkeys. Behavior suggests that the vision of *Aotus* may not be as acute at very low light levels (the dark of the moon) as the prosimian's vision. *Aotus* travels nearly twice as far on nights with bright moon, and many behaviors such as playing, fighting between groups, and intergroup calling only occur at high night-time light levels. Prosimians do not show this lunaphilia to such an extent. In general, prosimians are much more specialized in diet, locomotor capabilities, and even level of the forest utilized. In each guild of nocturnal prosimians there is variation in body size. *Aotus*, however, has remained more generalized in diet, locomotion and forest level used. This may partially be a result of lack of competition from other nocturnal primates. *Aotus* has the capabilities to change its diet from fruit to leaves, its locomotor patterns from quadrupedal to vertical clinger and leaper and even its activity cycle from strictly nocturnal to partially diurnal depending on the environmental circumstances.

254. Primates without blobs: The Distribution of Cytochrome Oxidase Activity in Striate Cortex in *Tarsius, Hapalemur*, and *Cheirogaleus*

Eve Lynn McGuinness, Colin T. McDonald, Martin I. Sereno, John M. Allman

Regularly spaced blobs (a.k.a. puffs or patches) of cytochrome oxidase have been seen in layers 2 and 3 of striate cortex of all higher primates studied as well as a prosimian, *Galago*. A comprehensive study of readily available laboratory primates by Horton (1984) suggested that this would be a feature common to all primates. We examined additional prosimian species expecting to see cytochrome oxidase blobs, but this has not been the case. The additional prosimian species studied are all either endangered or threatened with extinction and thus are not available for perfusion. Rare prosimians at the Duke University breeding colony that died of natural causes were frozen to -70°C and shipped to us. We fixed the brains by immersion in cold glutaraldehyde. We have also processed three frozen *Galago crassicaudatus* brains in exactly the same manner and have found in all three galagos distinct cytochrome blobs identical to those in the striate cortex of perfused galago brains.

We have examined brains from two *Tarsius syrichta* and one *Tarsius bancanus*, all of which died of natural causes and were frozen within minutes of death. In no tarsier brain have we seen any indication of blobs although the staining for cytochrome oxidase activity was excellent. Clear cytochrome oxidase bands can be seen in layers 4a, 4c, and 6a, but there is no evidence of blobs in layers 2 and 3. In one case the tarsier tissue was reacted at the same time and in the same solutions as an immersion-fixed galago brain that was found to contain clear-cut blobs. We have also found no evidence of blobs in two *Hapalemur griseus* and one *Cheirogaleus medius*.

Cytochrome oxidase blobs appear to delineate functional pathways in the visual cortex of higher primates and galagos. Their absence in *Tarsius, Hapalemur, and Cheirogaleus* suggests a different functional organization of visual processing in these prosimian primates.

We thank Dr. Patricia Wright and Dr. Elwyn Simons of the Duke University Primate Center for providing freshly frozen brains of *Tarsius, Hapalemur* and *Cheirogaleus* that died of natural causes and Dr. Martha Neuringer of the Oregon Primate Center for providing the freshly frozen *Galago* brains.

Reference:
PUBLICATIONS


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Summary: Work in our laboratory this past year has extended in several different but related directions as our multidisciplinary research group has grown. We have continued to study the properties of different populations of synapses in piriform cortex using in vitro brain slice methods and have shown that the property of synaptic facilitation in these synapses can be modified in an activity-dependent manner. We have also continued to develop and expand our ability to record extracellular activity from many neurons simultaneously and have devoted considerable effort to the elaboration of new analysis procedures for these...
data. Work has begun to substantially increase the power and sophistication of our neural network simulations by using the JPL/Caltech Mark III hypercube. This project was selected as one of the alpha test codes for this new machine and our simulation code was the second program to run successfully on the new Mark III. Our structural simulation of pyriform cortex has reached a fairly sophisticated state of development and is ripe for transport to the hypercube. In addition to these projects, which directly address the functional organization of neural networks, we have also undertaken several experiments designed to try to understand how these neural networks are organized during development. We were fortunate, in this regard, to have Karl Herrup, the Cornelius Wiersma Visiting Professor of Neurobiology, visiting our laboratory from Yale University for five months. With Karl's guidance, we have been studying the influence of Purkinje cell lineage on patterns of connectivity in the cerebellum. Overall, the last year has shown a considerable increase in the size, diversity and activity of our laboratory group.

255. The Modulation of Synaptic Facilitation in SynapsesAssociated with Different Fiber Systems in Piriform (Olfactory) Cortex of the Rat

James M. Bower, Mahendra Rao

The primary objective of these experiments is to study activity-dependent short- and long-term changes in synaptic effects on identified neurons in the piriform cortex of the rat. Using an in vitro brain slice preparation, our recent work has shown that single pyramidal cells receive excitatory inputs from facilitating and nonfacilitating synapses, each associated with different fiber systems. In particular, synapses on pyramidal cells associated with primary olfactory tract afferents facilitate while synapses on the same pyramidal cells arising from the intrinsic association fiber system do not (Bower and Haberly, 1986). The current experiments were designed to further study the facilitating properties of these two synaptic populations using the in vitro brain slice preparation. In these experiments, synaptic facilitation was first studied using a lowered concentration of calcium ion (1.2 mM) in the solution bathing the slice. In these conditions, we found that the association fiber synapses that did not facilitate in 2.4 mM Ca$^{2+}$ now did facilitate. We also have sought to modify the facilitating properties of the two different populations of synapses by preconditioning the synapses with five, 1 sec duration, 20 Hz trains of stimuli. Following this preconditioning, we found in several cells that a nearly complete suppression of the postsynaptic response to the second of two paired pulses was induced. This paired-pulse suppression lasted for up to 2 sec following an initial stimulation of the synapses. The suppressing effect was limited to the synapses actually preconditioned by the trains in that it did not affect the paired shock responses of unconditioned synapses on the same neuron. Additional evidence suggests that this effect, which has been observed for more than 4 hours after the conditioning trains, is not likely to be due to the enhancement of multisynaptic inhibitory events or to the simple depletion of transmitter stores. These changes therefore may reflect a synapse-specific, activity-dependent, long-term modification of the facilitating properties of these synapses, which would be an important aspect of any model of the dynamic function of this cortex.

References:

256. Analysis of the Dynamics of Activity in Ensembles of Neurons Recorded Simultaneously in Cerebellar Cortex

George J. Carman, Brian Rasnow, James M. Bower

A major focus of ongoing efforts in our laboratory is the development of methods to record and analyze the activities of many neurons simultaneously. We believe that these simultaneous extracellular recordings made from groups of single neurons provide the opportunity to investigate both the distributed spatial and temporal representations of sensory information as well as the dynamical behavior of these neurons. However, most analysis procedures currently available for studying extracellularly recorded spike train data are inherently unsuitable for studying neural dynamics. Accordingly, we have begun to develop new analysis procedures. To do this, we have applied dynamic methods to data obtained by recording simultaneously from multiple cerebellar Purkinje cells using experimental procedures previously described (Bower and Llinas, 1983). The analysis we have developed is
based on representing the activities of the $N$ single neurons at a time $t$ as a state $S(t) = (n_1, n_2, n_3, \ldots, n_N)$ where $n_i = 1$ or $0$ depending on whether cell $i$ fired at time $t$ or not. Using this convention, the instantaneous activity of $N$ neurons has a geometrical interpretation as a single point in an $N$-dimensional state space. The time series $(S(t_1), S(t_2), S(t_3), \ldots)$ can then be analyzed to determine the subset of the $2^N$ possible states which are occupied, as well as the subset of a combinatorially large number of cycles or trajectories which are followed between states. To aid in visualization, we are using the technique of simulated annealing (cf. Carman and Van Essen, 1985) to map the occupied regions of the $N$-dimensional space onto a space of reduced dimensions while preserving specific topological aspects of the original space (e.g., the Hamming distance within a neighborhood).

Preliminary results from this analysis technique reveal differences in the global activity of the same ensemble of Purkinje cells under different physiological conditions which may reflect functionally significant alterations in network performance.

References:

257. A Combined Genetic and Physiological Study of Neuronal Specificity in Mouse: Does Purkinje Cell (PC) Lineage Play a Role in Establishing Projection Patterns in Cerebellar Cortex?

Karl Herrup, James M. Bower

In addition to investigations of the functional organization of neural networks, our laboratory is also interested in the developmental organization of these networks. In particular, we are interested in knowing how the tremendous network specificity seen in the adult is set up and maintained. Recently, we have been investigating this issue as it applies to the establishment of the fine pattern of tactile mossy fiber projections to the cerebellar granule cell layer (see Bower and Woolston, 1983).

Several observations suggest that early in development, the arriving mossy fiber system might use intrinsic features of Purkinje cells (PCs) to help establish their patterns of connections. The mossy fibers are first present at a time when the PCs are present, but the granule cells with which they make exclusive contacts in the adult are still being generated. Further, recent results by Mason and Gregory (1985) have shown that mossy fibers make transient connections directly with the overlying PCs. To determine if there is a relationship between the physiologically defined map and the distribution of PCs with different lineages, we have mapped the tactile projections to the surface of several folia in mouse chimera and reconstructed a "lineage map" of the PCs overlying these inputs. β-Glucuronidase histochemistry was used to reveal the genotype of the cells, and a computerized microscope was used to reconstruct the two-dimensional map from a series of sagittal sections. As reported by others, there is an intermingling of PCs of the two genotypes in the chimera which gives the overall impression of randomness when viewed in single sections. Serial reconstructions, however, reveal regions where the ratio of the PC genotypes is strikingly different from the ratio in surrounding areas. Furthermore, we have found that the location of these regions is similar from one chimera to the next. Finally, the location of one such large region in the paramedian lobule consistently corresponds to the physiologically-defined map position of a large lower lip projection. This apparent correlation between a physiologically-defined projection area and PC lineage in the adult animal suggests that information of a cell-autonomous nature may be used to help establish the pattern of connections in this system.

References:

258. Neural Network Simulation on the JPL/Caltech Concurrent Processor (The "Hypercube")

Joan C. Horvath¹, James M. Bower

Structural simulations of neural networks will play an important role in our attempts to understand the functional organization of both cerebellar and piriform cortices. For example, by basing these simulations on the detailed anatomy and physiology of the real neural networks involved, this modeling can uncover the features of these networks most likely to be responsible for the complex patterns of neural activity we
record physiologically. We also anticipate that these simulations will eventually allow the direct investigation of the computations performed by these specific neural circuits. To accomplish these ends, we have begun to build general purpose neural network simulation tools on the newly developed JPL/Caltech "hypercube" concurrent processor. The hypercube, which is a supercomputer class machine, consists of an array of high speed microprocessors (a.k.a. "nodes") each capable of working on a different piece of a complex problem simultaneously. For example, in the case of neural network simulation, each node is made to represent one or many locally interconnected neurons. Within the hypercube, each node can transfer the results of its calculations to the other nodes, thus allowing neurons within one local group to influence neurons in other groups. The base code for the simulation software reflects this common aspect of brain and parallel computer organization. The code modeling the individual neurons encompasses properties like the dendritic structure of each cell and its membrane conductances, while the connectivity aspect of the code includes the neural connectivity matrix and transmission delays. A major part of this development effort involves building a "language" interface which will allow the modeler to easily define parameters without regard to the substructure of the computer or the code. These general modeling tools are currently being used to extend a structural simulation of piriform (olfactory) cortex (see the following abstract) and will also be used to implement a new structural simulation of cerebellar cortex.

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259. A Computer Simulation of Piriform Cortex

Matthew Wilson1, James M. Bower, Lewis B. Haberly2

Using parameters obtained from detailed anatomical and physiological experiments (Haberly, 1985), a computer model of piriform (olfactory) cortex has been developed that simulates physiological responses obtained from this cortex using a variety of recording methods. The model consists of a linear array of pyramidal cells interconnected by a highly divergent-convergent system of associational fibers. Excitatory input is delivered to the cells along a single afferent pathway with characteristics similar to the lateral olfactory tract projection to piriform cortex from the olfactory bulb. Two forms of inhibition are incorporated in the model: a feedforward, long latency, long duration, hyperpolarizing potential simulating a K+-mediated inhibitory process, 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, time constant estimates for inhibitory and excitatory events, and spatial variations in the distribution of different excitatory and inhibitory influences. This model has been shown to simulate a variety of known intracellular and extracellular responses. For example, in response to continuous odor-like stimulation, the model generates a series of oscillatory extracellular bursts comprised of a dominant 40 Hz sinusoid similar to that seen in unanesthetized animals. Single shock stimulation gives characteristic EPSP/IPSP intracellular responses and extracellular field potentials at appropriate latencies. In the presence of "noise," weak single shock stimulation produces damped oscillatory extracellular potentials. Both spontaneous and induced interictal/ictal epileptiform events can be generated after blocking inhibitory processes or strengthening specific associational pathways. The model suggests that the transition from interictal to ictal events can be controlled by the long latency, long duration, hyperpolarizing potential which simulates the K⁺-mediated potential observed in piriform cortex (Tseng and Haberly, 1986). Having successfully used this model to generate physiological responses characteristic of piriform cortex, the next objective will be to explore the significance of the specific structural and physiological features of this cortex for the processing of complex spatial and temporal olfactory information and their implications for general associative memory processes.

References:

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The work described in the following research studies tracing the information flow in the visual stereograms and cinematograms; 2) psychoanatomical statistical properties. Investigations cover four main topics:

1. The study of binocular depth and monocular 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 an axiomatic texton theory of preattentive vision and texture discrimination, using textures of constrained stochastic properties.

While most of these studies are done psycho-physically 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.

260. Texton Theory of Preattentive Vision

Bela Julesz, Dov Sagi, 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 based solely on the spatial density variation of a few conspicuous local features (called textons). These textons are elongated blobs, particularly line segments, and 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. 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, the only global operation by the preattentive visual system. This scaling operation is a fundamental process of early vision, and knowing how the aperture of focal attention varies with element size and with the texton-gradient strength is our main interest. We are extending these studies from static textons to moving ones. Since focal attention shifts can occur an order of magnitude faster than scanning eye movements, the study of moving textons (line segments) might explain how moving targets are not blurred even though the visual system does not use a "shutter."

References:

Summary: Connections and signals between neurons determine the coding scheme of the nervous system. Processing of information by the brain can be fully understood only by an approach that integrates behavioral, anatomical, and physiological methods. This idea underlies all of the work done in this laboratory. Barn owls and songbirds are particularly suitable for the above approach.

During the past year, immunohistochemical methods have provided a new clue that links anatomy and function in the owl's auditory system. An antiserum against a certain protein binds to neurons in a group of auditory nuclei involved in processing interaural time difference. An antiserum against glutamate carboxylase binds to the owl's brainstem nuclei in which neuronal inhibition is known to occur. Production and use of monoclonal antibodies have produced encouraging results in the study of the bird song system.

Behavioral analysis can guide neurophysiology. The converse is also true in this laboratory. Recent studies indicate how the auditory system solves some of the problems of using interaural time difference for sound localization. Neuronal responses in a song control nucleus of sparrows suggest that hearing and vocalization are intrinsically involved in the development of stimulus-selectivity of auditory neurons.

261. The Central Nucleus of the Inferior Colliculus as an Input Stage to the Map of Auditory Space in the Barn Owl

F. Hermann Wagner, Terry T. Takahashi, Mark Konishi

We studied the distribution of interaural time differences (ITDs) in the central nucleus of the inferior colliculus of the barn owl and the projection pattern of this tonotopically organized structure onto the non-tonotopic map of auditory space in the external nucleus of the inferior colliculus.

Neurons in the central nucleus that are sensitive to ITDs are sharply tuned to frequency. Their discharge to stimulation with tones or noise is a cyclical function of ITD indicating an ambiguity in time coding. By contrast, the neurons of the external nucleus, space-specific neurons, are broadly tuned. These neurons are selective for a single ITD if a sound contains more than one frequency.

In a penetration along the tonotopic axis of the central nucleus, the relative discharge level of different units at one particular ITD was independent of frequency and recording depth. The relative discharge level at the other ITDs varied with both frequency and recording depth. Thus, the neurons along such a penetration or "array" seem to express a single ITD. Horseradish-peroxidase retrograde tracing studies show that an array projects to neurons in the external nucleus expressing a similar ITD. We suggest that these arrays represent input stages to the space map and that the information conveyed to the space map by the convergence of many neurons contributes to the ability of space-specific neurons to respond selectively to a single ITD.

Mapping studies showed that the arrays are arranged topographically. ITDs near zero microseconds, which would result when sounds originate from frontal loci, were expressed anterior to ITDs that would result when sounds originate from eccentric loci. At a given anteroposterior level, ITDs that represent ipsilateral directions were expressed medial to those that represent contralateral directions. The range of the ITDs expressed by the arrays corresponds to the natural range of ITDs experienced by the barn owl. There is an increase in response latency from...
medial to lateral, but not from anterior to posterior. Short and long latencies correspond to arrays expressing ipsilateral and contralateral ITDs, respectively.

262. The Role of Stimulus Onset in Sensitivity to Interaural Time Differences in the Barn Owl

F. Hermann Wagner, Mark Konishi

Interaural time differences (ITDs) can provide information about the azimuth of a sound source. A sound signal contains ITDs in its envelope, especially at the on- and offset of the sound and in the continuing or "ongoing" waveform of the carrier. Many neurons in the auditory midbrain of the barn owl are tuned to ITDs. Moiseff and Konishi (1981) showed that the information contained in the ongoing waveform (ongoing ITD) was sufficient to explain the selectivity of these neurons to ITDs. It was not clear, however, whether the ITD at the onset (onset ITD) also plays a role.

We tested the importance of onset ITD for neurons in the inferior colliculus. The response to dichotic stimulation with correlated and uncorrelated noises was compared. In the uncorrelated paradigm, the signal to the two ears did not contain an ongoing ITD. The onset ITD was shifted within the barn owl's physiological ITD range. The onset amplitude varied randomly. The selectivity for a particular ITD was tested with a Rayleigh test. The period of the unit's best frequency provided the testing range.

Extracellular recordings were made from 21 single units. To stimulation with both paradigms, the units increased their spike rate over the spontaneous activity, even if they were not monaurally excitable. For each ITD, the units' responses were typically characterized by a transient (5-15 milliseconds) increase in spike rate after stimulus onset, followed by a less pronounced sustained response that lasted throughout the duration of the stimulus. The sustained response of all units to stimulation with a correlated noise was made equal to the number of repetitions in the uncorrelated paradigm. Then five out of eight neurons still showed tuning to ongoing ITDs.

These results suggest that onset ITDs do not play a role in the manifestation of ITD sensitivity in the barn owl's nervous system.

Reference:

263. An Immunohistochemical Marker for a Functional Pathway in the Auditory System of the Barn Owl

Terry T. Takahashi, Catherine E. Carr, Nicholas C. Brecha, Mark Konishi

Interaural time and intensity differences, the primary cues for sound localization in the barn owl, are processed in separate pathways. Nucleus laminaris (NL) is the site at which the timing of auditory signals from the two sides are compared. It is thus the origin of the pathway devoted to the processing of interaural time differences. We report here that vitamin D-dependent calcium-binding protein (CaBP)-like immunoreactivity is congruent with the terminal fields of NL and is therefore a useful marker for the time pathway.

Our antiserum was monospecific for CaBP. CaBP-positivity was assessed by staining with the peroxidase-antiperoxidase and avidin-biotin methods. Serial dilutions confirmed that the degree of staining is primary antibody dependent.

NL innervates the entirety of nucleus ventralis lemniscus lateralis (VL V) pars anterior and the central "core" of the central nucleus of the inferior colliculus (ICc). CaBP immunoreactivity, which consisted of a plexus of fibers ending in bouton-like swellings usually around ghosts of somata, conformed precisely to the terminal field of NL. By contrast, immunoreactivity was absent in the posterior division of VL V and from the "shell" which surrounds the core in ICc. The latter regions are innervated in their entirety by the projections of nucleus angularis, a cochlear nucleus and the origin of the pathway devoted to the processing of interaural intensity differences.

Somata of NL were also CaBP-positive. A restricted lesion in NL resulted in a localized loss of CaBP-positive terminals in those regions of ICc and cycles in the correlated noise was made equal to the number of repetitions in the uncorrelated paradigm. Then five out of eight neurons still showed tuning to ongoing ITDs.

These results suggest that onset ITDs do not play a role in the manifestation of ITD sensitivity in the barn owl's nervous system.

Reference:
VLV that are innervated by the lesioned area of NL. In adjacent sections processed by the Fink-Heimer method, degenerating axons were observed in the region of the deficit in immunoreactivity. The simplest explanation is that the immunoreactive fiber plexus is the terminals of axons originating in NL.

Earlier studies showed that injections of tritiated proline into the cochlea labels the targets of nucleus angularis and is thus a method for the delineation of the intensity pathway. The CaBP immunoreactivity described above provides a complementary technique for the delineation of the time pathway.

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264. GAD Immunoreactivity is Correlated with Functional Inhibition in the Auditory System of the Barn Owl

Catherine E. Carr, Nicholas C. Brecha1, Mark Konishi

Single unit studies have demonstrated that inhibitory processes mediate many binaural interactions. At least part of this inhibition may be GABAergic in nature, as inhibition in some auditory nuclei is correlated with the presence of immunoreactivity to the GABA biosynthetic enzyme, glutamate decarboxylase (GAD). In this study a specific antiserum to GAD was used to determine the distribution of GABAergic cell bodies and axon terminals in the owl's auditory brainstem and midbrain nuclei.

The barn owl uses binaural time and intensity differences for sound localization and processes these time and intensity cues in parallel channels. Eighth nerve afferents innervate the intensity-coding nucleus angularis and the time-coding nucleus magnocellularis. The intensity pathway includes the superior olive (SO), the ventral part of the nucleus of the lateral lemniscus (LLv), the posterior part of ventral nucleus of the lateral lemniscus (VLVp), and part of the central nucleus of the inferior colliculus (ICC). The projections of the time coding pathway are to nucleus laminaris (NL), the anterior part of the ventral nucleus of the lateral lemniscus (VLVa), and the inferior colliculus. Once in the inferior colliculus, the time and intensity pathways recombine to compute the location of the auditory stimulus in the space-mapped region (ICX).

Moiseff and Konishi (1983) classified neurons in the auditory system on the basis of response types and described inhibitory responses (EI, II, OI, OO) in VLV and IC. This physiological inhibition correlates with the distribution of GABAergic cell bodies observed in the present study (see Table 1).

Each auditory nucleus receives a characteristic innervation by GABAergic terminals. All the neurons of the time pathway receive large perisomatic GABAergic terminals of as yet unknown function, while the intensity pathway is characterized by more GABAergic neurons and finer terminals distributed throughout each nucleus. In addition, colchicine treatment has unmasked a number of GABAergic cell types in VLV, ICC, and ICX.

Reference:

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<table>
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<tr>
<th>Response</th>
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<th>Time</th>
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<td>GAD +v</td>
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265. Immunocytochemical Localization of Estrogen-binding Neurons in the Zebra Finch Brain

Manfred Gahr¹, Eugene Akutagawa, Mark Konishi

Administration of estrogen to a female finch chick induces her song control nuclei to develop male-like cytoarchitectonic attributes. Masculinization involves both the prevention of normally occurring cell death and the promotion of cell growth. Whether estrogen acts directly on the neurons of song nuclei is not known. We used monoclonal antibodies against the estrogen receptor to identify estrogen target cells in brain tissue. Many estrogen-binding cells were found in the preoptic area and the hypothalamus but not in the song system. Only the forebrain song nucleus HVc contained some estrogen-binding cells in its medial part. However, the medial and ventral neostriatum bordering HVc contained many estrogen-binding cells. Preliminary observations indicate gender differences in the number of these cells in adult zebra finches. In juvenile zebra finches, the number and distribution of estrogen-binding cells appear similar in the two sexes.

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266. Monoclonal Antibodies Selective for a Song Nucleus in the Zebra Finch

Miriam S. Zucker¹, To Ha Thai, Mark Konishi

Neurons of nucleus RA (Robustus archistriatalis), a song control nucleus, undergo atrophy and cell death in the female zebra finch unless rescued by estrogen implantation.

The plasticity of such neurons in response to hormonal cues may suggest the presence of molecular specializations that might mediate this differential development. Other molecules specific to the song system might be involved in axonal pathfinding or proper synapse formation.

Such molecules could be used as markers to identify and follow these neurons through development in vitro as well as in vivo. To this end, we have attempted to identify possible markers by raising monoclonal antibodies against antigens of nucleus RA. In cooperation with the Institute's Monoclonal Antibody Facility (see page 216), mice were immunized with homogenate of tissue from adult male zebra finch RA. In order to reduce the immune response to ubiquitous brain antigens, the mice were immunosuppressed after injecting them with tissue from an anterior, non-song part of the forebrain. Preliminary screening of the resultant monoclonals on forebrain tissue has yielded several antibodies that show specific binding in sets of song-system nuclei or in RA alone.

267. Development of Neuronal Selectivity in a Song-System Nucleus during Song Learning

Susan F. Volman, Mark Konishi

Many songbirds learn to sing in a two-stage process: they first memorize a song model; subsequently, they match their own vocalizations to this model by ear. In adult white-crowned sparrows (Zonotrichia leucophrys), auditory neurons in one of the song-control nuclei, HVc, show a consistent preference for a bird's own song (Margoliash, 1983; Margoliash and Konishi, 1983). The current study was undertaken to distinguish between two possible sources of this selectivity: it might reflect the process of storing a "template" of the song model or it might be a consequence of song matching.

We recorded from HVc in juvenile, presinging white crowns that had been "tutored" in the laboratory with a single song model from their home dialect. Neuronal responses to this song were compared with similar songs from the same geographic region (same dialect), songs of different dialects, reversed song, and songs altered by a computer system for song analysis and resynthesis. There was no evidence of a consistent preference for familiar song patterns in any of these birds, although at most recording sites, some stimuli elicited much stronger responses than others. One bird that had begun to vocalize was also tested. The song-like sounds this bird produced were shifted upwards in frequency due to the presence of a glass bead implanted within its syrinx (the avian sound-producing organ). In this bird there appeared to be a preference for the distorted sounds. These results support the hypothesis that selectivity for a bird's own song in HVc neurons arises during the process of song matching and not during memorization of the tutor song.

Single neurons in the juvenile birds often did respond preferentially to sounds with frequency and amplitude modulations of the sort present in white crown songs. Thus, as in adults, many HVc neurons
responded better to song than to tone or noise stimuli. This observation suggests that auditory feedback may selectively reinforce connections already present in the juvenile brain in order to form the adult pattern of neuronal responses.

References:

PUBLICATIONS


Summary: Neurons in the mammalian CNS that contain the transmitter dopamine (DA) can be visualized using histochemical methods. This approach has revealed that the somata of some of these neurons, localized in two structures of the ventral mesencephalon, the substantia nigra and the ventral tegmental area, give rise to three ascending systems referred to as the nigrostriatal, mesolimbic and mesocortical DA systems, depending on where the systems terminate. The anatomy, physiology, biochemistry and pharmacology of these neurons are now relatively well understood. Less progress has been made with respect to their function(s). However, it is believed that normal transmission in these systems is critical for cognition, arousal, attention, positive reinforcement, and motor activity. Malfunction in these systems is associated with schizophrenia, affective disorders, and Parkinson’s disease.

Animals implanted in CNS with chronic electrodes for stimulation of discrete sites can be trained to work to obtain a brief electric shock applied via the electrodes. A majority of the positive sites contain the somatas of DA neurons, or are traversed by their ascending axons, or are innervated by their terminals. This has led to the view that excitation of DA neurons is the critical factor for brain reward.

Animals in which the output of these DA neurons is increased via pharmacological manipulations are hyperactive and exhibit stereotypy. But decreased DA activity produced by neonatal damage also results in hyperactivity, while damage inflicted in adulthood is associated with hypoactivity. The basis for the neonatally-induced hyperactivity is not known, but it may be supersensitive DA receptors or aberrant development of inhibitory systems interacting with the DA systems.

The work in this laboratory concerns the reinforcing and motor functions of these DA neurons. For reinforcement, the question is whether DA neurons are in fact excited by the brief electric shock, and if so, whether this is the critical event. The available data are not free of conflict. For motor activity the question is whether the permanent hyperactivity induced by damage to the DA systems early in life is based on the supersensitivity of DA receptors. More information is necessary before this DA-linked motor dysfunction can be understood.

During this past year, the work of this laboratory has been extended to include non-DA neurons which receive direct or indirect input from the DA systems. The reason for this extension is that an understanding of the DA function in motor activity and reinforcement requires knowledge of the complete circuit, including DA and non-DA neurons responsible for these behavioral effects of DA.

Viewed in the context of behavior, the problems dealt with this past year concern the CNS basis of positive reinforcement and of drug-induced motor activity; viewed in the context of transmitters, they concern the functions of the ascending DA neurons.
268. The Function of Catecholamine Terminals and Local Intrinsic Neurons in Self-stimulation Behavior of Ventral Tegmentum

T. Takeichi¹, S. Kurumiya¹, Mamoru Umemoto¹, Marianne E. Olds

Several lines of evidence implicate the central DA systems in behavior maintained by positive stimulation of discrete brain sites. Mapping studies show that such behavior, designated as self-stimulation behavior (SS), can be obtained by stimulation of the nigrostriatal and mesolimbic-mesocortical DA systems. Lesion studies show that responding for the brain reward is depressed or abolished by damage to DA neurons. Pharmacological data also support the notion of DA neurons playing a critical role in brain reward. But the evidence is not free of conflict. In previous work concerned with the basis of reinforced behavior maintained by stimulation of medial frontal cortex, rats with neonatally-induced damage to the DA systems were tested for SS and found to be unaffected by the damage. These findings suggested that non-DA neurons or terminals or axons in frontal cortex were responsible for the brain reward. Since frontal cortex contains DA-terminal fields but not DA somatas, it seemed necessary to determine whether a similar lack of effects would result if the damage was inflicted in the ventral tegmental area, the region where the innervation of the frontal cortex originates and the SS behavior was maintained by stimulation in this structure. Groups of rats with neonatal damage to DA neurons were tested for SS in adulthood. No deficits were observed. Because it seemed possible that SS was supported in these rats by neurons that had not been damaged, the subjects were given injections of a DA-specific neurotoxin at the site of stimulation. Again, the animals tested showed no deficits. Yet, these same subjects given central injections of a neurotoxic substance damaging somatas at the stimulation site, but not fibers en passage, led to the suppression of responding. These effects could be due to 1) destruction of all somatas in the region, including DA and non-DA neurons; 2) induction of local epileptic activity which counteracted the rewarding effect of brain stimulation; and 3) the induction of a pathological state which made the cost of working for the brain reward too high for the behavior to be maintained. Although these data seem negative for the DA hypothesis, they require validation because of the toxic nature of the kainate which has been shown to induce a long-lasting pathological state. However, a viable alternative to the DA hypothesis of reinforcement is not available. Because of the toxicity of kainic acid, more data are needed to understand precisely the nature of the regulation of positive reinforcement by DA neurons.

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269. Activity of Reticulata Neurons during Amphetamine-induced Stereotypy

Marianne E. Olds

The substantia nigra contains the somatas of DA neurons projecting to the neostriatum, interneurons, and non-DA neurons projecting to premotor nuclei. The non-DA efferent neurons are believed to function as second-order DA output neurons. The available evidence indicates that DA and non-DA neurons in substantia nigra interact locally. The non-DA neurons are excited by drugs enhancing dopaminergic transmission and by DA iontophoresed in substantia nigra. Because these data were obtained in the immobilized animal, they fail to reveal the relationship between the excitatory neural responses and DA behaviors. The objective of these experiments was to gain insight into this relationship in order to determine the influence of DA neurons on the firing pattern of the non-DA neurons. In these experiments, unit activity in substantia nigra was recorded during episodes of hyperactivity or stereotypy induced by the indirect DA agonist, d-amphetamine, and the direct DA agonist, apomorphine. The results show that approximately 30-40% of the non-DA neurons in substantia nigra sampled show a dramatic increase in output during amphetamine and apomorphine-induced hypermotility and stereotypy.

Our next project involves the attempt to induce these DA behaviors by direct injection of amphetamine and apomorphine into neostriatum. The aim is to determine the contribution made by different populations or types of DA receptors to the excitatory responses of the non-DA
neurons in substantia nigra during DA hypermotility and stereotypy.

270. Investigation of the Relative Roles of Dopamine and Norepinephrine in Chronic Hyperactivity Induced by Neonatal Damage to Pathways Containing These Substances

Marianne E. Olds, Arthur Yuwiler

It is well established that damage to catecholamine (CA)-containing neurons in the neonatal rat injected intraventricularly with the CA-specific neurotoxin 6-hydroxydopamine (6-OHDA) leads to hyperactivity which may last throughout life. The development of experimental procedures for preferentially damaging either DA or NE neurons only, or both concurrently, has made it possible to examine the role played by either type of neuron in the hyperactivity that follows neonatal treatment with 6-OHDA. There is evidence that hyperactivity is induced when both DA and NE neurons have been damaged early in life or when only DA neurons have been damaged, but not when only NE neurons have been damaged. This has led to the view that the damage to the DA neurons constitutes the critical factor in inducing hyperactivity. But most of the evidence was obtained in developing animals. Development, however, is characterized by large changes in all transmitters so this may not be an appropriate period for a final assessment of whether NE neurons contribute to hyperactivity. Therefore, it seemed more appropriate to study the long-term effects of the neonatal damage on motor activity. Following the neonatal treatment, behavioral tests were given in adulthood and the data were correlated with levels of CA in different brain regions. The findings support the notion of noradrenergic facilitation of the hyperactivity associated with neonatal damage to the DA systems.

1 Chief, Neurobiochemistry Laboratory, Brentwood Neuropsychiatric Veterans Hospital, Los Angeles.

Meteoritic and 270. Investigation of the Relative Roles of Dopamine and Norepinephrine in Chronic Hyperactivity Induced by Neonatal Damage to Pathways Containing These Substances

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1 Chief, Neurobiochemistry Laboratory, Brentwood Neuropsychiatric Veterans Hospital, Los Angeles.

PUBLICATIONS


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Senior Research Associate: Charles R. Hamilton
Visiting Associates: Alice M. Cronin-Golomb, Polly Henninger Pechstedt, Eran Zaidel
Staff Associate: Betty A. Vermeire
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Summary: Research continues on hemispheric specialization and left-right cross integration in human subjects and in rhesus monkeys with surgical disconnection of the cerebral hemispheres. The presence of complementary hemispheric specialization in monkeys is now strongly indicated. It closely resembles hemispheric superiorities found with human subjects and, accordingly, should provide a valuable means for exploring the biological basis of lateralized abilities that previously seemed characteristic only of human beings. Other experiments with human patients and partially split-brain monkeys have begun to define specific functions in visual perception for different segments of the cerebral commissures. This approach offers the opportunity to study behaviorally the contributions to perception of different regions of visual cortex.

Related work is concerned with the theoretical implications of the split-brain findings in regard to consciousness. The emergent downward control concept of brain function that has made possible a new acceptance of consciousness as causative and functional in cognitive science is being explored as a more valid determinist paradigm for all science.
271. Stereopsis and the Cerebral Commissures

Charles R. Hamilton, Katrín M. Rodríguez, Betty A. Vermeire

The extent to which the cerebral commissures are necessary for stereopsis along the vertical meridian of the visual field has hardly been investigated even though the commissures are often presumed to be necessary. We have studied five split-brain (SB) and 20 normal (N) subjects for their ability to detect briefly-flashed dynamic random-dot stereograms at various positions in the visual field. The methods were detailed last year (Biology 1985, Abstract No. 271); the results have now been completed and conclusions may be drawn.

As a group, the SB subjects show significant ($p < 0.05$) deficits in global stereopsis within $2^\circ$ of the vertical meridian. These deficits are equally great at the point of visual fixation and at elevations of $\pm 5^\circ$, which contrasts strikingly with the excellent performance at lateral eccentricities of $\pm 5^\circ$. Because the deficits are associated with the vertical meridian, it is reasonable to attribute them to the surgical division of the cerebral commissures. The deficits were present for stimuli located in front of or behind the plane of fixation at disparities of $\pm 5, 10, 20, 40$ and $80$ seconds of arc. These disparities sample the fine and coarse range of local stereopsis. There was considerable variation between subjects in the magnitude of the deficit and in the disparities that were most affected.

The N subjects also showed some evidence of midline deficits ($p < 0.05$), although the magnitude was much less and it differed significantly from the SB deficit ($p < 0.05$). There was also considerable variability in the size of the midline deficit of the N subjects, with many subjects showing no loss and a few showing deficits similar to those of the SB subjects.

We conclude that the cerebral commissures play an important role in global stereopsis along the vertical midline. Future experiments should reveal if the commissures are equally important for local stereopsis and for depth based on different submodalities of vision. We also suggest that N subjects utilize "commissural stereopsis" to varying extents. It seems plausible to us that the variability reflects differences in early experience although genetic differences in commissural endowment are not precluded.

1 Undergraduate, California Institute of Technology.

272. Interhemispheric Transfer through Specific Commissural Segments

Charles R. Hamilton, Betty A. Vermeire

We have previously determined in macaque monkeys that a fairly precise anatomical separation of fibers from different cortical visual regions exists in the cerebral commissures. In the splenium of the corpus callosum, fibers from the V1/V2 border cross through the most posterior segment, preceded by fibers from V4, and next by fibers from MT and posterior IT. Anterior IT cortex sends its contralateral output through the anterior commissure. We have prepared four groups of partially split-brain monkeys, each completely split except for one of the four segments. They are now being tested for interhemispheric transfer through these segments on a variety of tasks including discrimination of patterns, movement, orientation, and facial features. Control groups include split-brain monkeys and partially split-brain monkeys with a split optic chiasm but with the entire splenium or the entire corpus callosum and anterior commissure preserved.

Results to date permit the following conclusions, which must remain tentative until more monkeys are tested and histological confirmation of the cortical areas left interconnected is obtained. First, all four commissural segments allow significant and approximately equal transfer of most discriminations. This in itself is surprising as several researchers have argued against transfer occurring from areas early in the hierarchy such as V1/V2 because of the restricted extent of intercortical connections or because of behavioral results with cortical lesions that show a lack of transfer if only V1/V2 cortex is left intact. Second, discriminations based on orientation result in reduced transfer through the anterior commissure, in keeping with the frequently found loss of interest of IT cells in orientation. Other suggestions of differential transfer that we have found require more data before they can be interpreted meaningfully. Third, a quantitative loss in transfer through the four segments is usually evident when compared to transfer through the entire splenium or through all the cerebral commissures, although the transfer is much greater than in the completely split monkeys. Overall, this approach to examining behaviorally the functions of different visual areas remains promising.
Complementary Hemispheric Differences in Monkeys

Charles R. Hamilton, Betty A. Vermeire

We continue to confirm last year's report (Biology 1985, Abstract No. 270) of complementary superiorities of the two halves of the brains of rhesus monkeys. The average left hemisphere of 26 monkeys was significantly better ($p < 0.001$) than the right at discriminating the orientation of tilted lines. We believe this represents lateralization of spatial ability because the left hemisphere excels in several other discriminations as well, all of which involve spatial relationships. By contrast, the average right hemisphere was dominant in learning, remembering, and generalizing the discrimination of facial features and expressions as described in the following abstract.

Twenty-four of these monkeys have learned both the spatial and facial discriminations. The difference in dominance for the two types of problems is highly significant ($N = 25, p < 0.05$), with spatial being relatively more left hemispheric than facial. Furthermore, the proportion of animals performing better with the left side on spatial and the right on facial ($16/24$) is significantly ($p < 0.005$) above the distribution expected by chance ($6/24$).

We are presently determining how similar these abilities are to those lateralized in human subjects. We manipulate variables, such as task difficulty, orientation of faces, spatial frequency of gratings, and extent of stimulus categorization, that are believed to affect the expression of hemispheric specialization in human beings. We expect that the monkeys will be similarly affected which will strengthen the claim that simian and human lateralizations are homologous. These findings indicate that hemispheric specialization evolved before the development of uniquely human cognitive abilities such as language and propositional thought.

Hemispheric Specialization for Discriminating Facial Stimuli in Split-brain Monkeys

Betty A. Vermeire, Charles R. Hamilton

For several years we have been studying differences in the abilities of the right and left hemispheres of split-brain rhesus monkeys to learn, remember, and generalize to new exemplars, photographs of monkeys’ faces. The monkeys were tested on eight go/no-go discriminations for food reward. Four problems involved discriminating one monkey from another with facial expression held constant and four additional problems required discriminating one facial expression from another, both performed by the same monkey. The experimental design was balanced for sex of the subjects, handedness, hemisphere of surgical approach, and problem order. Overall, the monkeys show a significant ($N = 25, p < 0.05$) right hemisphere advantage in learning the eight discriminations, they remember the problems significantly better with their right hemispheres ($N = 24, p < 0.05$), and they show better generalization of the learned discriminations to new exemplars with their right hemispheres ($N = 20, p < 0.01$). The magnitude of the laterality demonstrated was similar for discriminations of individual identity and of facial expression. Interestingly, females showed greater laterality on all three parts of the tests ($p < 0.02$ or less) than males.

These results confirm and extend results we reported last year (Biology 1985, Abstract No. 269). In addition, they suggest that the right hemisphere of monkeys may be homologous to the right hemisphere of human subjects in its superior ability to process facial and emotional information. We are undertaking two further investigations to see how close the homology is.

First, a special aspect of human laterality is being tested. Inversion of faces is especially disruptive to recognition, an effect that is much more pronounced with faces than with other stimuli that are usually seen upright, such as houses or cars. The monkeys are being tested for their ability to discriminate inverted monkey faces. If monkeys are like humans, the right hemisphere should be more disrupted than the left.

Second, eight additional discriminations have been selected to expand the range of difficulty. These are being learned by the monkeys to determine how increases in problem difficulty affect the expression of laterality. In human subjects, hemispheric differences may be more readily observed when harder tasks are used.

Differential Learning Rates for Facial Stimuli Involving Individual Identity or Facial Expression

Betty A. Vermeire

In the course of running the experiment on hemispheric differences in recognizing facial cues
described in the previous abstract, an interesting and unexpected result emerged. When the number of errors taken by the monkeys to reach a criterion of 90% correct performance was compared for the two types of problems, it was found that the monkeys learned discriminations of individual identity significantly faster \((p < 0.001)\) and remembered them better \((p < 0.01)\) than discriminations of facial expression. This was true for both the left and right hemispheres, even though the left hemisphere was less adept at learning and remembering the discriminations, as discussed in the previous abstract.

In contrast, 16 human subjects who were asked to sort the sets of photographs used in each discrimination into categories based on the appropriate relevant cue of identity or expression sorted faster \((p < 0.01)\) and made fewer errors \((p < 0.001)\) in sorting facial expressions than in sorting individuals. When quizzed on their strategies for forming the categories, human subjects reported using the rather obvious mouth position (open, closed, puckered lips, all teeth exposed, etc.) for discriminating expression, while resorting to rather subtle details of the face (hairline, coloration, nose, hair whorls, ears) in attempting to differentiate individuals.

Taken together, these results suggest that monkeys and human subjects evaluate information about monkeys' faces somewhat differently, although familiarity with the subject matter might be a contributing factor. In summary, it appears that for monkeys, it is more important to know who the other fellow is than to know what he is expressing.

276. Comprehension of Language by the Right Hemisphere

Charles R. Hamilton, Marie-Christine Nargeot
Betty A. Vermeire, Joseph E. Bogen

Studies with split-brain patients suggest greater participation of the right hemisphere in linguistic comprehension than traditionally has been believed. This conclusion has been challenged recently because several counter examples have been reported and, accordingly, claims made that right hemispheric linguistic comprehension reflects the abilities of only a few, select split-brain patients. Therefore, we tested and compared four split-brain patients: RY and AA, who have not been extensively tested for this ability, and NG and LB, who are known to possess right hemispheric comprehension.

The ability of the left hand to retrieve objects first felt by the right hand was determined for two conditions. In one, the subject named the object while feeling it with the right hand and, in the other, the subject did not name the object aloud. Retrieval by the left hand was significantly better when naming occurred, which indicates that the right hemisphere heard and understood the name and thereby correctly retrieved the object with the left hand. Alternative explanations based on ipsilateral sensory and motor connections, subcortical communication, or cross-cueing to the left hemisphere are implausible because these mechanisms would be equally available whether or not overt naming occurred.

The ability of the right hemispheres of the same patients to correctly match a word and picture was compared to the ability to name the same words and pictures. We found good performance on matching and poor performance on naming. Successful matching indicates that the right hemispheres could recognize the words and associate them with the pictures; inability to name the stimuli shows that the left hemispheres were not responsible for the matching.

In summary, both RY and AA, as well as NG and LB, show significant comprehension by their right hemispheres of simple spoken or written words. The results for RY are particularly interesting because his brain injury occurred after the period usually considered critical for the development of right hemispheric language. These results support the conclusion that the right hemispheres of split-brain patients usually possess some linguistic abilities. Patients who do not show these abilities may have sufficient damage to their right hemispheres to prevent comprehension. Although the upper limits of linguistic competence were not probed by these tests, it is clear that the right hemispheres were not as linguistically competent as the lefts, nor were the right hemispheres of RY and AA as linguistically proficient as those of NG and LB.

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2 Department of Psychology, University of California, Los Angeles.
277. Dissociation of Abstract Words and Concepts in the Right Hemisphere

Alice M. Cronin-Golomb

In a picture-matching task (Cronin-Golomb, 1986), the right hemisphere (RH) of complete commissurotomy subjects was found to be as proficient as the left hemisphere (LH) at understanding abstract concepts. In light of reports that the RH is limited in its comprehension of abstract words relative to concrete ones, it was inferred that abstract concepts and words may dissociate, with the latter being processed by the LH only. In order to assess the validity of this conclusion, the present experiment evaluates abstract word use by the separated hemispheres in a way that permits results to be compared more directly with the results from the picture-matching experiment.

Three complete commissurotomy subjects (LB, NG and AA) were tested. For each trial, two pictures were presented side by side. The subjects were known to be proficient at matching these same picture sets on the basis of a specific abstract concept (the picture-matching experiment). Directly beneath the pictures appeared a vertically-aligned array of six abstract words, one of which corresponded to the concept relating the pictures. All stimuli were presented simultaneously and within a single visual hemi-field (VF) using the lateral limits technique (Myers and Sperry, 1982). Subjects viewed the material for as long as desired and then pointed to the chosen word of the answer array. Twenty-four trials were presented first to the left VF (RH: left hand responding), then the right VF (LH: right hand responding) and finally in free vision. Five trials for which AA made incorrect matches under all conditions were eliminated from the analysis of his results.

The results for all subjects showed clearly that the RH, which had been successful previously at matching the stimulus pictures (related only through a particular abstract concept), was unable to match the pictures to an appropriate abstract word. The correct word was chosen by the RH in 5 of 24 (LB), 9 of 24 (NG), and 3 of 19 trials (AA) (chance = 4 of 24). By contrast, the LH was correct in 23 of 24 (LB), 19 of 24 (NG), and 15 of 19 trials (AA). In free vision, the abstract word was again chosen correctly for most trials: 24 of 24 (LB and NG) and 18 of 19 (AA).

The results support the conclusion drawn from the picture-matching experiment. Abstract words and concepts are dissociable: the RH of commissurotomy subjects is proficient at understanding abstract concepts that are associated with words it is not able to use.

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278. Split-brain Subjects Show Right Ear Advantage for Identifying Melodies

Polly Henninger Pechstedt

To compare right and left hemisphere processing of musical stimuli, three complete commissurotomy subjects (AA, LB, NG) were presented pairs of hummed, familiar nursery tunes (Three Blind Mice, etc.) dichotically and required to respond by pointing with the left hand to a picture representing the tune from an array presented in the left half visual field with the lateral limits technique (right hemisphere response) and by orally reporting the title (left hemisphere response). The test, designed by E. Peck, consisted of five binaural practice trials and 22 dichotic trials.

Subjects showed a right ear advantage for both responses, an unexpected finding when the response was restricted to the right hemisphere. It suggested that the melodies were being processed by the left hemisphere and information transferred to the right extra-callosally. Retesting with arrays in which pictures representing the right ear tunes were absent did not substantially increase left ear performance, suggesting that the left hemisphere controlled processing.

To test whether the binaural practice trials were informing the left hemisphere and enabling it to take control, a fourth subject (RY) was tested without these trials. Pointing in the left visual field, he performed less adequately and showed a left ear advantage. Apparently, the right hemisphere could do the task but less adeptly than the left. These findings suggest that if nonverbal stimuli evoke a mental representation of a word, the left hemisphere is likely to take control of processing. These results suggest that interhemispheric transfer is stimulated by left hemisphere awareness of
the stimuli and enables the left hemisphere to control processing. They reaffirm the early view of cerebral dominance that the left hemisphere is dominant and suggest caution in assuming that stimuli better processed by the right hemisphere are processed by that hemisphere. These stimuli may, however, result in increased interhemispheric collaboration.

These data suggest two independent laterized systems, a processing system and a response system, which function separately and are integrated within the responding hemisphere. Caution is suggested in interpreting results of experiments in which task and response demands are not isolated. The lateralized effects of cognitive activity may be obscured by those of response demands.

279. Split-brain Subjects Show that the Left Hemisphere Can Sing

Polly Henninger Pechstedt

Data from asphasic and left hemispherectomy patients who can sing suggest that the right hemisphere is responsible for the production of song. Although supported by evidence of a left ear advantage in children for identifying nursery tunes (Peck, 1976), this hypothesis has not been tested. Four complete commissurotomy subjects (AA, LB, NG, RY) were presented three or more pictures representing nursery tunes in the left, then the right, visual half fields with the lateral limits technique and asked to sing or hum the song that came to mind.

When the pictures were presented in the left visual field, the subjects did not sing the songs. Two attempted to do so but emitted either no sound or only a single note. Two said they were unable to identify the songs. When the pictures were presented in the right visual field, all subjects sang the songs correctly. Two subjects were subsequently retested in the left visual field. They sang two-thirds of the songs, but usually after a delay, suggesting interhemispheric transfer.

These results indicate that the left hemisphere can sing and suggest that under these conditions the disconnected right hemisphere cannot. It appears that the left hemisphere took control of processing and prevented right hemisphere expression. Perhaps in singing, the verbal requirements supercede the musical and the left hemisphere controls song production. Given the left ear advantage in children observed by Peck, it is possible that left hemispheric control of processing develops with increasing use of verbal mediation.

The results from retesting support the view that interhemispheric transfer can be stimulated by left hemisphere knowledge of the stimuli (see Abstract No. 278) and suggest that one of the functions of such transfer is to transfer control of processing. They suggest that the unity of purpose and behavior maintained by interhemispheric communication, via the corpus callosum in the normal brain and extra-callosal transfer in the bisected brain, may be done primarily through left hemispheric control.

Reference:
When the volume was equal, the dyslexics showed no ear advantage. When it was biased to the right, they showed a right ear advantage and when it was biased to the left, they showed a left ear advantage shifting to the right as the task load increased, as the clinical patients had. The normals showed the typical right ear advantage when the volume was equal or biased to the right and no ear advantage when it was biased to the left.

The dyslexics' absence of ear advantage in the volume-equal condition and ear advantages consistent with volume manipulations suggest that they are more susceptible to attentional factors than matched normals. Their shift in ear advantage with increasing task load suggests that 1) they do not use the right hemisphere preferentially for verbal processing, and 2) their performance is not totally under the control of attentional factors. With a sufficiently complex task, they overcome these factors and engage the appropriate hemisphere. These findings suggest that dysphonic dyslexia involves a deficit in the allocation and transfer of attentional resources and may result from a callosal dysfunction.

Reference:

1Doctoral Student at the University of Southern California.

PUBLICATIONS


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Summary: Our group is interested in how information is processed in the mammalian visual system. Our primary focus has been the study of extrastriate visual cortex in the macaque monkey using electrophysiological and neuroanatomical techniques. Recently, though, our efforts have diversified along several fronts: we have incorporated other approaches, from monoclonal antibodies to positron emission tomography, and we have begun to study other species, including humans, as well as other parts of the visual system, including the retina.

One area of progress has been in charting the location and extent of different visual cortical areas. In the macaque monkey, more than a dozen visual areas have been identified with a high degree of con-
idence, and there are many candidates for additional areas in regions that have yet to be fully explored. One of these regions is in the temporal lobe, where we have recently found an unexpectedly complex pattern of organization. Our evidence suggests that there may be six separate visual areas in the temporal lobe, rather than the three subdivisions previously suspected.

Another project within this general theme is an effort to chart the human visual cortex using positron emission tomography (PET). This non-invasive approach, which can be used to map visually evoked responses in normal human volunteers, holds great promise for providing a link between the wealth of information on non-human primates and our special interest in the organization of the human visual cortex. Our efforts in this collaborative venture are described in Abstract No. 252.

A second theme of our research has concerned the evidence for compartmental organization within individual visual areas. We have focused mainly on a tripartite pattern of stripe-like compartments within visual area V2. This year, in addition to continuing the combined physiological and anatomical approach reported last year, we have incorporated an immunohistochemical approach as well. We have used a monoclonal antibody, CAT-301, that selectively labels one of the compartments within V2, thereby providing an independent basis for its identification and characterization. We are also optimistic that another recently developed approach, in which voltage-sensitive dyes are used to monitor activity patterns across the cortical surface, will prove to be a valuable tool for analyzing functional aspects of compartmental organization.

The third theme of our visual studies concerns the way in which visual cortex processes information about complex visual patterns. Most neurons in nearly all visual areas respond to simple stimuli (spots, bars, and edges of light) and they are selective for very basic stimulus parameters (such as orientation, wavelength, and direction of motion). However, these properties generally reflect processing that takes place in primary visual cortex (area V1). In order to ascertain what types of analyses take place within the many different higher (extrastriate) visual areas, we use psychophysics as a guide, both for choosing the types of visual stimuli that are most appropriate to use and for interpreting results in a framework that can be linked to visual perception. Using texture patterns derived from psychophysical studies by Prof. Bela Julesz, we have found that many neurons in visual areas V1 and V2 respond in ways that may be useful for texture discrimination.

An independent facet of our research effort concerns the phenomenon of synapse elimination at the neuromuscular junction of neonatal mammals. Our broad goals are twofold: 1) to understand the cellular and molecular basis of the interactions that lead to the orderly elimination of all but one synapse upon each muscle fiber; and 2) to understand the rules that determine which synapses are to survive and which are to be removed. One intriguing issue has been the role of neural activity in determining the fate of synapses. It has been suggested that the developing neuromuscular junction acts as a classical "Hebbian" synapse, in which more active terminals are more likely to survive. We have made a direct test of this hypothesis by creating a situation in which there is differential activity among the synapses competing for survival at individual end plates. Our results clearly indicate that the more active synapses are not at an advantage and thus, contrary to the initial hypothesis, do not behave as Hebbian synapses.

We have also initiated a long-term project to generate a computer model of the processes associated with synapse elimination. Our rationale is based on the assessment that synapse elimination is likely to be controlled and modulated by many different cellular and molecular interactions, not by a single growth or survival factor. We are developing a model that will have the flexibility to incorporate all of these putative interactions and factors. By evaluating the performance of different versions of the model as it simulates the dynamics of a variety of developmental processes, we hope to understand the interplay among different hypothesized interactions in a way that cannot be achieved by intuition or introspection alone.

281. Multiple Topographic and Non-topographic Visual Subdivisions of the Temporal Lobe in Macaque Monkeys

Daniel J. Felleman, James J. Knierim, David C. Van Essen

Inferotemporal cortex (IT) of macaque monkeys is a large expanse of visual cortex situated between area
V4 posteriorly and the temporal pole anteriorly. IT has been implicated in various aspects of higher visual function ranging from complex form analysis to visual memory. On the basis of behavioral, anatomical and physiological data, IT has been previously partitioned into posterior (PIT) and anterior (AIT) subdivisions, neither of which was reported to have a topographically organized representation of the visual field. In a re-examination of these issues using various combinations of retrograde and anterograde tracers, we have obtained a substantially revised assessment of the organization of IT.

Our general strategy was to make tracer injections into V4, which provides the major ascending input to IT. Tracer injections were made into dorsal V4, representing lower fields ( \( n = 10 \) ), or ventral V4, representing upper fields ( \( n = 4 \) ), either alone or in various combinations, in nine hemispheres. In many hemispheres, the locations of labeled cell bodies in individual brain sections were digitized using a computer interfaced microscope, and the distribution of label was analyzed using two-dimensional, unfolded maps of extrastriate cortex and three-dimensional computer graphic reconstructions.

On the basis of the laminar pattern of labeling and the segregation or convergence of inputs from dorsal and ventral V4, we were able to distinguish three pairs of subdivisions located, respectively, in posterior, anterior, and medial portions of the temporal lobe. Topographically organized inputs, characterized by segregated regions of labeling, were observed in two separate regions within posterior IT: one within the superior temporal sulcus and the other on the pre-occipital gyrus. These zones were characterized by strong retrograde labeling in supra- and infragranular layers following fluorescent dye injections into V4. A second pair of subdivisions was located in anterior IT; one was in the superior temporal sulcus and the other on the inferotemporal gyrus. The third pair was located more medially, one in the occipitotemporal sulcus and the other near the medial edge of neo-cortex. Both these anterior and medial pairs of subdivisions were characterized by non-topographic inputs from V4, indicated by convergent, overlapping labeling from the upper and lower visual field and by a restriction of retrogradely labeled cells to the infragranular layers of cortex.

These data demonstrate several heretofore unrecognized subdivisions of the temporal lobe in macaque monkeys. In future studies it will be of interest to look for physiological correlates of these anatomically delineated subdivisions.

282. An Antibody, CAT-301, Identifies Distinct Pathways in Visual Cortex of the Monkey

Edgar A. DeYoe, Hideki Garren, Susan Hockfield, David C. Van Essen

An important strategy in trying to analyze the functional organization of the brain is to find ways of anatomically or chemically distinguishing between connectional pathways that are similar in some respects but, in fact, subserve different functions. We are using immunohistochemical approaches to try to identify pathways that contain distinctive chemical constituents that may reflect a commonality of function or development. Once identified in this way, the physiological properties of a pathway can be examined in hopes of gaining clues as to its unique functional contribution.

As reported last year (Biology 1985, Abstract No. 280), we have concentrated our efforts on a monoclonal antibody, CAT-301, that was originally targeted against cat spinal cord (Hockfield and MacKay, 1983). CAT-301 immunoreactivity reveals a remarkably interesting distribution in the visual system of macaque monkeys, including strong labeling of cells in the magnocellular division of the lateral geniculate nucleus (LGN) and layers 4B and 6 of striate cortex, which have a strong association with the magnocellular LGN. We have used CAT-301 to examine regions of cortex that, in turn, are the targets of projections from striate cortex. We found that within the second visual area, V2, CAT-301 reacted most strongly and most often with cells belonging to a particular compartment known as the "cytochrome-oxidase thick stripes." As the name implies, these stripes are recognizable by their high cytochrome oxidase activity and also by distinctive projections they receive from layer 4B in striate cortex. In turn, the "thick stripes" connect to two higher visual areas in the superior temporal sulcus, MT and MST, which are themselves strongly labeled by CAT-301. In contrast, at all stages along this chain from LGN to MST, there are additional, parallel pathways which are only weakly
labeled by the antibody. Interestingly, these pathways also differ in certain physiological properties such as contrast sensitivity, and spatial and temporal frequency preferences. The currently available data suggest that a common function of components in the CAT-301 labeled pathway may be the processing of visual motion. Early stages in the retina and LGN may be optimized to enhance the signal-to-noise ratio of rapidly changing light patterns whereas at higher stages, the velocity of small patches of light and eventually of whole objects and textured surfaces may be worked out. If CAT-301 could be conjugated to a useful neurotoxin, it might be possible to test these conjectures by selectively lesioning with immuno-magic-bullets.

Reference:

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283. Neurophysiological Correlates of the Perception of Texture Differences
Edgar A. DeYoe, James J. Knierim, David C. Van Essen, Bela Julesz

In our everyday observation of the world, most of us readily appreciate our visual ability to quickly locate differences in brightness, color or distance. Less obvious, though equally remarkable, is our ability to located differences in the texture of objects and surfaces. In fact, this ability is so common in nature that, to avoid detection, many prey animals have evolved complex skin or fur markings so as to closely match the texture of their natural habitat. However, if the animal moves, the camouflage is broken, such as when a brook trout suddenly darts across the stream bed.

To unravel the story of how the visual system processes texture patterns, we have used an animal model, the macaque monkey, whose visual abilities match our own in many ways. We have recorded the responses of individual neurons in the first and second visual areas, V1 and V2, while the animal viewed precisely controlled texture patterns presented on a video screen. Our first questions concerned whether neurons in V1 or V2 would actually respond selectively to texture differences. We used textures consisting of a field of small, elongated bars, taking advantage of the fact that cells in both V1 and V2 are known to respond selectively to the orientation and/or direction of motion of single elongated bars presented on a blank screen. Our first test was to present one such bar (target) within a field (surround) of bars having either the same or a different orientation, i.e., either matching or contrasting with the target. We found a variety of cells whose responses to the target bar were modified by the surround. For some of these, a good response was obtained only when the target and surround orientations differed, that is, only when a texture contrast was present. This suggests that these cells carry the same kind of texture information that, in a similar situation, allows a human observer to immediately locate the contrasting target. So far we have not found any striking differences in the occurrence of such cells in V1 and V2. A second test, however, has turned up a difference.

For the second test, we created a texture bar by embedding two rows of identically oriented line segments within a larger field of contrastingly oriented line segments. We could then make this "texture bar" move relative to the background texture and study how cells responded to the motion. In V1 few cells responded, but in V2 many not only responded but were selective for the direction of movement of the texture bar. Such cells carry information that could be used to break texture camouflage or even to specify the threedimensional shape of a textured object. If these differences between V1 and V2 are confirmed for a larger sample of cells, it would suggest that V2 plays a special role in the trout fisherman's ability to see where to cast his flies!

284. Computational Techniques for the Analysis of Anatomical and Physiological Organization of the Cerebral Cortex
George J. Carman, David C. Van Essen

We routinely perform anatomical and physiological experiments in order to study structure and function within and across cortical areas. In order to analyze and interpret the results, we must reconstruct data contained in many histological sections to reveal overall patterns of anatomical connections and physiological responses in relation to the two-dimensional cortical surface. Such reconstructions are made difficult by the fact that the cortex of higher
mammals is folded into a complex series of gyri and sulci. We circumvent these difficulties by reconstructing regions of interest as two-dimensional maps of the cortex, which provide an unfolded representation of anatomical and physiological features independent of the original plane of histological section. In order to overcome the limitations of the existing manual techniques (Van Essen and Maunsell, 1980), we have developed a set of computational algorithms that use the optimization technique of simulated annealing (Kirkpatrick et al., 1983) to produce such maps by computer (Carman and Van Essen, 1985).

Our algorithms have successfully generated maps of mathematical surfaces, demonstrating that they can maintain topological order while generating an unfolded, two-dimensional map of the surface of three-dimensional objects with arbitrarily small distortion. We have also used these procedures to generate an unfolded map of layer 4 of the entire striate cortex of Macaca fascicularis at a resolution of 0.5 mm. This map compared favorably with one generated using manual techniques from histological material from the same animal, with most differences between the two maps being attributable to (a) the limited accuracy of the manual technique, and (b) the limited sampling from intrinsically curved regions of the cortex, which tends to result in an underestimation of the area of these regions in the computer maps. Our computational algorithms yielded maps having a root mean squared error of less than 15 microns, suggesting that further improvements will be found in increased resolution of the data being mapped, rather than in increased precision of the mapping procedure.

The ability to produce such two-dimensional, unfolded maps of cortical surfaces contained within the three-dimensional volumes of the brain provides not only for the qualitative assessment of anatomical and physiological organization of the cortex, but also for subsequent quantitative analyses. The mapping of such data onto the plane is a prerequisite for the comparison of organization of a given cortical area across individuals, for the combination of data from several animals through normalization to an average map, and for many statistical procedures useful for comparisons both within and across cortical areas (Diggle, 1983). Such analyses provide important tools for the study of the anatomical and physiological organizations that underlie the cortical processes and representations responsible for sensory perception.

References:

285. Optical Studies of Electrical Activity in Visual Cortex

Harry S. Orbach, David C. Van Essen

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 photodetectors and measuring the photodetector outputs.

We are interested in applying this technique to mammalian sensory cortex with the aim of monitoring the activity of many sites simultaneously, measuring changes in electrical activity with submillisecond time resolution, having good spatial resolution, and having the ability to repeat the experiment on the same preparation with a variety of stimuli. In a sense, this would be the equivalent of real-time 2-deoxyglucose measurements.

We have set up such an optical system and have tested it, measuring signals from salamander olfactory cortex and rat visual cortex. We are currently examining the response of rat visual cortex to electrical stimulation with the aim of examining cortical connectivity. We have seen signals in response to focal electrical stimulation that spread over substantial regions of visual cortex (>1 mm). For some stimulus paradigms there is heterogeneity of the optical signal in the details of the spatial and temporal pattern.

We expect soon to turn our attention to the visual cortex of the squirrel monkey. We will then proceed to address questions of functional organization at a scale of about 100 µm. This is the scale at which a variety of functional parameters (e.g., orientation,
direction, and color) are mapped systematically across
the surface of the cortex. The optical technique is
uniquely qualified to examine the detailed arrange­
ment of such local mappings by presenting the animal
with various stimuli and seeing the resulting patterns
of cortical activity (analogous to determining the
functional properties of single cells by monitoring
spike activity in response to stimuli). We hope to use
the technique in topographic mapping of cortical
areas, in tracing connections within and between
areas, and in measuring dynamic features of cortical
activity.

286. A Computer Data Base for Visual Cortical
Connections in Macaque Monkeys

Terese N. Olllick, Daniel J. Felleman,
David C. Van Essen

Systematic studies of visual cortical connections in
macaque monkeys, carried out mainly over the past
decade, have revealed an extraordinary richness in the
number and diversity of distinct pathways. To date,
more than 50 major cortico-cortical visual pathways
have been firmly identified, and the list will ultimately
be several times larger. These data are drawn from
many laboratories often using different techniques and
varying criteria to identify individual brain areas.
Obviously, an organized data base system is needed to
keep track of the plethora of connectivity information.
Using commercially available software (DBASE III), we
have generated a computerized data base to facilitate
inquiry into existing cortical connections and allow for
easy extension of the data base as more experiments
are performed in this and other laboratories.

Using a recently devised format (Van Essen, 1985),
our data base stores and displays many different types
of information concerning each identified or suspected
cortico-cortical visual pathway in the macaque.
Among the major categories of information are the
origin and termination of the pathway, its certainty
and consistency of occurrence, and its density,
direction, and laminar organization. Additionally,
literature references are available for each pathway.
A search/sorting program has been developed which is
able to locate a pathway or group of pathways satisfi­
ing any combination of conditions specified by the
user. For example, the data base search/sorting
program can be instructed to indicate all connections
of area V2, or only those pathways that originate from
upper cortical layers, etc. The result of the search is
a listing of pathways meeting the specified conditions
and a listing of all literature references that support
the result of the search.

Future plans include provisions to continually
update the data base so as to include new cortical
areas and connections as they become identified.
Additional enhancements may include finer laminar
distinctions, analysis of the two-dimensional size of
efferent patches of projection cells, or perhaps some
morphological considerations of efferent cell types. In
addition, this data base will be extended to include
individual experiments from each laboratory, thereby
facilitating data analysis prior to and in preparation
for research publication.

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287. Sampling Strategies within the Primate Retina

David C. Van Essen, Charles Anderson

Visual information originating in the $10^8$ photore­
ceptors within the retina of the macaque monkey is
transmitted to higher centers by way of only $10^6$
retinal ganglion cells. This overall 100:1 convergence
is handled in highly non-uniform fashion, with a vastly
higher density of ganglion cells near the center of gaze
than in the periphery. Knowledge of the precise
density of ganglion cells subserving each point on the
retina is valuable because it imposes major constraints
on acuity and many other aspects of visual perception.
Determination of this relationship has been notoriously
difficult in the foveal region, however, owing to the
large displacement within the foveal pit between
ganglion cells and the cones they subserve.

We have addressed this problem by using published
values for the distribution of cones as well as ganglion
cells, and by making the plausible assumption that the
ratio of cones to their target ganglion cells changes
smoothly and monotonically with eccentricity in the
visual field. By this approach we corrected for the
physical offset in the fovea and estimated the
complete distribution of "shifted" ganglion cells. We
then separated the overall ganglion cell population into
its two major classes, P ganglion cells and M ganglion cells, which project, respectively, to the parvocellular and magnocellular layers of the LGN. Published values for dendritic field size as a function of eccentricity suggest that each cell type forms an orderly sampling network in which the spacing between sampling nodes increases linearly with eccentricity. By calculating the ratio of shifted ganglion cell density to the density of sampling nodes, we determined the "coverage factor" for each cell type. Interestingly, the coverage factor is not the same for P cells and M cells, and it varies with eccentricity for each cell type.

This analysis has provided the best available quantitative description of how the visual field representation is transformed at each stage of the retinogeniculate pathway. Moreover, it suggests a number of interesting implications and constraints with regard to psychophysical aspects of visual function that originate at the retinal ganglion cell layer.

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288. An Effect of Decreased Muscle Activity on the Rate of Neuromuscular Synapse Elimination
Edward M. Callaway, David C. Van Essen

Synapse elimination is a widespread phenomenon during normal development of the nervous system. In the neonatal rabbit soleus muscle, about three to five motor neurons innervate each muscle fiber at birth, and all but one of the synapses is eliminated during the first two weeks after birth. We are interested in the possible roles of activity in sculpting the adult state from the neonate. In the present study, the importance of muscle activity, as distinct from neuronal activity, has been investigated.

A number of previous studies have examined the role of activity in neuromuscular synapse elimination. These experiments have demonstrated that changes in overall activity have major effects on the rate of synapse elimination. In general, decreased activity levels result in decreased rates of elimination, while increased levels have the opposite effect. However, because such experiments generally alter activity levels of both axons and muscle fibers, the issue of whether activity effects are due to altered neuronal or muscle activity was not addressed.

We have shown that a decrease in muscle activity alone, induced by perfusion of the snake toxin alpha-bungarotoxin over the muscle, can dramatically slow the rate of synapse elimination while neuronal activity presumably persists at normal levels. Osmotic minipumps were implanted into 6-day-old rabbits to perfuse alpha-bungarotoxin over the soleus muscle at about two-thirds of the lethal dosage for a duration of two to five days. Such sublethal doses of bungarotoxin do not block muscle activity completely. About 80% of the muscle fibers in normal 6-day-old rabbit solei are polynervously innervated; this value drops precipitously to about 10% at day 11. Muscles treated with alpha-bungarotoxin from day 6 to days 8, 9, 10, and 11 averaged 69%, 65%, 54%, and 51% polynervous innervation, respectively. Thus the alpha-bungarotoxin-induced decrease in muscle activity dramatically reduced the rate at which synapses were lost. Because muscle activity was only partially blocked in these experiments, yet the effect of the block was highly significant, the results suggest that differences in muscle activity may have a graded effect on the rate of synapse elimination at each fiber. We plan to pursue this possibility further by assaying the fraction of receptors blocked in alpha-bungarotoxin-treated preparations and determining whether there is a direct relationship between this fraction and the rate of synapse elimination.

289. Activity and Spinal Position in the Regulation of Neuromuscular Synapse Elimination
Edward M. Callaway, James M. Soha, David C. Van Essen

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 (see Abstract No. 288). 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.

We have tested the possibility that more active neurons might be at an advantage during competition for retention of synapses. We rendered inactive a
small fraction of the motor neurons that innervate the rabbit soleus muscle by implantation of a small tetrodotoxin-laden plug into one of the spinal nerves. The nerve block was maintained for 4 days during the period of maximal neuromuscular synapse elimination. The number of muscle fibers innervated by active and inactive neurons was subsequently determined by measurement of twitch tensions resulting from stimulation of individual motor neurons. Inactive motor neurons were found to innervate an average of 19% more muscle fibers than the active neurons they competed against. Thus, when inactive neurons compete directly with active neurons, they are at no disadvantage. In fact, the results strongly suggest that the inactive neurons are at a slight advantage. We speculate that such an advantage could play an important role in sculpting a well known property of the adult neuromuscular system, namely, the correlation of motor neuron firing threshold with the number of muscle fibers innervated. We plan to test this hypothesis by comparing the degree of correlation of threshold and motor unit size at birth with the degree of correlation immediately following synapse elimination.

Most studies comparing the extent of synapse elimination by motor neurons from varying spinal cord positions have shown that cord position is not a major factor. Because we implanted tetrodotoxin-laden plugs into motor neurons from extreme spinal positions in the experiments described above, we also compared sizes of motor units from varying spinal positions in normal animals. To our surprise, we found that in 8- and 9-day-old rabbits, fast twitch motor units from extreme spinal positions (L7 and S2) were only 84% of the size of those from the main root, S1. There was no difference in size of slow twitch motor units from different spinal roots. We are currently studying both younger and older animals to test whether these highly significant differences in motor unit size at days 8 and 9 reflect a differential rate of synapse elimination according to spinal position or, alternatively, a smaller size of extreme-root fast motor units at birth.

290. Computer Modeling of Neuromuscular Synapse Elimination

James M. Soha, Edward M. Callaway, David C. Van Essen

The phenomenology of neonatal synapse elimination at the neuromuscular junction has been well studied over the past decade. Many physiological and anatomical characteristics of innervation patterns have been determined for normal development and following a variety of experimental perturbations. A number of different cellular and molecular interactions have been suggested to account for certain of these developmental events. Synapse elimination is likely to be a richly complex process that involves many such interactions, not just a single factor. In order to understand the dynamics of these interactions, we believe that it is important to generate models that can simulate different aspects of synapse elimination.

We have begun this effort by formulating a computer model of synapse elimination that emphasizes the nerve terminal interactions at individual endplates. Initialization consists of a random wiring of a specified pool of motor neurons onto a much larger pool of muscle fibers to achieve a specified level of polyinnervation. The current version of the model is driven by interactions associated with a hypothesized extracellular "scaffolding" molecule, which acts to stabilize a presynaptic nerve terminal by reducing the probability of terminal retraction and increasing the probability of terminal growth. In addition, presynaptic terminals induce a local accumulation of scaffolding that is synthesized by muscle fibers. These assumptions have proven adequate to simulate the time course of normal synapse elimination and the attainment of a uniform state of single innervation.

Many refinements and extensions of this basic model are planned. Included among these are the incorporation of activity-dependent effects, diffusible sprouting factors, and trophic factors that act globally by virtue of effects on the motor neuron cell body. We are hopeful that in addition to hypothesis testing, work with the model will suggest additional experiments that will help to further clarify the functions and mechanisms of synapse elimination.
PUBLICATIONS


NEUROGENETICS

Seymour Benzer
Summary: There is much to explore in the molecular landscape of the nervous system to provide the necessary groundwork for tracing its development from the genome. For a simple neural system that is amenable to analysis, we have chosen 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 send axons to the first optic ganglion (the lamina), whereas the axons of R7 and 8 pass through the lamina to synapse in the second optic ganglion (the medulla). This is, in effect, a very simple 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 clusters of five (R2, 3, 4, 5 and 8). R1, 6 and 7 are added shortly afterward (Ready et al., 1976).

Our group is applying genetic, antibody, and recombinant DNA methodology to identify the molecular specificities of the cell types, to trace from cell-specific antigens to their corresponding genes, and to analyze the molecular events in eye development (Zipursky et al., 1984).

A large fraction of the monoclonal antibodies (MAbs) made in this laboratory against the Drosophila nervous system cross react with various components of the human nervous system (Miller and Benzer, 1983). The availability of a large panel of cell-specific markers adds a new dimension to brain anatomy. Much of the mapping with antibodies in the past has been directed to known peptides or neural transmitters. These MAbs extend the range to unknown molecules, providing new markers for anatomical structures, cell types, and antigen molecules of the human brain. These experiments are being done in collaboration with Drs. Carol Miller and David Hinton who are Visiting Associates at Caltech. As Chief of Neuropathology at the University of Southern California Medical School, Dr. Miller has ready access to human brain material.

References:

291. Molecular Cloning of the sevenless Gene
Utpal Banerjee, Patricia J. Renfranz

Mutations at the sevenless (sev) locus in Drosophila result in the lack of a single neuron (photoreceptor cell No. 7) from the group of eight normally present in each ommatidium of the compound eye. Since the recruitment of cells into ommatidia in the eye disc is non-clonal (Ready et al., 1976), studying the sev gene and its gene product may help us understand the mechanisms underlying cell patterning in the eye disc.

The sev gene had previously been mapped to bands 10A1-2 of the X chromosome (Harris et al., 1976). We have isolated several P element-induced and EMS-induced alleles for this locus. A rapid "deep pseudopupil" (Franceschini and Kirschfeld, 1971) screening method was devised, in addition to a color-choice behavioral screen. Three P element-induced mutations were isolated from 23,500 male progeny. Five more
alleles were isolated by screening 30,000 progeny from EMS-treated flies.

The P-element insertion sites in one P allele (sev\(^{P1}\)) were localized on chromosome squashes hybridized in situ to P-element DNA. Approximately 50 P-insertion sites were seen throughout the genome, including one at 10A1-2. A genomic library of sev\(^{P1}\) DNA was constructed in the EMBL3 vector system and screened with P-element DNA, identifying 140 clones. The clone mapping to the sev region was identified by in situ hybridization of the phage DNA to Drosophila chromosomes, and localized between the deficiencies VM6 and 17Cc8 as defined by Zhimulev et al. (1981). Genomic Southern analysis indicated that this clone recognizes a restriction fragment length difference between an EMS-induced sev allele and other sev alleles of identical genetic background. This clone was used to isolate and map over 50 kilobases of wild-type DNA from this region. One of the genomic clones recognized major changes in restriction fragment size amongst the sev P alleles. Southern analysis also indicated that the mutant sev\(^{P1}\) probably arose from a deletion of DNA in the region.

DNA fragments from the original P clone as well as from wild-type clones have been used to probe Northern and RNA dot blots. At least two transcripts have been identified in the cloned region. One of these was ubiquitously expressed, but the other was expressed only in eye imaginal discs and adult heads. The latter transcript, about 2.5 kb in length, is more likely to correspond to the sev gene. We are currently mapping the transcripts within the cloned region and plan to use appropriate genomic fragments to rescue the sev phenotype by germline transformation.

References:

292. Tissue Localization of Transcription for the 24B10 Gene

John Archie Pollock

MAb 24B10 was isolated from mice immunized with homogenates of adult retina. The antigen and the gene that encodes it have been isolated in this laboratory. The antibody stains adult photoreceptor cells and their axons, the antigen appearing early in development of these cells during the third larval instar. Staining is also evident during embryogenesis, in the photoreceptor cells and axons of the larval photoreceptor organ. A genomic DNA clone was identified by immunopurifying the antigen, sequencing the amino terminus, synthesizing a corresponding oligonucleotide probe, and screening a genomic library. The gene has been cytologically mapped to band 100B of chromosome III (Zipursky et al., 1985).

By in situ hybridization, transcripts were localized in different stages of development. Whole mounts of third instar eye imaginal discs were prepared by a method similar to that of Kornberg et al. (1985). Two stages of pupal development and adult heads were analyzed following the procedure of Hafen et al. (1983).

The probes for hybridization were nick translated with tritiated nucleotides. For whole mount eye discs and frozen sections of pupae, a plasmid containing a 6.6 kb EcoRI fragment was used. For hybridization to adult heads, a nonoverlapping 600 bp PstI/EcoRI gel-isolated fragment was used, of which approximately 200 bp are coding sequence corresponding to the amino terminus of the antigen.

The tissue specificity correlated closely with the MAb 24B10 staining patterns, although the signal-to-noise ratio of in situ hybridization was considerably higher than with the antibody. In whole mount eye discs, hybridization was apparent posterior to the morphogenetic furrow, appearing first at the posterior edge of the disc. By five hours into pupation, when the morphogenetic furrow is fully extended, hybridization was apparent across the whole eye disc, up to its junction with the antenna disc. By 29 hours into pupation, the eye discs are everted; in tangential sections, hybridization was apparent over the centers of ommatidial clusters, not the surrounding pigment cells. In the adult eye, hybridization was similarly apparent only in retinular cell bodies. This indicates that the gene is transcribed in the same cells where the protein is expressed.

This method of localizing RNA transcripts is being employed to analyze various genomic and cDNA clones that are relevant to the development and function of the Drosophila visual system.
293. Mutations Altering Drosophila Photochoice Behavior

Dennis G. Ballinger

When given a choice between green ($\lambda = 550$ nm) and UV ($\lambda = 350$ nm) light in a T-maze photochoice test, wild-type flies show a marked (tenfold) preference for UV light. Flies carrying the sex-linked recessive mutation sevenless (sev) lack the R7 photoreceptor in each ommatidium of the adult eye and display a converse preference for green. Certain alleles of sev, like wild-type Drosophila, respond phototactically to these wave lengths of light, preferring each over darkness. Thus, sev mutant flies appear to have an alteration in visual processing that changes their photochoice.

In an attempt to identify genes that interact with sev in altering photochoice preference, flies carrying the sev mutation were mutagenized, and mutants that prefer UV light were isolated. Six lines contained autosomal dominant suppressor mutations that reverted the photochoice behavior of sev flies without affecting the absence of R7.

Three of these mutations, having the recessive phenotype of a 100-fold decrease in sensitivity to both UV and green light, were found to be in the same gene on the third chromosome (map position 3-52). This decreased sensitivity was accompanied by morphological changes, including smaller rhabdomeres (the light gathering specializations of the photoreceptor cells) and gaps between the pigment cells and photoreceptor cells. Of the other three mutations, determined to be on the second chromosome, two were of variable expression and were not studied further.

The final second-chromosome mutation causes no visible defect in retinal morphology. It appears to have truly altered the sign of photochoice in sev flies, i.e., flies carrying these two mutations prefer darkness to green light. The effect of this mutation on the pattern of photoreceptor innervation of the optic lobes is under investigation.

294. A Protein Associated with Particular Drosophila Sensory Organs

David R. Hyde, John Archie Pollock

Monoclonal antibody MAb 21A6, isolated from mice immunized with homogenates of adult retina (Fujita et al., 1982), stains material in the space between the rhabdomeres in the adult retina and ocelli. It also stains the larval photoreceptor organ. Furthermore, it stains the so-called "sense rods" that connect the sensory endings of the scolopophorous organs. These stretch receptor organs occur in the antenna, leg, wing, haltere, and other locations in the adult fly. The antibody also stains the developing scolopophorous organs in the embryo. Thus, this antigen occurs only in sensory organs, at sites that may have transducing functions.

The 21A6 antigen was immunopurified on a MAb 21A6-coupled Sepharose-4B column (Zipursky et al., 1984) from adult head homogenate extracted with 1% sodium deoxycholate and 1% sodium sarcosine before application to the column. The eluted proteins included approximately seven large (molecular weight $>200$ Kd) proteins. These protein bands were excised from SDS-polyacrylamide gels and individually used to immunize and boost BALB/c female mice. Three of the mice, immunized with three adjacent molecular weight protein bands of approximately 250-300 Kd, yielded sera that stained the intraretinular space of the adult Drosophila retina in a pattern like that of MAb 21A6, indicating that these bands contain the antigen, which can then be analyzed in an effort to elucidate its function in sensory organs.

References:

295. Proteins Recognized by Monoclonal Antibody 22G8

David R. Hyde

Monoclonal antibody 22G8 (MAb 22G8), isolated from a fusion of a mouse spleen immunized with Drosophila head homogenate (Fujita et al., 1982), stains the crystalline cones of the adult retina. It also highlights the morphogenetic furrow in the third larval instar eye disc (Zipursky et al., 1984). Since the
pattern and formation of the ommatidial clusters begins at or immediately adjacent to the morphogenetic furrow, the MAb was further investigated. It was found that, in addition to the morphogenetic furrow in the eye disc, MAb 22G8 also highlights folds in all the other imaginal discs. In cross sections, it was observed that staining was not confined to folds but was located in an inner membrane over the entire disc. Therefore, the antigen(s) recognized by MAb 22G8 are not transiently expressed molecules that migrate with the morphogenetic furrow but merely give that appearance due to the edge of the folded membrane in the furrow.

Western blots were performed with MAb 22G8 on Drosophila homogenates at stages from first larval instar through adult. They revealed three different molecular weight proteins recognized by MAb 22G8. A molecular weight 73 kd protein is expressed from early pupa through adult, a 44 kd protein is expressed at all stages except the third larval instar, and a 42 kd protein is expressed from third larval instar through adult. Therefore, of the three proteins recognized by MAb 22G8 on blots, only the 42 kd protein is expressed in the third larval instar, a crucial period in eye development.

Purification of the 22G8 antigen from third instar larva homogenates, using a MAb 22G8-coupled Sepharose-4B column (Zipursky et al., 1984) yielded the 42 kd but not the 44 kd or 73 kd proteins. Immunoaffinity purification from adult heads and early pupae yielded all three proteins. This suggests that the protein seen by MAb 22G8 in the inner membrane of imaginal discs is a developmentally specific 42 kd protein.

References:
cells in which it is expressed, its temporal expression, and cellular location.

298. Nuclear Proteins Involved in Drosophila Eye Development

Bruce A. Rabin

The developmental signals specifying the formation of the precise array of photoreceptor cells in the Drosophila compound eye are unknown. Elicitation of and responses to such signals may involve proteins acting on DNA within the nucleus. To identify trans-acting nuclear proteins involved in eye development, it is planned to isolate monoclonal antibodies (MAbs) that specifically recognize antigens localized to nuclei of the photosensory system. These MAbs will be used to immunopurify nuclear proteins to approach the following questions. Can one identify a set of nuclear proteins that vary in a cell-type-specific or photoreceptor-type-specific manner during development? Do the nuclear proteins bind specific DNA sequences? If so, can these sequences be identified and cytologically mapped to specific bands of polytene chromosomes? Can the regulated molecules be identified?

To generate MAbs specific for nuclei of the photosensory system, nuclei have been isolated from late third instar larval imaginal discs. Their purification was monitored by phase microscopy, as well as with the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI). The nuclear proteins were extracted with 4 M urea and purified free of insoluble material. Both of these fractions will be used as immunogens to isolate the nuclear-specific MAbs. Once these MAbs are raised, one or more of the antigens will be immunopurified.

Proteins localized to the nucleus may act by directly binding to DNA (Laughon and Scott, 1984; Hogness et al., 1985). To study the question of function in detail, one approach will be to use the proteins for in vitro DNA binding and DNA footprinting studies. While tissue-specific DNA binding proteins are likely to play a role in specifying the pattern of development, they may act by interacting with other proteins rather than directly with DNA. To analyze this possibility, the purified protein will be coupled to Sepharose and used as an affinity matrix for isolating specific proteins (Formosa et al., 1983).

References:

299. Antigenic Homologies between Mammalian and Drosophila Retina

David R. Hinton, Carol A. Miller

A panel of monoclonal antibodies (MAbs), originally made with Drosophila nervous system as primary immunogen, also cross reacts with selective components of the human nervous system (Miller and Benzer, 1983). For some of these MAbs, we have shown precise topographic homologies in the staining pattern between Drosophila and vertebrate retina.

MAb 24B10 stains Drosophila photoreceptor cells and their axons. Although 24B10 is nonreactive with the vertebrate retina, polyclonal antisera made with purified 24B10 antigen stains brightly the inner segment of vertebrate photoreceptor cells. This implies specific topographic homologies between a single neuronal cell type in both Drosophila and man. We plan to study this homology further by isolating and characterizing cDNA clones obtained by screening human retina and brain cDNA libraries with presently available 24B10 gene probes.

MAb 22C10 reacts with photoreceptor cells in the Drosophila larval eye imaginal disc. It also stains neurons and axons in the ventral nerve cord of the early embryo and the adult. In vertebrates, 22C10 expression is also developmentally regulated. In early development, the MAb reacts with a large set of neurons and their axons, but expression decreases with time such that, in the adult, only a limited number of neuronal cell types are stained, including the retinal photoreceptor cell. Western immunoblots of vertebrate central nervous system homogenates reveal strong bands at 60 and 86 kd, while in Drosophila there is a broad band of reactivity at 160-200 kd. We plan to study this antigen in the vertebrate by affinity purifying it, obtaining a partial amino acid sequence, and isolating corresponding cDNA clones.

Conservation through evolution of these developmentally modulated neural-specific molecules suggests that they may be functionally important in both...
vertebrate and invertebrate nervous systems.

Reference:

300. Organization of Human Nervous System Revealed by Monoclonal Antibodies

David R. Hinton, Victor W. Henderson¹, Carol A. Miller

Monoclonal antibody (MAb) probes were used to dissect the human nervous system at several fundamental levels of neural organization. A panel of 12 MAb, most prepared by Fujita et al. (1982), was used in conjunction with immunofluorescence and immunoperoxidase microscopy on cryostat sections of human neural and control tissues. CNS tissues were from the cerebral hemisphere, cerebellum, spinal cord, and retina. At the single cell level, MAb 3A4, 10G4, and 22C10 labeled, respectively, the outer segment, inner segment, and cell body of the photoreceptor cell; MAb 24B6 specifically stained the outer (photoreceptor-bipolar cell) synaptic layer. At a regional level of analysis, pyramidal neurons of the motor cortex, but not nonpyramidal or Betz cells, were labeled by MAb 3F12. Conversely, MAb 5A11 and 6A2 decorated nonpyramidal and Betz cells, but not pyramidal cells. MAb 6A2 stained a widely distributed functional system within the cord and cerebellum, including the dorsal nucleus of Clarke (origin of the spinocerebellar tract), Purkinje cells, and the emboliform nucleus. The results suggest that varying levels of organization within the human CNS are associated with unique molecular markers, which can be identified by MAb probes.

References:
¹Department of Pathology, University of Southern California Medical School, Los Angeles.

301. Neuronal Subset Degeneration in Alzheimer's Disease

Carol A. Miller, Maria Rudnicka¹, David R. Hinton

Alzheimer's disease is a dementing neurodegenerative disease with the histologic hallmarks of neuronal loss, neurofibrillary tangles and neurotic 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, we have examined autopsy tissues from the hippocampus, primary motor and premotor cortices (Brodman's areas 4 and 6) from five patients with Alzheimer's disease and five normal controls. We have identified three discrete neuronal subsets within the neocortex and hippocampus reactive with MAb 44.1, 3F12, and 6A2.

In all Alzheimer's disease tissues examined, a subset of pyramidal projection neurons reactive with MAb 3F12 is selectively lost within the hippocampus, especially in CA-1, subiculum and layers 2 and 3 of the entorhinal cortex. There is also loss of 3F12 reactive neurons primarily in layers 3 and 5 of the association cortex (Brodman's area 6).

MAb 3A4 identifies intracytoplasmic material only in Alzheimer's disease hippocampus and cortex and is distributed in a pattern coincident with neurons containing 3F12 antigen. Thioflavin S staining also identifies neurofibrillary tangles and neurotic plaques within the regions containing MAb 3F12-positive cells. MAb 3A4 does not generally co-localize 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 nonreactive after incubation of tissue with alkaline phosphatase. MAb 3A4 does not react with neurofilament triplet proteins on immunoblots of tissue homogenates nor with purified phosphorylated and nonphosphorylated neurofilaments.

Our results have defined a projection neuron in the hippocampus and neocortex that may be selectively vulnerable in Alzheimer's disease. We also have identified an intracellular antigen that may be unique to Alzheimer's disease, which may be a precursor of neurofibrillary tangles.

¹Department of Pathology, University of Southern California Medical School, Los Angeles.
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Summary: Our group is concerned with understanding how genes control the whole course of development of an organism starting with the earliest stages of the embryo. In segmented animals such as flies and human beings, there is an early worm-like stage in which the organism consists of little more than a linear array of body segments that are very similar to one another and quite undifferentiated. Much of the subsequent embryological development consists of a gradual unfolding of a gene-regulated program in which each segment takes on specific attributes that identify it as a head, thoracic or abdominal segment. We are studying a cluster of regulatory genes, known as the bithorax complex, which programs much of the development of the thorax and abdomen of the fly.

When the bithorax complex is totally removed from the organism by introducing a deficiency for the complex into both the maternal and paternal chromosomes, then the organism dies near the end of embryonic development and all of the abdominal segments revert back to a thoracic, or combined thoracic and head, type of development. By adding back in a sequential manner individual genes of the complex, the function of the normal (or "wild-type") genes can be deduced. A simple rule seems to hold for many if not all genes of the cluster; namely, their order along the chromosome parallels their order of derepression (or activation) along the body axis of the organism. We are exploring how well this rule applies over the entire gene complex and we are using genetic and molecular approaches to try to determine the mechanisms responsible for this rule. The major discovery we made this last year was to find that another rule, which we call the cis-overexpression (COE) effect, applies for many if not all of the genes of the complex. The COE rule states that certain mutants that result in a loss of function of a given gene result in a gain of function of the next most proximal gene in the complex.

302. Transabdominal: Misregulation within the Bithorax Complex

Susan E. Celniker

We have continued our investigation of the dominant gain-of-function mutant, Transabdominal (Tab), which puts stripes of abdominal tissue on the thorax. Genetic and cytological studies previously reported (Biology 1985, Abstract No. 293) showed that the dominant phenotype of Tab is due to the misregulation of the infra-abdominal (iab-6 and/or iab-7) genes. We have begun to analyze this misregulation on a molecular level. Using a genomic DNA fragment containing sequences surrounding the iab-7 homeobox as a probe, we have screened two λgt10 cDNA libraries: one from 3- to 12-hour embryos and the other from early pupae 5.5-7.5 days. These libraries were kindly provided by L. Kauvar. We obtained seven cDNAs, two pupal and five embryonic. All the cDNAs when used as probes to screen the wild-type BX-C walk show homology to restriction fragments spanning 17 kb. We have focused our studies on the longest (3 kb) pupal cDNA. This cDNA is specific for iab-7 by two criteria: 1) it hybridizes to salivary gland chromosomes at position 89E, and 2) under high stringency blot hybridization conditions, it does not hybridize to homeobox-containing sequences from Ultrabithorax (Ubx) or iab-2. We have used this cDNA as a probe for in situ hybridization to wild type and Tab 10- to 24-hour embryos and mass isolated imaginal discs. The hybridization pattern in the embryos is the same obtained by Harding et al. (1985), which showed transcript distributed in abdominal segments A5-A8. We see no other hybridization in the Tab embryos. However, the results from the studies with the imaginal discs are quite different. Both Tab and wild-type animals show strong labeling to genital discs and in addition Tab animals show labeling, albeit weak to the notal region of the wing discs. We are attempting to confirm these whole mount imaginal disc results using in situ hybridization to tissue sections from third
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instar larvae and plan to analyze Tab and wild-type transcripts from different developmental stages.

Reference:

303. Multiple Gene Dosages of the Bithorax Complex and the Phenomenon of Cis-Overexpression

Sarah M. Smolik-Utlaüt

The genes of the bithorax complex (BX-C) obey two primary rules for their cis regulation. The cis-inactivation (CIN) rule states that a mutation in a given gene tends to cause a loss of function in one or more genes immediately distal to it in the same chromosome. The cis-overexpression (COE) rule states that certain loss-of-function mutations in a given BX-C gene cause a slight dominant overexpression of the gene immediately proximal and in cis. One of the underlying assumptions used to interpret the COE rule is that in the wild-type animal, many if not all of the genes of the complex are weakly active one or more segments anterior to the segment in which they first become obviously expressed. We have tested this assumption by analyzing the phenotypes of animals carrying two, four, six and eight doses of the wild-type Ultrabithorax (Ubx) and bithoraxoid (bxd) genes. We first generated a tandem duplication of a duplication for these genes, namely, Dp(3;2)P10, which has the region from 89C to 89E up to and including bxdr duplicated and inserted into region 29C of the second chromosome (Lewis, 1978). In addition to this tandem duplication of DpP10, which we designate as Dp(P10)2, we use a tandem duplication for the entire complex, Dp(3;3)P5, to generate animals with multiple doses of Ubxr and bxdr. Animals with even eight doses survive as adults and presumably show a transformation of the dorsal third thoracic segment (T3) towards an abdominal one in the sense that the halteres shrink considerably in size and are often absent. Segment T3 of the larva shows a clear abdominal transformation: the denticles of the ventral setal bands of T3 become thicker and more hook-like, resembling those of the abdominal segments.

Reference:

304. An Improved Screen For Detecting Chromosomal Rearrangements in the Bithorax Complex

Rollin H. Baker, E. B. Lewis

We have previously reported on a global rearrangement or GR screen that permits us to disrupt the BX-C by detecting x-ray-induced chromosomal rearrangements that have one breakage point in the complex. The screen relies on the fact that rearrangements having breakage points between the centromere and the BX-C often cause a disruption of complementation between certain pairs of mutants of the BX-C. This phenomenon of pairing-dependent complementation was first discovered to occur in the bithorax complex and appears increasingly to be of fundamental importance for the understanding of the molecular events involved in the transcription and perhaps replication of genes. The GR screen has the advantage that it can detect virtually any chromosomal rearrangement having a breakage point within the BX-C, including cryptic ones which might not have any defect whatsoever in any BX-C gene function. The original GR screen suffers from the disadvantage that it detects not only breakages within the BX-C but also in a large region (comprising some 500 bands as seen in the salivary gland chromosomes) between the centromere and the BX-C; as a result less than 1% of the detected rearrangements have a breakage within the BX-C itself. The improved GR2 screen makes use of an inversion which brings the BX-C genes within a few bands of the centromere. When this chromosome is irradiated (and is recovered opposite a virtually identical inversion containing certain mutants that sensitize the detection of rearrangements, namely, the double mutant combination Cbr Ubr), approximately 596 of rearrangements are expected to have a breakage point in the complex. This screen is now being employed and with it we hope to saturate the complex with breakages that will allow us to identify new BX-C gene functions, to synthesize deletions for various separate genes of the complex and to identify any cryptic breakage points within the complex.

305. A Specific Morphological Effect of the Infra-abdominal Gene of the Bithorax Complex

Josephine Macenka, E. B. Lewis

Genes of the bithorax complex seem to share some functions in common while at the same time they may
express unique functions not found by any other gene of the complex, or in some cases only by immediately adjoining genes. Such effects on the organism provide sensitive indicators of specific gene functions and therefore provide a means of studying many of the highly unusual genetic phenomena that are associated with the BX-C genes. It has been known for some time that in the larval stage there are tiny dorsal triangular shaped hairs, or DTs for "dorsal triangles," in the posterior compartment of the abdominal segments but not in the thoracic segments (Lohs-Schardin et al., 1979). We have found that the DTs do not appear in the absence of the infra-abdominal-3 (iab-3) gene and appear in excess if there are either extra doses of the complex or if certain mutant blocks are present in the more distally located iab-4 gene. The latter effect is a new example of the COE effect cited in the Summary above. We are currently carrying out a scanning electron microscope study of the DTs in third instar larvae of a variety of genotypes.

Reference:

306. Protein Synthesis Pattern Following Heat Shock of Drosophila Embryos

Susan J. Eberlein

Very short heat shocks were administered to carefully staged early embryos of Drosophila melanogaster and the effects on protein synthesis pattern investigated. A low level of synthesis of the major heat shock proteins is visible in unshocked animals. A shock as short as two minutes will induce the heat shock response (reduction of normal protein synthesis, increased synthesis of the heat shock proteins) in syncytial blastoderm (2.5 hours of development) or later stages. Thus, the initial events of the heat shock response must occur within two minutes and not reverse upon rapid return to 22°. Survival following short shock is not strictly correlated with high level of heat shock response. Pre-blastoderm embryos do not produce significant heat shock protein but survive a two-minute 43° heat shock better than do heat shock response competent blastoderm. The protein synthesis pattern prior to blastoderm stage is very stable, possibly enhancing survival following short heat shock. Shocks of three minutes or longer are more detrimental to pre-blastoderms than to later stages, confirming the role of the heat shock response in survival of longer shock. Induction of stage-specific developmental defects (phenocopies) by heat shock is apparently correlated with the disruption of normal protein synthesis pattern (probably resulting from altered gene expression). Use of very short heat shocks to induce the response will be valuable in identifying the precise time at which a specific defect can be induced.


Susan J. Eberlein

A rule-based expert system shell suitable for use on microcomputers has been developed and specialized for solving problems related to Drosophila genetics. The system is written in the language FORTH, but knowledge of this language is not required for use of the system. Rules for the reasoning process are entered in English-like syntax. Reasoning proceeds on the basis of facts entered by the user, or answers to questions posed by the computer program. Specialized rule bases have been developed to demonstrate basic genetic principles (recessive/dominant genes, sex linkage, recombination), using Drosophila as an example. In one example program, the system asks for input data and runs simple statistical tests to determine the probable parental genotypes in a genetic cross. These example programs are designed as demonstrations and teaching aids. The system may be readily specialized for other applications. For example, known information about an organism may be entered, causing the system to search a data base to find the probable identity of the organism. Such specializations could be valuable in experimental design or medical diagnosis.

308. Regulation during Development of the Bithorax Gene Complex (BX-C) in Drosophila

E. B. Lewis

Transvection provides a way of studying how the genes of BX-C are triggered to turn on, presumably sequentially, so as to produce substances that then regulate batteries of genes outside of BX-C. Genetically-derived data from transvection experiments involving chromosomal rearrangements, a
tandem duplication, Dp(3;3)P5, and zeste-a mutant alleles, suggest that within BX-C a special class of regulatory molecules is generated, possibly sense or anti-sense RNAs. These molecules would migrate along the chromosome to produce the cis-polar effects observed within BX-C. At the same time, they would be able to influence significantly genes in the adjoining BX-C when that complex is in the somatically paired homologous chromosome or when it is in the adjacent section of a tandem duplication.

**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.

309. The Structure and Regulation of the Actin 5C Gene of Drosophila

Beverley J. Bond, Norman Davidson

Support: National Institutes of Health, USPHS

At least six developmentally regulated mRNAs are made from the Drosophila actin 5C gene. We have examined the structure of these RNAs in detail and have determined that they are heterogeneous at both their 5' and 3' ends. At the 5' end, there are two nonhomologous leader exons which are alternatively spliced to the remainder of the gene. At the 3' end of the gene, there are three different sites of polyadenylation. This 3' variation is the principal cause of the length diversity observed in the transcripts. Our data indicate that in whole animal RNA the two leader exons are used with approximately equal probability in all stages of development and with all three polyadenylation sites. There is some developmental variability in the use of the three polyadenylation sites. We have begun to study the sequences necessary for the initiation of transcription from each start site. We made fusions between putative actin 5C promoter sequences and the bacterial CAT gene and tested these by transient transformation experiments in the Kc line of Drosophila cultured cells. We found that both exon 1 and exon 2 are preceded by separate, functional promoters. 2.5 kb of upstream sequences are necessary for optimal expression from exon 1. Exon 2 can be efficiently transcribed with as little as 450 bases of upstream sequence present.

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310. Mouse Acetylcholine Receptor γ Subunit: Gene Expression during Differentiation

Lei Yu, Norman Davidson

Support: California Foundation for Biochemical Research
National Institutes of Health, USPHS

The activity of the majority of eukaryotic genes is regulated both temporally and spatially, making possible the various types of cells and tissues necessary for a multicellular organism. One of the characteristics of mammalian skeletal muscle is the high abundance of acetylcholine receptor (AChR) molecules on the cell surface. The AChR on the postsynaptic membrane is an integral membrane protein complex composed of four subunits, α, β, γ, and δ. It functions as an agonist gated ion channel upon the binding of acetylcholine molecules and plays an important role in muscle contraction. Using a cDNA clone coding for the mouse AChR γ subunit (Yu et al., 1986), we studied the γ gene expression pattern in two mouse myogenic cell lines, BC3H-1 and C2C12. These cells grow and proliferate in rich medium under tissue culture condition. When proliferation is restricted by confluence and/or exposure to a less rich medium, the cells stop dividing and undergo differentiation, producing large amounts of muscle-specific proteins in the process. To study the AChR γ gene expression during differentiation, total cellular RNA samples were isolated from undifferentiated and differentiated cells, fractionated by gel electrophoresis, transferred to nylon membranes, and hybridized with radioactively-labeled RNA probes. The γ subunit probe hybridizes strongly to two RNA species with estimated molecular lengths of 1.95 kb and 2.1 kb, respectively. These RNA species are present at low levels in undifferentiated cells and become relatively abundant when the cells differentiate. The induction ratio is approximately 28 fold for BC3H-1 and 50 fold for C2C12. This up-regulation of AChR γ messages upon differentiation correlates well with the observation that the large amounts of AChR molecules appearing on the muscle surface during differentiation were synthesized de novo rather than being stored in the cytoplasm before differentiation and suggests that the control of expression is mainly at the transcriptional level.

Reference:

1Division of Chemistry and Chemical Engineering, California Institute of Technology.
311. Binding of the Drosophila HSTF to the hsp 70 Promoter: Evidence for Dynamic Interactions

David J. Shuey, Carl S. Parker 1

Support: American Cancer Society
National Institutes of Health, USPHS

A Drosophila heat-shock gene transcription factor (HSTF) has been shown to bind to three domains upstream from the TATA homology on a hsp 70 gene. The domain closest to the TATA homology consists of two contiguous binding sites with different binding affinities. Occupancy of the TATA homology proximal site (site 1) coordinates HSTF binding to the neighboring site (site 2) in a cooperative manner (Topol et al., 1985).

We have used alkylation interference and protection experiments to determine which residues within the binding sites are closely contacted by the HSTF. The contacts inferred from these studies included the residues present in the consensus sequence found in all HSTF binding sites and exhibit rotational symmetry, suggestive of a multimeric HSTF. By employing a gel electrophoresis separation technique, we were able to resolve two protein-DNA complexes consisting of site 1 occupancy (complex A) and sites 1 and 2 occupancy (complex B). Analysis of these discrete species reveals that a subset of contacts within site 1 change upon HSTF binding to site 2, suggesting that a conformational change in the protein-DNA complex occurs (Shuey and Parker, 1986).

That this apparent conformational change may be important for hsp 70 gene activation follows from in vivo and in vitro transcription studies with deleted templates that reveal a strict requirement for site 2 sequences even in the presence of a fully functional site 1. Thus, a clear correlation exists between cooperative HSTF binding at site 2, apparent conformational changes, and activation of transcription.

References:

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

312. HSTF-induced DNA Bending at the Drosophila hsp 70 Promoter

David J. Shuey, Carl S. Parker 1

Support: American Cancer Society
National Institutes of Health, USPHS

Template deletion studies performed in this laboratory and others have identified a region known to be critical for the heat-inducible transcription of a Drosophila hsp 70 gene. This domain, spanning sequences from approximately -60 to -95, consists of two contiguous HSTF binding sites (sites 1 and 2) that are occupied in a cooperative manner. Recent alkylation interference and protection studies suggest that a conformational change occurs in the protein-DNA complex at site 1 upon sequential HSTF binding at site 2. These results prompted us to investigate the possibility that an altered DNA structure occurs in this region as a result of HSTF binding.

As discussed by Wu and Crothers (1984), the mobility of identically sized DNA fragments containing a specific DNA bend is dependent upon the position of the bend relative to the ends of the fragment. In accordance with gel electrophoresis theory, when a bend is located near the end of the molecule it will migrate faster than a molecule with the bend centrally located. Hsp 70 binding sites 1 and 2 were cloned between direct repeats of a pBR322 restriction fragment. Following restriction with five enzymes that cleave within the pBR322 sequences, we were able to isolate five identically sized DNA molecules having variant relative positions of the HSTF binding loci. The gel electrophoretic mobilities of these fragments were indistinguishable. Upon formation of HSTF-DNA complexes, however, the anomalous migration characteristic of "bent" DNA was observed. We conclude that by the criteria of anomalous gel mobility, HSTF binding to a single site or to both sites results in the introduction of a specific DNA bend that maps to the binding domain of the hsp 70 promoter (Shuey and Parker, 1986).

References:

1Division of Chemistry and Chemical Engineering, California Institute of Technology.
313. Homogeneous Purification of HSTF from S. cerevisiae and from D. melanogaster


Support: American Cancer Society
National Institutes of Health, USPHS
Helen Hay Whitney Foundation

The heat-shock transcription factors from both yeast and Drosophila have been purified to total homogeneity using a four-step protocol that includes an ion-exchange column and three affinity columns. When the purified HSTF from either organism is subjected to SDS-PAGE, seven discrete polypeptides of molecular weights 37 kd, 40 kd, 43 kd, 48 kd, 70 kd, 92 kd and 155 kd are visible. Excepting the 155 kd polypeptide, each of these polypeptides can be excised from the gel, renatured, and shown to bind the heat shock elements on the hsp promoters. Furthermore, HSTF purified from one organism can footprint the heat shock element on the other organism's promoter. We have extended this analysis and have demonstrated that HSTF purified from HeLa cells can bind to the heat shock element of yeast hsp promoters.

We believe that the smaller polypeptides are proteolytic products derived from a larger precursor protein. We have begun obtaining amino acid sequence information from some of the polypeptides and soon will be able to identify those amino acids in the non-proteolyzed form of the HSTF that are responsible for binding to DNA. The sequence information will also enable us to clone the HSTF gene.

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

314. Biochemical Analysis of the Fushi Tarazu and Ultrabithorax Promoter Regions

Joanne Topal, Gregory J. Wiederrecht*, Sean Tavtigian, Carl S. Parker

Support: National Institutes of Health, USPHS
Helen Hay Whitney Foundation

We have analyzed the fushi tarazu (ftz) and Ultrabithorax (Ubx) promoter regions in vitro using nuclear extracts from Kc cells and 0-12 hour embryos. Footprinting assays with crude and partially fractionated extracts identified a number of sequence-specific DNA binding proteins. There are at least 12 binding domains within 400 base pairs (bp) upstream of the transcriptional start site and within the untranslated leader region of the ftz promoter. Since the information necessary for generating the "zebra" pattern of ftz expression is encoded in the 740 bp sequence upstream of the ftz translational start site (Hiromi et al., 1985), it seems likely that at least a subset of our binding proteins plays a role in controlling the spatial distribution of ftz transcripts in embryogenesis. The location of several Ubx promoter binding domains within 200 bp of the startpoint of transcription also suggests that they are involved in the transcriptional control of Ubx.

We extended this study by investigating ftz and Ubx promoter activity in vitro. The cloned ftz gene has a very active promoter in a Kc cell in vitro transcription system and in extracts made from 0-12 hour embryos. The Ubx promoter is tenfold weaker than the ftz promoter in Kc cell extracts and is generally inactive in embryonic extracts.

We constructed Bal31 5' deletion sets for both the ftz and Ubx promoters and assayed the efficiency of these altered promoters in vitro. For the ftz promoter, deletion of upstream binding sites reduced transcription by no more than threefold in Kc cell extracts and no effect was observed in embryonic extracts. In all extracts tested, the promoter was nonfunctional when the most proximal TATA box was removed. The Ubx promoter behaved in a similar manner: deletion of upstream binding sites marginally affected in vitro transcription with Kc cell extracts, and although there is no canonical TATA box in the Ubx promoter, deletion of an AT-rich sequence located at -25 bp inactivated the promoter in vitro.

Reference:

1Division of Chemistry and Chemical Engineering, California Institute of Technology.
CELL SORTING FACILITY

Supervisor: Ellen Rothenberg
Staff: Patrick F. Koen, Rochelle A. Diamond

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.
MONOCLONAL ANTIBODY FACILITY

Supervisor: Paul H. Patterson  
Staff: To Ha Thai, Kathleen Fisher

During the past year we have collaborated with several investigators to produce monoclonal antibodies (MAbs) for use in their experiments. We have generated MAbs that stain chromaffin and possibly SIF cells from newborn rats (P. H. Patterson), neuronal markers for the adult male zebra finch song system (M. Konishi), subsets of neurons in the rat brain (J. Bower), and MAbs that distinguish human alpha transforming growth factor (aTGF) from human epidermal growth factor (EGF) (L. Hood). We have also attempted to generate MAbs that recognize a transcription factor from Drosophila that is specific for heat-inducible genes (C. Parker) and MAbs that identify Xenopus RNA-binding proteins (B. Wold). In addition, we have introduced several new methods, including in vitro sensitization, a new strain of mice (RBF/Dn), and a new line of myeloma cells (HLI-653).

MICROCHEMICAL FACILITY

Supervisor: Suzanna J. Horvath  
Staff: Peter C. Gaines, Christine A. Hill, Synthia J. Ross, Ron H. Sweet

We have continued our effort to improve and extend the application of state-of-the-art microchemical techniques in biological research. For members of the Caltech community, we routinely synthesize DNA and protein fragments and very soon we will also be able to provide protein sequence analysis.

Our two automated DNA synthesizers, which have the capacity to synthesize six different DNA fragments simultaneously, provided more than a thousand deoxyoligonucleotides for a large variety of applications, such as primer-directed DNA sequencing, gene assembly, site-directed mutagenesis, etc. We have increased our flexibility regarding small (0.2 µM), regular (1 µM), large (10 µM) scale synthesis and for special requests we have carried out several syntheses with chemically modified bases at certain positions.

For a limited number of research projects, we have chemically synthesized over a hundred small peptides (up to 32 amino acids in length) and conjugated to carrier proteins (KLH, ovalbumin). These peptide-protein conjugates have been used to immunize rabbits or mice to give conventional heterogeneous antisera or monoclonal antibodies with the desired specificities. The laboratory has one automated peptide synthesizer, which operates with one reaction vessel synthesizing one peptide at a time. In order to extend the capacity of our peptide synthesis, we have also been synthesizing peptides manually. Our HF apparatus is set up to deprotect and cleave two peptides simultaneously from their solid phase support.

For peptide and protein sequence analyses, we are in the process of setting up a gas-phase sequencer with on-line PTH-amino acid analysis and automatic sequence determination, data acquisition and analysis.
GRADUATES

Twenty-six students in Biology were awarded the B.S., M.S. or Ph.D. degree in June 1985. Names, degrees conferred and titles of doctoral theses are as follows:

Bachelor of Science

Kenneth D. Bell III, B.S.
Karyn Jean Betzen, B.S.
Felice Faye Borisy, B.S.
Sean Roberts Eddy, B.S. with honor
Hideki Garren, B.S. with honor
Edward N. George, B.S.
George Garing Gibbs, B.S. with honor

Bachelot of Science

Daniel Richard Kegel, B.S.
Santosh Narayanan Krishnan, B.S.
Janice Diane Pata, B.S. with honor
Janice Fusae Sakai, B.S. with honor
Se Jung Shin, B.S.
Steve S. Shin, B.S.
Robin Katherine Wilson, B.S.

Master of Science

Arie M. Michelsohn, M.S.

Doctor of Philosophy

Mark Knowles Bennett, Ph.D. Brain Type II calcium and calmodulin-dependent protein kinase: Purification, characterization and molecular cloning.

Madeline Anne Crosby, Ph.D. Regulation of the Drosophila glue gene Sgs-3; Sequences required for puffing and transcription.

Douglas Arthur Fisher, Ph.D. Class I genes of the major histocompatibility complex: Structural studies on genes of the Tla locus.

George Loweree Gaines III, Ph.D. The control of RNA synthesis in isolated HaLa cell mitochondria.

Boning Gao, Ph.D. The structure and expression of the gene coding for Bindin, a species-specific sea urchin protein.


Katherine Mixter Mayne, Ph.D. Structural and functional studies on the subunits of the nicotinic acetylcholine receptor.

Paul Wells Meyer, Ph.D. The avoidance response of Phycomyces in a controlled environment.

Robert Edwin Pruitt, Ph.D. Characterization of the genome of Arabidopsis thaliana.

Yi Henry Sun, Ph.D. Organization and evolution of the class I genes in the murine major histocompatibility complex.

Astar Winoto, Ph.D. Structure and function of the murine T-cell receptor genes and the murine class I genes of the major histocompatibility complex.
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Uptake of FITC-dextran by vacuolar protein targeting (vpt) mutants

The photographs depict vacuolar morphology and the uptake of FITC-dextran by yeast cells which are defective in the delivery of certain proteins to the vacuole. The cells were visualized using Nomarski (left) or fluorescence (right) optics. All photographs are enlarged ~1000X. The cells shown on the top represent one class of vpt mutants, which are identical to wild-type cells in vacuolar morphology. The cells pictured on the bottom are characteristic of a second class of vpt mutants which have an altered vacuolar morphology, as defined by Nomarski optics and FITC-dextran uptake criteria (see Abstract Nos. 182 and 183).