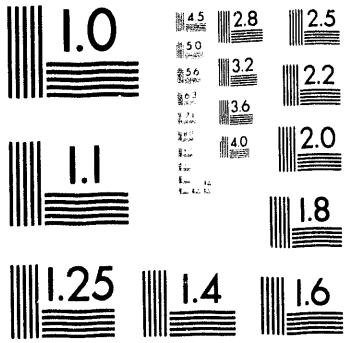
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For Period of October 1, 1991 - September 30, 1993

Date Published: October 1993

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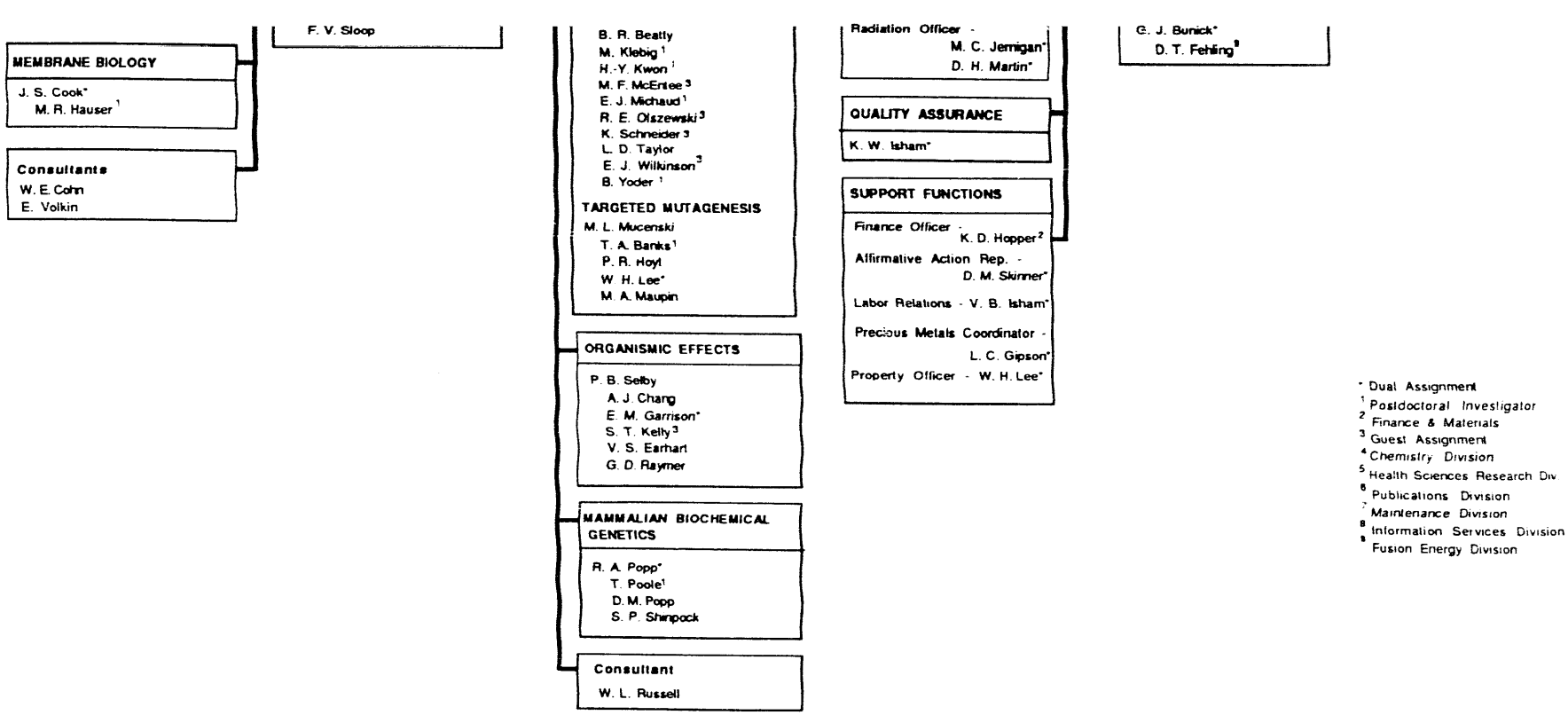

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[6] Publications Division
[7] Maintenance Division
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PREFACE

This Progress Report summarizes the research endeavors of the Biology Division of the Oak Ridge National Laboratory during the period October 1, 1991, through September 30, 1993. The report is structured to provide descriptions of current activities and accomplishments in each of the Division's major organizational units. Lists of information to convey the entire scope of the Division's activities are compiled at the end of the report.

FOREWORD

The Biology Division of the Oak Ridge National Laboratory is a part of the Department of Energy's intramural program in life sciences. Accordingly, about 70% of the Division's total budget is derived from the Department of Energy through its Office of Health and Environmental Research. With respect to experimental biology, congressionally mandated missions of this Office are to define and acquire comprehension of energy-related health effects and to map and ultimately sequence the entire human genome.

Within the framework of these broad missions, predominant programs of the Biology Division include mammalian genetics and mutagenesis, genomic organization and expression, and protein structure and function. Among these programs, interactions of animals, cells, and macromolecules with their respective environments reflect a unifying theme. Goals of studies concerning genetic and somatic effects of radiation and chemicals include identification and quantification of these effects, elucidation of pathways by which the effects are expressed, assessment of risks associated with radiation and chemical exposures, and establishment of strategies for extrapolation of risk data from animals to humans. Concurrently, fundamental biological research continues to illuminate the intricacies of normal life processes as prerequisites to comprehending mutagenic and carcinogenic effects of environmental agents.

The premier challenge to the Division for the past decade has been to maintain the diversity and unassailable quality of research programs, despite chronic budgetary erosion with consequential hardships, as required to adapt to evolving needs of sponsors and society. To meet this challenge, the Division has greatly expanded and integrated its activities in genome research, which includes physical and functional gene mapping, new strategies for DNA sequencing, and informatics. Also relevant to genome research, expertise has been acquired in embryonic stem cell technology which enables construction of transgenic mice in which preselected genes are targeted. Transgenic animals represent unique resources for genome mapping and for designing models of human diseases and offer new avenues for the diagnostics and treatment of genetic disorders. During the present reporting period, the Division has also succeeded in renovating and equipping the protein crystallography laboratory and thereby strengthening the core program in protein engineering.

Research grants and contracts from agencies other than DOE, secured through initiatives of principal investigators, comprise the remaining portion of the funding base for the Biology Division. Collectively, these grants augment and enhance the DOE-supported activities and provide positions for students,

postdoctoral investigators, and research associates, who contribute enormously to the Division's total research efforts.

The Progress Report is intended to provide both broad perspectives of the Division's research programs and synopses of recent achievements. Readers are invited to contact individual principal investigators for more detailed information, including reprints of publications.

Fred C. Hartman

Superscripts after Staff Names on Research Summaries

[1]Postdoctoral Investigator

[2]Consultant

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RESEARCH ACTIVITIES

Mammalian Genetics and Development Section

Section Overview - L. B. Russell

A major theme of the Section's research is the utilization of mutations (agent-induced, insertional, or targeted) as valuable tools in the study of the DNA structure and/or function of specific genes or genomic regions. Analysis of the disruption by mutations of normal developmental processes can serve to dissect bewildering developmental complexities. Because of the existence of many regions of conserved synteny between the mouse and human genomes, detailed physical/functional analyses, aided by mutation resources in the mouse, can predict functions in a corresponding human genomic segment, can determine the organismal roles played by newly identified DNA sequences, and can identify mouse models for the study of human genetic diseases. Section investigators are also major participants in a new Genome Mapping Program, which is summarized under a separate heading.

Use of Agent-Induced Mutations for Structure/Function Analysis of Specific Genes and Genomic Regions

The Molecular Genetics Group is continuing its analysis of genomic regions that are associated with overlapping deletion mutations generated in past and ongoing specific-locus mutagenesis experiments, and which, in aggregate, cover 2-3% of the genome. The mapping of DNA clones to, and within, these regions is greatly simplified by the availability of maps generated in earlier and ongoing genetic studies (the Genetic Analysis Group) that identify functional units in the region surrounding the locus and delineate complementation groups. Recent concentration on the region surrounding the *p* (pink-eyed dilution) locus has contributed to the dissection of the complex Prader-Willi and Angelman human disease syndromes. These are examples of "contiguous gene syndromes," whose total, and sometimes variable, phenotypes could be due to the deletion of variable sets of closely linked genes. In addition to demonstrating similarities between certain mouse *p*-deletion phenotypes and features of the human diseases, a fine-structure homology map of the region has been constructed in the two species. The *p*-deletion complex has also been used successfully to identify and localize a gene for cleft palate *(cp1)* close to *p*. An exciting outcome of the deletion-mapping exercise is the finding that a neurotransmitter receptor, coded for by the *Gabrb3* gene, is expressed during fetal facial development and may be involved in palate morphogenesis.

To improve fine-structure functional mapping of selected regions, the Molecular Genetics Group, using a point-mutation inducer (ENU), has saturated the wild-type regions that correspond to large *c*- and *p*-locus deletions with presumed single-locus mutations of developmental significance. Since, by virtue of the characterized deletion complexes, such mutations can readily be located on the molecular/physical map of the region, positional cloning is facilitated. Among the genes that have been identified in the saturation-mutagenesis studies is one affecting embryonic ectoderm development, another controlling erythrocyte morphology, and a highly pleiotropic gene, mutation in which causes reduced fitness/nervous behavior/male sterility. The studies are also yielding estimates of the minimum number of genes within defined-length regions that are mutable to biologically significant phenotypes.

In addition to the *p* and *c* regions, the agouti *(a)*-locus region has received considerable attention during this reporting period. Molecular access to this region was achieved by the Insertional Mutagenesis Group through exploitation of a complex radiation-induced chromosome rearrangement (generated by the Chromosomal Damage Group) with probes derived from transgenic mice. The *a* region has now been cloned, and the molecular structure of numerous *a*-locus mutations (genetically characterized by the Genetic Analysis Group) is under investigation. The classical yellow-lethal *(A^y)* mutation has been found to cause ubiquitous over-expression of the agouti gene and is providing molecular insights into Type-II diabetes traits. A closely linked gene, *raly*, has been identified that may be directly associated with the preimplantation lethality of A^y. Others of the specific-locus regions are yielding important information in collaborative studies. Thus, the short-ear *(se)* gene has been identified as that coding for bone morphogenetic protein-5; and the piebald *(s)* locus has been placed in a fine-structure map relative to microsatellite markers.

Another approach to positional cloning is being made through the use of reciprocal translocations. Such translocations, which have been, and are being, generated in mutagenesis experiments of the Chromosomal Damage Group, are being systematically examined for phenotypic effects only in both hetero- and homozygotes. For translocations exhibiting such phenotypes, several of which resemble human disorders, cytogenetic localization of the breakpoints is being done by high-resolution banding techniques. Based on this preliminary mapping, DNA probes are chosen to localize breakpoints more closely by the use of fluorescent *in situ* hybridization (FISH), thus providing prerequisites for positional cloning of the gene(s) disrupted by the chromosome rearrangement.

Mouse Models of Human Disease

In addition to the several examples of mouse equivalents to human genetic diseases that have been uncovered in the studies of genomic regions surrounding specific loci (Prader-Willi/Angelman, type-II oculocutaneous albinism, cleft palate,

type-II diabetes, etc. -- see above), a number of individual mutations producing diseases with possible human parallels are under intensive study. The X-linked mutation scurfy *(sf)* causes an immune-system disorder that the Genetic Analysis Group has found to result from faulty thymic "education" of T lymphocytes. Transplantation of fetal thymi has demonstrated that the gene acts quite early; and monoclonal antibody treatments, as well as the genetic introduction of *sf* into certain transgenic lines, have led to the hypothesis that the expansion of self-reactive clones of immune cells within the body allows for the overproduction of cytokines that lead to scurfy disease.

A highly useful model of human disease has been provided by the insertional mutation Tg737, which, in homozygotes, produces polycystic kidney disease closely resembling human ARPKD (autosomal recessive polycystic kidney disease) that is found with appreciable frequency. The Insertional Mutagenesis Group has cloned and mapped a cDNA corresponding to the mouse gene whose expression is interrupted by the Tg737 insert, and has sequenced the homologous human gene. Studies on the histopathology and immunohistochemistry of the mutant have gone hand-in-hand with the molecular characterizations. Among other insertional mutations that have produced phenotypes promising to provide mouse models of human disease conditions is one that produces hearing abnormalities and a defect in the organ of Corti.

Several transgenic lines (including Tg737) from the Insertional Mutagenesis Group are being examined for possible skeletal or cataract phenotypes by the Organismic Effects Group. Insertional mutations with such disorders will be readily accessible to molecular analyses. A number of the skeletal mutations that have emerged from this group's mutagenesis studies with radiations or chemicals have been found to have phenotypes closely resembling those in certain human genetic disorders. In addition, this group continues to study dominant-mutation interaction phenotypes that may mimic recessive inheritance.

A mouse model for sickle-cell anemia has been constructed by the Mammalian Bicchemical Genetics Group by making appropriate crosses to breed the Hb S Antilles transgene into a mutant line that produces high-oxygen-affinity hemoglobins. The red-cell sickling and the secondary organ pathology of this mouse model will facilitate studies on the natural progression of the disease and on possible therapeutic intervention. A virus-induced immunodeficiency syndrome in a congenic mouse is also under study by this group.

A number of human birth defects of the type that are usually of unknown etiology have been mimicked by exposures of early embryonic (pre-organogenesis) stages to certain chemicals. A working hypothesis proposed by the Chromosomal Damage Group is that the normal pattern of early gene expression has been disrupted by epigenetic mechanisms.

Mutagenesis

In addition to continuing to provide the genetic reagents for structure/function analyses of specific genes and regions, the mouse models for human genetic disease, and the tools to aid in genome mapping, mutagenesis studies are directed toward assessing genetic risk and toward clarifying mechanisms of mutagenesis. Increased capabilities for analyzing the nature of mutations not only by genetic and cytogenetic but also by molecular techniques have provided evidence about nuclear states conducive to the induction of either intragenic or large DNA lesions. This evidence, in combination with knowledge gained about mutation frequencies induced by different agents in different germ-cell stages, allows the choice of appropriate mutagenesis protocols for the induction of appreciable frequencies of developmentally significant deletion mutations throughout the genome; such mutations are increasingly amenable to positional cloning as the mouse genome becomes populated with microsatellite and other markers.

An interesting finding made during this reporting period by the Mouse Mutagenesis Group is that a small priming treatment given 24 h prior to a challenging dose can alter the response of stem-cell spermatogonia to resemble that of postspermatogonial stages with respect to the production of primarily large lesions.

The Chromosomal Damage Group is continuing to increase the resource of cytogenetically-mapped mouse translocations. Some of these translocations have been, and others will be, useful for positional cloning of genes of developmental interest. Other translocations of the type that yields late-surviving unbalanced segregants are dependable sources of specific malformations that are models of human congenital anomalies, and they provide information about chromosome segregation.

The group has also continued to identify mutagens that are female-specific and has hypothesized that the relatively diffuse nature of oocyte chromatin permits intercalations or specific bindings that are not possible in postspermatogonial stages of the male. Attempts have been made by the Mouse Mutagenesis Group to enlarge the currently meager specific-locus-mutation data base for chemical exposures of females. A considerably improved historical control rate for females has been derived through re-interpretation of cluster mutations.

Information about the nature of mutations induced by different mutagens in different germ-cell stages has formed the basis for a large-scale experiment by the Organismic Effects Group to assess what types of mutations are most likely to produce dominant phenotypes. The Assessment of Dominant Damage (ADD)approach used by this group combines the study of numerous dominant end points and methods of data analysis.

The large-scale effort by the Insertional Mutagenesis Group to generate insertional mutations in transgenic mice (using first the CAT and then a tyrosinase transgene) has been slowed down and replaced by detailed follow-up studies on a number of mutations of special interest (see above). A transgenic mutation data base ("TBASE") has been put on line. Transgenic-mouse procedures are being used for experiments that test hypotheses about actions of specific genes, e.g., by attempting to correct the phenotype of mutants by transfer of the corresponding wild-type gene. The capability for making specific gene "knock-outs" is now available through the establishment of the Targeted Mutagenesis Group.

Interactions and Service Functions

The Genetic Analysis Group, in addition to performing research in basic genetics and cytogenetics, maintains hundreds of mutant stocks (as well as inbred, congenic, and specially constructed strains, and the several very large stocks used for mutagenesis studies). The value of having preserved mutant stocks generated over a period of decades has become amply apparent through our structure/function analysis of specific genes and genomic regions (see above). Mutations that continue to arise in ongoing mutagenesis studies are tested genetically, analyzed cytogenetically, and assayed with available DNA probes -- to be added to the available mutation resource. An embryo-freezing program involving a number of stocks has been operating for several years. Among other resources of the Section are its cytogenetic capabilities for conventional mitotic and meiotic preparations (including synaptonemal-complex studies, in collaboration with T. J. Ashley, Yale University), as well as for fluorescent *in situ* hybridization (FISH) techniques.

While each group of the Mammalian Genetics and Development Section has its own active program, there are numerous interactions between groups, some examples of which are apparent in the preceding sections of the Overview. The multiple interactions enhance the output of individual groups and produce a fine spirit of cooperation in the Section. In addition, active collaborations are in progress with numerous laboratories throughout the world. Over the past 4 years, for example, over 80 laboratories have utilized our mouse stocks.

MOLECULAR GENETICS AND MOUSE-GENOME STUDIES

E. M. Rinchik, C. T. Culiat[4], M. D. Potter[4], D. K. Johnson, K. J. Houser, M. J. Walkowicz[4], D. A. Carpenter, C. L. Long

The underlying theme of the research of the Molecular Genetics Group is the combination of mouse genetics and germline mutagenesis with the techniques of molecular biology and DNA analysis for the study of genomic regions associated with heritable mutations. In the course of such investigations, we try to develop the mouse as a significant experimental system with which to address many questions and problems to be encountered as the Human Genome Program evolves. Moreover, because many regions of the mouse and human genomes manifest significant homologies both at the level of DNA sequence and at the level of linkage conservation, we make a directed effort to exploit the mouse as a model system for the study of human genetics and human genetic diseases.

Germ-cell mutagenesis experiments conducted at ORNL over the past 40 years have generated numerous radiation- and chemical-induced mutations, many of which are still maintained in breeding stocks. These mutations vary widely in degree of phenotypic complexity, in severity of effect, and in structure at the DNA level. An important subset is comprised of radiation-induced lethal mutations, available for several specific regions within the mouse genome. Many of these mutations are chromosomal deletions of varying lengths, and we estimate that, in aggregate, they cover approximately 2% of the genome. The use of these heritable deletion mutations as biological "reagents" for addressing questions of structure and of functional complexity of the mammalian genome as well as for exploiting the mouse in the analysis of complex human genetic diseases has become the common denominator of the group's interests.

Use of Mouse Mutations for the Dissection of Complex Genetic Syndromes in Humans

Over the past few years (in collaboration with R. D. Nicholls at the University of Florida), we have mapped a number of DNA clones derived from human chromosome (HSA) 15q11-q13 on a panel of radiation-induced deletions that include the mouse pink-eyed dilution (*p*) locus. HSA 15q11-q13 is the region implicated in the Prader-Willi and Angelman Syndromes (PWS and AS), which are genetic, multisystem disorders in children and which are often associated with cytogenetically detectable deletions of this region of the human genome. PWS is characterized by delayed development, hypotonia, feeding problems in infancy, growth retardation, hyperphagia and obesity, small hands and feet, and hypogonadism. AS patients manifest severe mental retardation (no speech), ataxia, seizures, and hyperactivity. Mild facial dysmorphisms, as well as hypopigmentation and problems with internal organs, are sometimes observed in

both syndromes. These two syndromes are among the many so-called "contiguous-gene" syndromes that have been identified in human genetic diseases. It is likely that the total (and variable) phenotype observed in individuals is due to the deletion of a number of closely linked genes. Interestingly, several of the phenotypes observed in *p*-deletion homozygotes are provocatively similar, albeit on a gross level, to those observed in children with PWS (e.g., hypopigmentation, hypogonadism, and growth retardation) or with AS (hypopigmentation, hyperactivity, and ataxia).

We have been quite excited by the HSA 15q11-q13--mouse *p*-region homology, as we hope to use members of the panel of *p*-locus deletions as models for some of the effects associated with these human syndromes. For example, we recently made use of mutations at *p* itself in the mouse, along with predictions of how the *p* gene would be expressed in mouse skin synthesizing different types of pigment, to identify a human fetal brain cDNA clone that is the homologue of *p*. We were also able to discover that this gene, when mutated, is responsible for type II oculocutaneous albinism in humans and is the likely cause of the hypopigmentation sometimes observed in PWS or AS children who carry a cytogenetically detectable deletion of HSA 15q11-q13. Moreover, more detailed molecular-mapping exercises have allowed the construction of a fine-structure homology map of this genomic region in the two species, thereby paving the way for evaluating the use of specific mouse mutations (and phenotypes) as models for other syndrome components in the future.

Neurotransmitters and Craniofacial Development

Anomalies in facial development are among the most common developmental defects observed in human populations, and characteristic facies comprise striking, diagnostic components of numerous clinical syndromes. Diverse factors contribute to the etiology of these types of developmental defects; for example, it is known that both environmental factors acting during embryogenesis and genetic components can be involved in the generation of facial malformations. Dissection of the genetic component of particular facial developmental defects in humans (e.g., cleft palate with or without cleft lip) has proved to be complex. In this regard, the mouse is an exceptional model system for studying the effects of teratogenic and/or intrinsic genetic factors in palate development.

Our studies with radiation-induced *p*-locus deletions over the past few years have led us to an interesting observation on the potential role(s) and importance of neurotransmitters and their receptors in craniofacial development in the mammalian fetus. Mice homozygous for any one of a number of *p* deletions have an isolated cleft of the palate and die at birth. We have proposed that these *p* deletions remove a closely linked gene, *cp1* (cleft palate-1), that is necessary for normal palate formation. Over the past year, we have used a panel of *p*-region

deletions for the genetic and molecular mapping of *cp1*, which resulted in defining a specific genetic interval containing *cp1*. We noted a striking correlation (20/20) between the inability of a given *p* deletion to complement the *cp1* defect and alterations at the gene (*Gabrb3*) encoding the ß3 subunit of the type-A γ-aminobutyric acid receptor. These results, in combination with earlier evidence that γ-aminobutyric acid or its pharmacological agonist diazepam interfere with palate development *in vivo* or *in vitro*, have led us to the unexpected hypothesis that a specific γ-aminobutyric acid type-A receptor subtype containing the ß3 subunit, which is expressed in non-neuronal cell types during facial development, may be intimately involved in the genetic control of palate morphogenesis in the mid- to late-gestation mouse fetus. This hypothesis is currently being tested by transgenic-mouse experiments, in which we are attempting to correct the phenotype of these mutants by transfer of the wild-type *Gabrb3* gene to mutant embryos.

Development of Fine-Structure Functional and Physical Maps of Deletion-Associated Regions

Over the past six years, we have been involved in large-scale mutagenesis experiments designed to "saturate," with presumed point mutations, specific regions of the mouse genome associated with long, radiation-induced deletion mutations. These experiments are designed: (1) to establish an estimate of the minimum number of genes within a defined-length region that are mutable to specific, biologically significant phenotypes; (2) to provide, for several regions of the genome, a fine-structure functional map, based on a series of heritable point mutations with characteristic phenotypes, which can subsequently be correlated with a detailed molecular/physical map currently being developed for these same regions; and (3) to use these fine-structure mutation maps as surrogates with which to explore the functional composition of homologous regions of the human genome.

One experiment involves a large (6- to 11-centimorgans) deletion of the albino (*c*) locus in chromosome 7. A two-generation-cross protocol places highly mutagenized [*N*-ethyl-*N*-nitrosourea (ENU)-treated], genetically marked chromosomes 7 opposite this large *c* deletion. New recessive mutations (either lethals or viable visibles) mapping to the deleted region of chromosome 7 can, therefore, be detected in one of the resultant classes of progeny. Further, any new mutation, including lethals and other detrimental variants, can be propagated in a breeding stock derived from another phenotypic class present in the progeny of these crosses.

Testing of 4525 such pedigrees has, so far, detected a total of 25 new mutations, defining at least 8 loci. Six new loci are defined by lethal mutations, one locus (*fit-1*) is defined by a series of alleles that specify a runting syndrome, and a number of repeat mutations have been detected at the

inner-ear-development locus shaker-1 (*sh-1*). Each locus has been mapped with respect to breakpoints carried by members of the panel of radiation-induced deletion mutations, thereby rapidly placing these presumed "point" mutations within the gross functional map and the emerging molecular map of this 6- to 11-cM region of chromosome 7. Identifying on a physical map those deletion breakpoints that bracket loci defined by new ENU-induced mutations will set the stage for the most fine-structure physical/functional mapping exercise yet: the location of a presumed small, intragenic, developmentally significant mutation on a molecular map. For example, at least a portion of the *fit-1* gene has been localized to a $\leq$ 300-kilobase region between the distal breakpoints of two distally extending, prenatally lethal *c* deletions. Therefore, cloning of this gene by positional-cloning techniques is imminent. *fit-1* will be a particularly interesting gene to clone, because we have recently discovered that mutations in this gene result in abnormal hematopoiesis. Mutant animals appear to exhibit deficiencies in several types of hematopoietic stem cells, and they possess erythrocytes having significantly altered morphology.

Other ENU-induced *c*-region mutations are no less interesting. One locus, defined by three non-complementing mutations, is required for development of the embryonic ectoderm in the gastrulating embryo. This locus has likewise been placed into an interval of the physical map by virtue of its initial mapping between specific *c*-deletion breakpoints. Three other mutations, all affecting early postimplantation development, have likewise been positioned between breakpoints in other regions of the deletion complex. Several of these mutations are quite interesting because they differ in severity of effect; for example, one mutant allele at one of the loci is associated with death of embryos shortly after uterine implantation, whereas another allele of the same locus causes a mild, almost imperceptible runting syndrome that has little effect on the adult. Molecular isolation of these genes, along with the analysis of the mutant gene and gene product in each case, should be quite useful in elucidating the function of these gene products within their corresponding developmental pathways.

We have been applying a similar strategy to recover ENU-induced mutations within the region covered by *p*-locus deletions. This particular mutagenesis experiment is valuable in the context of the homology between the mouse *p* region and the human 15q11-q13 region discussed above. Because a number of syndromes, including PWS, AS, and others, have been associated with alterations in HSA 15q11-q13, point mutagenesis of the homologous *p* region in the mouse may aid in defining some of the individual loci that contribute to these complex syndromes. To date, screening of 1178 pedigrees has already identified 11 mutations. One interesting set of five non-complementing mutations results in a syndrome of reduced fitness, nervous behavior, and male sterility, similar to that observed in certain *p*-deletion homozygotes. Because ENU-induced mutations are likely to be intragenic, single-gene lesions, it follows that these independent mutations identify one highly pleiotropic gene, rather than a contiguous-gene

syndrome (which might have explained the original deletion phenotype). Thus, we are also able to study the flip-side of contiguous-gene pleiotropy, by having available mutations at a single locus that result in single-gene pleiotropy.

We anticipate that, in addition to contributing to a fine-structure functional maps of genomic regions associated with deletions, these new (presumably) intragenic mutations will also be important for future use as function-deficient (or function-altered) hosts for receiving segments of cloned, wild-type DNA via transgenic-mouse technology. These types of correction-of-phenotype experiments will comprise an important strategy of gene identification for expressed DNA sequences derived from these regions, and they will be important in the end game of correlating physical maps and DNA sequence with a functional map. Furthermore, we hope that such high-efficiency mutagenesis strategies will allow the identification of developmentally significant loci in the mouse genome that might also be found in homologous regions of the human genome (e.g., as in HSA 15q11-q13 syndromes described above). In this way, perhaps specific organismal function(s) can be assigned to human DNA sequences that might otherwise be characterized only at the level of DNA sequence or biochemical function.

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GENETIC ANALYSIS

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Genetic and Functional Analyses of Chromosomal Regions Surrounding Specific Loci: Interactions with Molecular Studies

The products of germline mutagenesis studies carried out over several decades include sets of deletion mutations, each centered around one of the seven marker loci of the specific-locus test, as well as mutations centered around the Steel (*Sl*) locus. Genetic and cytogenetic analyses of the deletion complexes, carried out under this program, furnish biological "reagents" of high utility for intensive physical/functional mapping, as well as for addressing questions about the functional complexity of the mammalian (mouse and human) genome (see report by Rinchik *et al.*).

Recent analyses and the generation of strategic genetic constructs have involved several regions.

(1) For the pink-eye *(p)* region in Chromosome 7, tests with flanking markers and complementation data for most of the 1035 possible combinations between 46 *p*-locus mutations have at last revealed a relatively clear pattern that identifies 8 functional units and locates them relative to each other. The 46 mutations can be ordered into at least 22 complementation groups. Studies reported by the Molecular Genetics Group have identified striking homologies with human chromosomal regions associated with a number of disease conditions. The cleft-palate phenotype that has been associated with a limited physical region of the deletion complex corresponds to the "neonatal-lethal" function identified in the complementation studies.

(2) Genetic analyses in the agouti *(a)*-locus region are integrated with the molecular studies of R. P. Woychik's group. Nearby markers have been screened (with negative results, to date) for possible involvement in *a*-locus deletions. Genetic constructs have been made involving rearrangements that produce an *a*-locus phenotype, and a complementation map is being generated; two functional units for prenatal survival have been located on this map.

(3) Collaborative studies with D. M. Kingsley (Stanford University School of Medicine), involving our short-ear *(se)* mutations led this year to the cloning of the short-ear gene, which was shown to code for bone morphogenetic protein-5. The complementation map greatly facilitated the mapping of critical probes, and the final identification of the *Bmp-5* gene was

accomplished by one of our viable *se* mutations that was found to involve an 84 kb deletion.

(4) Among dilute *(d)*-locus mutations, we found some to be deletions long enough to involve staggerer *(sg)*, which controls certain neurological functions. Collaborative studies have been initiated.

(5) Over 60 mutations involving the Steel *(Sl)* locus are being analyzed in an ongoing project that is providing genetic reagents for molecular studies by L. J. Stubbs. The phenotype associated with several of these mutations differs with regard to viability, pigmentation, and/or fertility pattern from the original Steel prototype. At least 6 (and possibly as many of 11) of the *Sl* mutations are translocations, suggesting that the locus (located in 10D1.32) may represent a mutational "hot spot" in the mouse genome. Each *Sl* translocation involves (in addition to Chromosome 10) a different autosome.

Complementation and other genetic analyses involving albino *(c)* and dilute *(d)*-locus mutations were completed earlier, but stocks are maintained and supplied for collaborative studies. Genetic analysis of brown *(b)*-locus mutations has been carried out by E. M. Rinchik's group. Mapping of the piebald *(s)* locus relative to microsatellite probes is being carried out in collaboration with S. M. Tilghman (Princeton University).

Other Studies in Basic Genetics and Cytogenetics

(1) A pericentric inversion, a very rare type of aberration in the mouse, was generated in a mutagenesis experiment and found to involve breakpoints at 8A1 and 8A4, with consistently nonhomologous synapsis in the inverted segment. This inversion has been characterized by fluorescent *in situ* hybridization (FISH). The results indicate that telomeric sequences are promoting recombination not only within these sequences themselves but over a distance of >200 kb.

(2) SXR-stock males carry, in addition to the normal *Sry* (testis-determining factor) sequences in the proximal portion of the Y chromosome, also a second copy of *Sry* in the very distal portion of the Y which undergoes obligatory crossovers with the X, producing XX males. Within this stock, we discovered exceptional females that had an XY karyotype but were fully fertile. While our original hypothesis was that the proximal *Sry* sequences had been deleted in these females, FISH studies with an *Sry* probe showed that this was not the case and suggested instead that an inactivating mutation had occurred; carrier females are designated XY*. A cytogenetic study of the progeny of XY* females indicates that about one-half are products of X-Y* nondisjunction. Thus it appears that there may be an absence of X-Y* pairing in meiosis.

(3) Male sterility, a dominant effect of certain mutagenic treatments, is usually observed with considerably higher frequency than are dominant visible phenotypes. In a long-range project, sterile sons from mutagenesis experiments are routinely analyzed by histological and cytogenetic techniques. The great majority of the relatively frequent sterile sons derived from postspermatogonial treatments have been found to carry chromosome rearrangements that either involve a sex chromosome, with breakpoints located randomly, or two (or more) autosomes, with breakpoints that are close to telomeric and/or centromeric regions. By contrast, the rare sterile sons found in the progenies of mutagenized spermatogonial stem cells are almost always karyotypically normal, the sterility resulting from occlusion of spermatic ducts.

Outreach and Service

The valuable mutant resource was used to varying extents by most of the other investigators in the Section (see publications listed under other reports in this volume). In addition, over the past 4 years, over 80 outside laboratories (including 10 in foreign countries) utilized our mouse stocks for either collaborative or independent research, many of them receiving multiple stocks. Three major categories of stocks are being propagated at ORNL: (1) large stocks used either directly in mutagenesis studies, or employed for the generation of other stocks used in such studies; (2) standard or specially constructed inbred, recombinant-inbred, or congenic strains, or stocks combining specific marker utilized for genetic experiments or molecular studies; (3) hundreds of small stocks propagating mutations that have arisen (and continue to arise) in mutagenesis experiments over the past decades, and whose value for genome analysis is becoming increasingly recognized. An embryo-freezing program involving numerous stocks is under way, but new stocks arise at a faster rate than they can be cryopreserved.

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GENOME ANALYSIS

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Developing Animal Models for the Study of Human Birth Defects

In recent years, genes responsible for a number of serious human hereditary disorders have been isolated and characterized on the molecular level, opening new avenues of early diagnosis and treatment for each disease. Despite this remarkable progress, the causes of most common human birth defects remain unknown. Although many birth defects are clearly not inherited, most are likely to involve the perturbation of genes or gene products required for the normal progression of specific developmental pathways. One way to gain access to some of these genes is to study animals expressing heritable disorders that closely resemble birth defects commonly seen in human infants, since these related mouse and human disorders are likely to result from disturbance of the same or similar series of morphogenetic events.

Work in our laboratory is focused on the analysis of a collection of mutants that have been generated and maintained at Oak Ridge. These mutants, which arose as part of an ongoing mutagenesis program conducted by W. M. Generoso, (see report by Chromosomal Damage Group), express a wide variety of profound developmental disorders which resemble human birth defects of serious medical consequence. Each of these animals carries a reciprocal chromosome translocation which visibly marks the site of mutation, and greatly simplifies our search for the disrupted genes. As part of Generoso's program, each mutant has been subjected to cytological examination, allowing the breakpoints of each translocation to be assigned to specific chromosomal subregions. These cytological studies represent the first crucial step toward the mapping and positional cloning of genes disrupted by each translocation.

Our goal is to gain access to the developmentally-essential gene(s) disrupted in each of these mutants, to provide access to morphogenetic pathways that are most sensitive to genetic and environmental perturbations in man. To this end, we have developed a general strategy for the localization of genes disrupted by a translocation, based upon the use of DNA probes from the cytologically-determined breakpoint region in fluorescent *in situ* hybridization (FISH) analysis of normal and mutant chromosome spreads. This project has been initiated only recently, and is therefore in its early stages. We are currently concentrating our efforts on FISH mapping of breakpoints associated with three specific mutations: (1) 1Gso, a 2;14 translocation which produces a dominant neurological defect and recessive embryonic lethality; (2) 11Gso, a 5;16 translocation, resulting in pronounced hydrocephalus which kills homozygotes

before three weeks of age; and (3) 9Gso, a 4;9 translocation producing severe defects of the lower spine and extremities in the small number of homozygous animals that survive to birth. As time progresses and the project takes on momentum, we expect to apply these mapping methods to the analysis of new translocations arising from Generoso's ongoing studies. These cloned genes will provide new means of tracing specific factors, both genetic and environmental, contributing to similar anomalies in human infants. An understanding of specific gene products which must be expressed during critical stages of mammalian development will provide the means through which intelligent methods of prevention and treatment of these devastating defects may finally be identified.

INSERTIONAL MUTAGENESIS IN TRANSGENIC MICE AND THE MOLECULAR ANALYSIS OF MOUSE MUTATIONS

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Molecular analysis of mutations in the mouse is an efficient way of establishing structure/function relationships between individual genes and complex developmental traits in the whole organism. Insertional mutations in transgenic mice are particularly useful for this purpose because the mutant locus is "tagged" with the exogenously added DNA and can be readily characterized at the molecular level. Over the past five years we have identified a number of different insertional mutations with a range of phenotypes that include embryonic lethalities, fertility defects, skeletal deformities, inner ear defects and kidney disease. Additionally, we have generated a number of transgenic lines with unusual patterns of coat pigmentation, all of which were produced with the same tyrosinase minigene as a transgene. These lines with unusual pigmentation may prove to be useful as "enhancer traps" to identify important developmental regulatory sequences. Overall, we are now directing most of our attention to the characterization of several different mutants at the molecular level.

Molecular Analysis of Insertional Mutations

In the Tg737 insertional mutant, homozygous animals on the FVB/N inbred background develop a complex phenotype that involves bilateral polycystic kidney disease (PKD). Our analysis of the developmental pathology in this line revealed that the mutant phenotype has a remarkable similarity to autosomal recessive polycystic kidney disease (ARPKD) in humans; hence, we believe that this mutant line will serve as a useful animal model for studying ARPKD in humans. We have now identified, cloned and mapped a cDNA that corresponds to a gene whose

expression is interrupted in the Tg737 mutant animals. Moreover, we have also cloned and sequenced the homologous human gene, which is >90% identical with the mouse gene. In collaboration with Dr. Steve Reeders' laboratory at Yale University School of Medicine, we are currently in the process of determining whether the human homolog of the Tg737 gene is directly associated with human ARPKD.

In another line, called Tg370, a recessive insertional mutation causes the development of a characteristic skeletal defect involving an undulation of the distal vertebrae of the spine. We have now cloned and completely characterized the structure of the mutant locus in this line. Additionally, utilizing evolutionarily conserved regions within the sequences flanking the transgene integration, we have cloned a region of the human genome that corresponds to the mutant locus of the Tg370 line. The Tg370 integration site maps to the proximal section of chromosome 5 in the mouse, very close to the dominant Thick-tail (*Tht*) mutation. We are currently in the process of investigating whether the Tg370 insertional mutation is a recessive allele of *Tht*.

In the Tg2742 line, a recessive insertional mutation causes a vigorous circling behavior and hearing defects in animals homozygous for the transgene. Analysis of the inner ear in the mutant animals revealed that the organ of Corti has characteristic defects that are likely to be associated with the circling behavior and hearing abnormalities. We are currently in the process of further characterizing the mutant phenotype, and will continue our experiments directed at cloning the mutant locus.

Characterization of The Mouse Agouti Locus

We are also using probes derived from transgenic mice for the molecular characterization of other mutations in the mouse. For example, one such mutation, called Is1Gso, arose in one of W. M. Generoso's radiation experiments. In this mutation, new alleles of limb deformity (*ld*) and agouti (*a*), two loci normally separated by 20 cM on Chromosome 2, arose simultaneously. Utilizing molecular reagents from the *ld* insertional mutant, we were able to clone and characterize the structure of the Is1Gso mutant; this ultimately allowed us to clone the agouti region on chromosome 2. We are now in the process of studying the structure of the numerous agouti locus mutations that are currently being maintained within the mutant stocks in the Mammalian Genetics Section. We have also determined that two of the dominant mutations at the agouti locus cause the agouti gene to be ubiquitously over-expressed, which provides molecular insight into the obesity and type II-diabetes traits that are exhibited by these mutant animals. Additionally, reagents from the agouti region have been used to clone another gene mapping close to agouti that we believe is directly associated with the preimplantation embryonic lethality of Lethal Yellow, an agouti locus mutation that has been

extensively analyzed with embryological techniques. Efforts are currently under way to establish the role of this new gene in preimplantation mouse development.

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MURINE TARGETED MUTAGENESIS

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The major focus of the Targeted Mutagenesis Group is to determine the biological function of genes *in vivo*, utilizing embryonic stem (ES) cell technology. Murine ES cells can be manipulated *in vitro* to disrupt an endogenous gene by homologous recombination. Targeted clones are identified and these cells are introduced into blastocysts (3.5-day-old mouse embryos). The blastocysts are then implanted into pseudopregnant female mice to resume normal development. During development, the targeted ES cells may contribute to all tissues of the embryo, including the germ-line. Mice in which the targeted ES cells have made a contribution are referred to as chimeric. Through appropriate breeding strategies, mice are generated that are homozygous for the mutated gene of

interest. The characterization of these null mutants helps to gain important insight into the biological function of the mutated gene in the context of the whole animal system. Furthermore, these animals become important reagents for more detailed analysis of gene function. Most importantly, the creation of null mutants of genes that are homologous to those associated with diseases in humans can provide murine models for these genetic diseases.

A majority of our efforts have been spent establishing a targeted-mutagenesis laboratory within the Biology Division. M. L. Mucenski disrupted the *Evi-1* proto-oncogene while a postdoctoral fellow at Children's Hospital, Cincinnati, Ohio, and brought this project with him to ORNL. *Evi-1* was identified as a common site of retroviral integration in murine myelogenous leukemias of the AKXD-23 recombinant inbred mouse strain. Biochemically, *Evi-1* encodes a sequence-specific DNA-binding protein that contains 10 zinc-finger domains that are similar to those found in TFIIIA, a *Xenopus* RNA III transcription factor. Seven targeted clones have been identified and over 20 chimeric animals generated. Two animals have proved to be germline chimeras, but neither has passed the altered *Evi-1* allele to any progeny.

Another proto-oncogene disrupted by M. L. Mucenski while he was a postdoctoral fellow is *c-Myb*. Studies of *c-myb* expression are now being continued in the Biology Division. The *c-Myb* proto-oncogene encodes a sequence-specific DNA-binding protein that is predominantly expressed in immature hematopoietic cells. As these cells differentiate, *c-Myb* expression decreases. Whereas heterozygous *c-Myb* mice are indistinguishable from wild-type animals, homozygous mice die at 15.5 days of gestation due to severe anemia. The homozygous mutant animals are apparently unable to switch from embryonic-yolk-sac to fetal-liver erythropoiesis. Additional hematopoietic lineages are also affected by the lack of *c-Myb* expression. Current studies include the detailed analyses, using a variety of *in vivo* as well as *in vitro* methodologies, of the hematopoietic potential of mutant fetuses in comparison to phenotypically normal littermates at various days of gestation. Transgenic animals are being generated that express the *c-Myb* cDNA using ubiquitous or lineage-specific heterologous promoters. Through appropriate breeding strategies, the transgenes will be expressed in mutant animals in an attempt to rescue the mutant phenotype. These studies will provide important insight into the biological function of the *c-Myb* proto-oncogene in mice.

Targeting constructs have been made to study the function of three cytokines, which are low-molecular-weight proteins that are secreted by antigen-activated cells of the immune system. The primary function of cytokines is to regulate immune-cell interactions through both direct and indirect modes of action. The ability of some cytokines to trigger a cascade of pleiotropic effects has made it extremely difficult to delineate their precise mode of action. Targeting constructs have been made for gamma interferon (γ-INF), tumor necrosis factor

alpha (TNF-α), and tumor necrosis factor beta (TNF-β). Targeted clones have been identified for γ-INF and TNF-β. Chimeric animals are currently being generated using several TNF-β clones. Recently, cells have been electroporated with the targeted construct for TNF-α, and colonies are currently being analyzed to identify targeted events.

CHARACTERIZATION OF MUTANT MOUSE STOCKS

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Studies on the Immune-Deficient Mutant, Scurfy

We have characterized the pathology of the X-linked recessive mutation scurfy which causes exfoliative dermatitis, anemia, cachexia, and death at approximately 24 days of age. The disease is associated with a lymphohistiocytic proliferation in the liver, skin, and lymphoid organs. By a number of experiments, we have generated evidence that the lesions in scurfy mice are mediated by T lymphocytes which must be generated in a genetically scurfy thymic microenvironment. Our studies involving fetal thymic transplants have demonstrated that the surfy defect acts upon the fetal thymic environment very early in its development, committing T cell precursors to initiate the disease phenotype.

Further studies have implicated the $CD4^+CD8^-$ helper T cell subset as critical to disease initiation and progression. Treatment of scurfy mice with anti-CD4 monoclonal antibodies (mAbs) doubles the lifespan of the animals while anti-CD8 mAbs have no effect. Similar results are seen when the scurfy gene is bred into transgenic mouse lines that lack CD4 or CD8 cells. In order to determine whether disease in the scurfy mouse is associated with a failure in thymic negative selection, we have tested the ability of *sf*/Y mice to thymically delete T cell clones expressing TCR V_β elements reactive with endogenous *Mls* and *I-E* antigens. In both cases, the thymi of *sf*/Y mice failed to delete the appropriate clones. We currently hypothesize that expansion of these (or similar) self-reactive clones within the body — and their subsequent interactions with other cells of the immune system — allows for an overproduction of cytokines which, in turn, leads to scurfy disease and death. Cytokine mRNA expression by scurfy splenocytes *in vitro* was assayed by RT-PCR. Messenger RNA levels for IL-4, IL-6, IL-10, and TNF-α were all found to be increased in comparison with levels in normal control splenocytes.

Studies on Insertional Mutants

Additional characterization of the Tg737 insertional mutant by electron microscopy and immunohistochemistry demonstrated marked similarities between lesions in this mouse and those seen in autosomal recessive polycystic kidney disease (ARPKD) in humans. Allelism testing was done to compare Tg737 to an unmapped spontaneous mutation in BALB/c mice (*bpk*) that also produces polycystic kidneys; the mutations were not allelic. Histologic sections from other transgenic lines showing reproductive failure were examined. Lesions noted included aspermia, interstitial cell hyperplasia, failure of ovulation, failure of implantation, malformation of tubular structures (uterus, cervix), and one pseudohermaphrodite.

Pathogen Identification

A previously undescribed *Corynebacterium* sp. was isolated in the 9211 barrier colony in May 1992. This organism caused severe dermatitis in suckling nude mice with 100% mortality. Since that time, a similar organism has produced disease in mouse colonies at several universities across the country. Collaborative studies are under way to identify this pathogen more accurately.

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MOUSE MUTAGENESIS

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The specific-locus test, which has gained considerable utility as a result of recent genetic and molecular analyses of the marker loci and surrounding regions (see other sections of this Progress Report), is being used to study all germ-cell stages in the male (from spermatogonial stem cells to mature spermatozoa), and, to a more limited extent, in the female.

Enlarging the Germ-Line Mutagenicity Data Base

One objective of this program has been to enlarge the germ-line data base for different chemical mutagens in order to explore whether certain patterns of response might be demonstrable. Since the time of the last report, we completed large-scale experiments with glycidol, AZT, and bleomycin. Our protocol has included procedures that also yield accurate comparative *productivity* results, which can be interpreted with regard to induction of presumed dominant lethals (i.e., major chromosome-breakage and -loss events) and/or germ-cell cytotoxicity, thus adding these outcomes to gene-mutation-rate data.

Expansion of the specific-locus-mutation data base has allowed us to make an examination of the effects of germ-cell stage on both quantity of mutation yield and nature of mutations (for the latter, see below). For chemicals mutagenic in poststem-cell stages, three patterns have been identified according to the stages in which they elicit maximum response: (1) early spermatozoa and late spermatids; (2) early spermatids; and (3) differentiating spermatogonia.

The ability to generate large numbers of mutations, and a high proportion of deletions and other rearrangements among them, makes a mutagen valuable for studies seeking to recover developmentally significant new variants that are amenable to molecular access. We have shown chlorambucil (CHL) and melphalan (MLP) to be such mutagens. In collaboration with other laboratories, CHL is being used to generate phenotypically detectable new recessive viable and sublethal mutations throughout the genome.

We have augmented the Oak Ridge historical specific-locus control rate for males by over 100,000 offspring, increasing sensitivity in statistical calculations of induced rates. A new historical control rate for females has been derived that is considerably superior to the rate previously used (see below).

Factors that Affect the Nature of Induced Mutations

The numerous mutations involving each of the specific loci that have been accumulated over the past several decades have facilitated intensive physical/functional mapping of regions surrounding the marker loci (see reports by Molecular Genetics and Genetic Analysis Groups). In turn, the knowledge gained has improved our capability for characterizing the nature of mutations.

Based on recently gained information concerning the phenotype of the ablated condition at each locus, we carried out a retrospective analysis of published and unpublished data that classified mutations on the basis of their phenotype as LL (large lesions), IG (intragenic), or OL (other lesions). This analysis indicated that, regardless of the nature of the chemical, very high frequencies of LLs are induced in postmeiotic stages, but low frequencies in either stem-cell or differentiating spermatogonia. In the data used for this analysis, however, the contribution of mutations derived from different germ-cell stages was different for different chemicals, somewhat limiting the validity of the comparisons. More recently, we have analyzed mutations induced by chemicals that are active in both pre- and postmeiotic stages, using molecular as well as genetic and cytogenetic criteria. These analyses confirmed the low incidence of IGs and high frequency of LLs for mutations induced in postspermatogonial stages, and the reverse for spermatogonially-induced mutations.

An exciting result came from our comparison (using direct molecular analyses as well as retrospective criteria) between mutations induced in previously undisturbed spermatogonial stem cells and those induced in "sensitized" spermatogonia, i.e., those that received a challenging dose of X rays 24-h following a priming dose. The latter were found to have a considerably higher LL/IG ratio, similar to the ratio observed for mutations induced in poststem-cell stages. Earlier studies had shown a major augmentation (above additivity) in mutation rate to result from 24-h fractionation treatments. Our new finding of a qualitative difference indicates that the additional mutations are the result of the second (challenging) dose and that the mutation-rate-augmenting effects are not due, merely, to an increase of a normally responsive component of the spermatogonial population. The finding that the additional mutations are primarily large DNA lesions suggests that the nuclear state of sensitized stem-cell spermatogonia may be different from the state of previously undisturbed spermatogonia and similar to that of postspermatonial stages.

Spontaneous Mutation Rate in Females

Earlier data on specific-locus mutations in the progeny of untreated females presented two problems. First, the total number of mutational events was low (only 3 independent mutations in 210,993 offspring), yielding wide confidence limits. Second, one of the three mutations was recovered as a sizable cluster that,

for years, has complicated interpretations of a spontaneous rate for females and has resulted in alternative spontaneous rates being used in different calculations.

Because oocytes, all of which are already present at birth, do not undergo any mitotic divisions, clusters can arise only from mutation in pre-oocyte stages. We used evidence from *visibly mosaic* mutants recovered in the F_1 generation of specific-locus-test (SLT) experiments (i.e., the generation that is scored for mutants) to address the question of whether spontaneous mutations occur with equal frequency at each pre-oocyte cell division. This evidence strongly suggests that an appreciable percentage of spontaneous mutations arise preferentially in a single DNA strand in a post-mitotic stage of gametogenesis.

Because the cluster that has complicated the literature thus clearly did not arise in a parental oocyte (but probably as a single-strand mutation in the gamete of a *grand*parent of the generation that is being scored for mutations), we concluded that it is appropriate to use only the "single" mutations as control for mutations that are *induced* in oocytes. For calculations of spontaneous rate, we further added results from several experimental groups that involved treatments proved to be ineffective. The lowering of the rate (via removal of the cluster), as well as the increase in sample size, both increase the chance of statistically detecting a mutagenic effect in a given experiment. The newly derived spontaneous specific-locus mutation rate for females, 1.6×10^{-6} per locus [6/(536,207 x 7)], is highly significantly below the spontaneous mutation rate in males.

Induced Mutations in Females

Compared with data for males, relatively little information exists concerning the mutagenic effects of chemicals in females. For radiation-induced specific-locus mutations in females, however, a large body of data has been accumulated. We recently reviewed this with respect to the information it provides about biological and physical factors that influence mutation yields in the hope that this will lead to a more knowledgeable interpretation of past and ongoing chemical mutagenesis studies.

Of only five chemicals so far explored for their effect in oocytes (the study of a sixth chemical, bleomycin is currently under way, see below), two (ethylnitrosourea, ENU, and triethylenemelamine), and possibly a third (procarbazine hydrochloride), are mutagenic -- with at least one of these (ENU) mutagenic in arrested as well as maturing oocytes. However, the mutation rate is, in each case, lower than for treated male germ cells. By contrast, we found the ENU-induced mutation yield for the maternal genome of the zygote to be an order of magnitude higher than that for the zygote's paternal genome or for spermatogonia (in which ENU has been identified as a "super-mutagen"). A high proportion of mutants derived from chemical treatment of oocytes (including the

oocyte genome in zygotes) are mosaics, probably owing to lesions affecting only one strand of the DNA.

Specific-locus mutation studies are under way with bleomycin, a chemical that is female-specific (i.e., negative in males) in dominant-lethal tests. It appears that specific-locus mutations are induced in females during the period of maximum dominant lethality (weeks 2-4), but the rate may be difficult to determine with accuracy because of the very low survival for these stages.

A characteristic of specific-locus mutations induced in oocytes is that they include a considerably higher percentage of large (multi-locus) lesions (LLs) than do mutations induced in spermatogonia. For each germ-cell type, the frequency of LLs appears lower for the chemicals so far tested than for radiations.

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CHROMOSOMAL DAMAGE

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Chemical and radiation mutagenesis research in mice has proved to be a driving force in advancing fundamental mammalian biology. Its contribution comes primarily from studies of the underlying mechanisms of mutation induction and from molecular and developmental analyses of valuable mutants that are generated in mutagenesis experiments.

Possible Effects on Gene Expression from Exposure of Pregastrulation Embryos

Progress to be made in the prevention of human birth defects hinges, for the most part, on understanding the etiologies of a large class of developmental anomalies whose causes are not known. There is strong likelihood that many of them originate from events that occur during the pre-organogenesis stages of embryonic development. This group has been the pioneer in exploring developmental toxicity during early embryogenesis. It is now amply evident in mice that exposure of pre-organogenesis stages to certain agents can lead to fetal abnormalities, many of which are analogous to those in humans that are of unknown etiology. Our working hypothesis has been that the normal pattern of gene expression in early embryogenesis is disrupted by epigenetic mechanisms. A recent highlight from this project is the induction by retinoic acid of a novel set of malformations that feature homeotic phenotypes such as supernumerary limbs, ectopic organs, and duplication of the lower body segment. These anomalies were induced by treating mouse embryos in the blastocyst stage, providing evidence that axis and pattern formations are determined prior to gastrulation.

Female-Specific Mutagens

We have found a number of intercalating and alkylating chemicals to be mutagenic in females but not in males. In humans and in all mammals, the chromosomes in the majority of oocytes of adult females are arrested in the diplotene stage of meiosis with the chromatin in a diffuse state. In adult males, on the other hand, all stages of spermatogenesis are present simultaneously, and in all cells that are postmeiotic, the chromosomes are relatively more condensed than they are in arrested oocytes. We hypothesize that the female-specific response found by us is attributable to the diffuse nature of the oocyte DNA which allows molecules either to intercalate between base pairs or to bind to specific sequences.

Creation of a Translocation Resource for Morphogenetic and Molecular Studies

Chromosome rearrangements provide highly favorable reagents for molecular access to genes of developmental interest. Translocations with detectable phenotypes promise to be important tools for physical mapping of the breakpoints and for relating DNA structure to normal and abnormal development. They have been a dependable source of specific malformations and functional deficiencies that are used as models of human disorders and provide materials for morphogenetic studies that could not be conducted in human subjects.

Because chromosome rearrangements have such a high potential for contributing to the progress to be made in molecular developmental biology, and because our laboratory is the only one producing this class of mutations in appreciable numbers during the course of large-scale mutagenesis experiments, we have embarked on a systematic search for translocations and other rearrangements that produce potentially useful phenotypes in homozygotes or heterozygotes: we are establishing a repository for such rearrangements, and are mapping their breakpoints by high-resolution G banding.

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ORGANISMIC EFFECTS

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Assessment of Dominant Damage for Radiation, Chlorambucil (CHL), and Ethylnitrosourea (ENU)

An important gap in knowledge regarding genetic risk estimation for chemicals and radiation is the degree of correlation between specific-locus mutation frequencies and the frequencies of induction of dominant mutations that cause serious organismic damage in first-generation progeny. The first large-scale attempt to provide an understanding of this relationship is under way in experiments in our laboratory using the Assessment of Dominant Damage (ADD) approach. First-generation progeny are being examined for several types of dominant organismic damage following exposure of their fathers to treatments expected to induce either large DNA lesions, such as deletions and other rearrangements (chlorambucil to early spermatids or sperm), small intragenic lesions (ENU to spermatogonial stem cells), or a mixture of lesions (X- or γ-rays to spermatogonial stem cells). All groups have concurrent controls. The types of dominant organismic effects that are studied in the progeny are skeletal malformations, cataracts in the lens of the eye, stunted growth, dominant visibles, and survival to 11 weeks of age. It is estimated that several thousand genes can mutate to dominants that cause the many types of organismic damage that we observe.

With analyses now complete on about 40% of the contemplated total progeny of 16,000, only the ENU treatment shows a significant increase in any of the four types of skeletal analyses thus far applied, and it shows an increase in all four analyses. Also, only the ENU treatment has thus far been shown to induce a significant increase in serious cataracts, and it is also yielding by far the highest specific-locus mutation frequency of the four treatments. Even with ENU, however, the frequency of dominant damages is rather low (e.g., 1–2% for serious skeletal anomalies.) When one considers the large number of recessive mutations that must be induced in the entire genome (based on specific-locus mutation frequencies), it is obvious that few induced gene mutations are capable of causing serious organismic damage in heterozygotes.

Despite the high frequency of specific-locus (recessive) mutations that has been demonstrated for CHL (Russell *et al.*, *PNAS* **86**: 3704-3708, 1989), none of the four skeletal analyses in the ADD experiments gave any clear suggestion of induction of dominant mutations. It thus appears that the DNA lesions induced by CHL are poorly correlated with dominant organismic damage.

There is thus far no suggestion of any more than a very slight effect, if any, for the protracted radiation exposure. Some of the analyses for the acute radiation exposure group suggest a slight effect. Our large current experiment that is using gamma radiation will provide data on induced dominant organismic damage for the first time for a low dose rate, and it also incorporates many advances over our earlier experiment that has been applied in risk estimation. It thus seems likely that it will provide the basis for a major improvement in genetic risk estimation for radiation. The findings in both radiation experiments already strongly suggest that there is no large error of underestimation in UNSCEAR's (United Nations Scientific Committee on the Effects of Atomic Radiation) direct estimate of genetic risk following paternal irradiation, even if that estimate is applied to all genetic disorders causing serious handicaps by young adulthood, including those of complex etiology.

Development of a More Powerful Test for Mutagenicity

Our finding that ENU induces enough mutations to cause a statistically significant shift in the proportions of offspring with many of the more common (and non-serious) skeletal anomalies suggests that samples of only 100 control and 100 experimental offspring would often be large enough to permit demonstration of statistically significant induction of dominant mutations by a mutagenic treatment as strong as the one with ENU, even if one restricts analysis to just the more common anomalies. The data from the ADD experiments provide rich material for developing a mutational test that will have the significant advantages of (1) dealing directly with dominant mutations, (2) requiring production of markedly fewer animals, and (3) having a close relationship with one of the two major methods of genetic risk estimation.

Findings Relevant to Claims by Others that Radiation Induces High Frequencies of Dominant Tumor Mutations

This year, in an analysis of data collected many years ago by several members of the Biology Division, we investigated two other types of dominant organismic damage, namely longevity reduction and incidences of major diseases, including neoplasms, as detected by a careful autopsy at the time of natural death (Cosgrove *et al.*, in press). Offspring derived from stem-cell spermatogonia of males exposed to 600 R of acute X radiation were compared to concurrent controls. No hint of any effect of the treatment was found for either longevity or the incidences of major diseases.

Nomura (see, for example, *Nature* **296**: 575-577, 1982) and others have reported high frequencies of tumors in offspring of treated males and have assumed that these are the result of dominant mutations. Results of our recent ADD experiments (see above) provide a possible explanation for the discrepancy with our findings. The ADD data clearly indicate a strong negative correlation between litter size and body weight. In experiments in which high frequencies of dominant lethals are induced, the offspring are therefore presumably considerably larger than in experiments in which litter sizes have remained approximately normal. Anomalies that are positively correlated with body weight, such as neoplasia (as has been reported for mice and rats) would thus be expected to be found mostly in the former types of experiments. It is noteworthy that the increased F_1 tumor incidence reported by Nomura and others followed treatments that induce substantial dominant lethality. By contrast, the ORNL experiments of Cosgrove *et al.* involved irradiation of spermatogonial stem cells (see above) and therefore caused virtually no litter-size reduction.

Organismic Effects of Insertional Mutations

We are examining homozygotes from approximately 200 transgenic lines of mice (generated in the program of R. P. Woychik) for skeletal malformations and cataracts. Two recessive insertional mutations have been found to have especially interesting skeletal features. One of these, Tg737, causes a large number of skeletal abnormalities. A detailed characterization of this mutation is in progress, with one of the most interesting features being that homozygotes for this mutation often have too many molars if they are on the FVB/N genetic background and too few molars if they are on the C3Hf genetic background. Work in progress is expected to associate at least a few additional transgenes with skeletal effects or cataracts of clinical and/or developmental importance.

Studies of Individual Dominant Mutations

As part of our interest in learning more about the nature of induced dominant mutations, we studied the interactions between the radiation-induced dominant skeletal mutations cleidocranial dysplasia (*Ccd*) and short digits (*Dsh*). Seven synergistic interactions and three antagonistic interactions were demonstrated (Selby *et al.*, in press). Under certain circumstances, such interaction phenotypes of dominant mutations could be confused with recessive inheritance. *Dsh* homozygotes exhibit the remarkable feature of completely lacking vertebrae. In spite of this, on at least one genetic background, these short-limbed dwarfs have normal viability until the time of birth. In collaboration with E. M. Rinchik and with L. Flaherty (SUNY, Albany), we have recently shown the *Dsh* gene to be located near the proximal end of chromosome 5.

Radiation-Induced Specific-Locus Mutation Frequencies in Oocytes Present Near Time of Birth

We completed an extensive analysis of specific-locus data collected from female mice irradiated near the time of birth and from their controls (Selby *et al.*, 1991). A dose-rate effect was demonstrated both for mutation induction and for oocyte killing, as judged from fertility. This experiment provided a large body of data on a major group of oocytes additional to the two groups (mature and maturing, and immature) that had earlier been studied extensively in adults. The results in all three groups suggest that the genetic hazard from radiation is considerably smaller for women than for men. The state of the nucleus of mouse oocytes present around the time of birth (this study) may be the one most comparable to that of the human oocytes that are predominantly at risk to radiation.

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MAMMALIAN BIOCHEMICAL GENETICS

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Our group has continued studies on animal models of human diseases that involve abnormal hemoglobins and virus-induced immunodeficiencies. Clinical hematology, biochemical and immunological methods, and molecular biology are being used to characterize induced mutations, altered gene products, and viruses that cause pathophysiological effects in mice. Results of our recent studies are summarized below.

Development of a Mouse Model of Sickle Cell Disease

We have developed an improved transgenic mouse model of sickle cell disease. The Hb S Antilles transgenes from a previously described transgenic line of mice (Tg58Ru) were bred onto the genetic background of a recently developed line of mice (MHOAH) that produces mutant, high-oxygen-affinity hemoglobins (P_{50} of 24.5 mm Hg) rather than normal mouse hemoglobins (P_{50} of 40 mm Hg). The electrophoretic patterns of hemoglobins separated on Titan III cellulose acetate plates established that the red cells of these transgenic sickle cell mice contain a mixture of the high-oxygen-affinity forms of mouse hemoglobin, the low-oxygen-affinity Hb S Antilles (P_{50} of 40 mm Hg), and hybrid hemoglobins comprised of human and mouse globins. These transgenic Hb S Antilles mice exhibit many hematological and pathological symptoms commonly found in patients with sickle cell anemia. The peripheral blood contains a higher (15%) than normal (2.5%) frequency of reticulocytes, and numerous misshapen erythrocytes that resemble irreversibly sickled red cells. The spleen is enlarged, congested, and laden with stainable iron. Splenic erythropoiesis is expanded to compensate for the shortened life span of the misshapen erythrocytes in blood of these transgenic mice. The kidneys are congested, and glomerular atrophy, thickening of the capsular epithelium and iron deposits in the proximal tubules all indicate that vascular damage has occurred in the glomerulus. The lungs are contracted, and the alveolar septa are thickened and congested. Several factors may contribute to the red cell sickling and secondary organ pathology in this improved transgenic mouse model of sickle cell disease. In the venous circulation, the difference between the low-oxygen-affinity of Hb S Antilles and the high-oxygen-affinity of the mutant forms of murine hemoglobins favors preferential deoxygenation of Hb S Antilles; the deoxygenated form of Hb S Antilles polymerizes to cause red cell sickling, red cell membrane damage, water loss and the formation of irreversibly sickled red cells, and the organ pathology observed in these transgenic sickle cell mice. We believe that these transgenic sickle cell mice will provide a valuable experimental animal model for additional studies on the natural progression of and possible therapeutic intervention against sickle cell disease.

Identification of Retrovirus-Infected Lymphoid Cells in B10.F Mice

A unique ecotropic murine retrovirus (B10FV) infects newborn B10.F congenic mice via the milk, but the B10 inbred partner is resistant to B10FV. B10FV readily integrates into the DNA of somatic cells in lymphoid organs of B10.F mice and causes splenic hyperplasia, lymphocyte subset perturbations, immunodeficiency, and life shortening in B10.F mice. The lymphoid organs of B10.F mice contain high titers of B10FV. We have obtained a rat-anti-MuLV monoclonal antibody, which is specific for a MuLV envelope glycoprotein, from Dr. Leonard Evans, Rocky Mountain Laboratory. We wanted to determine whether the antibody would detect MuLV antigen on the surface of B10FV-infected cells in order to characterize the target cells of B10FV in neonatal B10.F mice. A FACStar^{PLUS} flow cytometer and fluorochrome-conjugated antibodies were used to analyze cellular suspensions of thymus, mesenteric lymph node and spleen of neonatal and young B10.F mice. MuLV antigen was detected on approximately 30% of the cells obtained from lymphoid tissues of B10.F neonates but MuLV antigen was not detected on similar cells obtained from B10 mice, which suggested that the rat-anti-MuLV monoclonal antibody was able to detect a retroviral envelope glycoprotein on the surface of B10FV-infected B10.F lymphoid cells. A higher percentage of virally infected cells was present in the mesenteric lymph node and spleen than in the thymus of neonatal mice, which is consistent with the hypothesis that newborn B10.F mice acquire the viral infection via their mother's milk. Combined use of the rat-anti-MuLV and other cell surface antibodies showed that B10FV is expressed on the surface of $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes, but additional studies will be required to determine whether one or all of these lymphocyte subsets is a target cell for the infection of B10FV in newborn B10.F mice. The immune deficiency syndrome, accelerated ageing, and early death of viremic B10.F mice represent the best cluster of signs described to date for an animal model of AIDS in humans. We believe that B10.F mice could be a useful experimental animal for studies on methods to modify subsets of lymphoid cells to reduce the risk of retroviral infections in newborn mice, to increase the resistance to viral replication and the onset of immune deficiency in infected mice, and hopefully to extend the results of such studies to HIV-infected humans.

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Genome Mapping Program

Overview - L. B. Russell

In mid-1992, we initiated a multi-task project on mapping in mouse-human homology regions designed to begin construction of directly comparable, fine-structure physical and functional maps that will make it possible to exploit the mouse more efficiently as a model genetic system. Two of the tasks have now been under way for a year, namely, the physical mapping of mouse chromosome (Mmu) 7, and the application of computer technology to problems of data analysis and data management. Two additional tasks to be considered for the future involve development of a large targeted-mutagenesis program for determining organismal function of selected human cDNA sequences, and development of an embryonic-stem-cell-based system for the *in vitro* generation of germline deletion complexes throughout the mouse genome.

The mapping project has been conducted by the Genome Analysis Group in the large region between the *p* (pink-eyed dilution) deletion complex and the Mmu-7 centromere, 20 centimorgans of which is homologous to a large portion of human chromosome (HSA) 19q. Since the Lawrence Livermore National Laboratory (LLNL) has concentrated on the physical mapping of HSA 19, we are expanding our studies to other murine regions with significant HSA-19 homologies (e.g., Mmu 8, 9, 10, 17) in order, eventually, to optimize the ability for exploiting the mouse in exploring functions of HSA-19 genes. A 2,000-kb region surrounding *p* has been isolated as a contiguous series of yeast artificial chromosome (YAC) clones, which are also aiding in the analysis of the deletion complex (see Mammalian Genetics and Development Section above). Furthermore, the short region just proximal to that, which is homologous to HSA 11p15, has also been analyzed physically.

Based on the rationale that genes and other functionally-significant DNA sequences are most likely to have been conserved throughout evolution (whereas non-gene regions may vary tremendously between species), a collaborative project has been initiated with LLNL to identify genes in cloned human DNA by using conserved sequences in murine YACs or PACs (P1-based artificial chromosomes) to "trap" similar sequences from human cosmids.

In addition to applying computer technology to management of the data being generated by the Mmu-7 mapping project, the Computational Biology Group is focusing on DNA-sequence analysis. They have developed an artificial intelligence system that can be trained to integrate a number of patterns in DNA sequences that are characteristic of gene-coding regions and, once trained, to recognize coding sequences in anonymous DNA. The method has been 92%

successful in identifying coding sequences with which it was previously unfamiliar, and more recently has been used by a number of laboratories to identify putative gene sequences for various human heritable diseases. Computer-assisted analyses of this sort will be an essential adjunct to the Human Genome Initiative in distinguishing genes from non-coding DNA. "Recognition modules" are being developed for identifying different types of biologically significant features in DNA-sequence data, such as protein-coding regions. In addition, systems are being developed that are capable of assembling features of the sequence data into models describing the structure of whole genes.

MAPPING HUMAN-MOUSE GENOMIC HOMOLOGIES

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Close comparisons between the genetic maps of mouse and man have revealed striking similarities in the genomes despite the obvious biological differences between these two species. Mouse and human chromosomes resemble each other closely within large blocks carrying variable numbers of related genes. Within these homologous segments, mapping data obtained in the mouse may often be extrapolated from one species directly to the other; the location of conserved sequences or functional genes in the mouse can thus be used to predict the positions of related sequences in man, and vice versa. The large number of existing murine mutants, and our ability to create new mutations by experimental means, make the mouse the most promising assay system for studying the organismal functions of human genes. In order to exploit the mouse more efficiently as a model genetic system, however, existing man-mouse comparative maps must be expanded and refined, to more clearly reveal the similarities and subtle differences existing between genomes of the two species.

Our group has focused its efforts upon generating highly detailed comparative genetic and physical maps within selected regions of the mouse genome, especially within a large chromosomal segment extending from the murine pink-eyed dilution (*p*) locus to the centromere of mouse chromosome 7 (Mmu7). This region of the chromosome has been targeted for several reasons. First, this proximal Mmu7 region carries a number of interesting murine mutations, especially those resulting from overlapping deletions centered at *p* (see reports by the Genetic Analysis and Molecular Genetics Groups). In collaboration with E. M. Rinchik's group, we have worked to establish a detailed physical map of the *p* region, and to generate data and reagents required to precisely locate and isolate the genes associated with certain deletional phenotypes. As part of this work, we have generated a long-range restriction map spanning 2 million

basepairs (2 Mb) surrounding the *p* locus itself, and have isolated the entire region as a contiguous series of yeast artificial chromosome (YAC) clones. Mapping information obtained to date suggests that these related regions of the mouse and human genomes are very similarly organized, inviting the prediction that some of the phenotypes expressed by *p* region mutants may represent murine equivalents of PWS/AS symptoms.

The proximal region of Mmu 7 carries homology with two other well-mapped human regions. Just proximal of the *p* region lies a relatively small segment of homology to HSA 11p15; this segment borders a much larger region representing, possibly, the entire q arm of HSA19. Our studies have indicated that all of the seven genes mapping to this proximal Mmu7 region and related to sequences located on HSA11p15 (*Ldh-1, Ldh-3, Saa-1,-2, and -3, Tph, and Myod-1)* are tightly clustered within a maximum distance of 500 kb; this segment may thus be very limited in total length, although the positions of its proximal and distal borders have not yet been defined. Our most recent studies have concentrated upon comparative mapping of the large (ca. 20 cM) region of homology to HSA 19q. Work within this large homology region is being conducted in close collaboration with members of the Human Genome Center at the Lawrence Livermore National Laboratory (LLNL), who have concentrated their efforts on genetic and physical mapping of human chromosome 19. Very recently, we have expanded our efforts to include the analysis of all murine regions with significant HSA19 homology, including segments of mouse chromosomes 8,9,10, and 17. By coordinating efforts and sharing resources and information with the LLNL team, we hope to construct a comparative map that maximizes potential for direct interspecies comparisons, and optimizes our ability to exploit the mouse in exploring the functions of HSA19 genes.

Strategies for the Identification of Evolutionarily-Conserved DNA Sequences

As part of a distinct but closely related program, we have recently begun to explore new methods to exploit mouse-human genomic relationships for the identification of new human genes. This project has been designed to capitalize upon LLNL's collection of contiguous cosmid and YAC clones, which, with some gaps, span the length of human chromosome 19. Most cloned HSA19 genes and DNA markers have been localized to specific cosmid clones, but many more, as-yet-unidentified genes can be expected to be scattered throughout each contig. In collaboration with Elbert Branscomb and other members of the LLNL team, we have recently begun to design and test methods for the rapid and efficient identification of genes and other functionally-significant DNA sequences from cloned human DNA.

Our approach relies upon the fact that DNA sequences with important biological functions are most likely to be conserved throughout evolution. Because of this fact, the genes of mouse and man are, on the whole, very similar in DNA

sequence; non-gene regions, by contrast, will generally vary greatly between two such highly divergent species. We are currently exploring several different means of selectively cloning the sequences that are most similar in mouse and human DNA. These approaches focus upon using conserved sequences in murine YAC or PAC (P1-based artificial chromosomes) clones to "trap" similar sequences from human cosmids that represent homologous human regions. Our present efforts are part of a pilot study to examine the feasibility of such an approach, and are focused upon a limited number of well-characterized regions known to be similar in man and mouse. Ultimately, we intend to apply these methods to the identification of genes along the length of HSA19.

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AN INTELLIGENT SYSTEM FOR DNA SEQUENCE INTERPRETATION

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A major challenge facing the Human Genome Project is to develop the technology for recognizing and interpreting the information contained in genomic DNA sequence data. Several laboratories are already producing hundreds or thousands of kilobases/year of DNA sequence. These efforts would benefit greatly from reliable computational methods to locate exons and other gene components because experimental methods used to localize genes, such as exon trapping, are labor-intensive and time-consuming. Until recently, these have represented the only viable methodology since computational methods have been largely inadequate for sequence interpretation.

We are developing an integrated artificial intelligence system to identify and interpret biologically significant features in genomic DNA sequence data. The project combines expertise in molecular biology and genetics in the Biology Division with concurrent-processing and intelligent systems expertise in the Engineering Physics and Mathematics Division. We are focusing on two central areas of DNA sequence analysis: (1) improvement of the basic pattern recognition

technology used to locate sequence regions which correspond to genes and other biologically relevant features, and the corresponding development and distribution of reliable feature recognition tools to aid experimental activities; (2) development of integrated systems for the intelligent automated assembly of recognized genetic features into hypothetical models for gene structure and function.

(1) Feature recognition tools. We are currently developing a set of recognition tools, termed "recognition modules," to identify different types of biologically important features in DNA sequence data. These are based on a unique approach to sequence pattern analysis, developed at ORNL, which integrates multiple sensors with neural networks. As an example, the module which locates protein coding regions in DNA sequences uses several "sensors" that report attributes of the DNA sequence to a neural network which, in turn, has been trained to interpret these signals and recognize protein coding regions. This coding recognition module has unprecedented accuracy, correctly localizing more than 90% of coding regions greater than 100 bases long with a very low false positive rate. Since the summer of 1991, the coding recognition module, together with a rule- based expert system which interprets its output, has been available through GRAIL, an e-mail server with over 600 users world-wide. Investigators searching for the causes of human genetic diseases have used the system to identify such genes as the one responsible for X-linked adrenoleukodystrophy.

(2) Intelligent system development. We are also developing artificial intelligence inference systems capable of assembling features in DNA sequence data into models describing the structure of whole genes. These systems, which include the Gene Assembly Program (GAP), as well as facilities for searching protein sequence databases for proteins related to the inferred gene product, are being incorporated into a graphical client-server computer interface so they can be made available to the genome research community.

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Molecular, Cellular, and Cancer Biology Section

Section Overview - S. K. Niyogi

The projects in this Section are directed toward understanding at the most fundamental level the mechanisms of cellular processes that relate to the overall mission of the Biology Division. Programs are concerned with the structure of DNA and its organization in the eukaryotic genome, the structure of chromatin, messenger RNA (mRNA) turnover and its enzymatic mechanisms, all of which problems underlie the cellular functions and the genetic responses of organisms to environmental factors. Other programs of the Section, also relating organisms to their environment, are concerned with genetically engineered proteins for the study of structure/function relationships, mechanisms of carcinogenesis, regulation of transport of metabolites by mammalian cells, and the maintenance of viable cells by freezing. The latter program also has an applied component in the preservation of valuable mutants. Some of the significant findings of the various groups are encapsulated here to demonstrate how the ongoing work is not only of interest as basic science but also relates to the programs of the DOE.

Structure/Function Relationships of Biologically Important Proteins

After the mapping, isolation, and sequencing of a gene, the elucidation of its true biological function requires studying the gene product. The Protein Engineering and Chemistry Group is the largest unified cohort in the Section that seeks to address this aspect of biological function with studies of a variety of important proteins. Its members utilize site-directed mutagenesis and other sophisticated genetic, biochemical, and chemical techniques to explore structure/function relationships of proteins of both plant and animal origin. Although the proteins form a diverse group, they are each of significance to various aspects of the researches of the Biology Division and the programmatic missions of the DOE. The proteins under study include (1) energy-related enzymes, namely, ribulosebisphosphate carboxylase/oxygenase (Rubisco) and phosphoribulokinase (PRK), biocatalysts that regulate photosynthesis and thereby biomass yield, with additional relevance to the global CO_2 problem (greenhouse effect); (2) epidermal growth factor (EGF) and its receptor, proteins that regulate cell growth and are therefore of importance to cancer biology.

With the three-dimensional structure of Rubisco now published, mechanistic studies of the enzyme have taken on added significance. Many of the Protein Engineering and Chemistry Group's findings have now been validated, and new questions regarding biological and physicochemical aspects of Rubisco can be adequately addressed in a more systematic manner.

A significant finding of the Group is the ability to restore by chemical modification the activity that was crippled by site-directed mutagenesis of an active site of the protein. Such restoration of activity is achievable by both covalent and noncovalent modification. Utilizing this breakthrough, mechanistic questions regarding intersubunit interactions and each step of the biochemical pathway of CO_2 fixation are being analyzed in a methodical fashion.

Besides carboxylation, Rubisco catalyzes an energy-wasteful process by oxygenation of the substrate ribulosebisphosphate. Through replacement of an active-site serine residue with alanine, the Protein Engineering and Chemistry Group has succeeded in enhancing the carboxylation/oxygenation ratio in Rubisco even though the catalytic rates are diminished. This achievement lends credence to prospects for increased plant yield by judicious genetic manipulations of Rubisco -- a long-sought goal of plant biologists.

Another plant protein under intensive study is the light-regulated enzyme PRK which catalyzes the production of ribulosebisphosphate, the obligatory substrate for CO_2 fixation. Various aspects of the catalytic and regulatory mechanisms of this important enzyme are being elucidated utilizing both site-directed mutagenesis and protein chemistry. Recent achievements include pinpointing of the regulatory cysteines, discerning the basis of catalytic incompetence of the oxidized form of the enzyme, and defining interactions between PRK and thioredoxin (the *in vivo* regulator).

Growth factors and their specific cell-surface receptors, acting in harmony, play key roles in regulating cell growth. Moreover, many receptors are also protooncogenes, and either overexpression or mutation to oncogenes leads to various growth abnormalities including cancer. The Protein Engineering and Chemistry Group is studying EGF, a prototypical growth factor that initiates cell proliferation by the stimulation of the tyrosine kinase activity of its receptor. The last two years have seen considerable progress in the identification and characterization of the receptor-binding residues of the EGF ligand. Most of these residues are hydrophobic with the exception of a highly conserved electrostatic residue (arginine 41) which is nestled between two hydrophobic domains. A highly significant observation is that the receptor-binding residues act independently of one another with multiple mutations leading to cumulative effects on receptor binding affinity. It therefore becomes feasible to develop EGF superagonists that could be useful in clinical applications, for example, wound healing, treatment of burns, ulcers, and cataracts. On the other hand, identification of the EGF residues critical for receptor binding and activation makes it feasible to design EGF mutants that could act as growth inhibitors.

Utilizing several of their novel EGF analogues as cross-linking agents, the Protein Engineering and Chemistry Group has started mapping the ligand-binding domain of the EGF receptor, preparatory to a full-scale mutagenesis scheme to

identify specific receptor residues intimately involved in this crucial biological interaction. Mutant receptors could then be expressed in recipient cells, both *in vitro* and *in vivo,* for an in-depth study of structure-function relationships of the EGF receptor.

Messenger RNA Turnover in Eukaryotes

The mechanism of posttranscriptional RNA processing and turnover represents a major challenge for molecular biologists and biochemists. The RNA Metabolism Group has been studying various aspects of eukaryotic mRNA turnover, focusing on several enzymes including two 5′→3′ exoribonucleases that degrade decapped mRNA, the latter arising from the action of a decapping enzyme. All these enzymes were discovered by the RNA Metabolism Group. The 5′→3′ exoribonuclease 1 (XRN1) from yeast has been cloned and sequenced, and the disrupted gene introduced into cells to analyze the physiological consequences. The studies indicate that this enzyme and the mRNA decapping enzyme, in concert, catalyze mRNA turnover. Exoribonuclease 2 (XRN2) is coded by an essential gene in yeast and is therefore of great interest. Understanding how these enzymes regulate mRNA processing is important because, as an example, some cancers are believed to develop from increased stability of certain oncogene mRNAs. On the other hand, manipulation of the activity of these key enzymes might lead to the stabilization of mRNAs that produce beneficial enzymes and proteins.

Crystallographic Analysis of Nucleosomes and Proteins

The Structural Biology Group has analyzed in detail their 8 Å crystallographic solution of the nucleosome core particle structure and now has diffracting crystals that will allow a <3 Å solution. For this purpose they have reconstituted preparations of precisely positioned nucleosomes that are each homogeneous in their histone composition and in the associated DNA sequence, and they have shown that the reconstitutions conform to theoretical predictions. The use of palindromic DNA sequences further enhances crystal homogeneity. The DNA wrapping around the protein core of the nucleosome does not form a smoothly curved superhelix but at several locations makes sharp histone-induced bends related to the primary DNA sequence at those points. These are putative binding sites for DNA binding proteins. The analysis has the promise of establishing the structural basis for the regulation of transcription and other DNA-dependent functions of the cell. This group is also investigating the crystal structure of pyrophosphatase and, in collaboration with the Protein Engineering and Chemistry Group, has initiated a study of the crystal structures of spinach phosphoribulokinase and human epidermal growth factor.

DNA Sequencing Technology

In genome-related studies, several new methods for sequencing are being developed or explored by the DNA Sequencing Technology Group, including the use of stable isotopes as DNA labels, sequencing by hybridization, scanning probe microscopy for mapping single DNA molecules, and analysis by laser desorption mass spectrometry. The activity of this group involves collaborations through different disciplines and various Divisions of ORNL. A major accomplishment in this reporting period has been the development of photosensitive reagents for the synthesis of microchip arrays of oligonucleotides for use in sequencing by hybridization.

Chromatin Structure/Function

The group studying Genome Structure and Organization has extended their fine analysis of the relation of DNA primary sequences in their model system (a complex satellite of a crustacean) to mutation hotspots, showing that regions of sequence-dependent distortion of the helix are regions of high mutation frequency. The satellite sequence is transcribed in specific organs at specific growth stages, a fact that has been demonstrated both biochemically and histologically by cytological hybridization. The regulation of such transcription is under current investigation. The extent of DNA methylation appears to be a significant factor in establishing which sequences are transcribed.

Alternative approaches to exploring the structure of chromatin have been adopted by the Chromosome Chemistry Group. They have been examining the structure as well as the transcription and replication characteristics of short linear DNA molecules, possibly one gene per fragment, which exist in the macronucleus of a ciliated protozoan. In addition, they have continued their development of electron microscope tomography and its application to resolving three-dimensional structures, focusing on the Balbiani rings engaged in RNA synthesis in the polytene chromosomes of the insect *Chironomus*. Using an electron-dense stain developed in their laboratory, they have been visualizing the mRNA synthesized on the giant chromosome puffs. These studies are expected to lead to a better understanding of the packaging, replication, and transcription of DNA in eukaryotic chromosomes.

Cellular Events at the Membrane Level; Transport and Growth Regulation

The Membrane Biology Group has continued its studies on the regulation of transport systems in relation to mammalian cell growth and differentiation. The major projects are concerned with co-expression of genes for transepithelial hexose transport in differentiating renal proximal tubule cells in culture, and cellular responses, including both mitogenicity and transport modulation, to engineered hEGF variants developed by the Protein Engineering and Chemistry Group.

Receptors, Carcinomas, and Cell Targeting with Antibodies

Underlining the importance of the role of specific receptors in pathogenesis, the Molecular Immunology Group has been investigating the integrin $\alpha 6\beta 4$, found on the cell surface in a number of carcinomas. The group is focusing on its tissue distribution, molecular characterization, and attempts to identify its specific ligand. Other studies concentrate on monoclonal antibodies that are specific to endothelial cell-surface glycoproteins in the lung. They have demonstrated that a major lung endothelium-specific antigen is thrombomodulin, a protein that plays a significant role in the prevention of intravascular clotting. A goal of this group is to develop a system utilizing antibody-containing liposomes that will be targeted to lung and other tissues and thus will be potentially useful in tissue-specific drug delivery. This group has provided monoclonal antibodies to over 200 laboratories worldwide.

Radiation-induced Cell Transformation

A major effort of the Radiation Carcinogenesis Group has been an integrated study of myeloid leukemia in RFM mice at the molecular, chromosomal, and whole-animal levels, as well as skin carcinogenesis by both ionizing and non-ionizing radiation. Neoplastic progression in rat tracheal epithelium is also being studied by this group, using an *in vivo/in vitro* model that they have developed. Their studies focus on identifying the critical target cells for carcinogens, including various types of radiation, and the influence on progression of cell-cell interactions mediated by diffusible growth factors. In the Radon Program, a recent finding of particular interest is that α particles, which may be lethal to cells they penetrate, do not of themselves cause transformation to precancerous cells, but they interact strongly and synergistically with β and γ radiations to induce cell transformation. This finding takes on special significance in view of the fact that radon is not only an α emitter, but it and its daughters also emit both β and γ radiation. The Environmental Protection Agency has cited radon as the second leading etiological agent in lung cancer in the United States.

Cryobiology: Basic Studies and Practical Applications

The Fundamental and Applied Cryobiology Group has continued basic studies on the cryobiology of human and mouse sperm and applied studies in freezing of embryonic mice and *Drosophila* for the cost-efficient, long-term banking of mutant stocks with the benefit of avoiding genetic drift that accompanies continuous breeding. Although human sperm has been successfully cryopreserved for decades, the methods have been based on trial-and-error techniques with little being known about the underlying mechanisms. In contrast, mouse sperm resists cryopreservation, but cryopreservation would be a major aid in maintaining the large number of transgenic stocks that are being developed worldwide. This year the group has succeeded in preserving *Drosophila* embryos with 25% recovery of viable, fertile organisms, an achievement that has been recognized with an

R&D 100 award, a citation for the 100 most significant technological achievements in research and development in the past year. The cost of maintaining the flies by continuous breeding in all *Drosophila* laboratories has been estimated as upwards of $6M, underscoring the potential economic significance of this accomplishment.

Collaborative Endeavors

It is important to stress that the various research groups in the Molecular, Cellular, and Cancer Biology Section are not islands of activity, rather they represent intellectual and research endeavors that span not only the Division but extend into the scientific community at large. A few examples of such activity with the outside community are given below. The interactions within the Division and with other Divisions in ORNL are too many to enumerate.

The Protein Engineering and Chemistry Group has extensive interactions with DuPont, Michigan State University, Rutgers University, University of Illinois at Urbana, Chiron Corporation, Institute for Physiological Chemistry (Hamburg, Germany) and other institutions. Clones of Rubisco, PRK, and EGF have been made available to investigators in both the public and private domain. The RNA Metabolism Group is collaborating with investigators at University of North Carolina and University of Arizona. The DNA Sequencing Technology Group has numerous collaborations, both in-house and external, with the scientific community. In addition to providing antibodies in response to hundreds of requests, the Molecular Immunology Group interacts, on a regular basis, with scientists at University of Pittsburgh, University of Texas at Galveston, the University of Rome, and others. Extensive collaborations between the Fundamental and Applied Cryobiology Group and geneticists at the University of Chicago have led to joint research proposals funded by NSF, and similarly, collaborations with the University of Oregon and the University of Indiana have led to grants funded by NIH. The Radiation Carcinogenesis Group has collaborations at NIEHS, New York University, and Colorado State University. The list is indeed quite long!

PROTEIN ENGINEERING AND CHEMISTRY

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The unifying theme in this group is the use of site-directed mutagenesis and chemical approaches to elucidate structure/function correlations of proteins. Investigations focus on four proteins with quite diverse functions: ribulose bisphosphate carboxylase/oxygenase, an enzyme whose activity is a major

determinant of biomass yield; phosphoribulokinase, a photosynthetic enzyme whose activity is regulated by light; epidermal growth factor, a hormone that regulates cellular growth and differentiation; and epidermal growth factor receptor, a cell surface glycoprotein having tyrosine kinase activity that mediates the growth promoting properties of epidermal growth factor.

Ribulose Bisphosphate Carboxylase/Oxygenase (Rubisco)

Introduction

Ubiquitous among photosynthetic organisms, Rubisco is essential for net conversion of atmospheric CO_2 into carbohydrates. Thus, this enzyme, a major cornerstone of living processes, is crucial to biomass yield and is also relevant to the global CO_2 issue (*i.e.*, the greenhouse phenomenon). The enzyme is bifunctional: in addition to catalyzing the carboxylation of D-ribulose-1,5-bisphosphate (ribulose-P_2) to yield two molar equivalents of D-3-phosphoglycerate (the CO_2-fixation reaction), it also catalyzes the oxidation of ribulose-P_2 by molecular oxygen to yield one molar equivalent each of phosphoglycolate and 3-phosphoglycerate. Although multiple substrate specificities among enzymes are not unusual, the bifunctionality of Rubisco is perhaps unprecedented in that the two reactions catalyzed are the initial steps in competing metabolic pathways — photosynthetic assimilation of CO_2 and photorespiration, the latter an energy-wasteful process which results in the release of previously fixed CO_2.

Our goals are (a) to understand the mechanism of this complex enzyme, especially the precise catalytic roles of active-site residues, and (b) to evaluate the feasibility of improving the carboxylase/oxygenase activity ratio and thereby providing an approach to enhancing biomass yields.

In contrast to Rubisco from higher plants, which is comprised of two gene products (eight large and eight small subunits per molecule of enzyme), the functionally analogous enzyme from the purple, non-sulfur photosynthetic bacterium *Rhodospirillum rubrum* is a homodimer and the product of a single gene. This enzyme has thus been used for many of our mutagenesis studies. However, an expression system for an L_8S_8 enzyme has been optimized recently so that mutagenesis is being pursued with both forms of the carboxylase.

The three-dimensional structure of Rubisco with a bound substrate analogue has been published recently by Dr. Carl Brändén and colleagues at the Swedish University of Agricultural Sciences, thereby revealing intricate details of the active site and subunit-subunit interactions. This structure validates many of our conclusions concerning structure-function relationships derived collectively from affinity labeling, chemical cross-linking, comparative sequence analyses, and site-directed mutagenesis.

Development of a New Assay Procedure for the Oxygenase Activity of Rubisco

Because of our interest in defining the structural features of the enzyme that dictate the partitioning ratio between the carboxylation and oxygenation reaction pathways, access to a reliable, facile oxygenase assay (long available for the carboxylase activity) is imperative. Historically, other laboratories have relied on an oxygen electrode for measuring oxygen consumption in the oxygenase pathway. Oxygen electrodes are notoriously insensitive and subject to false signals; these technical difficulties seriously undermine the reliability of the assay and preclude detecting small changes in the partioning ratio, which nevertheless might be of functional significance. The other historical approach for monitoring oxygenase activity has been the use of dually-labeled ribulose-P_2 as substrate and the eventual chromatographic quantification of glycolate (an indicator of oxygenase activity) and glycerate (an indicator of carboxylase activity). This assay is highly accurate but cumbersome because of the necessity to dephosphorylate the actual reaction products prior to chromatographic analysis.

Our new method is more direct and utilizes singly-labeled [1-^{3}H]ribulose-P_2, which is convenient to synthesize from commercially available [2-^{3}H]glucose. The kinetic characteristics of the enzyme which determine relative rates of carboxylation and oxygenation (v_c/v_o) can best be appreciated as a ratio of the catalytic efficiencies (V_{max}/K_m) for the two activities, $(V_c/K_c)/(V_o/K_o)$. This latter term defines the substrate specificity factor, denoted as the constant τ, and readily allows v_c/v_o to be expressed as a function of the relative concentrations of the two gaseous substrates: $v_c/v_o = \tau \bullet ([CO_2]/[O_2])$. When [1-^{3}H]ribulose-P_2 is used as substrate, the distribution of tritium between phosphoglycolate (derived from the oxygenation pathway) and phosphoglycerate (derived from the carboxylation pathway) is a direct measure of flux between the two pathways (v_c/v_o). We can resolve phosphoglycolate from phosphoglycerate directly by high-performance anion exchange chromatography on a mono Q column.

Chemical Rescue of Site-Directed Mutants of Rubisco

Despite its revolutionary impact on enzymology, site-directed mutagenesis, as a means for altering structure, is generally restricted to the twenty amino acids normally occurring in proteins. Thus, reliance on homologous series of compounds to establish structure-reactivity correlations, a hallmark of mechanistic studies with non-enzymic catalysts, has not been possible with enzymes. This limitation is partially overcome by the demonstration by Professor Jack Kirsch and colleagues at Berkeley that an enzyme, crippled because of an active-site substitution, can be partially rehabilitated ("rescued") merely by the addition of exogenous organic compounds that mimic the missing side chain. For example, the virtually inactive K258A mutant of aspartate aminotransferase is stimulated by primary amines; the degree of stimulation, after correcting for steric effects, correlates with the pK_a of the amine in accordance with the Brønsted relationship.

These observations provide strong, direct evidence that the ϵ-amino group of Lys258 is the catalytic base that abstracts the α-carbon proton from the aldimine intermediate as postulated from earlier crystallographic studies.

Chemical rescue of deficient site-directed mutants can also be achieved through covalent chemical modification as developed in our laboratories, thereby expanding the diversity and subtlety of structural changes that can be effected through mutagenesis. Examples include substitution of lysyl with aminoethylcysteinyl residues (net replacement of the γ-methylene group with a sulfur atom), substitution of glutamyl with carboxymethylcysteinyl residues (net insertion of a sulfur atom between the β- and γ-methylene groups with lengthening of the side chain by $\sim$1 Å), and substitution of arginyl with homoarginyl residues (net insertion of a methylene group with lengthening of the side chain by $\sim$1 Å).

We are utilizing both covalent and noncovalent chemical rescue to address a number of structure/function issues of the carboxylase. One issue entails the significance of intersubunit, electrostatic interactions at the active site between Glu48 and Lys329 found only in the activated form of the enzyme and between Glu48 and Lys168 in the nonactivated form of the enzyme. Our studies have shown that even conservative amino acid replacement of each of these three residues leads to virtual abolishment of carboxylase activity. Each mutant was isolated in high yield as a stable dimer; hence, elimination of either a Glu48---Lys168 or Glu48---Lys329 salt bridge does not preclude subunit-subunit interactions. Furthermore, each mutant, although inactive in the overall carboxylation reaction, displays substantial activity in the first step in catalysis — the enolization of ribulose-P_2. The observed catalytic competence of these mutants, even in a partial reaction, provides dramatic evidence of intact subunit structures, normal activation chemistry, and substrate binding.

By *in vivo* hybridization of catalytically-deficient site-directed mutants of the carboxylase, we showed that the active sites are created by interacting domains from adjacent subunits. The large COOH-terminal domain comprises an eight-stranded β,α-barrel and contains most active-site residues, including Lys168 and Lys329. The smaller NH_2-terminal domain from the adjacent subunit extends partially across the top of the barrel and includes active-site residues Glu48 and Asn111. Lys329 is located within the flexible loop 6 of the β,α-barrel; in the nonactivated form of the enzyme, this loop is so mobile that it cannot be seen in the electron density map. However, in the activated form of the enzyme with a reaction-intermediate analogue bound, loop 6 folds over the top of the barrel and becomes immobilized, in part, by electrostatic interactions between Lys329 and Glu48 of the adjacent subunit and between Lys329 and the carboxylate of the bound analogue. One might readily imagine that loop 6 and the NH_2-terminal segment of the active site, due to their positioning, are involved in controlling ligand access to the active site and in precluding dissociation of reaction

intermediates from the active site. Recent findings that support such a postulate follow:

(i) Substitution of Glu48 by Gln (*i.e.*, mere replacement of the oxyanion of a carboxylate sidechain by NH_2) dramatically decreases the τ-value (the CO_2:O_2 specificity factor) from 10 to 0.5. Thus, the negative charge provided by Glu48 and/or its salt bridge to Lys329 greatly influences the relative reactivity of the enediol intermediate in favor of CO_2.

(ii) E48Q exhibits a far greater propensity than wild-type enzyme to misprotonate the enediol intermediate, with the consequent formation of xylulose-1,5-bisphosphate. Thus, the active site of the mutant enzyme is more accessible to bulk solvent due to destabilization of subunit-subunit interactions at the mouth of the β,α-barrel leading into the crevice for ligand anchoring.

(iii) Reinsertion of a carboxylate at this position by carboxymethylation of E48C, which restores ~5% of the original wild-type carboxylase activity, elevates the τ-value relative to that of E48Q by more than 3-fold.

(iv) Treatment of K329C with 2-bromoethylamine partially restores enzyme activity as a consequence of selective aminoethylation of the introduced thiol group. The catalytic constant (k_{cat}) of this novel carboxylase, which contains a sulfur atom in place of a specific lysyl γ-methylene group, is 2.5-fold lower than that of the wild-type enzyme. This detrimental effect by such a modest structural change underscores the stringent requirement for a lysyl side chain at position 329. In contrast, the aminoethylated mutant protein exhibits K_m values for CO_2 and ribulose-P_2 that are unperturbed relative to those for the wild-type enzyme. Clearly, major reductions in k_{cat} with unaltered K_m values argue for a direct role of Lys329 in catalysis, as reinforced by recent characterization of the mutant protein in catalysis of partial reactions. More importantly, as regarding partitioning between the carboxylation and oxygenation pathways, our new chromatographic assay has permitted an evaluation of the specificity factor of the aminoethylated rescued enzyme; we observe that the τ-value is reduced 2-fold relative to that of wild-type enzyme.

(v) The virtually inactive K329A is subject to effective chemical rescue by ethylamine. However, the τ-value of the rescued enzyme is about 2-fold lower than that of wild-type enzyme.

Thus, a variety of structural changes and chemical manipulations focused on the Glu48---Lys329 intersubunit salt bridge render major alterations in ligand accessibility and gaseous substrate specificity. This work defines both specific regions of the polypeptide chain and structural features for future microsurgery in pursuit of understanding specificity determinants and ultimately of designing a catalytically superior enzyme.

An Active-Site Mutant with Enhanced Carboxylase Specificity

The CO_2/O_2 specificity factor (τ) of Rubisco partially determines the efficiency of photosynthetic carbon assimilation; hence, an evaluation of whether τ can be altered by genetic or chemical manipulation is highly relevant to considerations of biomass yield. Heretofore, engineered alterations of the enzyme have only decreased the selectivity for CO_2 utilization. We now observe that alanyl replacement of active-site Ser368 of the *Rhodospirillum rubrum* carboxylase enhances the carboxylation selectivity ~1.6-fold over the wild-type level. This enhancement reflects a greater relative decline in oxygenase efficiency than in carboxylase efficiency. In contrast to wild-type enzyme, the carboxylase activity of the Ser368 mutant protein is not perceptibly inhibited by O_2, perhaps indicative of a change in the rate-limiting step in the overall reaction pathway. The greatest significance of this study is the demonstration that a modest structural change of a single side chain at the active site of Rubisco can elevate the specificity factor. This observation would appear to validate the feasibility of tailored optimization of the enzyme's catalytic properties.

Mutagenesis of Hexadecameric Rubisco from the Cyanobacterium Anabaena

We had previously established an efficient expression system for hexadecameric Rubisco from *Anabaena*, which is dependent upon coexpression of the GroESL chaperonin proteins to assist the proper assembly of the L_8S_8 Rubisco. The organization of the operon encoding Rubisco in *Anabaena* has also been addressed. The native *Anabaena rbc* operon has a 558-bp "spacer" between the large subunit (*rbcL*) and the small subunit (*rbcS*) genes. An earlier report suggested that the majority of operon transcripts terminated within this spacer region, causing a deficiency of small subunits. Examination of the spacer region did not reveal any potential Rho-independent terminator sequences. Deletion of this spacer region had no effect on the yield of active carboxylase. The spacer region contains an open reading frame that could represent a third member gene of the operon. This gene, tentatively identified as *rbcX*, is 397-bp in length and would encode a 132-residue peptide of 15188 Da. Searching Genbank did not reveal any closely related peptide sequences, although a short segment of identity (L--WLHTFS--K) with arabinose isomerase from *E. coli* was noted. This gene is apparently translated in *E. coli*, as indicated by β-galactosidase production from an in-frame *rbcX'::'lacZ* fusion.

The purification and general characterization of the recombinant L_8S_8 Rubisco of *Anabaena* 7120, as prerequisites to mutagenesis, have been completed. Site-directed mutagenesis is now focused on the Thr66 and Trp67 (the secondary ligands for the C-1 phosphate that correspond to Thr53 and Asn54 of the *R. rubrum* enzyme) which influences the τ-value. Modest perturbations of τ-values were associated with the T66A and T66S mutants in parallel with properties of corresponding *R. rubrum* mutants. The S380A and S380C mutants

were also constructed and purified. Unlike the *R. rubrum* cognate (S368A), S380A has a deleterious effect on r, suggesting that compensatory structural rearrangements that enhance r in the L_2 enzyme are not available in the L_8S_8 active site.

Phosphoribulokinase (PRK)

Our interest in this enzyme derives from two considerations: PRK catalyzes the formation of ribulose-P_2 (from ribulose-5-P and ATP), the requisite CO_2 acceptor in photoautotrophs, and PRK is regulated by light via the ferredoxin-thioredoxin electron transfer chain. Thus, our studies focus on mechanisms of both catalysis and regulation. We have examined the function of Lys68, whose involvement in catalysis was implicated by Dr. Henry Miziorko and colleagues at Medical College of Wisconsin, utilizing affinity labeling of the bacterial enzyme with ATP analogues. The equivalent position is occupied by lysyl or arginyl residues in the PRK from both prokaryotic and eukaryotic sources suggesting a requirement for a basic residue at this location. We have replaced Lys68 of spinach PRK with arginyl, glutaminyl, alanyl, and glutamyl residues. All of the mutant enzymes retained significant activity indicating that Lys68 is not required for catalytic activity. Neither does the replacement of Lys68 by arginine or glutamate cause any significant perturbation to the K_m for either ATP or ribulose-5-P. These results suggest that though Lys68 may be near the active site of PRK, it is not required for catalysis or substrate binding.

We showed earlier by chemical modification that the intrasubunit, regulatory disulfide of the homodimeric PRK from spinach is constituted by Cys16 and Cys55 and that these two residues are located at the active site. Thus, either direct consequences of removal of a catalytic group or indirect consequences of conformational changes could account for the complete loss of PRK activity that accompanies oxidation. Site-directed mutagenesis has been used by us to explore the former possibility. Replacement of Cys16 by serine is without effect on catalytic activity, while identical substitution for Cys55 decreases k_{cat} ~ 10-fold; the double mutant also retains significant activity. Since neither sulfhydryl is required for catalysis, the lack of a free sulfhydryl at the active site can only partially explain the catalytic incompetence of the oxidized enzyme. Even though intrinsic fluorescence of both oxidized and reduced PRK is very similar, the microenvironment of the only two tryptophanyl residues (Trp 155 and Trp 241) would not necessarily be sensitive to localized conformational changes restricted to the regulatory site.

As an alternate approach to detecting conformational differences between the two activation states of PRK, we have examined the inter-residue distance between Cys16 and Cys55 in the reduced enzyme with chemical crosslinking reagents. Spinach PRK is rapidly inactivated by stoichiometric levels of 4,4'-difluoro-3,3'-dinitrodiphenylsulfone (FNPS) or 1,5-difluoro-2,4-dinitrobenzene

(DFNB), which span 9 Å and 3.5 Å, respectively. ATP, but not ribulose 5-phosphate, retards the rate of inactivation, suggesting that modification has occurred at the nucleotide binding domain of the active site. Sulfhydryl modification is indicated by partial reversibility of inactivation as effected by exogenous thiols. Tryptic mapping by reverse-phase chromatography of [^{14}C]carboxymethylated enzyme, subsequent to its reaction with either FNPS or DFNB, demonstrates modification of Cys16 and Cys55 by both reagents, and formation of only one major chromophoric peptide in each case. Based on the sequence analysis of the purified chromophoric peptides, Cys16 and Cys55 are crosslinked by both FNPS and DFNB. Thus, the intrasubunit distance between the β-sulfhydryls of Cys16 and Cys55 is dynamic rather than static. Diminished conformational flexibility upon oxidation of the regulatory sulfhydryls to a disulfide may be partially responsible for the concomitant loss of enzymatic activity.

Epidermal Growth Factor (EGF)

Background

Because of their crucial role in the regulation of growth and differentiation and the abnormalities resulting from their malfunction, growth factors have attracted considerable interest. EGF, a 6-kDa protein with three internal disulfide bonds, exhibits high-affinity binding to its specific cell surface receptor. Upon binding EGF, the receptor undergoes autophosphorylation on tyrosine residues by its intrinsic protein-tyrosine kinase activity, which also phosphorylates endogenous substrates. Tyrosine phosphorylation triggers a cascade of biochemical events including increased glycolysis and protein synthesis and increased transcription of specific genes, which ultimately leads to a stimulation of DNA replication and cell proliferation.

Research Goals and Approach

The major objective of our research is the elucidation of structure/function correlations of human EGF by protein engineering. Utilizing the human EGF gene, cloned in this laboratory as a bacterial secretory protein, targeted amino acid residues were substituted by oligonucleotide-directed mutagenesis. The selection of amino acid residues for mutagenesis was based on (a) their interspecies homologies; (b) published solution structures of mouse and human EGFs, which are based on 2D-NMR and computer modeling; and (c) previous studies of EGF structure/function in our laboratory. Mutant proteins were purified to homogeneity and tested for (a) specific binding to the EGF receptor, (b) stimulation of the protein-tyrosine kinase activity of the EGF receptor, (c) stimulation of DNA synthesis and growth of mammalian cells in culture (see Cook *et al.* Membrane Biology), and (d) possible structural alterations as determined by NMR spectroscopy.

Receptor-Binding Residues of EGF

(i) *Hydrophobic Residues.* Our studies have indicated the importance of several hydrophobic residues in EGF for binding to its receptor. These include Leu15, Ile23, and Leu26 in the N-terminal domain, and Leu47 in the C-terminal domain. Replacement at any of these positions led to drastic reduction in EGF's biological activity. The most severe losses in receptor affinity occurred upon replacement by polar residues, indicating the importance of hydrophobic "bonding" in EGF-receptor interaction. The role of these hydrophobic residues in serving as possible contact points with the receptor is further underscored by the fact that substitutions at these positions led to very minor alterations in the native structure of EGF that could not account for the drastic reduction in receptor affinity.

(ii) *Electrostatic Residues.* In general, replacement of polar residues in EGF led to only modest or no reduction in receptor affinity. The residues studied include Glu40, Gln43, Arg45, and Asp46, all in the C-terminal domain. A clear exception is the highly conserved Arg41. Both nonconservative and conservative (to lysine) replacements led to dramatic reductions in activity, while structural analysis by NMR revealed negligible conformational alterations. The essentiality of the guanidinium moiety at position 41 was confirmed by protein chemistry: chemical modification of Lys41 to homoarginine by treatment with *O*-methylisourea restored full activity.

(iii) *Tyrosine 13.* This residue was implicated as playing a role in receptor binding due to its close proximity to Arg41, as well as its high degree of conservation in EGF and EGF-like proteins that can bind to the EGF receptor. Removal of the hydroxyl moiety by substitution with phenylalanine had little effect on the binding, indicating that it is not involved in crucial hydrogen bonding with the receptor or with other regions of the EGF molecule. Substitution with the non-polar leucine decreased receptor affinity only slightly, indicating that aromaticity at this site is also not critical. Replacement with other hydrophobic residues resulted in diminished receptor affinity as a function of decreased hydrophobicity. Substitution with the polar residues markedly lowered receptor affinity. CD spectral analysis revealed only minor structural alterations. Overall, the results indicate the functional importance of hydrophobicity of Tyr13 in receptor binding.

(iv) *Independent Interactions of EGF Residues with the Receptor.* Single-site EGF mutants Y13H, Y22D, I23T, and L26G were genetically combined with the L47A variant to produce a series of double-site mutants having alterations simultaneously in separate domains of the growth factor. Similarly, the combination of Y13H and I23T generated a double-site mutant having two mutations within the same domain. Finally, combination of I23A with L26A altered two side chains located in close proximity to each other. Analysis of the relative receptor affinities of the single- and double-site mutants showed that mutation at any one site does not substantially alter the effect of mutation at the second site

in the EGF molecule. The cumulative effect of simultaneous mutations on receptor affinity indicates that each of the separate sites functions essentially independently in the interaction of the EGF molecule with its receptor. Overall, our results indicate that EGF-receptor interaction involves critical residues that are interspersed throughout the EGF ligand.

Epidermal Growth Factor Receptor (EGFR)

Background and Rationale

The identification of critical residues in the EGF ligand that are needed for receptor binding and kinase activation has generated great interest in the ligand binding residues of EGFR. In the case of the EGFR family of proteins, the tertiary structure is not yet known; however, the complete amino acid sequences of EGFR from both human and avian sources are available. The sequences of several other transmembrane receptor proteins having moderate to high homology with EGFR are also known. A comparison of the sequences of the extracellular domains of these related receptor proteins has provided important clues about structural motifs. However, with such a large protein as the EGFR (170 kDa), the selection of which residues to mutate within the ligand-specifying variable region of the extracellular domain can be directed more reasonably and efficiently following the detection of those receptor residues located in close proximity to the ligand binding site(s), using ligand-directed affinity labeling. Preliminary studies from other laboratories have utilized deletion and replacement mutagenesis in an attempt to localize the ligand binding region(s) on the receptor. The results provide evidence for the potential participation of two broad regions of the receptor in EGF binding. Cross-linking of mouse EGF, bearing the single reactive N-terminal α-amine, has distinguished two residues in the receptor's extracellular domain (Lys-336 and Tyr-101) as being in close proximity to the EGF α-amine. The presence of relatively few reactive groups, however, limits the usefulness of native mouse or human EGF in most cross-linking studies designed to map residues throughout the receptor's ligand binding domain. Receptor-ligand cross-linking studies can be greatly extended by the use of EGF analogues specifically engineered to introduce lysine amines, which are reactive with various cross-linking agents, at specific sites throughout the ligand molecule.

Cross-Linking Studies

A baculovirus expression system for the EGFR ectodomain is currently being utilized to obtain sufficient quantities of the protein for cross-linking studies. Two cross-linking reagents are being tested: (1) N-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA); and (2) sulfosuccinimidyl-2-(p-azidosalicylamido) ethyl-1,3'-dithiopropionate (SASD). EGF mutants, in which reactive amines (lysines) have been introduced selectively near sites in the ligand we have identified as essential for optimal receptor-ligand interaction, will be reacted with

the radioiodinated form of NHS-ASA or SASD to generate a series of ligands with radiolabeled, photoreactive moieties introduced selectively within the EGF molecule. The radiolabeled, photoreactive biomolecule will be incubated with the receptor protein and cross-linked by photolysis at 270-305 nm. The receptor-ligand complex can be analyzed by autoradiography followed by digestion with trypsin to generate a series of receptor fragments terminated by lysine or arginine, some of which should be radiolabeled. Sequence analysis of the "tagged" receptor fragments should permit identification of residues located at or near the ligand binding site. We hope to make substantial progress in the near future.

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RNA METABOLISM

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Regulation of the cytoplasmic stability of mRNAs has been found to be a major control mechanism which modulates mRNA levels in a variety of eukaryotic and prokaryotic systems. An important part of unravelling the mystery of mRNA turnover is to analyze in detail the enzymes that may be involved. RNA processing reactions which convert precursor RNA species to mature RNA molecules also involve unique ribonucleases. Studies of ribonucleases, two 5′→3′ exoribonucleases and an mRNA decapping enzyme, and their possible role in mRNA turnover and processing have been continued in this laboratory.

Analysis of the Metabolic Role of 5′→3′ Exoribonuclease-1 of *Saccharomyces Cerevisae*

General characteristics. Characteristics (cell size, generation time, and levels of macromolecules) of a yeast strain lacking an active *XRN1* gene have been examined and compared to a wild-type (wt) control strain. The *xrn1* cells have an average cell volume 1.5 to 1.8-fold larger. By both Coulter counter analysis of average diameter and by microscopic examination, the cells are more diverse in size and more pear-shaped. The generation time is 1.9 to 2.1-fold that of wt cells

and cell number/A_{650} is about 55%. The protein level per cell is increased 1.5 to 1.7-fold. The cellular rRNA (18S and 25S) content is approximately 0.9-1.0 that of wt cells. The synthesis rate of protein and RNA, measured by labeled precursor incorporation, is as predicted from the cellular level and generation time; thus, total cellular protein synthesis ([^{3}H]leucine incorporation) occurs at 80-90% of the rate of wt cells. Poly(A)$^+$ RNA is also synthesized at a rate 80-90% of the rate of wt cells, measured by the rate of [^{3}H]adenine incorporation into oligo(dT)cellulose-bound RNA.

Protein levels and synthesis rates. Since a major activity of XRN1 is 5′→3′ exoRNase activity, a defect in mRNA synthesis or turnover in cells lacking the enzyme might affect protein levels and synthesis rates. To analyze protein levels, extracts were made of wt and *xrn1* cells and examined by one-dimensional denaturing polyacrylamide gel electrophoresis. From the results of densitometer scans of protein bands, the ratios of the amounts of protein band level/total protein level found in the *xrn1* extract as compared to the wt extract were calculated. The protein bands varied as much as 2.2-fold in relative amounts in the *xrn1* extracts. To determine if the perturbations of protein band content were due to altered protein synthesis rates, wt and *xrn1* cells were pulse-labeled with [^{35}S]methionine for 2.5 and 5 min. There was as much as a twofold difference in ^{35}S content found when PAGE-resolved protein bands from wt extracts were compared to *xrn1* cell extracts. The results showed that the rates of synthesis of PAGE-resolved protein bands were measurably altered in the *xrn1* cells, suggesting a possible alteration in specific mRNA levels.

Analysis of alterations in mRNA turnover. To evaluate more directly perturbations in mRNA levels in the *xrn1* cells, possibly resulting from altered mRNA synthesis or turnover rates (or both), the levels and half-lives of the mRNAs, *CYC1* and *RP51A* (short half-lives) and *ACT1* and *PGK1* (long half-lives), were measured. For half-life determinations, transcription was inhibited by thiolutin (Thl) or 1,10-phenanthroline (Pht), and mRNA content upon subsequent incubation was quantitated by Northern analysis. The results with Thl at 30°C showed that the half-life of *CYC1* mRNA is increased from 12 min (wt) to 28 min (*xrn1*). The half-life of *RP51A* mRNA is increased from 15 min to 35 min. *ACT1* mRNA decayed at a slightly reduced rate in the *xrn1* cells. No difference in *PGK1* mRNA turnover in experiments involving 120 min exposure to Thl was found. Similar decay rates for *CYC1* and *RP51A* mRNAs were found using Pht as the transcription inhibitor at 30°. At 36°C with Pht, the half-lives of *CYC1* and *RP51A* mRNAs in the wt strain were less than at 30°C, 7 and 8 min, respectively. No significant change of the half-life values at 30°C was found with the two mRNAs in the *xrn1* cells at 36°C, and the half-lives for *CYC1* mRNA and *RP51A* mRNA were 3.1 to 4.0-fold longer, respectively, than wt values.

The levels of specific mRNAs in wt and *xrn1* cells were measured by Northern analysis, and the results showed that the cellular *CYC1* and *HIS3* mRNA

levels are two- to three-fold that of the wt value, and the relative cellular levels (e.g. *CYC1/ACT1*) for the mRNAs of the *xrn1* cells vary twofold in amount. The *CYC1* mRNA value is that predicted from a near normal synthesis rate and a turnover rate of about 40% of wt.

Poly(A) and cap structure-deficient mRNAs. Analysis of the slowed turnover rates of short-lived mRNA species and the higher cellular levels of some of these mRNAs in yeast cells lacking XRN1 showed the accumulation of essentially full-length mRNAs that do not bind to oligo(dT)-cellulose. Quite high levels (50% or more) of the mRNAs of *xrn1* cells are poly(A) deficient. Examination of the cap structure status of the accumulated poly(A)-deficient mRNAs in *xrn1* cells by measurements of m^7G content of total poly(A)$^-$ RNA showed that this RNA fraction contains only slightly more cap structure than the same fraction from wt cells. The rate of XRN1 hydrolysis of specific full-length mRNA species also showed that approximately 50% of the *xrn1* poly(A)-deficient mRNAs lack the cap structure since they are susceptible to XRN1 hydrolysis. The enzyme hydrolyzes capped mRNA poorly, if at all. No hydrolysis of wt poly(A)-deficient mRNA species was found, as was also true of both wt and *xrn1* poly(A)$^+$ mRNA species.

Primer extension analysis of the 5′-terminus of *RP51A* mRNA of wt and *xrn1* cells was carried out to determine if the chains of *xrn1* poly(A)$^-$ mRNAs were further modified (shortened) at the 5′-terminus. The results showed that 30-40% of the *xrn1* poly(A)-deficient *RP51A* mRNA molecules are slightly shortened (predominantly 3-4 nt) at the 5′ end. The accumulation of poly(A)-deficient mRNA species (especially the short-lived mRNAs) modified at the 5′-termini, together with the restriction of the rate of their turnover in cells lacking the enzyme, suggest a role for XRN1 in the turnover process. That such mRNAs could be formed due to processing defects does not appear likely because the polysome distribution of mRNA species in the *xrn1* cells is very similar to the same in wt cells. Also, pulse-labeling of cells shows that long poly(A) chains are formed in *xrn1* cells as in wt cells.

5′→3′ Exoribonuclease-2 is Encoded by the *HKE1* Gene

In 1991-1992, sequencing of an essential yeast gene cloned in three laboratories on the basis of different functional characteristics of temperature-sensitive mutants showed that the gene has substantial homology (37% over a 414 amino acid stretch) to the *XRN1* gene. In collaboration with Michael Douglas' laboratory at the University of North Carolina, we were able to show that the gene encodes a second 5′→3′ exonuclease, XRN2. It was shown that exonuclease-2 activity parallels *HKE1* gene dosage in yeast. Antibody to a fusion protein made on the basis of the sequence of the *HKE1* gene immunoprecipitates XRN2 purified in this laboratory. The immunoreactive protein is approximately 116 kDa as

predicted from the gene sequence. The array of phenotypes of yeast cells with a *ts* mutation in the gene for XRN2 suggests that the protein may function in several different reactions in yeast cells.

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STRUCTURAL BIOLOGY

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This group is investigating the three-dimensional structures of important biological macromolecules. One major objective is to determine the structural organization of genomic DNA and associated histone proteins by pursuing a medium-to-high resolution X-ray crystal structure of the nucleosome core particle. Several other proteins are also being studied, including inorganic pyrophosphatase, epidermal growth factor, O^6-methylguanine-DNA methyltransferase, and phosphoribulokinase.

Nucleosome Core Particle Crystal Structure

The nucleosome core particle structure has been determined to a resolution of 8 **Å** by X-ray crystallographic methods. Features present in this structure

include the histone organization, the double helical DNA structure, numerous protein-DNA interactions, and distortions in the path of the DNA around the protein core.

Nucleosomes reconstituted from homogeneous histone octamers and cloned specific-sequence DNA are necessary in order to determine a higher resolution (~ 3 Å) crystal structure. Several DNAs that form precisely positioned nucleosomes have been developed. We are currently concentrating on a 146 bp palindrome that has been engineered based on one-half of the human α-Satellite sequence. A source of disorder in the crystals occurs because two possible orientations, related by a 180° rotation about the nucleosome's dyad axis, exist for nucleosomes to be incorporated into the crystal lattice. Non-palindromic DNA does not have true dyad symmetry at the base level, whereas palindromic DNA makes the two halves of the nucleosome precisely equivalent and thus removes the two-fold disorder.

Nucleosomes containing the α-Satellite palindrome sequence have been prepared by salt-gradient dialysis reconstitution and have been crystallized. The crystals have dimensions of approximately 3.0 x 0.25 x 0.25 mm. Diffraction experiments show reflections between 2.8 and 2.6 Å in the c-axis direction, while the resolution limit is approximately 3.0 and 3.2 Å in the general direction of the a and b axes, respectively.

Yeast Inorganic Pyrophosphatase (PPase)

Yeast inorganic pyrophosphatase is a dimeric 64 kDa enzyme that catalyzes the hydrolysis of pyrophosphate. This highly exergonic reaction drives to completion numerous otherwise reversible inorganic pyrophosphate-yielding reactions, including nucleic acid biosynthesis and fatty acid biosynthesis. The high resolution structure of yeast inorganic pyrophosphatase is being determined in collaboration with D. Voet at the University of Pennsylvania. A new room temperature data set at 2.7 Å resolution has been collected and refined using XPLOR installed on a Cray Y-MP.

O^6-Methylguanine-DNA Methyltransferase (MGMT)

O^6-Alkylguanine lesions in DNA are repaired by *in situ* dealkylation in a stoichiometric suicide reaction by O^6-methylguanine-DNA methyltransferase which accepts the alkyl group in one of its cysteine residues. Studies are in progress to obtain crystals of this repair enzyme suitable for X-ray crystal structure determination. Our initial experiences, using published purification methods and standard crystallization screening protocols, led us to conclude that existing purification methods yield MGMT contaminated with variable lengths of DNA. Therefore, a new purification procedure was developed which produces pure

protein. Crystallization screening experiments and solution small-angle scattering studies are in progress.

Epidermal Growth Factor (EGF)

Epidermal growth factor (EGF) is a 6 kDa protein consisting of 53 amino acids with 3 internal disulfide bonds. EGF has been crystallized in monoclinic and tetragonal space groups. Data collected on the tetragonal form indicate cell constants of a=b=53.7 Å, c=182.5 Å with 6 molecules per asymmetric unit. Several milligrams of EGF that contain seleno-methionine in place of methionine have been prepared for use as an intrinsic heavy atom derivative.

National Resource Instrumentation

The purpose of this activity is to revitalize national resource instruments associated with the neutron scattering program at the High Flux Isotope Reactor (HFIR) and the X-ray small-angle scattering program. Two major objectives are the rebuilding of principal instruments to increase their capabilities in structural biology research and establishing a structural biology user program as a necessary prelude to the ANS.

Each of the national resource instruments has undergone various improvements and testing. The 30-meter SANS was improved in several areas including data analysis software, instrumentation safe-guards, and the addition of new sample cells and apertures. These upgrades have improved the instrument's capabilities, especially in the area of structural biology. The Huber four-circle diffractometer has been relocated to a dedicated beam line at the HFIR. Experiments to test the diffractometer and to characterize the neutron beam on this port are in progress. The diffractometer control electronics and computer are being upgraded. The 10-meter SAXS is in the final stage of extensive rebuilding. The detector, beam stop assembly, beam collimation system, transmission device, and sample holders have been rebuilt. The instrument is in routine operation for most types of samples, and it is being frequented by outside users. The detector electronics interface and software are being upgraded now to achieve optimal performance with weakly scattering biological samples. The number of structural biology groups interested in using the facilities has increased in the last year from none to approximately 20.

Small-angle neutron scattering (SANS) was used to measure the radius of gyration (R_g) of solutions of phosphoglycerate kinase (PGK) in a variety of substrate environments in D_2O. The R_g of 24.1 Å was measured for native PGK. A decrease in Rg (more compact conformation) was observed for the following: 23.7 Å for PGK+sulphate, 23.6 Å for PGK+CrATP, 23.3 Å for PGK+PGA(3-phosphoglycerate)+CrATP, 23.0 Å for PGK+CrATP+sulphate, and 22.7 Å for PGK+PGA+CrATP+sulphate. The statistical error was about ±0.3 Å. These

results are consistent with the proposed catalysis model of a hinge bending motion of the enzyme, and since CrATP is not hydrolysed, these results represent the conformational states of the bound products. The second virial coefficient was also measured for this system and is consistent with that calculated from the protein volume.

Two other small-angle scattering experiments have been completed, one studying fullerenes and one investigating the solution structure of glyceraldehyde-3-phosphate dehydrogenase. Experiments are under way to characterize O^6-methylguanine-DNA methyltransferase and a bacterial cellobiohydrolase.

NEW METHODS FOR DNA SEQUENCING

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Mapping and sequencing a genome is a slow process at present. To develop faster and more accurate methods for sequence determination, four different approaches are being pursued, one employing gel electrophoresis and the other three eliminating the electrophoresis step.

One approach is to utilize stable isotopes as DNA labels and to detect them on electrophoresis gels by resonance ionization spectroscopy, a method that is very selective and very sensitive. Eight stable isotopes of tin have been incorporated into triethylstannylpropanoic acid and attached to oligodeoxynucleotides that have, in turn, been used as primers for the Sanger sequencing procedure and also to produce PCR products. A cage compound for rare earth elements has also been synthesized and attached to DNAs; there are ~50 isotopes that could be used as DNA labels from this set of elements. Multiple isotopes are advantageous since each can be assigned to a different DNA and multiple DNAs can be assayed simultaneously. The gel electrophoresis procedure itself has also been modified, by making thinner, open-faced gels and by using discontinuous buffers; both modifications shorten the time needed for DNA analysis. Resonance ionization spectroscopy has been shown to detect two different tin isotopes that were on DNAs that had been separated by gel electrophoresis. Tin-labeled DNAs produced in the Sanger sequencing and polymerase chain reaction procedures have also been successfully detected after gel electrophoresis.

In a second approach, sequencing-by-hybridization derives the DNA sequence by determining the ability of the DNA to hybridize to a set of

oligonucleotides, e.g. 8-mers, in which all possible sequences of interest are represented. Methods have been developed to synthesize that set of oligonucleotides directly on a glass surface by a reaction scheme that greatly reduces the number of chemical steps needed to prepare the oligonucleotide matrix. At the same time, in collaboration with K. L. Beattle of the Houston Advanced Research Center, the matrix is also being constructed from presynthesized oligonucleotides. Studies on the accuracy of hybridization have been initiated. The DNA labels may be either fluorescent or stable isotope types; both are being examined, along with the instruments to detect these labels.

Third, scanning probe microscopy is being explored as a means of mapping single DNA molecules in collaboration with R. J. Warmack of the Health Sciences Research Division. Short DNAs and proteins that bind to specific sequences in the DNA molecule are being examined to detect their positions on the DNA after binding. In this manner a map of significant sequences in a single DNA molecule may be obtained. The attachment of the restriction endonuclease EcoR1 is currently under investigation to demonstrate its presence at known binding sequences on plasmid DNAs. The atomic force microscope has been particularly effective in these studies; the scanning tunneling microscope is also being tested.

Finally, the characterization of DNA sized accurately by mass spectrometry could replace gel electrophoresis for this measurement since the results could be obtained far more quickly. In collaboration with C. H. Chen of the Health Sciences Research Division, a tunable vacuum ultraviolet ionizer has been introduced into a time-of-flight mass spectrometer. This new instrument has proved effective in analyzing oligonucleotides by the process of matrix-assisted laser desorption mass spectrometry. Oligonucleotides as long as 100 have been detected. Labeling the DNAs with ferrocene, a DNA label developed in the gel electrophoresis stable isotope project above, may increase the range of DNA sizes and the sensitivity of the method.

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GENOME STRUCTURE AND ORGANIZATION

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We are defining the organization and function of very highly repeated but nevertheless complex DNAs in the genomes of higher eukaryotes. Our model system is the genome of the Bermuda land crab, *Gecarcinus lateralis*, with particular emphasis on its G+C-rich satellite DNA, which has the most complex satellite sequence known. The satellite (16,000 copies per genome) has a repeat unit of ~2.1 kb comprised of conserved domains that share 85 to 96% similarity, range in size from 50-670 bp, and are interspersed with divergent domains. This satellite DNA is much more complex than other satellite DNAs; we are investigating its transcription.

Molecular and Cellular Biology of a DNA Satellite

Cytoplasmic localization of transcripts of a complex G+C-rich crab satellite DNA. As a probe for satellite transcripts we have used a 368 bp *Eco* RI fragment from the very highly conserved 3' end of the satellite; the probe was amplified and either radiolabeled or biotinylated by PCR as described below. We have analyzed polysomal poly(A)$^+$ mRNAs on Northern blots with the satellite probe; mRNAs from midgut gland (hepatopancreas), limb bud papillae and claw muscle revealed the presence of transcripts. Characteristically different patterns were seen at different stages of the intermolt cycle. The isolation of the mRNAs from polysomes provides strong evidence that the transcripts that had reached the cytoplasm were being translated. We have therefore investigated *in situ* hybridization of the satellite probe with cytoplasmic RNAs. Biotinylated probes were applied to sections of midgut gland, limb bud papillae, ovary, or testis of anecdysial crabs. Retention of the target mRNAs was improved by UV-irradiation prior to hybridization. Transcripts were abundant in the cytoplasm of all tissues except testis. Sections of crab midgut gland treated with RNase A prior to hybridization and sections of mouse pancreas served as controls; neither showed any signals with the probe.

Denaturants or cosolvents increase the specificity of amplification by PCR of a G+C-rich DNA by genetically engineered DNA polymerases. During amplification by PCR, the DNA probe for the hybridizations described above was to be simultaneously biotinylated or radiolabeled. Therefore, it was essential that a pure product be obtained. Under standard conditions, the DNA was not amplified specifically but contained several nonspecific bands on gel electrophoresis. We established conditions that facilitate the specific amplification of G+C-rich (57% G+C) DNA by PCR. We used two genetically engineered enzymes, Ampli*Taq* DNA polymerase and Ampli*Taq* DNA polymerase, Stoffel Fragment. Addition of certain denaturants or cosolvents to PCR mixtures resulted

in the production of the single specific band of the size expected. Reagents that improved specificity of the amplified product were formamide, glycerol, DMSO, Tween 20 and Nonidet-P40; urea, ethanol and 1-methyl-2-pyrrolidone (NMP) inhibited amplification. Of the two enzymes, Ampli*Taq* DNA polymerase, Stoffel Fragment, was the more specific and efficient.

The Molecular Biology of Crustacean Molting

Intermolt cycle correlated changes in glycosylation of exoskeletal proteins demonstrated by lectin binding. In order to explore the activation of specific structural genes associated with growth, we have been investigating proteins of the crustacean exoskeleton. The "typical" crustacean exoskeleton consists of four-layers: (from outside in) an epicuticle, exocuticle, endocuticle and membranous layer. We have developed means for isolating these layers independently. In *G. lateralis*, these four layers account for 15, 8, 75, and 2% of the dry weight of the exoskeleton, respectively. Protein concentration and percent organics increase progressively in each deeper layer.

In this project, we have examined glycosylation as an indicator of post-translational modification. Proteins extracted from the layers of exoskeleton during anecdysis and late proecdysis as well as from the layers of exuviae of *G. lateralis* were electrophoresed on SDS-polyacrylamide gels and blotted to PVDF (polyvinylidene difluoride) membranes after which they were reacted with biotinylated lectins. A total of seven lectins [Concanavalin A (Con A), soybean agglutinin (SBA), wheat germ agglutinin (WGA), *Dolichos biflorus* agglutinin (DBA), *Ulex europeus* agglutinin (UEA), *Ricinus communis* agglutinin (RCA 120) and peanut agglutinin (PNA)] were tested. Of the seven, more binding occurred with Con A (indicating the presence of mannose) and SBA (indicating the presence of D-galNAc) in crabs as also occurred in insects. Specificities of Con A and SBA were demonstrated by inhibition experiments with methyl-α-D-mannopyranoside and D-galNAc, respectively. With the exception of a few of the smaller M_r (< 31 kDa) protein bands, most of the binding occurred with higher M_r bands during anecdysis; in late proecdysis, small as well as high M_r proteins were bound as was the case with the layers of exuviae. Binding of protein bands by SBA suggests the presence of α- or β-linked D-galNAc; poor binding of DBA and little or no binding of RCA 120 or PNA indicated that D-galNAc is β-linked. The presence of fucose residues was indicated by the binding of UEA. These observations indicate the occurrence of post-translational modifications of exoskeletal proteins; however, their functional significance is as yet unknown.

Sequence similarities in proteins of crab (*G. lateralis*) exoskeleton and insect cuticle. Analyses of proteins from the four exoskeletal layers by PAGE revealed an abundance of small M_r proteins with acidic pIs like those of insect cuticles. Further similarities of crab exoskeletal proteins to insect cuticular proteins were shown immunochemically and by lectin binding as described above.

Sequences of 15-30 N-terminal amino acids of ten proteins have been determined. Several proteins (42, 27, and two of 25 kDa) were selected because of their similarity to insect cuticular proteins demonstrated by immunochemical binding. Others were chosen because of their presence in all four layers of the exoskeleton. Proteins and their sources were: 42 kDa, epicuticle; 10.9 and 10.2 kDa, exocuticle, endocuticle, and membranous layer; 25 and 27 kDa, membranous layer. The 10.9 kDa proteins from the three layers had sequences identical to each other as did the three 10.2 kDa proteins. Sequences of the 10.9 and 10.2 kDa proteins were similar, though not identical. On 2D gels, the 25 kDa protein separates into two isoelectric variants; most of the first 20 N-terminal amino acids of the two variants were identical. The sequences of several of the crab exoskeletal proteins were similar (32 to 47% identity) to some of the cuticular proteins from five insect species, suggesting a common ancestry of members of the two arthropod classes. The similarity but not identity of the proteins of the various layers suggest subtle changes in gene activation during the synthesis of the exoskeleton.

Tubulin genes of *G. lateralis*. A chicken tubulin gene (1.9 kb) was used as a probe to screen the *G. lateralis* cDNA library, constructed earlier from poly(A)$^+$ RNA isolated from regenerating limb buds, for clones containing tubulin genes under high stringency conditions. Fifteen positive clones were identified; five were selected for further characterization.

The genes for the five α-tubulin isotypes (α1-α5) of *G. lateralis* differ from each other by the presence of nucleotide substitutions within both the coding region and the untranslated region. Two clones (α1 and α2) contained complete coding sequences with start and stop codons. All five cDNA clones contained an open reading frame; their sequences were very similar in their coding regions but not in their untranslated regions. Identity between the nucleotide sequences in the coding regions of the five clones was over 82% and identity between the predicted amino acid sequences was over 83%. The calculated lengths of α1 and α2 were 451 amino acids. DNA sequences of all five clones were highly homologous to insect (*Drosophila melanogaster*) α-tubulin genes. Predicted amino acid sequences of clones were highly (93%) homologous to vertebrate (e.g *Mus musculus*) α-tubulins. Much of the divergence of the crab α-tubulin polypeptides occurred near the carboxy terminus. Strong biases were observed in the case of codon usage; the third position was usually C or G. Poly (A) tails in the five clones ranged from 21 to 40.

The total number of tubulin genes in the crab genome was estimated as 5 to 6 by Southern blotting of main component DNA digested with *Bam* HI, *Eco* RI, *Hind* III or *Spe* I and probed with a 156 bp 5' segment of tubulin α1 that had been radiolabeled by PCR. Northern blot analysis of polysomal poly (A)$^+$ mRNA using the untranslated region of tubulin α1 showed that α1 gene expression is tightly regulated during the intermolt cycle in epidermis and limb bud. In epidermis, the intensity of the signal was very high during anecdysis and gradually decreased as

proecdysis progressed, indicating that tubulin synthesis precedes the changes in cell structure. By contrast, in limb bud the signal increased with the advancement of proecdysis.

Actin genes of *G. lateralis*. Actins, structural proteins that are highly conserved during evolution, polymerize into filaments that are important for different forms of cellular motion. These include the marked changes in the shape of epidermal cells that occur during proecdysis as the cells enlarge prior to synthesizing the new exoskeleton and partially degrading the old exoskeleton. The role of actins in intracellular motility and muscular contraction as well as the structure and mechanical properties of the cytoplasmic matrix is well known. Eukaryotes contain several different actins and their correspondingly distinct genes. There are three distinct groups of actins, α, β and γ. β and γ actins are usually present in cytoplasm. Cytoplasmic actins are similar in all eukaryotes; all the actins of invertebrates and plants are in this class. Expression of invertebrate actin isoforms is tissue-specific and developmental stage-specific.

The human β actin gene (2 kb; from Clonetech) was used as a probe to screen our *G. lateralis* regenerating limb bud cDNA library for actin clones under high stringency conditions. Nineteen positive clones were identified; four were selected for further characterization. The four clones ranged in size from 1 kb to 2.3 kb, including poly A tails that ranged from 6 to 55 bases. From their partial nucleotide sequences, it is clear that three of the four clones are very similar. The crab actin clones have been compared to those of actin isoforms from other organisms contained in the Genebank/EMBL database using FASTA. The three related clones are in the class of β-actin, whereas the fourth is a γ actin.

The total number of actin genes encoded in the crab genome was estimated as 7 to 8 by Southern blotting of main component DNA digested with *Bam* HI, *Eco* RI or *Hind* III and probed with the human β actin gene.

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CHROMOSOME CHEMISTRY

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The major goal of this group is to analyze and understand the macromolecular structure of eukaryotic chromosomes and their relation to DNA packaging, transcription, and replication. Our laboratory employs a wide range of biophysical, biochemical, and ultrastructural techniques to work towards detailed macromolecular models.

Chromatin Structure in a Hypotrichous Ciliated Protozoan

Ciliated protozoa exhibit nuclear dimorphism, i.e., the existence of a transcriptionally-active macronucleus (MAC) in the same cytoplasm with an inactive micronucleus. Hypotrichous ciliates possess features that distinguish them from other ciliates: (1) MACs consist of a "bag" of highly polyploid (ca. 10^4-fold), short (ca. 2-3 kbp), linear DNA molecules — each fragment corresponding to an individual structural gene and regulating flanking sequences; (2) MAC DNA replication is localized in a Replication Band (RB) that migrates along the nucleus during S phase. Several MAC genes of *Euplotes* have been cloned and sequenced. The list includes 8 genes: 5 S RNA; polyubiquitin; two versions of HSP 70 (heat shock proteins); two versions of histone H3; one histone H4 and the sole histone H1. These genes were sequenced from telomere to telomere. For most, the copy number per macronucleus and the nucleosome positions were also determined. *In situ* hybridization studies with 5S RNA and H1 gene probes revealed that the many gene copies are randomly arranged throughout MACs. Thus, DNA replication within RBs does not appear to initiate with any particular genes, but rather progresses through the MAC synthesizing whatever DNA lies within its path. Studies on the *in vivo* activity of RBs have indicated that numerous cell culture parameters can turn off replicational activity, including crowding of cells, heat shock, aphidicolin and cAMP phosphodiesterase inhibitors. Under such circumstances the region of DNA synthesis within the RB dramatically decreases incorporation of [^{3}H] dT and structurally collapses. We are currently exploring *in*

vivo controls on the activity of RBs and the post-translational modifications of RB chromatin proteins.

Chromatin Structure in the Cellular Slime Mold

Starting in 1992, we initiated research with a new biological system, the cellular slime mold *Dictyostelium discoideum*. This organism has many advantages for problems of chromosome structure and function, primarily arising from the ease of molecular genetic techniques available. Two projects have been started: (1) site-directed mutagenesis of histone H1; (2) 3-D architecture of mitotic chromosomes. In the first project, we have cloned and sequenced the *Dictyostelium* H1 gene. There appears to be only one type with a single-copy gene. Plasmids for knock-out experiments and anti-sense mutations will soon be constructed. In the second project, the seven mitotic chromosomes were visualized in the electron microscope (EM) after specific staining for DNA, employing a stain developed in our laboratory (see description of stain below).

Three-Dimensional Reconstruction of Electron Microscope Tomography (EMT)

We have been interested in the 3-D reconstruction of asymmetric organelles, specifically of chromosomal structures during transcription, replication, and higher-order packaging. Most attention has been focused upon a chromosomal region of RNA synthesis, the Balbiani Rings (BR) of *Chironomus* salivary gland cells. This gene is present on highly polytene chromosomes (ca. 10^4 endoreplicated), and, when active, generates a "puffed" region in the chromosome body. In the electron microscope, electron-dense nascent ribonucleoprotein granules (RNP) can be observed surrounding the chromatin axis. Recent studies have emphasized analysis of the folded 3-D structure of the 37 kb mRNA within the BR granule. This has been accomplished by combining three new techniques: (1) specific nucleic acid staining by osmium ammine B, developed in our laboratory; (2) energy-filtered imaging of the stained sections on a Zeiss EM902; and (3) reconstruction by EMT. Data have been collected on seven BR granules, some of which have been reconstructed. These calculated structures are displayed on a Silicon Graphics workstation and volume-rendered using a software package, "VoxelView." When enough BR granules have been reconstructed, attempts will be made to arrive at a consensus 3-D structure of mRNA folding. This sequence of operations is now quite repeatable and will eventually be applied to other nuclear structures, such as the mitotic chromosomes of *Dictyostelium discoideum*.

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MEMBRANE BIOLOGY

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The transport of metabolites across cell surfaces or across epithelial sheets of cells is studied by this group in mammalian cell systems. Cloned cells in culture are used because they ensure physiological and genetic homogeneity as well as experimental control over the chemical environment (hormones, growth factors), thus permitting the unambiguous identification of cell types and the environmental factors to which they respond. Recent work has focused on cell lines derived from pig kidney (LLC-PK_1) or opossum kidney (OK) both of which differentiate *in vitro* into populations with the properties of proximal tubule cells, and on fibroblast or mouse-skin lines responsive to epidermal growth factor (EGF). The goal of this group is to develop an understanding of the regulation of specific transport systems in relation to cell growth and differentiation. Three projects are outlined for this report period.

Differential Expression of Genes for Hexose Transport in Differentiating Kidney Cells

As in the proximal tubule of the mammalian kidney *in vivo*, at the apical (luminal) surface of differentiated LLC-PK_1 and OK cells is a Na^+-coupled glucose transporter (NaGT) which co-transports Na^+ and hexose. The electrochemical driving force on Na^+ from lumen to cell interior can energize the NaGT to drive glucose against its chemical gradient to high intracellular levels. Once in the cell, glucose exits over the basolateral Na^+-independent glucose transporter (GT), completing its transepithelial transport. In parallel, the Na^+/K^+-ATPAse at the basolateral surface extrudes the co-transported Na^+, maintaining the Na^+ gradient.

Several literature reports have led us to the following working hypothesis: in proliferating cells a low K_m, high affinity, GT is expressed that is functionally adapted to the cells' need for glucose as a metabolite; for these cells glucose is required to support growth, and the hexose that is taken up is immediately phosphorylated and enters the metabolic pathways and is not again released as native glucose. In differentiated cells expressing the apical NaGT, on the other hand, the basolateral GT is a high K_m, low affinity transporter functionally adapted to allowing free glucose to exit from the basolateral surface, thereby accomplishing transepithelial transport. We have obtained kinetic transport evidence that supports the low K_m-to-high K_m shift on differentiation in both cell types. A major difference between the cell types is the 2:1 stoichiometry for Na^+:glucose transport on the LLC-PK_1 NaGT and the 1:1 stoichiometry for the NaGT in OK cells, evidence that the cells derive from different segments of the proximal tubule in the species of origin.

We also have obtained from Harvey Lodish (MIT) suitable cDNA probes with which we are exploring this shift in gene expression at the mRNA level. Both high- and low- K_m GT genes are present in the genomic DNA of LLC-PK_1 cells (Southern blot analysis) and the patterns of mRNA expression are compatible with the hypothesized shift (Northern blot analysis). The detailed temporal relationships in comparison with the (co-)expression and of the NaGT, and the sensitivity of mRNA expression to compounds like tumor promoters and epidermal growth factor, both of which inhibit differentiation while promoting proliferation, are under investigation.

Transport of Polyamines

Polyamines are a group of compounds including putrescine, spermidine, and spermine, containing two to four amino groups. They are required for cell growth and differentiation, possibly through an interaction with exposed phosphate groups in DNA. In LLC-PK_1, polyamines are taken up by transporters that are separate from the well-known renal transporter for organic bases. The two polyamine transporters are distinguished in that one is Na^+-dependent and one Na^+-independent, the former with a K_m of $\sim 10\ \mu M$, which is in the physiological range of the blood level. In differentiated renal cells, putrescine is either transported across the sheet of cells in a direction suggesting that *in vivo* it is recovered from the glomerular filtrate and restored to circulating blood plasma, or it is oxidatively deaminated to form γ-aminobutyraldehyde; very little is metabolized to the higher polyamines. A provocative finding is that the aminoglycoside antibiotics gentamicin, neomycin, and kanamycin, clinically used antibiotics that are nevertheless toxic to the kidney *in vivo*, compete with and can block polyamine uptake by the Na^+-dependent transported in kidney cells.

Cellular Responses to EGF Mutants

The Membrane Transport Group has also been exploring the responses of EFG-dependent mouse epidermal cells (strain MK) to mutants of human epidermal growth factor (hEGF), engineered by site-directed mutagenesis in the laboratory of S. K. Niyogi. These mutants have been characterized by Niyogi's group with respect to their binding to EGF receptors *in vitro* and their effects on the V_{max} of the receptors' tyrosine kinase activity. We have been extending these studies to the cell biological responses, focusing initially on stimulation of [^{3}H]thymidine incorporation into DNA and the phosphorylation of cellular proteins *in vivo*. In general, we find that the cells respond in the direction expected from the biochemical studies, i.e., poorly binding mutants are less effective and tightly binding mutants are more effective than native hEGF when assayed on whole cells, but the quantitative differences in effectiveness are much less than those observed *in vitro*, more than two orders of magnitude in some cases. At sufficient concentrations, mutants that stimulate tyrosine kinase activity to a lower V_{max} *in vitro* can nevertheless elicit the maximum degree of [^{3}H]thymidine incorporation in intact cells. We have been able to confirm that aromaticity at position-37 on hEGF is not essential for activation of cells; seven other amino acids in this position were also effective mitogens, and only two showed even (slightly) reduced dose-response activity. Similarly, the importance of the guanidinium group on arginine-41, demonstrated by *in vitro* binding to the receptor, was also confirmed in whole cells (see Protein Engineering and Chemistry).

The quantitative differences between the *in vivo* and *in vitro* studies appear to be rate effects related to differences in kinetics of binding to the receptor or to the rate of signal transduction, hypotheses that are still being explored. We are extending these studies to other responses of cells to hEGF, including phospholipase C activation and lipocortin phosphorylation (seconds), Na^+/H^+ antiport stimulation (minutes), and induction of new mRNA for hexose transporters (hours).

MOLECULAR IMMUNOLOGY

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Basic Carcinogenesis

Cell surface proteins mediate interaction between cells and their environment. In addition to uptake of nutrients, cells receive specific stimuli from hormone and growth factor-receptor interaction at the cell surface and specific attachment to the extracellular matrix. The expression of these molecules can be

altered in tumor cells and the alteration may affect growth and metastatic characteristics of the cells.

A tumor surface protein complex, identified on cells of several lung and mammary carcinomas of BALB/c mice, has recently been identified as the integrin $\alpha 6\beta 4$ heterodimer. Even though integrins are involved in attachment to other cells and to the extracellular matrix, no specific ligand has been identified for $\alpha 6\beta 4$.

Analyses of human tissue show that $\alpha 6\beta 4$ is expressed at low levels in normal organs but is elevated in some carcinomas, i.e., colon, lung, and larynx. In contrast, no detectable levels of this integrin were found in primary human breast carcinomas. Expression of $\beta 4$ protein in normal tissue is restricted to certain squamous epithelia, particularly of skin and colon, and some subsets of endothelium.

The cDNA coding for the murine $\beta 4$ protein has been cloned and sequenced. It shares about 90% identity with the human $\beta 4$ sequence. An RNA splice variant containing a 159 bp insert is present in some normal murine tissues and in tumors. The biological significance of this form is being studied. The region of genomic DNA upstream from the transcriptional start site of murine $\beta 4$ is being cloned and sequenced to determine gene regulation.

Monoclonal Antibodies to Lung Endothelium

MAbs to normal mouse lung cells have been developed to study the role of normal cells as precursors of different types of lung cancer and to probe the interaction among cells during metastasis to the lung. Rat MAbs to mouse lung macrophages, Type I alveolar cells and endothelial cells have been identified and characterized. The most remarkable of these are two antibodies that recognize different epitopes on a 112 kDa glycoprotein (P112) expressed extensively on lung endothelial cells. This is the first demonstration that endothelial cells in the lung are different from those in other organs in that they express a unique surface glycoprotein. The target protein has been identified as thrombomodulin by amino acid sequence, cDNA sequence, and enzymatic activity.

The MAb to thromobomodulin may be useful for organ-specific drug delivery, for studying the interaction between tumor cells and endothelial cells, and for "negative imaging" of tumors for diagnosis. Recent studies using liposomes targeted with these MAbs have shown for the first time that liposomes can be localized efficiently *in vivo*. Liposomes of optimal lipid composition and antibody concentration have been formulated and tested. Growth of lung tumors can be retarded by targeting isotope or cytotoxic drugs to the lung. In addition, immunohistochemical staining of sections of mouse embryos indicates that thrombomodulin may play an important role in embryogenesis.

Monoclonal Antibodies for Drug Targeting

Targeting of drugs to tumors as a specific means of chemotherapy has long been an appealing idea. Development of tumor-selective MAbs in the 1980's kindled enthusiasm for this approach. Unfortunately, localization of MAbs in solid human tumors has been inefficient. In the best of studies, less than 0.1% of the injected dose accumulates in tumor. Model studies in our laboratory and elsewhere have shown that targeting in the vascular space is much more efficient. Efforts are under way to produce MAbs that bind selectively to tumor blood vessels. Studies involve both human renal cell carcinomas and a model rat tracheal cell carincoma. It is anticipated that identification of specific, or at least selective, MAb for tumor endothelium may increase targeting efficiencies at least tenfold.

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RADIATION CARCINOGENESIS

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There are not adequate data for estimating the risk to humans of excess cancer as a result of low-dose-rate low-linear energy transfer (LET) irradiation or exposure to high-LET radiation. The aims of this program are to develop adequate models for describing the effects of both low- and high-LET radiation in relation to dose, dose rate and fractionation. The specific aims have involved obtaining data for dose-response relationships for the induction of cancer, in particular myeloid leukemia, in RFM mice exposed to ^{37}Cs γ rays and to fission-spectrum neutrons. In a collaborative study with Lawrence Berkeley Laboratory, both fluence and dose-response relationships are being obtained for heavy ions varying in energy. These data have been used to model the dose-response relationships of high-LET radiations for tumor induction. The data from the studies of myeloid leukemia induction have been used to test the hypothesis that the effects of low doses of low-LET radiation, that are so difficult to quantify directly, can be estimated from the data obtained with low-dose-rate irradiation and multiple

small fractions. The results indicate that the hypothesis, which depends on a linear-quadratic dose-response relationship, is valid. It has also become clear that the size of the dose fractions that are required to estimate the initial slope of the single dose-response curve varies between tissues by over a factor of 25. The results of these experiments have demonstrated that repair is involved when doses either are given at a low dose rate or are fractionated, and that the number and perhaps size of the targets for cancer induction vary markedly among different tissues. Since the induction of myeloid leukemia by radiation involves a specific chromosomal aberration in chromosome 2, either an interstitial translocation or a deletion, the results imply that either the probability of the DNA lesion occurring is decreased or that the lesion is repaired with low-dose-rate or fractionated exposures.

In the collaborative study on cancer induction by heavy-charged particles, it has been shown that the effectiveness for cancer induction increases up to a maximum that appears to be when the LET reaches about 100 *K*eV/μm but does not decrease significantly with beams with LET levels as high as 400 *K*eV/μ. This latter finding is in contrast to cell killing and mutation. The finding is also not consistent with the recently proposed relationship of quality factor to LET.

In another collaborative study with R. D. Ley and colleagues at the Lovelace Medical Foundation, we have elucidated the pathogenesis of fibrosarcomas and hemangiosarcomas in the stroma of the corneas of *Monodelphis domestica* induced by ultraviolet radiation (UVR). The tumors occurred in animals in the experiments that established that cyclobutane pyrimidine dimers were the specific DNA lesion in the induction of squamous cell carcinoma by UVR. The pathogenesis of the corneal tumors is of interest because the first change observed in the stroma of the animals exposed to UVR was neovascularization, followed by proliferation of what had been assumed to be terminally differentiated fibroblasts, the only cell type found in the stroma of the cornea. These and other findings suggest that the release of fibroblast growth factor, which is known to also initiate angiogenesis, is induced by UVR. Subsequent exposures to UVR neoplastically transform the proliferating fibroblasts. The sequence of events in this example of induced tumorigenesis is very different from other systems; for example, angiogenesis usually occurs after cells have been neoplastically transformed and considerable growth has occurred. The sequence of molecular events involved and the nature of the growth factors involved are now under study.

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NEOPLASTIC PROGRESSION IN RAT TRACHEAL EPITHELIUM

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In solid cancers there is good evidence that while the initiating events are a prerequisite for carcinogenesis, they do not determine the probability that a cancer develops. This fact makes it imperative to study the factors influencing the expression of initiation or neoplastic transformation. Studies carried out with a combined *in vivo* and *in vitro* rat trachea model are designed to evaluate (1) critical target cells for development of respiratory cancers; (2) the influence of direct cell-cell contact on neoplastic development following exposure to high- or low-linear energy transfer (LET) radiation; and (3) the influence of cell-cell interactions mediated by diffusible growth factors, such as transforming growth factor type β, on the evolution of respiratory neoplasias.

In the model system, tracheal cells are isolated, manipulated, and grown *in vitro*. They can subsequently be used to re-populate denuded tracheas that have been implanted *in vivo*. A brief summary of specific projects is given below.

Modulation of TGF-β Induced Growth Inhibition in the Intact Tissue

During the course of neoplastic progression, rat tracheal epithelial cell lines become less sensitive to growth inhibition by TGF-β in culture. For this reason it has been proposed that this loss of sensitivity may be critical to evolution of the neoplastic state. However, the relevance of this observation to tumor development *in vivo* needs to be confirmed. Preliminary data obtained with normal tracheal cells indicate that the response to TGF-β is modified in the presence of other cell populations. Current experiments are designed to evaluate the response of preneoplastic, neoplastic and normal cells to TGF-β in repopulated tracheal grafts *in vivo*.

Alpha-Particle-Induced Alterations in Exposed Rat Tracheal Mucosa

Given the well-defined energy spectrum and penetrability of alpha particles in tissues, we are attempting to better define the critical target cells for neoplastic development in irradiated respiratory tissues. Two basic approaches are being utilized. The first involves separation of different subpopulations of tracheal cells prior to irradiation, and the second, irradiation of the intact tissue while systematically varying the distance from the alpha-particle source to different subpopulations within the intact trachea. The parameters of interest are cell survival and the development of preneoplastic or neoplastic lesions in cell populations irradiated under systematically varied conditions. There appear to be critical interactions between alpha particles and low energy gamma emitted by ^{241}Am and ^{238}Pu. We will be focusing our efforts on unraveling the mechanism(s) whereby alpha particles interact with other types of ionizing radiation to induce preneoplastic/neoplastic alterations in respiratory epithelium.

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FUNDAMENTAL AND APPLIED CRYOBIOLOGY

P. Mazur
K. W. Cole
P. D. Schreuders[1]
J. W. Hall

Cryobiological Preservation of Drosophila Embryos

Because of its genetic importance, there now exist over 20,000 mutant lines of *Drosophila*, and the number is accelerating. The maintenance of these lines by standard generational breeding is costly. Equally or more important, the frequent generational cycles accelerate genetic drift. Cryopreservation would be an obvious solution, but intermittent attempts by *Drosophila* geneticists over the 20 years since we succeeded in freezing mouse embryos failed. In 1985, Dan Lindsley and NSF organized a meeting of *Drosophila* people and cryobiologists. As a consequence of that meeting and subsequent proposals, NSF awarded a grant to our group, in collaboration with Anthony Mahowald (University of Chicago), to work on this formidable problem.

Permeabilization. The standard approach to cryopreservation requires that cells or organisms be (a) permeated with molar concentrations of a cryoprotectant like glycerol and (b) cooled slowly enough so that they osmotically dehydrate to their equilibrium water content before reaching the temperature at which ice formation can occur in the organism, generally -10 to -30°C. Thus, to be successfully cryopreserved, the cell or organism must be permeable to both water and the cryoprotectant. Unfortunately, *Drosophila* embryos are permeable to neither because of waxes in the vitelline membrane that surrounds the embryo proper. Consequently, for any hope of cryopreservation, they must be permeabilized. The effective treatment is drastic: An outer chorion is first removed by exposure to 50% Clorox. The surrounding water is then replaced by pure alcohol to provide a solvent miscible with the next step, which is exposure to an alkane like heptane. In 1991-1992, we identified several factors critical to effectively permeabilizing the embryos without killing them. First, the embryos must be exposed to alkane containing low and precisely known concentrations of alcohol for precise times; 0.3% butanol in n-heptane for 90 seconds. If the concentration of alcohol is <0.3%, permeabilization is poor. If the concentration is >0.4%, the permeabilization procedure becomes seriously damaging. A second important point is that the effectiveness of the permeabilization and its benignness depends critically on the developmental stage of the embryos (see below). At the proper stage, some 80% are permeabilized and over 90% are viable. On the basis of their osmotic response in hypertonic solutions, the permeabilized embryos are permeable to both water and the cryoprotectant ethylene glycol.

Preventing Chilling Injury. In theory, it should be possible to preserve cryobiologically such permeabilized embryos by a classical slow freezing approach. Unfortunately, this proved not to be the case. We found that if

embryos are cooled slowly enough to prevent intracellular freezing (<1°C/min), they are all killed by about -25°C even in the total absence of extra- or intra-embryonic ice. We determined, in other words, that they are extremely sensitive to chilling injury, and that the rate of death from chilling increases sharply with decreasing temperature. Calculations based on the activation energy of the process suggested that the only way to avoid death from chilling was to cool and warm the embryos at very high rates. But the required high cooling rates would, under ordinary circumstances, produce lethal intracellular ice.

Vitrification. There is a potential route around this problem, namely, to induce the water in cells to solidify by forming a glass instead of crystallizing. The only way to achieve such vitrification during cooling and prevent devitrification during warming is to introduce very high concentrations (i.e., >50% by wt) of glass-inducing solutes in and around the embryos. The solutes must not only be permeating, but they must have low toxicity.

In 1987, W. Rall (a former graduate student in the group), found that the conflicting aims of very high concentrations and toxicity could be reconciled in mouse embryos by adding a glass-inducing solute like glycerol in two steps. (1) The embryos are allowed to fully permeate with a concentration like 2 M that is tolerated. Then (2) the internal concentration is raised to the desired very high level by osmotically dehydrating the "loaded" embryos by exposing them for short times to high concentrations of glycerol at 0°C, at which temperature permeation is low. P. Steponkus' group at Cornell, in collaboration with Rall, applied the same strategy to permeabilized *Drosophila* embryos, except that they used ethylene glycol instead of glycerol because permeation of the latter was too slow. When they then cooled 13-h embryos at ~25,000°C/min in a mixture of liquid and solid nitrogen at -205°C and warmed them at comparable rates, 18% of the embryos survived (i.e., hatched). This finding, reported in 1990 in *Nature*, was of major importance, since it showed that some survival could be obtained. However, only 3% of those that hatched went on to form adult flies for an overall efficiency of 0.5%, too low to constitute practical cryopreservation.

Rall had shown that ~50 wt % cryoprotectant concentrations induced vitrification in mouse embryos even with cooling rates as low as 10°C/min; yet Steponkus *et al.* had reported that *Drosophila* embryos failed to survive cooling at several thousand degrees/min. The difference, they implied, was the need to outrun chilling injury. But in early 1992, we made the important finding that survival is much more dependent on very high warming rates than it is on very high cooling rates. Survival is reasonably high if cooling is slow provided that warming is very rapid (-200 to 0°C in 0.1 sec). But none survive in the reciprocal case where cooling is very rapid, but warming is slowed to even 2000°C/min (-200 to 0°C in 6 sec). If chilling injury were the major factor, time during cooling should

have equivalent effects to time during warming. The lack of reciprocity favors the view that the critical factor is whether vitrified cytoplasm remains glassy during warming or undergoes devitrification (i.e., crystallization). It is considerably easier to induce vitrification during cooling than it is to prevent devitrification during warming. Whether or not a vitrified solution devitrifies depends on (a) the concentration of glass-inducing agent and (b) the warming rate. The two interact strongly. We are using 55% by weight ethylene glycol. Data from G. Fahy of the American Red Cross shows that a warming rate of ~500°C/min should prevent devitrification. If devitrification occurs, the heat of crystallization released can be detected by calorimetry. Using differential scanning calorimetry, Paul Schreuders, a postdoctoral fellow in our group, and Brian Sales (ORNL Solid State Division) have found that our ethylene glycol-PVP devitrification solution does not devitrify when warmed at 200°C/min. Why then do we have to warm at 100,000°C/min? We believe it is because not all parts of the embryos see 55 wt %. If they see only 5% less, Fahy's data show that the warming rate required to prevent devitrification increases ~5,000 fold to ~200,000°C/min.

Critical Dependence of Survival on Developmental Stage. In spite of attention to avoiding devitrification, only an average of 18% of vitrified 12-h embryos hatched and only 5% of the surviving larvae developed into adult flies, values similar to those reported by Steponkus for 13-h embryos. The *Drosophila* embryo takes 21 h to hatch. Initially, we had considered using very early stage embryos because the experience of cryobiologists has been that simpler systems are better able to survive freezing. We quickly abandoned this approach after discovering that young (3-h) eggs were far more sensitive to chilling injury than even the sensitive 12-h stage. The use of embryos older than 14 h had been precluded by the finding that such older embryos permeabilized poorly and consequently would not survive vitrification. For these reasons primarily, we concentrated our efforts on 12-h embryos. Embryos at this stage, however, are undergoing the critical steps of head involution and dorsal closure, and older embryos might be inherently less sensitive. Consequently, we reexamined the responses of permeabilization and vitrification of embryos older than 12 h with an important difference. Rather than timing from egg laying, we timed development from a zero-point that could be accurately defined developmentally, namely, the time when 50% of the embryos were so-called Stage 14 and 50% stage 15. This point, which can be determined within 15 min, corresponds to 12.5 h from egg laying. When we then examined the permeabilization of embryos and survival after vitrification of embryos ranging from 11.5 to 17.5 h ("zero" -1.0 to "zero" + 5 h), we obtained the results reported in *Science* (Dec. 18, 1992), namely, survival peaks sharply at 14.5 to 15.0 h. At that developmental stage, some 60-70% hatch and some 40% of the resulting larvae develop into fertile adults. The resulting overall efficiency of 20-25% is high enough to constitute effective practical cryopreservation of the Oregon-R wild type strain under study. The procedures that yield these results were presented at a workshop on *Drosophila*

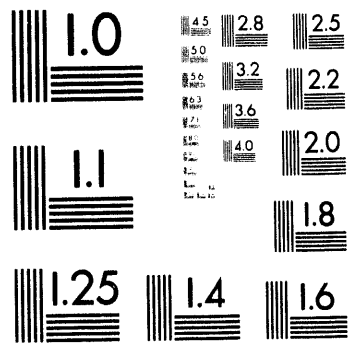
cryopreservation at the Drosophila Research Conference in April 1993, and a detailed protocol is being made available to interested stock centers and individuals.

Fundamental Cryobiology of Human and Mouse Spermatozoa

Although sperm were among the first mammalian cells to be successfully frozen, surprisingly little is known about the fundamental cryobiology of these important cells. In collaboration with John Critser and Frederick Kleinhans of Methodist Hospital, Indianapolis, we have been studying this matter in human spermatozoa and we have initiated an analogous study of the cryobiology of mouse sperm under the auspices of an ORNL Seed Money grant. The rationale for studying the cryobiology of human sperm is that present empirical methods of freezing yield only 25-50% functional survival with high individual variability. Because of the AIDS crisis, it is becoming mandatory that donor sperm be stored a minimum of six months before being used in artificial insemination. The ultimate purpose of the study on mouse sperm is to devise methods for cryopreserving the sperm of transgenic strains of mice, the number of which is accelerating. Cryopreserved sperm have the potential of being substantially more cost-effective than cryopreserved embryos. Unfortunately, investigators to date have not been able to reproducibly cryopreserve the sperm of this species. The reason for the difficulty is unknown.

Knowledge of the permeability of a cell to water and to cryoprotectants can be a powerful tool in predicting the likely optimum values for the major steps involved in freezing. From the value of the permeability coefficient for the cryoprotectant, one can compute the optimum procedure for adding and removing the cryoprotectant without osmotic shock. From knowledge of the permeability of the cell to water and its temperature coefficient, one can predict the cooling rate likely to be low encugh to preclude lethal intracellular freezing. Accordingly, the first step of our research on human sperm has been to determine the permeabilities to water and glycerol at temperatures between 0 and 30°C.

To apply the permeability equations, we need to know that the sperm behave as ideal osmometers in the sense of their volume being a linear function of the reciprocal of the external osmolality of nonpermeating solutes. Electron spin resonance (ESR) provides a powerful tool for measuring the intracellular aqueous volume. To a suspension of sperm in an anisotonic solution of a nonpermeating solute in physiological saline are added tempone and chromium oxalate (CrOx). Tempone is an aqueous spin probe which labels all aqueous regions to yield a sharp, narrow ESR signal. The magnetic broadening agent CrOx is membrane impermeable and only affects extracellular regions where it broadens the tempone signal to near extinction. Then by comparing the amplitude of the remaining sharp intracellular signal with a standard and knowing the cell concentration, one can compute the average cell aqueous volume. Experiments using the technique on


1.0
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5.0
5.6
6.3
7.1

2 of 2

human and bovine sperm confirm ideal osmotic responses under both hypotonic and hypertonic conditions.

If sperm are placed in sufficiently hypoosmotic solutions of non-permeating solutes, they will swell until they undergo spermolysis (gross membrane leakiness), and the time taken to reach the spermolytic point is a measure of their permeability to water (Lp). If sperm or other cells are placed in a solution that is hyperosmotic with respect to a permeating solute like glycerol but hypoosmotic with respect to nonpermeating salts or sucrose, the cells will initially shrink due to the hyperosmotic glycerol, but will then swell progressively as glycerol and water enter. If the salt solution is sufficiently hypoosmotic, the swelling will continue until the sperm lyse. In this case the time taken to reach the spermolytic point is a measure of the permeability of the sperm to glycerol (Pg).

We assess the critical spermolytic volume by labeling the sperm with the fluorescent molecule carboxyfluorescein and by then observing with a laser cell sorter the time at which the fluorescence disappears as a consequence of the development of membrane leakiness. The critical tonicity for human sperm based on this procedure is 0.06 osmolal. Since the sperm behave as ideal osmometers, the volume of intracellular water at the critical tonicity relative to the isotonic volume will be reciprocally related to the osmolality, i.e., 0.29/0.06 or about 5X isotonic. The reason that sperm can withstand swelling to 500% of their normal water volume is that the plasma membrane in this cell type (which apparently is fixed in area) surrounds a highly elongated ellipsoid that can be converted to a sphere of 5X greater volume without increase in surface area.

The information on critical tonicity and spermolytic volume has permitted us to determine the permeability coefficients of sperm to water (Lp) and glycerol (Pg) by measuring the time to hypotonic spermolysis as just described. Pg was also measured by the ESR method. The value of Lp is approximately equal to that of the human red cell, heretofore the highest known. The value of Pg is about 10X that of the human red cell, which itself is one of the higher values known.

From the value of Lp and its activation energy, one can calculate the cooling rate required to induce lethal intracellular freezing; that is, one can calculate the cooling rate at which the sperm are unable to dehydrate to their equilibrium water content before reaching their nucleation temperature of about -25°C. The calculated cooling rate in human sperm is about 10,000°C/min. In most cells studied (e.g., mouse embryos), the rate calculated to produce intracellular ice is in good agreement with the experimentally determined rate. That appears not to be the case in human sperm. Because of their small size, we have not yet been able to directly determine the cooling rate dependence of intracellular freezing in sperm, but inferential estimates based on the cooling rate dependence of survival indicate that intracellular freezing begins to occur at cooling rates between 10 and

100°C/min (Henry *et al.*, submitted) – a 100- to a 1000-fold discrepancy with the calculated rate.

A major problem in achieving h' 'n survivals of frozen sperm is that the maximum concentrations of glycerol tolerated are one-half to one-third of those generally considered optimal. The current limitations could be osmotic. The introduction of cryoprotectants like glycerol causes initial cell shrinkage and the removal of cryoprotectant can cause extensive cell swelling. The extent and duration of the shrinkage or swelling depends on Pg and depends on the rate at which cryoprotectant is added to or removed from the external medium. Gao *et al.* have shown that osmotic shrinkage in nonpermeating media (NaCl or sucrose) is exceedingly damaging to human sperm. As expected from the high value of Pg, the abrupt addition and removal of hyperosmotic glycerol produces little loss in membrane intactness, although it is somewhat more damaging than is slow addition and removal. On the other hand, motility is substantially more sensitive to the rapid removal of glycerol. Since motility depends on the existence of functioning mitochondria and on an intact and functioning motor apparatus (axoneme), its greater sensitivity to the abrupt removal of glycerol suggests that the permeability of these internal structures to glycerol may be substantially lower than the permeability of the plasma membrane. An analogous difference between the water permeability of organelles and the plasma membrane could also account for the large differences noted in the preceding paragraph between the cooling rates calculated to induce intracellular freezing and those inferred experimentally to do so.

The proposed strategy for studying mouse sperm is similar to that for human sperm. Work in the past year has emphasized the development of techniques for extracting the sperm, and assessing their motility and their membrane intactness using the fluorophors carboxyfluoroscein diacetate and propidium iodide and the Division's Fluorescence Activated Cell Sorter. Like human sperm, the osmotic response of mouse sperm based on ESR measurements has been found to be ideal over a wide range of hypo- and hypertonicities. Preliminary estimates have been made of their critical tonicity and water permeability. The values are similar to those for human sperm, although mouse sperm are about twice as large.

Mouse Embryo Banking Projects

There are several reasons to preserve mouse genetic and experimental lines in the form of frozen embryos: (1) As insurance in case the breeding line is lost by reproductive failure, disease, or fire (which occurred at Jackson Labs in 1947 and 1989); (2) to study, control, or reduce genetic drift in inbred lines; (3) to permit experiments that otherwise could not be undertaken, such as a comparison on the same day between progenitors and genetically selected descendants; and (4) to provide a method of maintaining genetic stocks that is cheaper than conventional

breeding in terms of manpower, space, and facilities. For the past several years we have been engaged in a mouse embryo banking project that was funded by a grant from the National Institute on Alcohol and Alcohol Abuse to John Crabbe at Oregon Health Sciences University. This project involves the banking of mouse embryos at various generations in the genetic selection of two lines that exhibit differences in response of body temperature to alcohol.

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EDUCATIONAL ACTIVITIES

Doctoral and Postdoctoral Training Programs

The University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences was established in 1965 through a joint effort of the Biology Division of the Oak Ridge National Laboratory and The University of Tennessee, so that the scientific talents and research facilities of the Laboratory could be utilized more fully in graduate education. The School accepted its first class of seven graduate students in the fall of 1967. Since then it has grown and developed steadily. It now has a graduate enrollment of 26 students and is a recognized center of quality education in the southeast. The University of Tennessee supports 16 students with research assistantships; 1 student each is supported by an Oak Ridge Associated Universities Fellowship, a University of Tennessee Hilton Smith Fellowship, and a National Science Foundation predoctoral appointment; 2 students are supported by National Institutes of Health predoctoral appointments. As of September 1993, a total of 142 students have been awarded Ph.D. degrees through the Graduate School of Biomedical Sciences. Nearly all continue today in careers in research or research/teaching, and some have won distinction for their research contributions. Although intended primarily for Ph.D. training, the School has awarded a total of 20 M.S. degrees. This component of the program was added to meet the needs of UT and ORNL employees.

The Graduate School offers courses and laboratory experience in many aspects of biomedical science, emphasizing biochemistry and molecular biology, carcinogenesis and radiation biology, mammalian genetics and development, and structural and cellular biology. It has one full-time faculty member from The University of Tennessee (D. E. Olins) and two research professors (A. L. Olins and L. J. Hauser) supported through extramural research funds. An adjunct faculty of 20 members from the Biology Division and other divisions of the Oak Ridge National Laboratory and 2 members each from the Oak Ridge Associated Universities and the University of Tennessee fulfill most of the obligations for teaching and directing thesis research. The Graduate School of Biomedical Sciences is a component of the Graduate School of The University of Tennessee but is housed within the Biology Division of the Oak Ridge National Laboratory. Both classroom teaching and laboratory research training utilize Biology Division facilities. The School's Director is employed by both institutions and reports to the Dean of the Graduate School at the University, the Biology Division Director, and the Associate Director for Environmental, Life, and Social Sciences at the Laboratory.

The overall objective of the Graduate School of Biomedical Sciences is to develop a high quality, multidisciplinary graduate training program in the

Biomedical Sciences. The program begins with a core curriculum that emphasizes the multidisciplinary and quantitative aspects of modern biology while providing a diversity of laboratory research experiences. Advanced students take courses and tutorials in specialized areas, participate in research seminars, and pursue dissertation research under the direction of a faculty preceptor.

The students form a very active group of investigators as reflected by 15 coauthorships, 11 as first authors, during the last 20 months. This represents a significant contribution to the productivity and excellence of ORNL's Biology Division. In return, the students receive superb guidance and training by staff members of the Biology Division.

Postdoctoral training is another important activity of the Biology Division. The program is administered by The University of Tennessee and by Oak Ridge Associated Universities. Support for 5 postdoctoral fellows was provided by a Postdoctoral Training Grant in Carcinogenesis Research from the National Cancer Institute, and 15 postdoctoral fellows were supported by research grants and contracts secured by individual principal investigators. After a two- or three-year period of research in the Biology Division, trainees obtained positions in universities, industries or other government laboratories.

Undergraduate Training Programs

The Biology Division participated in four undergraduate training programs: (i) Great Lakes Colleges Association/Associated Colleges of the Midwest (GLCA/ACM Science Semester), (ii) Oak Ridge Associated Universities Student Research Participation (SRP), (iii) Science Engineering Research Semester (SERS), and (iv) Minority Summer Intern. Under the auspices of these organizations and in cooperation with Oak Ridge National Laboratory, outstanding college juniors and seniors are offered opportunities for independent research in the life sciences. In the past 24 months, there were 16 students, possessing the educational qualifications and the potential for a successful scientific career, who spent several weeks performing research under the guidance of Biology Division staff members.

Although the principal purpose of the programs is to provide training experience for the students, it often allows Division staff members an opportunity to broaden their areas of research. Upon completion of their research activities in the laboratory, students prepare a formal scientific paper and present a talk on their work. The programs, in which over 573 students have participated during the past 27 years, have received the enthusiastic endorsement of the students, their colleges, and the members of the Biology Division.

APPENDICES

Advisory Committee — FY 1993 -1994

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Center for Cancer Research, E17-543
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Dr. H. Ronald Kaback
Howard Hughes Medical Institute
Professor, Department of Physiology
University of California at Los Angeles
405 Hilgard Avenue
Los Angeles, California 90024-1570

Extramural Activities

Society Committees

G. J. Bunick	-	Special Interest Committee on Small-Angle Scattering, American Crystallographic Association (1991—); chair (1993)
J. S. Cook	-	Chairperson, Publications Committee, American Physiological Society (1989—1992)
R. J. M. Fry	-	History Committee, Radiation Research Society (1982—); Finance Committee (1990—)
F. C. Hartman	-	Awards Committee, American Society for Biochemistry and Molecular Biology (1992—1993)
P. Mazur	-	Chairman, Publications Committee, Society for Cryobiology (1974—) Board of Governors, Society for Cryobiology (1967—)
A. L. Olins	-	Minorities Committee, American Society for Cell Biology (1985—1991) Faculty Senate, University of Tennessee (1989—1992)
E. M. Rinchik	-	Chairman, Committee for Mouse Chromosome 7, International Mammalian Genome Society (1990—1992)
L. B. Russell	-	Twenty-Fifth Anniversary Committee, Environmental Mutagen Society (1993—)
D. M. Skinner	-	Board of Governors, The Crustacean Society (1987—) Selection Committee, Miller Research Fellows, Miller Institute for Basic Research in Science, University of California, Berkeley (1987—) Chair, Membership Recruitment (National), Association for Women in Science (1992); co-chair, Nominating Committee (1992—) Steering Committee, Women in Cell Biology of the American Society for Cell Biology (1992—)

Advisory Committees

G. J. Bunick - Applications Software Working Group, National Energy Research Supercomputer User Group (1991–)

J. S. Cook - Member of the Corporation, Mount Desert Island Biological Laboratory (1962–)
External Review Committee, Biomedical Sciences Program, Wright State University (1991)

R. J. M. Fry - Scientific Committee 40, National Council on Radiation Protection and Measurements (1977–)
Honorary Member, National Council on Radiation Protection and Measurements (1993–)
Scientific Committee 75 (Chairman), National Council on Radiation Protection and Measurements (1983–)
Committee 1, International Commission on Radiological Protection (1985–)
Member, Aerospace Medicine Advisory Committee, National Aeronautics and Space Administration (1988–1993)
Chairman, Radiation Discipline Working Group, National Aeronautics and Space Administration (1992–1995)
Chairman, Subcommission on Radiation Biology, Committee on Space Research (1990–1994)
Visiting Committee, Medical Department, Brookhaven National Laboratory (1991–)

W. M. Generoso - Committee on Toxicology, National Research Council (National Academy of Sciences) (1993–1996)
Advisory Board, Second International Conference on Environmental Mutagens in Human Populations at Risk (1993–1995)

F. C. Hartman - Executive Committee, Division of Biological Chemistry, American Chemical Society (1989–1991)
USDA Grant Review Panel (1993–)

S. J. Kennel - Study Section on Health Effects Research, U.S. Environmental Protection Agency (1982–)
Ad hoc member, National Cancer Institute Site Visit Committees (1992–1993)

S. K. Niyogi - Scientific Expert, United Nations Industrial Development Organization (1989—)
External Advisor to Indian Jute Industries Research Association, Government of India (1989—)
Advisor to Indian Institute of Chemical Biology, India (1986—)

A. L. Olins - Member of the Corporation, Marine Biological Laboratory, Woods Hole (1983—)
National Science Foundation, Cell Biology Program, Review Panel (1990—1993)

D. E. Olins - Member of the Corporation, Marine Biological Laboratory, Woods Hole (1983—)

R. A. Popp - Mouse Hemoglobin Nomenclature Committee (1984—)
Ad hoc Member, Sickle Cell Center Review Panel, National Institutes of Health (1992)

E. M. Rinchik - Ad hoc Member, Cellular Biology and Physiology Study Section -1, National Institutes of Health (1993)

L. B. Russell - Environmental Health Institute, Fellow (1987—); Awards Nomination Committee (1991—)

P. B. Selby - Scientific Advisor and Member of the United States Delegation to UNSCEAR (United Nations Scientific Committee on the Effects of Atomic Radiation) (1984, 1986—)
Member, Technical Committee, Environmental Mutagen Society (1992—)

D. M. Skinner - Member, Visiting Professorships for Women Panel, National Science Foundation (1993)
Member of the Corporation, Marine Biological Laboratory, Woods Hole (1971—)
Member, Advisory Committee, Department of Biology, Georgetown University (1983—)
DOE Review of Laboratory Programs for Women (1992, 1993)

A. L. Stevens - Biomedical Sciences Study Section, National Institutes of Health (1985—)

M. Terzaghi-Howe - Member, Metabolic Pathology Study Section, National Institutes of Health (1990—)

R. P. Woychik - Committee on Transgenic Nomenclature, Institute of Laboratory Animal Resources (ILAR), National Academy of Sciences (1991—1992)
Member, National Institutes of Health-DOE Genome Working Group on the Mouse (1991—)
Member, Planning Committee for the Renal Biopsy and Genetic Material Data Bank (1992—)
Member, Organizing Committee, NIEHS Workshop Series on Devlopmental Biology and Developmental Toxicology (1991—)
Member, Planning Committee for National Academy of Sciences, Board on Biology, Workshop on the Sharing of Biological Resources (1992—1993)

Editorial Boards

J. S. Cook - *American Journal of Physiology* (1987—1996)
Current Topics in Membranes and Transport, Advisory Board (1983—)
News in Physiological Sciences, Associate Editor (1986—)
Chair, Managing Board (1989—1992)

R. J. M. Fry - *Radiation Research*, Editor-in-Chief (1988—)
Advances in Radiation Biology (1990—1993)

W. M. Generoso - *Teratogenesis, Carcinogenesis, and Mutagenesis* (1979—)
Mutation Research (1985—)

F. C. Hartman - *Journal of Protein Chemistry* (1982—1996)
Journal of Biological Chemistry (1992—1996)

P. Mazur - *Cryobiology* (1967—)

D. E. Olins - *Chromosoma* (1990—)

L. B. Russell - *Mutation Research* (1976—)

D. M. Skinner - *Gene* (1986—)
Physiological Zoology (1989—)

Awards, Honors

B. R. Beatty	-	Martin Marietta Energy Systems, Inc., Technical Support Award (1992)
G. M. Guinn	-	Martin Marietta Energy Systems, Inc., Technical Support Award (1993)
V. L. Godfrey	-	Martin Marietta Energy Systems, Inc., Technical Support Award (1992)
M. R. Harpel & F. C. Hartman	-	Martin Marietta Energy Systems, Inc., Publication Award (1993)
K. B. Jacobson	-	Martin Marietta Energy Systems, Inc., Operations and Support Team Award (1992)
	-	Fellow, American Association for the Advancement of Science (1992)
P. Mazur	-	R&D 100 Award (1993)
	-	1993 Distinguished Service Award, American Association of Tissue Banks
D. J. Moore	-	Martin Marietta Energy Systems, Inc., Administrative and Office Support Award (1993)
R. J. Mural	-	R&D 100 Award (1992) (with E. C. Uberbacher & R. C. Mann, Engineering Physics and Mathematics Division)
R. A. Popp	-	Martin Marietta Energy Systems, Inc., R&D Accomplishment (1993)
L. B. Russell	-	Environmental Mutagen Society Award (1993)
	-	Marjory Stoneman Douglas Award (1993)
	-	"Woman of Impact" Award, American Association of University Women, Oak Ridge Branch (1993)
A. Stevens & F. W. Larimer	-	Martin Marietta Energy Systems, Inc., Publication Award (1992)
M. Terzaghi-Howe	-	Martin Marietta Energy Systems, Inc., R&D Accomplishment (1993)

Invited Presentations at Major Conferences
October 1, 1991 - September 30, 1993

Fry, R. J. M.

"The Biological Basis for Dose Limitation to the Skin." International Conference on Radiation Effects and Protection, Mito, Japan, March 18-20, 1992.

"New Risk Estimates at Low Doses." Twenty-Eighth Annual Meeting of the National Council on Radiation Protection and Measurements, Arlington, VA, April 1-2, 1992.

"Radiation-Induced Cancer: Risks and Mechanisms." International Conference on Effects of Low Dose Ionizing Radiation: Implications for Human Health, Bournemouth, UK, May 18-20, 1992.

"Deep Space Missions: Radiobiological Research Needs." 29th Plenary Meeting of the Committee on Space Research at the World Space Congress, Washington, DC, August 28-September 5, 1992.

"The Radiation Protection Problems of High Altitude and Space Flight." Workshop on Radiation Protection toward the Turn of the Century, Paris, France, January 11-13, 1993.

Hartman, F. C.

"Use of Site-Directed Mutants of Rubisco to Explore Structure-Function Relationships." First Scandinavian Photosynthesis Congress, Karlslunde, Denmark, November 25-29, 1991.

"Functional Significance of Intersubunit Electrostatic Interactions at the Active Site of *R. Rubrum* Ribulose-Bisphosphate (RuBP) Carboxylase/Oxygenase." American Society for Biochemistry and Molecular Biology Symposium on Protein Engineering Approaches to Enzyme Mechanism, San Francisco, CA, April 6-9, 1992.

Jacobson, K. B.

"Structural Characterization of Underivatized Pteridines by Fourier Transform Mass Spectroscopy." Sixth International Conference on Pteridines and Related Biogenic Amines and Folate, Seoul, Korea, June 7-13, 1992.

"Development of Mass Spectrometric Methods for DNA Sequencing and Genome Analysis." Future Technologies for DNA Analysis, Washington, DC, October 26-27, 1992.

"Development of Faster, More Accurate Methods for Genome and DNA Analysis that use Stable Isotopes of Tin and Other Metals." Biomedical Optics '93 Meeting, Los Angeles, CA, January 16-22, 1993.

Mazur, P.

"Mouse and *Drosophila* Embryos as Illustrations of Two Sets of Cryobiological Problems and Principles." Conference on Theoretical Basis of Cryopreservation, Hardec Kralove, Czechoslovakia, October 6-9, 1992.

"Workshop on Cryobiological Preservation of *Drosophila*." Annual Drosophila Research Conference, San Diego, CA, April 3, 1993.

"Vitrification Based Cryopreservation of *Drosophila* Embryos." Cryogenic Engineering Conference, Albuquerque, NM, July 12-16, 1993.

Popp, R. A.

"*In Vivo* Sickling of Red Cells and Pathobiology in a Newly Developed Transgenic Hb S Antilles Line of Mice." 18th Annual Meeting on Sickle Cell Disease, Philadelphia, PA, May 22-25, 1993.

Rinchik, E. M.

"Fine-Structure Functional and Physical Mapping of Germline Deletions in the Mouse: Experimental Models for the Human Genome Program." Human Genome III Meeting, San Diego, CA, October 20-23, 1991.

Russell, L. B.

"Contributions of the Specific-Locus Test (SLT) to Structural Characterization of Lesions Induced by Different Mutagenic Treatments in Mmouse Germ Cells." Environmental Mutagen Society Annual Meeting, Reno/Sparks, NV, March 15-19, 1992.

"Effects of Spermatogenesis Stage on Quantity and Quality of Mutations." International Conference on Male-Mediated Developmental Toxicity, Pittsburgh, PA, September 16-19, 1992.

"Structural Differences Between Specific-Locus Mutations Induced in Two Different Populations of Spermatogonial Stem Cells of the Mouse." Environmental Mutagen Society Annual Meeting, Norfolk, VA, April 17-22, 1993.

Stubbs, L.

"Genetic and Physical Mapping Within Mouse Chromosome 7: Foundations of a Chromosome-Wide Comparative Physical Map, and Detailed Analysis of Selected Regions with Special Biological Interest." Third DOE Contractor-Grantee Workshop of the Human Genome Program, Santa Fe, NM, February 7-11, 1993.

Terzaghi-Howe, P.

"Effects of Radiation on Rat Respiratory Epithelial Cells: Critical Target Cell Populations and Importance of Cell Interactions." 29th Plenary Meeting of the Committee on Space Research at the World Space Congress, Washington, DC, August 28-September 5, 1992.

"Mutagenesis and Carcinogenesis in Rodents and Humans." Radiation Research Society, Dallas, TX, March 22, 1993.

Woychik, R. P.

"Genome-Wide Insertional Mutagenesis and the Molecular Analysis and Mapping of Disease-Related Genes in Humans and Mice." Third Contractor-Grantee Workshop for the DOE Human Genome Program, Santa Fe, New Mexico, February 7-11, 1993.

Abstracts for Technical Meetings
October 1, 1991 - September 30, 1993

Affholter, K. A., S. J. Henderson, G. J. Bunick, G. D. Wignall, R. E. Haufler, and R. N. Compton. Small-angle neutron scattering of C_{60} and C_{70} in CS_2. American Crystallographic Association Annual Meeting, Albuquerque, NM, May 23-28, 1993.

Allison, D. P., T. Thundat, L. A. Bottomley, K. B. Jacobson, R. P. Woychik, J. J. Schrick, and R. J. Warmack. Scanning tunneling microscope imaging of DNA chemically attached to gold surfaces. American Vacuum Society's 39th National Symposium and Topical Conferences, Chicago, IL, November 9-13, 1992.

Allison, D. P., T. Thundat, K. B. Jacobson, L. A. Bottomley, and R. J. Warmack. Scanning tunneling microscopy; image contrast of DNA. Scanning Microscopy 1993 Meeting, Los Angeles, CA, May 8-13, 1993.

Allison, D. P., T. Thundat, T. L. Ferrell, M. J. Doktycz, K. B. Jacobson, and R. J. Warmack. Scanning probe microscopy of complete plasmids. Third Contractor-Grantee Workshop for the DOE Human Genome Program, Santa Fe, NM, February 7-11, 1993.

Allison, D. P., T. Thundat, L. A. Bottomley, K. B. Jacobson, R. P. Woychik, J. J. Schrick, and R. J. Warmack. Imaging DNA chemically attached to gold surfaces with the scanning tunneling microscope. 59th Meeting of the Southeast Section of the American Physical Society, Oak Ridge, TN, November 12-14, 1992.

Alpen, E. L., P. Powers-Risius, R. DeGuzman, and R. J. M. Fry. *In vivo* carcinogenesis of the mouse Harderian gland by charged particle radiations. Third Annual Investigators Meeting on Space Radiation Research, Houston, TX, April 14-16, 1992.

Arlinghaus, H. F., M. T. Spaar, N. Thonnard, K. B. Jacobson, M. J. Doktycz, G. W. Kabalka, and R. C. Switzer. Development of laser resonance ionization for quantitative imaging of trace elements in biological materials. 39th National American Vacuum Society Symposium and Topical Conferences, Chicago, IL, November 9-13, 1992.

Bishop, J. B., R. W. Morris, K. T. Cain, L. A. Hughes, L. B. Foxworth, and W. M. Generoso. Alterations in the reproductive patterns of female mice by exposures to xenobiotics. Sixth International Conference on Environmental Mutagens, Melbourne, Australia, February 21-26, 1993.

Bishop, J. B., J. C. Seely, R. W. Morris, K. T. Cain, and W. M. Generoso. Alterations in ovarian histology and reproductive patterns of female mice exposed to xenobiotics. Environmental Mutagen Society Annual Meeting, Norfolk, VA, April 17-22, 1993.

Blair, P. J., J. E. Wilkinson, and V. L. Godfrey. Fetal lymphoreticular disease is established early in thymic development in the scurfy (*sf*) mouse. Federation of American Societies for Experimental Biology, Anaheim, CA, April 5-9, 1992.

Brandes, H. M., F. W. Larimer, M. K. Geck, C. D. Stringer, P. Schurmann, and F. C. Hartman. Functional analysis of active-site cysteinyl residues of thioredoxin *f* by use of site-directed mutants. American Society for Biochemistry and Molecular Biology Symposium, San Diego, CA, May 30 - June 3, 1993.

Brandes, H. M., C. D. Stringer, and F. C. Hartman. Crosslinking of the regulatory sulfhydryls of phosphoribulokinase (PRK). Joint Meeting of the American Society for Biochemistry and Molecular Biology and Biophysical Society, Houston, TX, February 9-13, 1992.

Brown, G. M., D. P. Allison, T. Thundat, K. B. Jacobson, R. J. Warmack, and T. L. Ferrell. Adsorption of DNA on chemically modified surfaces. American Vacuum Society's 39th National Symposium and Topical Conferences, Chicago, IL, November 9-13, 1992.

Bultman, S. J., E. J. Michaud, and R. P. Woychik. Molecular characterization of the murine agouti locus. 34th Annual Southeast Regional Developmental Biology Conference, Clemson, SC, May 22-24, 1992.

Bultman, S. J., E. J. Michaud, and R. P. Woychik. Molecular characterization of the mouse *agouti* locus. Mouse Molecular Genetics, Cold Spring Harbor, New York, August 26-30, 1992.

Campion, S. R., and S. K. Niyogi. Cumulative effect of double-site mutations of human EGF on receptor binding. American Society for Biochemistry and Molecular Biology, DBC -American Chemical Society Joint Meeting, San Diego, CA, May 30 - June 3, 1993.

Chathadi, K. V., M. J. Doktycz, and K. B. Jacobson. Development of new procedures for multiple labeling of DNA with stable tin isotopes for sequencing. Seventh National Conference on Undergraduate Research, Salt Lake City, UT, March 12, 1993.

Doktycz, M. J., H. F. Arlinghaus, and K. B. Jacobson. Electrophoresis and detection of tin-labelled DNAs on open-faced gels. Annual Meeting of the Electrophoresis Society, Research Triangle Park, NC, June 22-24, 1992.

Doktycz, M. J., J. L. Doyle, W. A. Gibson, R. C. Allen, H. F. Arlinghaus, and K. B. Jacobson. Electrophoresis optimization for analysis of tin-labeled DNA by resonance ionization spectroscopy. Genome Sequencing and Analysis Conference IV, Hilton Head, SC, September 26-30, 1992.

Doktycz, M. J., J. L. Doyle, W. A. Gibson, H. F. Arlinghaus, R. C. Allen, and K. B. Jacobson. Modification of elctrophoresis conditions for optimizing analysis of stable isotope labeled DNA. Third Contractor-Grantee Workshop for the DOE Human Genome Program, Santa Fe, NM, February 7-11, 1993.

Du, J., F. W. Kleinhans, P. Mazur, and J. Critser. Permeability of the human spermatozoa to glycerol determined by EPR. American Physical Society, Indianapolis, IN, March 16-20, 1992.

Du, J., F. W. Kleinhans, P. Mazur, and J. K. Critser. Osmotic behavior of human spermatozoa studied by EPR. Annual Meeting of Society for Cryobiology, Ithaca, NY, June 14-19, 1992.

Du, J., J. Tao, F. W. Kleinhans, P. Mazur, and J. K. Critser. Mouse spermatozoa water volume determined by EPR. American Society of Andrology Annual Meeting, Tampa, FL, April 16-19, 1993.

Du, J., J. Tao, F. W. Kleinhans, P. Mazur, and J. K. Critser. The osmotic response of mouse spermatozoa measured by EPR. Annual Meeting of Society for Cryobiology, Atlanta, GA, July 19-23, 1993.

Foote, R. S., J. B. Davidson, R. J. Mural, R. A. Sachleben, K. P. Stengele, and E. C. Uberbacher. Oligonucleotide arrays for DNA analysis. Third Contractor-Grantee Workshop for the DOE Human Genome Program, Santa Fe, NM, February 7-11, 1993.

Foote, R. S., R. A. Sachleben, and K. B. Jacobson. DNA sequence analysis by solid-phase hybridization. Third Contractor-Grantee Workshop for the DOE Human Genome Program, Santa Fe, NM, February 7-11, 1993.

Fry, R. J. M. Deep space missions: Radiobiological research needs. World Space Congress, Washington, DC, August 28 - September 5, 1992.

Fry, R. J. M. The biological basis for dose limitation to the skin. International Conference on Radiation Effects and Protection, Mito, Japan, March 18-20, 1992.

Fry, R. J. M. Update on guidance on radiation received in space activities. Third Annual Investigators Meeting on Space Radiation Research, Houston, TX, April 14-16, 1992.

Fry, R. J. M. Radiation-induced cancer: Risks and mechanisms. International Conference on Effects of Low Dose Ionizing Radiation: Implications for Human Health, Bournemouth, UK, May 18-20, 1992.

Galloway, P. J., and J. S. Cook. Epidermal growth factor and glucose transport development in the opossum kidney cell line, OK. Federation of American Societies for Experimental Biology Meeting, Anaheim, CA, April 5-9, 1992.

Galloway, P. J., and J. S. Cook. Basolateral glucose-transport development in porcine kidney (LLC-PK_1) and opossum kidney (OK) cell lines. Society of General Physiologists Annual Meeting, Woods Hole, MA, September 10-13, 1992.

Gao, D., E. Ashworth, P. F. Watson, P. Mazur, and J. K. Critser. Osmotic tolerance of human spermatozoa. Society for Cryobiology Annual Meeting, Ithaca, NY, June 14-19, 1992.

Gao, D., L. Mark, L. McGann, P. Mazur, and J. K. Critser. Prevention of osmotic injury of human spermatozoa during addition and removal of cryoprotective agent (CPA): Modeling and optimization. Society for Cryobiology, Atlanta, GA, July 19-23, 1993.

Gao, D. Y., E. E. Noiles, F. W. Kleinhans, P. Mazur, and J. K. Critser. Glycerol permeability and its activation energy of human spermatozoa. 1992 Annual Meeting of American Society of Andrology, Bethesda, MD, March 28-30, 1992.

Generoso, W. M. Periods of susceptibility to mutagenic insult in female mice. Environmental Mutagen Society Annual Meeting, Norfolk, VA, April 17-22, 1993.

Handel, M. A., E. Goldberg, W. Zhou, L. Lameier, and E. M. Rinchik. Genetic approaches to analysis of LDH-C expression during spermatogenesis in the mouse. 25th Isozyme Conference, St. Thomas, Virgin Islands, December 5-7, 1991.

Harp, J. M., A. E. Roberson, E. C. Uberbacher, and G. J. Bunick. Crystallization of nucleosome core particles containing a DNA palindrome. American Crystallographic Association Annual Meeting, Albuquerque, NM, May 23-28, 1993.

Harp, J. M., A. E. Roberson, E. C. Uberbacher, and G. J. Bunick. Crystallization of nucleosome core particles containing a DNA palindrome. American Society for Biochemistry and Molecular Biology, DBC - American Chemical Society Joint Meeting, San Diego, CA, May 30 - June 3, 1993.

Harpel, M. R., and F. C. Hartman. Engineering an improved CO_2/O_2 specificity for *R. rubrum* ribulose bisphosphate (RuBP) carboxylase/oxygenase. American Chemical Society, San Francisco, CA, April 5-10, 1992.

Harpel, M. R., and F. C. Hartman. Chemical rescue of the catalytically-deficient K329A mutant of *R. rubrum* ribulose-bisphosphate (RuBP) carboxylase/oxygenase. Sixth Symposium of the Protein Society, San Diego, CA, July 25-29, 1992.

Harpel, M. R., and F. C. Hartman. Chemical rescue of the catalytically-deficient K329A mutant of *R. rubrum* ribulose-bisphosphate (RuBP) carboxylase/oxygenase. 13th Enzyme Mechanisms Conference, Key Largo, FL, January 6-10, 1993.

Harpel, M. R., F. W. Larimer, E. H. Lee, R. J. Mural, T. S. Soper, and F. C. Hartman. Functional significance of intersubunit electrostatic interactions at the active site of *R. rubrum* ribulose-bisphosphate (RuBP) carboxylase/oxygenase. American Society for Biochemistry and Molecular Biology Symposium on Protein Engineering Approaches to Enzyme Mechanism, San Francisco, CA, April 6-9, 1992.

Harpel, M. R., F. W. Larimer, G. H. Lorimer, and F. C. Hartman. Functional analysis of His287 of *R. rubrum* ribulose bisphosphate (RuBP) carboxylase/oxygenase by site-directed mutagenesis. Seventh Symposium of the Protein Society, San Diego, CA, July 24-28, 1993.

Harpel, M. R., E. H. Lee, and F. C. Hartman. Use of site-directed mutants of Rubisco to explore structure-function relationships. First Scandinavian Photosynthesis Congress, Karlslunde, Denmark, November 25-29, 1991.

Hauser, M. R., S. R. Campion, R. Matsunami, D. A. Engler, S. K. Niyogi, and J. S. Cook. Variants of human epidermal growth factor stimulate rapid phosphorylation of lipocortin-1 in permeabilized murine epidermal keratinocytes. Federation of American Societies for Experimental Biology Meeting, Anaheim, CA, April 5-9, 1992.

Hauser, M. R., S. K. Niyogi, and J. S. Cook. Differential responses of Na+/H+ exchange and mitogenesis in mouse keratinocyte cells (MK) to low concentrations of EGF and EGF variants. Experimental Biology 93, New Orleans, LA, March 28 - April 2, 1993.

Henry, M. A., P. Mazur, and J. K. Critser. Sperm cell concentration following cryopreservation using various cooling and warming rates. The American Fertility Society, The Canadian Fertility and Andrology Society, Montreal, Canada, October 9-14, 1993.

Henry, M., E. E. Noiles, P. Mazur, and J. K. Critser. Cryopreservation of human spermatozoa: The effects of cooling rate and warming rate on the maintenance of motility, plasma membrane integrity and mitochondrial integrity. American Society of Andrology Annual Meeting, Tampa, FL, April 16-19, 1993.

Jackson, I. J., J. A. Bell, B. Cattanach, E. M. Rinchik, and L. Stubbs. Mapping of deletions of chromosome 4 in the region of the mouse brown locus; identification of genes which play a role in embryonic growth and development. Sixth International Workshop on Mouse Genome Mapping, Buffalo, NY, October 11-15, 1992.

Jacobson, K. B., H. F. Arlinghaus, M. J. Doktycz, R. A. Sachleben, G. M. Brown, and F. W. Larimer. Development of faster, more accurate methods for genome and DNA analysis that use stable isotopes of tin and other metals. Biomedical Optics '93 Meeting, Los Angeles, CA, January 16-22, 1993.

Jacobson, K. B., H. F. Arlinghaus, M. J. Doktycz, R. A. Sachleben, G. M. Brown, F. W. Larimer, and R. P. Woychik. Development of faster, more accurate methods for genome and DNA analysis that use stable isotopes of tin and other metals. Biomedical Optics '93 Meeting, Los Angeles, CA, January 16-22, 1993.

Jacobson, K. B., C. H. Chen, M. V. Buchanan, and S. A. McLuckey. Development of mass spectrometric methods for DNA sequencing and genome analysis. Future Technologies for DNA Analysis, Washington, DC, October 26-27, 1992.

Jacobson, K. B., and R. L. Hettich. Structural characterization of underivatized pteridines by Fourier transform mass spectroscopy. Sixth International Conference on Pteridines and Related Biogenic Amines and Folate, Seoul, Korea, June 7-13, 1992.

Jacobson, K. B., R. A. Sachleben, G. M. Brown, M. L. Garrity, F. V. Sloop, M. J. Doktycz, J. L. Doyle, H. F. Arlinghaus, and N. Thonnard. Stable isotopes as DNA labels: Development of labeling, separation, and detection methods. Cold Spring Harbor Meeting on Genome Mapping and Sequencing, Cold Spring Harbor, New York, May 6-10, 1992.

Jacobson, K. B., R. A. Sachleben, G. M. Brown, F. V. Sloop, M. L. Garrity, M. J. Doktycz, H. F. Arlinghaus, R. S. Foote, F. W. Larimer, R. P. Woychik, and N. Thonnard. Analysis of iron, tin and lanthanide labeled DNA by resonance ionization spectroscopy (RIS) for genome mapping and sequencing. Third Contractor-Grantee Workshop for the DOE Human Genome Program, Santa Fe, NM, February 7-11, 1993.

Kumari, S. S., and D. M. Skinner. Cuticular proteins (CPs) of a crab (*Gecarcinus lateralis*) cross react with antibodies against insect CPs. American Society of Zoologists Annual Meeting, Atlanta, GA, December 27-30, 1991.

Kumari, S. S., and D. M. Skinner. Sequence similarities in proteins of crab (*G. lateralis*) exoskeleton and insect cuticles. Annual Meeting of American Society of Zoologists, Vancouver, Canada, December 27, 1992.

Larimer, F. W., M. R. Harpel, and F. C. Hartman. Site-directed mutation of the C1-phosphate binding-site of ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco). Seventh Symposium of the Protein Society, San Diego, CA, July 24-18, 1993.

Mazur, P. The role of unfrozen fraction in cell survival. Annual Meeting of Society for Cryobiology, Ithaca, NY, June 14-19, 1992.

Mazur, P. Mouse and *Drosophila* embryos as illustrations of two sets of cryobiological problems and principles. Conference on Theoretical Basis of Cryopreservation, Hardec Kralove, Czechoslovakia, October 6-9, 1992.

Mazur, P., K. W. Cole, and A. P. Mahowald. Permeabilization of *Drosophila* embryos by alcohol-alkane mixtures. *Drosophila* Research Conference, Philadelphia, PA, March 12-15, 1992.

Mazur, P., K. W. Cole, and P. D. Schreuders. Exposure to ethylene glycol vitrification solution limits the survival of Drosophila embryos vitrified at the optimum developmental stage. Society for Cryobiology, Atlanta, GA, July 19-23, 1993.

Mazur, P., K. W. Cole, P. D. Schreuders, and A. P. Mahowald. Survival of *Drosophila* embryos after cooling to -200°C. *Drosophila* Research Conference, Philadelphia, PA, March 12-15, 1992.

Mazur, P., K. W. Cole, P. D. Schreuders, and A. P. Mahowald. Relative contribution of cooling and warming rate to the survival of *Drosophila* embryos cooled to -205°C. Annual Meeting of Society for Cryobiology, Ithaca, NY, June 14-19, 1992.

Mazur, P., P. D. Schreuders, and K. W. Cole. Injury to Drosophila embryos during the removal of ethylene glycol vitrification solution. Society for Cryobiology, Atlanta, GA, July 19-23, 1993.

Metallinos, D. L., A. J. Oppenhiemer, V. Tsui, W. Dietrich, E. M. Rinchik, L. B. Russell, and S. M. Tilghman. Genetic analysis of the *piebald* locus. Sixth International Workshop on Mouse Genome Mapping, Buffalo, NY, October 11-15, 1992.

Moyer, J. H., R. P. Woychik, J. E. Wilkinson, and V. Godfrey. Molecular characterization of a new mouse model of recessive polycystic kidney disease that closely resembles the human disorder. 25th Annual Meeting of The American Society of Nephrology, Baltimore, MD, November 15-18, 1992.

Mural, R., X. Guan, J. R. Einstein, and E. C. Uberbacher. Combining neural networks and expert systems to identify features in DNA sequences. Eleanor Roosevelt Institute Transcribed Sequences Workshop, San Francisco, CA, October 31 - November 1, 1991.

Mural, R. J., X. Guan, and E. C. Uberbacher. Grail: An on-line service for locating protein coding regions in anonymous DNA sequences. Cold Spring Harbor Laboratory Genome Mapping and Sequencing Meeting, Cold Spring Harbor, New York, May 6-10, 1992.

Nicholls, R. D., K. M. Avidano, W. Gottlieb, P. E. Newumann, B. Horsthemke, M. P. Colombo, and E. M. Rinchik. Mapping of loci deleted in Angelman and Prader-Willi Syndromes identifies conservation of synteny in mice and possible animal models. International Congress of Human Genetics, Washington, DC, October 7-11, 1991.

Nicholls, R. D., S. J. Bultman, R. A. Spritz, S-T. Lee, K. M. Strunk, B. Horsthemke, M. T. C. Jong, W. Gottlieb, P. E. Currier, M. F. Waters, L. B. Russell, and E. M. Rinchik. Mouse models for genomic imprinting and phenotypic features in Prader-Willi and Angelman Syndromes. American Society of Human Genetics, San Francisco, CA, November 9-13, 1992.

Nicholls, R. D., D. J. Driscoll, P. K. Rogan, R. A. Spritz, W. Gottlieb, M. Jong, K. Avidano, M. F. Waters, C. C. Glenn, C. A. Williams, R. T. Zori, B. Horsthemke, W. Robinson, A. Schinzel, S. Saitoh, N. Niikawa, L. B. Russell, S. J. Bultman, and E. M. Rinchik. Mapping of loci in human chromosome 15q11-q13 and a region of conserved synteny in mouse chromosome 7, including a candidate imprinted genet (D15S9) and the pink-eyed dilution (p/D15S12) gene. Chromosome 15 Mapping Workshop, Tucson, AZ, June 18-19, 1992.

Nicholls, R. D., W. Gottlieb, K. Avidano, M. Jong, D. J. Driscoll, M. J. Mascari, P. K. Rogan, B. Horsthemke, L. B. Russell, and E. M. Rinchik. Mammalian genomic imprinting Prader-Willi and Angelman Syndromes and mouse models. NIH Conference on Genomic Imprinting, Bethesda, MD, April 12-13, 1992.

Nicholls, R. D., M. T. C. Jong, K. M. Avidano, S. J. Bultman, R. A. Spritz, S-T. Lee, K. M. Strunk, B. S. Horsthemke, L. B. Russell, and E. M. Rinchik. Genetic dissection of complex human syndromes by the study of homologous mouse models: Albinism and genomic imprinting as two examples. Third Miami Children's Hospital Research Institute Symposium, Miami, FL, December 7-9, 1992.

Niyogi, S. K., S. R. Campion, D. K. Tadaki, M. R. Hauser, and J. S. Cook. Mapping the receptor binding/activation residues of human EGF by protein engineering. 1993 American Physiological Society Conference: Signal Transduction & Gene Regulation, San Francisco, CA, November 17-20, 1993.

Noiles, E. E., P. Mazur, F. W. Benker, F. W. Kleinhans, R. P. Amann, and J. K. Critser. Critical osmolality, water, and glycerol permeability coefficient determination of equine spermatozoa. 25th Annual Meeting of Society for the Study of Reproduction, Raleigh, NC, July 12-15, 1992.

Noiles, E. E., P. Mazur, H. D. Boldt, F. W. Kleinhans, and J. K. Critser. Hydraulic conductivity (Lp) and its activation energy (Ea) in human sperm. Annual Meeting of the American Society of Andrology, Bethesda, MD, March 28, 1992.

Noiles, E. E., P. Mazur, F. W. Kleinhans, and J. K. Critser. Water permeability of human sperm in the presence of intracellular glycerol. Annual Meeting of Society for Cryobiology, Ithaca, NY, June 14-19, 1992.

Olins, A. L. Electron microscope tomography: Thick sections of transcribing chromatin. Electron Microscopy Society of Southern Africa, Capetown, South Africa, December 4-6, 1991.

Olins, A. L. Electron microscope tomography: The substructure of RNA in hnRNP particles. Scanning 92, Atlantic City, NJ, April 1-3, 1992.

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Stevens, A., F. W. Larimer, and C. L. Hsu. Characterization of 5'→3' exonuclease-1 and yeast cells lacking the enzyme. 1993 Keystone Symposium on Nucleases: Structure, Function & Biological Roles, Tamarron, CO, February 23 - March 1, 1993.

Stubbs, L., and E. M. Rinchik. Toward a restriction map of mouse chromosome 7: Determination of linkage and physical distances in regions of homologous to human 11p15. Third International Workshop on Chromosome 11, La Jolla, CA, September 14-16, 1992.

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Tadaki, D. K., S. R. Campion, D. A. Engler, and S. K. Niyogi. Evaluation of the roles of electrostatic residues of human EGF in receptor binding. Physiological Roles for the EGF System: EGF, TGF-Alpha, and the EGF Receptor, Nashville, TN, November 7-9, 1992.

Tadaki, D. K., and S. K. Niyogi. Functional importance of hydrophobicity at position 13 of human epidermal growth factor (hEGF). American Society for Biochemistry and Molecular Biology, DBC - American Chemical Society Joint Meeting, San Diego, CA, May 30 - June 3, 1993.

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Turner, J. E., and R. J. M. Fry. High-LET radiation carcinogenesis: What do we know and what do we need to know? Eleventh Symposium on Microdosimetry, Gatlinburg, TN, September 13-18, 1992.

Uberbacher, E., J. R. Einstein, X. Guan, D. Buley, and R. J. Mural. Gene recognition and assembly in GRAIL system. The Third Contractor-Grantee Workshop for the DOE Human Genome Program, Santa Fe, NM, February 7-11, 1993.

Wang, T. H., W. K. Yang, H. I. Saavedra, D.-M. Yang, D. M. Popp, P. R. Hoyt, L. Y. Ch'ang, and M. Y. Yang. Effect of retroviral long terminal repeat (LTR)-

containing transgenes on thymic lymphoma induction in FVB/N mice neonatally treated with azacytidine. Annual Meeting of American Association for Cancer Research, San Diego, CA, May 23-26, 1992.

Watson, P. F., E. E. Noiles, M. R. Curry, P. Mazur, J. K. Critser, and R. H. Hammerstedt. Response of spermatozoa to hyposmotic stress reflects cryopreservation success. International Congress on Animal Reproduction, The Hague, Netherlands, August 23-27, 1992.

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Woychik, R. P., B. R. Beatty, S. Bultman, E. J. Michaud, J. Moyer, H. Kwon, and M. L. Klebig. Genome-wide insertional mutagenesis and the molecular analysis and mapping of disease-related genes in humans and mice. Third Contractor-Grantee Workshop for the DOE Human Genome Program, Santa Fe, New Mexico, February 7-11, 1993.

Yang, M. Y., D. M. Popp, and R. A. Popp. Flow cytometric identification of cellular MuLV expression on lymphoid cells from one-month-old-B10.F mice. The American Association of Immunologists/Chemical Immunology Society Joint Meeting, Denver, CO, May 21-25, 1993.

Yang, W. K., T-H. Wang, D-M. Yang, D. C. Henley, and L-Y. Ch'ang. Test for the tumorigenicity of retroviral vectors in mice. Effects of animal age and prior chemical carcinogen treatment. Keystone Symposium, Keystone, CO, April 12-18, 1993.

Yen, C-P., D.-M. Yang, S. G. Shinpock, and W. K. Yang. Mitochondrial rhodamine-123 retention property of human cancer cells: Genetic analysis by fluorescent-activated cell sorting. 83rd Annual Meeting of the American Association for Cancer Research, San Diego, CA, May 23-26, 1992.

Grants

Principal Investigator	Title	Inclusive Dates
G. J. Bunick	Structural Studies of Nucleosomes (NIH)	01/91 – 12/95
F. C. Hartman	Characterization of Phosphoribulokinase (USDA)	10/86 – 09/94
A. L. Olins	Chromatin Structure (NIH)	11/90 – 03/94
D. E. Olins	Development of Electron Microscope Tomography (NIH)	05/91 – 07/95
	Structure and Function of the Replication Band (NSF)	01/89 – 06/95
E. M. Rinchik	Saturation Mutagenesis (NIH)	09/88 – 08/93
D. M. Skinner	Degradation and Synthesis of Crustacean Tissues and their Control (NSF)	09/89 – 09/94
M. Terzaghi-Howe	Cell Interactions: Expression of Preneoplastic Markers (NIH)	05/83 – 06/96
R. P. Woychik	Insertional Mutagenesis in Transgenic Mice (NIH)	05/89 – 12/93

Contracts

Principal Investigator	Title	Inclusive Dates
W. M. Generoso	Chromosome Aberration Effects in Mice (NIEHS)	06/82 — 09/95
	Environmental Mutagenesis Workshop (EPA)	06/90 — 06/93
	Mutagen-Induced Fetal Anomalies Subsequent to Exposure of Mouse Zygotes (EPA)	03/89 — 09/93
P. Mazur	Cryopreservation of Mouse Embryos (OHSU)	06/88 — 04/95
	Frozen Embryos (NSF)	04/87 — 09/91 07/93 — 03/94
	Fundamental Cryobiology of Human Sperm (NIH)	09/89 — 09/92
R. A. Popp	Mutant Hemoglobins that Allow Hb S to Sickle (NIH)	07/89 — 04/94
L. B. Russell/ R. P. Woychik	Chemical Mutagenicity Studies in Mice (NIEHS)	05/81 — 06/95
R. P. Woychik	Epithelial Cells within the Liver of Mutant Mice (P&G)	04/93 — 04/94

Financial Summary and Personnel Distribution

Total Biology Division
FY 1992

Funding Source	Funding in Thousands	Percent of Total Budget	Scientific/ Technical Person-Years*
Department of Energy	10,536	75.5	41.0
National Cancer Institute	625	4.5	2.2
National Institute of Child Health and Human Development	292	2.1	2.3
National Institute of Environmental Health Sciences	1,853	13.3	8.6
National Center for Human Genome Research	246	1.7	1.6
Department of Agriculture	27	0.2	---
Environmental Protection Agency	85	0.6	0.1
University of Tennessee	254	1.8	0.7
Oregon Health Sciences University	14	0.1	---
Methodist Hospital of Indiana	29	0.2	0.2
	13,961	100.0	56.7

*Does not include ~52.0 person years: 46.5 PY Distributed (administration and clerical, animal caretakers, histology, and kitchen); 5.5 PY supported by other divisions and ORNL seed money.

Financial Summary and Personnel Distribution

Total Biology Division
FY 1993

Funding Source	Funding in Thousands	Percent of Total Budget	Scientific/ Technical Person-Years*
Department of Energy	11,900	81.0	43.0
National Center for Human Genome Research	299	2.0	2.4
National Cancer Institute	200	1.4	1.2
National Institute of Child Health and Human Development	330	2.2	1.3
National Institute of Environmental Health Sciences	1,387	9.4	6.8
Environmental Protection Agency	55	0.4	---
University of Tennessee	411	2.8	1.9
Oregon Health Sciences University	35	0.2	0.3
Methodist Hospital of Indiana	23	0.2	0.2
University of Texas	60	0.4	0.3
	14,700	100.0	57.4

*Does not include ~ 51.0 person years: 44.0 PY Distributed (administration and clerical, animal caretakers, histology, and kitchen); 7.0 PY supported by other divisions and ORNL seed money.

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