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BIOLOGY DIVISION PROGRESS REPORT

For Period of October 1, 1989 - September 30, 1991

Date Published: October 1991

Fred C. Hartman, Director

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L. B. Russell, Mammalian Genetics and Development

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October 1991

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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, 1989, through September 30, 1991. 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, the congressionally mandated mission of this Office is to study adverse health effects of energy production and utilization.

Within this broad mission, predominant research themes of the Biology Division include interactions of animals, cells, and molecules with their respective environments. Investigations focus on genetic and somatic effects of radiation and chemicals. Goals 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. Concurrent basic studies in genetics, biochemistry, molecular biology, and cell biology illuminate 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 expanded and integrated its activities in genome research, which includes physical and functional gene mapping, new strategies for DNA sequencing, and informatics. During the present reporting period, the Division has also strived to upgrade protein crystallography to complement 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.

This 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

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RESEARCH ACTIVITIES

Molecular, Cellular, and Cancer Biology Section

Section Overview - J. S. Cook

The various projects in this Section are directed toward understanding at the most basic level the mechanisms of life 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, DNA-repair processes, and enzyme mechanisms, all problems underlying 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 mechanisms of carcinogenesis, regulation of transport of metabolites by mammalian cells, and the responses of cells to 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.

The Protein Engineering and Chemistry Group is the largest unified cohort in the Section. They use site-directed mutagenesis and other sophisticated biochemical and genetic 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 mission of the Biology Division and include energy-related enzymes, growth factors important to cancer biology, and repair enzymes that function in the responses of cells to physical and chemical mutagenic agents.

Members from the Protein Engineering and Chemistry Group, collaborating with investigators in the Engineering Physics and Mathematics Division, 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.

The DNA Repair and Mutagenesis Group focuses on the mechanisms of mutation induction in human cells and repair of alkylated DNA by human repair enzymes. The group has successfully cloned the cDNAs for two human enzymes that repair alkylation damage. They are the first cDNAs to be cloned for human repair enzymes of known biochemical activity. The selection strategy is based on the repair of alkylation damage in repair-deficient *E. coli*; when the human repair proteins encoded by the cDNAs are expressed in the otherwise repair-deficient bacteria, bacterial survival is enhanced and the cDNA-containing plasmids can be recovered from the colonies that grow up.

The RNA Metabolism Group has been studying the regulation of eukaryotic messenger RNA synthesis and turnover, focusing on several enzymes including a 3' → 5' exoribonuclease that degrades decapped mRNA. The interactions of the activities of these enzymes appear to play important roles in regulating mRNA concentration and translation. In addition, 5' → 3' exoribonuclease 1 (XRN1) from yeast has been cloned and sequenced, and the disrupted gene has been introduced into cells to analyze the physiological consequences.

The Structural Biology Group has analyzed in detail their 8 Å crystallographic solution of the nucleosome core particle structure and now has 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 will further enhance 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 Group, has initiated a study of the crystal structure of human epidermal growth factor (hEGF).

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, and 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.

The Membrane Biology Group has continued its studies on the regulation of transport systems in relation to mammalian cell growth and differentiation. Three major projects are concerned with the role of protein kinase C in the regulation of amino acid transport, 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 Group.

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; a goal is to develop a system utilizing antibody-containing liposomes that will be targeted to lung and will thus possibly be useful in tissue-specific drug delivery.

The 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.

The group studying Molecular Genetics of Cancer is exploring the hypothesis that germline-integrated retroviral and retrotransposable genes are particularly susceptible to environmental carcinogenic insults. Transgenic mice, both germline and somatic, are being used as experimental models in which the investigators are attempting to activate silent transgenes with known carcinogens.

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*.

The Fundamental and Applied Cryobiology Group has continued fundamental studies on the cryobiology of human and bull sperm and applied studies in freezing of embryonic mice and *Drosophila* for the cost-efficient, long-term banking of mutant stocks. This year they have defined the means for

reproducible permeabilization of *Drosophila* embryos to cryoprotectants and water while maintaining high viability. The control of this step is essential to the follow-on: reproducible freezing with high viability in the recovery of cryopreserved specimens.

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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 O^6-methylguanine-DNA methyltransferase and *N*-methylpurine-DNA glycosylase, proteins that repair alkylated DNA.

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, serves a critical role in the production of biomass for energy and is 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 to yield two molar equivalents of D-3-phosphoglycerate (the CO_2-fixation reaction), it also catalyzes the oxidation of ribulose bisphosphate by molecular oxygen to yield one molar equivalent each of phosphoglycolate and 3-phospho-glycerate. 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 (1) to understand the mechanism of this complex enzyme, especially the precise catalytic roles of active-site residues, and (2) 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, until recently, for all of our mutagenesis studies. However, an expression system for an L_8S_8 enzyme has been optimized recently so that mutagenesis can be pursued with either form 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 earlier conclusions concerning structure-function relationships derived from affinity labeling, chemical cross-linking, comparative sequence analyses, and site-directed mutagenesis. Confirmed findings include the presence of Lys166, Lys329, and Glu48 at the active site; the interfacial location of the active site with side-chains from adjacent subunits required for catalysis, the activation-state dependence of the Lys166-Lys329 proximity, and the role of Lys329 in facilitating the reaction of CO_2 with the enediol of ribulose-P_2 (the initial intermediate in overall catalysis).

Roles of Active-Site Residues

Asn111. Crystallography suggests that Asn111 may contribute to the binding of Mg^{2+} and/or the phosphorylated substrate. Replacement of this residue by Gln, Asp, Ser, or Lys results in total loss of carboxylase activity. However, the mutant proteins are able to form quaternary complexes with CO_2, Mg^{2+}, and carboxyarabinitol-P_2 (the transition-state analogue), thereby demonstrating that Asn111 is not required for carbamylation (activation) nor binding of phosphorylated ligands (*i.e.,* ribulose-P_2). Despite the lack of carboxylase activity, the mutant proteins retain the ability to catalyze enolization of ribulose-P_2, which represents the first step in overall catalysis. Thus, Asn111 must participate primarily in some step beyond enolization.

Prior to the elucidation of the three-dimensional structure, we showed that the active site is created by interacting domains from adjacent subunits and that catalysis requires amino acid residues from both domains. The formation of enzymatically active heterodimers by the *in vivo* hybridization of an inactive position-111 mutant with an inactive position-166 mutant is consistent with the crystallographic assignment of Asn111 to the NH_2-terminal domain of the active site.

Pro167. The active site of Rubisco includes the species invariant segment Lys166-Pro167-Lys168 in which both lysyl residues are catalytically functional. Electron density maps reveal the Lys-Pro peptide bond to be of *cis* configuration. *cis*-Peptide bonds are very unusual; of the 33,000 peptide bonds recorded in the entire Brookhaven Protein Data Bank, only 0.05% are *cis*, while 6.5% of those involving prolyl residues are *cis*. To investigate the significance of this rare *cis*-prolyl residue, it has been replaced with Gly, Ala, or Ser. Surprisingly, the mutant proteins exhibit substantial carboxylase activity — up to 70% of wild-type activity in the case of the seryl substitution. The K_m for ribulose-P_2 is increased 50-fold, perhaps reflecting lesser constraint in the relative positioning of the two lysyl ϵ-amino groups. We believe that folding constraints must dictate a *cis*-peptide bond at position-167, because a *cis* to *trans* alteration would be anticipated to result in major conformational disruptions.

Lys168. Evolutionary conservation, structural location and site-directed mutagenesis suggest an important role for Lys168 of Rubisco from *Rhodospirillum rubrum*. Evaluation of mutant Rubiscos in which Lys168 has been replaced by a variety of amino acids indicates that while these mutants lack overall carboxylase activity, they do serve as catalysts for the enolization of ribulose bisphosphate, as measured by exchange of the C3 proton with solvent. These observations, as well as ligand-binding properties of the mutant proteins, are consistent with Lys168 facilitating a catalytic step subsequent to enolization.

His321 and Ser368. Numerous chemical, kinetic, and mutagenesis experiments implicated Lys166 as the base that initiates overall catalysis by promoting the enolization of ribulose-P_2. The three-dimensional structure of the enzyme complexed with the transition-state analogue (which may not be equivalent to the enzyme complexed with ribulose-P_2) indicates that Lys166 is too far away from C-3 of substrate to play such a role. Solely on structural grounds, His321 and Ser368 were suggested as candidates for the base that initiates catalysis. Mutant enzymes with replacement of His321 by Ser, Ala, Gln, or Asn are competent in both net carboxylation and the initial enolization step of catalysis (3-10% of the wild-type rates) but exhibit moderate increases in K_m values for ribulose-P_2. All of the mutant enzymes bind the transition-state analogue stoichiometrically, although the stabilities of these complexes are decreased relative to the wild-type enzyme. Thus, His321 is not required for catalysis

although it likely is important in stabilizing a transition state. Similar studies with substitutions for Ser368 likewise demonstrate that this residue is not required for catalysis (*e.g.,* the alanyl mutant exhibits ~2% of the wild-type carboxylation rate).

Concerted Site-Directed Mutagenesis and Chemical Modification

A subtle structural alteration that can be introduced by chemical modification in conjunction with site-directed mutagenesis is conversion of a glutamyl residue to a carboxymethylcysteinyl residue. The glutamyl residue is first replaced by a cysteinyl residue using site-directed mutagenesis. This new cysteinyl residue can then be carboxymethylated with iodoacetate. The net changes are replacement of the ß-methylene of the glutamyl side-chain with a sulfur atom and insertion of an additional methylene group in the glutamyl side-chain so that the carboxylate is about 1.5 Å further removed from the α-carbon (polypeptide backbone).

This strategy has now been successfully executed at Glu48, a previously identified active-site residue of the carboxylase, with a concomitant profound change in substrate specificity. The Glu48→Cys protein displays ≤0.05% of wild-type carboxylase activity, like the Glu48→Gln protein characterized previously. However, upon treatment of the Glu48→Cys protein with iodoacetate, the specific carboxylase activity is restored to 4-6% of the wild-type value. Consistent with the putative carboxymethylation of Cys48, isoelectric focusing of the treated mutant protein reveals two new species with pI values lower than that displayed by the untreated mutant protein. One of these novel species coincides with wild-type enzyme, while the second is equally represented in an identically treated preparation of wild-type enzyme retaining 85% of its initial carboxylase activity. The samples of treated mutant and wild-type proteins are in fact indistinguishable, and the net effect of the program of concerted mutagenesis and chemical modification as applied here may be regarded as the lengthening of the glutamyl side chain through the insertion of a sulfur atom between the ß- and γ-carbons. Of particular significance, we find that this subtle active-site alteration results in a dramatic, fivefold decrease in specificity factor. The pronounced relative enhancement of oxygenase activity is readily visualized qualitatively by the formation of substantial levels of phosphoglycolate by the carboxymethylated mutant protein at a high concentration of bicarbonate that almost completely suppresses phosphoglycolate formation by wild-type protein. This represents the first example in which a change in substrate specificity can be correlated with a programmed structural modification of a known active-site residue. Moreover, this result affirms active-site characterization as a viable basis for engineering the carboxylase/oxygenase activity ratio, and may offer an empirical precedent towards optimizing the photosynthetic yields through direct, active-site manipulation of the carboxylase.

Chemical Rescue

Dr. Jack Kirsch and colleagues at UC Berkeley have described a new approach, which they designate "chemical rescue", to discern the roles of active-site residues. A putative active-site residue is replaced by site-directed mutagenesis, thereby rendering the enzyme deficient in catalysis. In some cases, the crippled mutant protein can be partially rehabilitated merely through noncovalent interaction with an exogenously added compound. For example, the virtually inactive Lys258→Ala 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.

We have applied chemical rescue to a long-standing issue concerning the mechanism of Rubisco; namely, the basis of the requirement for protein carbamylation. Both activities are absolutely dependent on nonenzymic derivatization of an active-site lysyl residue (Lys191 of the *Rhodospirillum rubrum* enzyme) by CO_2 to form a carbamate, which is stabilized by the catalytically essential Mg^{2+} cation. As predicted by EPR and NMR spectroscopy and recently proven by crystallography, the oxyanion of the carbamate provides one ligand for the Mg^{2+} ion. If the only role of the carbamate is to assist in coordination of Mg^{2+}, the obligate dependence of catalytic competence on carbamate formation appears enigmatic, because equivalent coordination of the metal cation could conceivably have been achieved more directly by means of an aspartyl or glutamyl side chain. Invoking a catalytic demand for the carbamate nitrogen directly, however, argues that a protein carboxylate would prove inadequate in supporting catalysis.

Our recent investigation of the properties of the Lys191→Cys mutant of Rubisco provides indirect evidence of a role for the carbamate that transcends metal-ion binding. Surprisingly, the mutant protein, lacking the carbamate-forming lysyl residue, retains the capacity to bind tightly the reaction intermediate analog carboxyarabinitol-P_2 in a CO_2/Mg^{2+}-dependent manner normally indicative of carbamate formation and proper coordination of metal ion. Despite its manifestation of the ligand-binding properties which closely mimic those of the wild-type enzyme, the mutant protein is devoid of detectable carboxylase activity, thereby implicating the carbamate nitrogen *per se* in catalysis. To explore this possibility more fully, we have evaluated the ability of exogenous amines to "rescue" the mutant protein.

Significantly, the Lys191→Cys protein manifests ribulosebisphosphate-dependent fixation of $^{14}CO_2$ when incubated with aminomethanesulfonate but not ethanesulfonate. This novel activity reflects a K_m value for ribulosebisphosphate

which is not markedly perturbed relative to wild-type enzyme, a K_m for Mg^{2+} which is in fact decreased ten-fold, and rate-saturation with respect to aminomethanesulfonate (K_d = 8 mM). Chromatographic and spectrophotometric analyses reveal the product of CO_2 fixation to be D-3-phosphoglycerate, while turnover of [1-^{3}H]ribulosebisphosphate into [^{3}H]phosphoglycolate confirms oxygenase activity. We conclude that aminomethanesulfonate restores activity to the Cys191 mutant protein by providing a nitrogenous function which satisfies a catalytic demand normally met by the carbamate nitrogen of Lys191. Our new study thus explains the unique dependence of carboxylase activity on carbamate formation. Lys191 emerges as an active-site residue of multifaceted catalytic importance. Its role in enzyme activation and coordination of the essential metal ion is clear; its precise role or roles in the catalytic mechanism await determination.

Cloning of an L_8S_8 Rubisco

As a prerequisite for site-directed mutagenesis of a L_8S_8 form of Rubisco, the *rbc* operon from *Anabaena* 7120 was placed under control of the *tac* promoter and expressed in *E. coli* MV1190. Substantial amounts of insoluble large subunit were produced, but not active enzyme, suggesting that the carboxylase was not being correctly assembled *in vivo*. Coexpression of *rbc* and the operon encoding the GroESL complex (heat-shock proteins) from a compatible plasmid resulted in high levels of active, soluble enzyme. The recombinant carboxylase, purified to homogeneity, was similar to the enzyme from the authentic cyanobacterium. An initial mutational survey of active site residues has targeted Glu61, Lys176, Lys202, and Lys335 (equivalent to *R. rubrum* residues Glu48, Lys166, Lys191, and Lys329).

Vector Development

Expression systems that rely on the *lac* operator on plasmids are prone to loss of repression due to repressor titration. When strict control is warranted, we have mobilized the *lacI^q* gene on both ColE1 and p15A plasmids. However, even in the presence of adequate levels of repressor, the *tac* promoter is subject to only ~20-fold repression. Normally, the *lac* repressor binds cooperatively to the *lac* operator and one of two pseudo-operators, located 92-bp upstream and 401-bp downstream from the operator. With the operator and one pseudo-operator present, ~400-fold repression is achieved. The *tac* promoter (consisting of the -35 region of the *trp* promoter, the -10 region of the *lac* promoter, and the *lac* operator) has no pseudo-operators. We have constructed a prototype *tac* promoter/operator with a synthetic pseudo-operator located 5' to the promoter. The Rubisco expression vector pFL245 exhibits only 5-fold repression in the presence of a single copy of *lacI^q*. When modified with the synthetic pseudo-operator, 20-fold repression ensues, accompanied by full inducibility. We are evaluating the effect of multiple copies of *lacI^q* on the pseudo-operator constructs.

To simplify mobilizing desirable accessory genes, i.e., *lacI* and *groESL*, we are inserting these genes into a variant of the IncQ plasmid pKT240. This plasmid confers kanamycin resistance and is maintained at approximately 15 copies per cell. The IncQ origin is compatible with ColE1- and p15A-based plasmids.

Phosphoribulokinase (PRK)

PRK, an enzyme unique to the reductive pentose phosphate cycle, catalyzes the ATP-dependent phosphorylation of D-ribulose-5-phosphate to form D-ribulose-1,5-bisphosphate, the requisite CO_2 acceptor in photosynthetic organisms. The light-coupled regulation of PRK activity in higher plants via the thioredoxin-mediated reversible oxidation/reduction of sulfhydryls/disulfides is well established. Among several chloroplast enzymes which respond to the thioredoxin system, PRK is unusual in that the site for modulation of activity constitutes a part of the catalytic site. Our earlier chemical modification studies of the homodimeric enzyme from spinach have shown that both participants of the regulatory intrasubunit disulfide, Cys16 and Cys55, are located at or near the nucleotide-binding domain of the active site.

The loss of kinase activity concomitant with disulfide bond formation could be due to oxidation of a sulfhydryl group that facilitates catalysis, to steric blockage of the active site, or to conformational changes that accompany the oxidation. Major conformational changes would appear unlikely given the proximity of Cys16 and Cys55 as predicted from their common domain location. Furthermore, the demonstrated binding of ATP by the oxidized form of PRK suggests that steric effects are not the sole basis of catalytic incompetence. Attention is currently focused on the possible contributions, direct or indirect, of Cys16 and Cys55 to catalysis.

The cysteinyl-16 sulfhydryl group of PRK appears catalytically passive, as its selective methylation reduces k_{cat} only 2-fold. The hyperreactivity of Cys16 complicates the evaluation of the role of Cys55 through chemical modification. Preferential cyanylation of Cys55, after methylation of Cys16, has been achieved resulting in >95% loss of kinase activity inherent to the methylated enzyme. Whether PRK derivatized solely at Cys55 would exhibit one-half of wild-type activity or would be inactive has been unclear.

To determine the possible contribution of the two regulatory residues (Cys16 and Cys55) to catalysis, site-directed mutagenesis has been used to replace each of them in the spinach enzyme with serine or alanine. The only other cysteinyl residues of the kinase, Cys244 and Cys250, were also replaced individually by serine or alanine. A comparison of specific activities of native and mutant enzymes reveals that substitutions at positions 244 or 250 are inconsequential. The position 16 mutants retain 45-90% of the wild-type activity

and display normal K_m values for both ATP and ribulose-5-phosphate. In contrast, substitution at position 55 results in 85-95% loss of wild-type activity, with less than a 2-fold increase in the K_m for ATP and a 4- to 8-fold increase in the K_m for ribulose-5-phosphate. These results are consistent with moderate facilitation of catalysis by Cys55 and demonstrate that the other three cysteinyl residues do not contribute significantly either to structure or catalysis. The enhanced stability, relative to wild-type enzyme, of the Ser16 mutant protein to a sulfhydryl reagent supports earlier suggestions that Cys16 is the initial target of the oxidative deactivation process.

Epidermal Growth Factor (EGF)

Background

Because of their crucial role in the regulation of growth and differentiation and the abnormalities resulting from their malfunction, there is considerable interest in the study of growth factors. EGF, a 6-kDa protein with three internal disulfide bonds, exhibits high-affinity binding to its specific cell surface receptor. In response to 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, increased transcription of specific genes, ultimately leading 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, recently 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 and (b) published solution structures of mouse and human EGFs, which are based on 2D-NMR and computer modeling. Mutant proteins were purified to homogeneity and tested for (1) specific binding to the EGF receptor, (2) stimulation of the protein-tyrosine kinase activity of the EGF receptor, (3) stimulation of DNA synthesis and growth of mammalian cells in culture, and (4) possible structural alterations as determined by NMR spectroscopy.

Another goal of the research is the development of EGF analogues which even upon saturation binding to the EGF receptor, are unable to elicit the full tyrosine kinase stimulation needed for cell proliferation. Such analogues could be useful as inhibitors of cell growth and might possess antitumor activity. It might

also be possible to develop superagonists that may be more effective than EGF in its clinical applications, namely, treatment of wounds, burns, ulcers, etc.

Analysis of Research Results

1. Replacement of aromatic (tyrosine) residues in EGF led to a decrease in receptor affinity. In general, the reduction in activity appears to be due to alterations in the native structure of EGF. An exception is Tyr13 in the N-terminal domain of EGF. Tyr13, located close to Arg41 which is a critical residue, appears to play a functional role in receptor binding.

2. The interaction of EGF with its receptor appears to primarily involve hydrophobic "bonding" rather than electrostatic interactions. Therefore, replacement of hydrophobic residues in either the N-terminal or C-terminal domain of EGF led to drastic reduction in biological activity. These studies have also led us to conclude that both the N-terminal and C-terminal domains of EGF are required for biological function.

3. A clear exception to the above is the role of the highly conserved Arg41 in EGF. Both nonconservative and conservative (to lysine) replacements led to drastic reductions in activity. Structural analysis of the mutants by NMR spectroscopy revealed negligible conformational alterations. The importance of the guanidinium group at position 41 is further underscored by the fact that chemical modification of Lys41 to Homoarginine41 led to a complete restoration of receptor binding ability.

4. The replacement of hydrophobic amino acids – Ile23, Leu26, and Leu47 – not only led to a drastic reduction in receptor affinity, but also produced EGF mutants that are deficient in the activation of the receptor tyrosine kinase. Moreover, such mutants are able to displace wild-type EGF and inhibit the latter's stimulation of the receptor's tyrosine kinase activity. Therefore, these mutants are promising as potential growth inhibitors, and are currently being tested as possible antitumor agents in breast cancer and bladder cancer cells that are dependent on EGF for their growth.

Conclusions

Amino acid substitutions by protein engineering have provided crucial information about the degree and nature of importance of specific residues in EGF. Certain mutants appear promising as anti-EGF and therefore antigrowth substances, and could be useful for potential cancer therapy. On the other hand, certain mutants are biochemically more active than native EGF. Such superagonists could be superior to EGF in its clinical applications.

Mammalian DNA Repair Proteins for Alkylation Damage

O^6-methylguanine-DNA methyltransferase (MGMT) and *N*-methylpurine-DNA glycosylase (MPG) are the two key, and ubiquitous proteins responsible for the removal of carcinogenic and mutagenic O^6-alkylguanine and toxic *N*-alkylpurines, respectively, induced in DNA by simple alkylating mutagens. MGMT is a stoichiometric protein which accepts the alkyl group directly from DNA on a unique cysteine residue in a suicide reaction. MPG is an enzyme which removes the alkyl purines from DNA leaving apurinic sites that are subsequently repaired. The *E. coli* MGMT and MPG genes have been cloned elsewhere some time ago. We have recently cloned the cDNAs of human and mouse MGMT and MPG. However, very little is known about the reaction mechanisms of these classes of proteins. In order to understand the role of specific amino acid residues and domains involved in enzyme catalysis and base specific recognition, we compared the deduced amino acid sequences of the human, mouse, *E. coli*, and *B. subtilis* MGMTs. The sequence Pro-Cys-His-Val-Arg containing the alkyl acceptor cysteine is invariant in these proteins. Additionally, in the neighboring regions one arginine and two tyrosine residues are conserved along with several hydrophobic residues and an asparagine residue in the six different polypeptides. These residues should be candidates for site-directed mutagenesis for elucidating their role in alkyl transfer reaction.

In contrast to MGMT, a sequence comparison of human, rat and *E. coli* MPG polypeptides did not reveal any conserved aspartic, glutamic and histidine residues which are expected to be involved in catalysis in an alignment for best fit. As expected, human and rat MPGs share extensive homology but very little sequence similarity exists between *E. coli* and mammalian proteins. However, a glutaminyl residue present in all MPGs may be involved in the recognition of 3-alkyladenine and an arginine present only in the MPGs that utilize *N*-alkylguanines also as substrates may be responsible for the recognition of 3- and 7-alkylguanine. We plan to test the possible roles of these residues by site-directed mutagenesis.

We have been able to overproduce the human and mouse MGMTs by cloning their cDNAs in a T7 expression vector system. This involved creation of an *Nde*I site spanning the start codon of the protein and we utilized oligonucleotide primer-mediated inverse polymerase chain reaction (PCR) for the site-directed mutation. The pure human and mouse MGMTs will be produced in milligram quantities for crystallization studies in G. Bunick's laboratory in this Division.

PCR-mediated site-directed mutagenesis was also carried out to delete a tetrapeptide sequence of mouse MGMT present only in rodent MGMTs but not

in the human protein. Our preliminary experiments indicate that this additional sequence is not necessary for the activity of MGMT.

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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 to recognize and interpret the information contained in genomic sequence data. A number of laboratories are already producing 10s or 100s of kilobases of DNA sequence and would greatly benefit from reliable computational methods to locate exons and other gene components. Experimental methods used to localize genes, such as exon trapping, are labor-intensive and time-consuming, but until now have represented the only viable methodology since standard computational methods are vastly inadequate to the task of 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. To accomplish our goal, 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 expert systems methods for intelligent automated assembly of recognized genetic features into hypothetical models for gene structure and function.

(1) Feature Recognition Tools. We are currently developing an improved set of recognition tools, termed "recognition modules," to identify each type of biologically important feature 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 seven 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 92% of coding regions. It is already being used in a number of major laboratories searching for genes related to human diseases, and has successfully located a number of new genes. An automated version of this tool is available on Internet, allowing researchers to discover genes in their sequence data almost instantly.

(2) Intelligent System Development. The second goal of the project is to develop an artificial intelligence inference system which is largely capable of

replacing the intuition that human investigators use to "assemble" features that constitute genes within the DNA sequence data to describe the structure of whole genes. Our intent is to integrate the recognition tools we are developing into an expert system environment capable of intelligent automated assembly of gene components.

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DNA REPAIR AND MUTAGENESIS

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DNA repair is a universal and vital phenomenon. It is essential for maintaining the integrity of the genome, which is subject to alterations, both spontaneous and those induced by diverse chemical and physical agents. The repair is neither perfect nor complete. The persistence of unrepaired lesions as well as error-prone repair lead to mutations, tumors, and toxicity. In this laboratory, our involvement in both DNA repair and mammalian mutagenesis is summarized as follows.

DNA Repair

Our work is exclusively focused on the repair of alkylation damage in DNA in human and other mammalian cells. Simple alkylating agents induce two major types of DNA adducts, namely O^6-alkylguanine, a mutagenic and possibly carcinogenic lesion, and *N*-alkylpurines (mostly 3-alkylguanine, 3-alkyladenine and 7-alkylguanine), which are likely to be toxic and carcinogenic if not repaired

properly. O^6-alkylguanine in DNA is repaired by *in situ* dealkylation in a stoichiometric reaction in which the repair protein, O^6-methylguanine-DNA methyltransferase (MGMT), accepts the alkyl group on a unique cysteine residue. *N*-alkylpurines are removed catalytically by *N*-methylpurine-DNA glycosylase (MPG) leaving abasic sites in DNA which, if not repaired, are likely to be mutagenic and toxic. Both MGMT and MPG are well regulated in mammals just as in bacteria, although the phenomenology of MPG regulation has not been extensively studied. An understanding of the molecular basis of regulation of these proteins will help in identifying the factors that affect both somatic and germ cell mutations induced by alkylating mutagens. This information also has practical implications in that MGMT antagonizes certain alkylating drugs and is overexpressed in drug-resistant tumor cells. Following our successful cloning of a cDNA of human MGMT, we expressed the human protein in *E. coli* for its subsequent purification and use as antigen. We have also cloned the mouse MGMT cDNA and purified the protein. The gene has also been mapped on the long arm of human chromosome 10 and the distal end of mouse chromosome 7. Regulation of MGMT has been found so far to occur at the level of transcription. Our current research involves studies on regulation of MGMT gene, its inducibility and amplification, and the molecular mechanism of its extinction in Mex^- cells which are extremely sensitive to alkylating agents.

We have cloned the human MPG cDNA and subsequently the cDNA from mouse cells. Our current studies involve construction of mammalian expression vector of MPG and studies of its regulation in human and rodent cell lines.

Mutagenesis

Our mutagenesis studies involve ionizing radiations of both low (X ray) and high (α particle) LET types as mutagens as well as mutations induced by site-specific insertion of alkylated bases in a target DNA. In the last report, we described the shuttle plasmid system used as a surrogate for studying human mutagenesis. We have continued to use the plasmid pZ189 for determining the yield and type of such mutations induced by X rays and α particles. These studies are extremely important in view of the paucity of information on the molecular bases of mutations in general and on radiation-induced mutations in humans in particular. The availability of a small target in the plasmid pZ189, which allows phenotypic screening for mutation, has enabled us to quantitate mutational yields following *in vitro* irradiation of plasmid DNA with X rays and α particles and to determine mutation distribution at the nucleotide level. The disadvantage of the small target is that mutations involving large-scale sequence changes cannot be identified. At the same time, such targets provide the major advantage that the data on the molecular nature of mutations, not available so far, can be readily obtained.

In the last Progress Report, we reported the unusual features of mutants induced by both X rays and α particles. Further analysis of a larger set of mutants confirmed the earlier conclusions that not only the point mutants are localized in hot spots involving mostly GC pairs, but the GC pairs are predominantly present in 5'-TC/3'-AG doublet sequences. It appears that whatever the mechanism, the neighboring bases have a profound effect on the outcome of the mutations during DNA replication.

We investigated the effects of free radical scavengers on the quantity and quality of mutations in the shuttle plasmid and observed that at equi-mutagenic dose the type and hot spots of mutations were unaffected by the presence of scavenging agents. In a comparison of mutations in the same X-irradiated target gene, we made the remarkable observation that while the hot spots remain the same, the mutations induced in *E. coli* were mostly transversions while a majority of the mutations induced in human cells were of transition type.

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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 regulates 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 and their possible role in RNA turnover and processing have been continued in this laboratory.

Characterization of a 5′ → 3′ Exoribonuclease (XRN1) Gene of *Saccharomyces cerevisiae*

Cloning and disruption of the *XRN1* gene. The *XRN1* gene of *Saccharomyces cerevisiae*, cloned as described previously, was found to be contained in a 6.5-kb *Xba*I - *Xho*I DNA fragment of chromosome VII. To test the essentiality of the *XRNI* gene, a plasmid was made in which a *Bgl*II-*Bgl*II fragment of the gene was deleted and replaced with the *URA3* gene of yeast. A larger fragment within the gene, containing the altered fragment, was used for integrative transformation of two haploid yeast strains. Transformants were obtained, and Southern blotting analyses of each strain confirmed that homologous integration had occurred at the correct chromosomal locus. Both enzyme activity and immunoreactivity of XRN1 were absent from the disrupted strains. The measurements of enzyme activity involved chromatography of the extracts on hydroxylapatite columns, and the results showed a second peak of activity that was coincidental in chromatographic elution profile to that found for a partially-purified second 5′ → 3′ exoribonuclease. There was no indication from the studies of gene-copy number of a closely-related gene, and several less stringent hybridization conditions were tried. The *XRN1* gene mutants grew considerably more slowly (a 55% reduction in cell growth rate at 30°C and 66% at 36°C in one case), and that the decreased growth rate is due to lack of the *XRN1* gene was tested by comparing the growth rates of the mutant and wild-type strains after transformation with an *XRN1* gene-containing plasmid. Upon transformation, the growth rates of the mutant strains returned to normal.

Sequencing of the *XRN1* gene. Six internal *Eco*RI fragments along with the terminal *Xho*I-*Eco*RI and *Xba*I*Eco*RI fragments of the *XRN1* gene were subcloned individually into plasmid pBS+. These clones were used to produce single-stranded DNA template for dideoxy sequencing. Double-stranded DNA of pFL305 was prepared as double-stranded template. Sequencing of each subclone was initiated using the "universal primer" and extended when necessary using primers synthesized ad hoc. Double-stranded template (using custom primers) was used to provide overlapping sequence to link specific fragments, and for opposite strand sequencing as required.

The DNA sequencing of the *XRN1* gene shows an open reading frame of 4584-bp, capable of encoding 1528 amino acids. By comparison with GenBank sequences it was found that *XRN1* is 99.9% identical to the yeast *DST2* gene, encoding a DNA strand transferase. Very recent reports show its identity to the *SEP1* gene, the product assayed also as a DNA strand transferase, to the *KEM1* gene, affecting nuclear fusion, and to the *RAR5* gene, affecting chromosome stability. The high-level 5′ → 3′ exoribonuclease activity of the gene product, as studied in this laboratory, shows that the protein has a distinct enzymatic activity and studies of its function have been pursued as described below.

Analysis of the role of XRN1 in pre-rRNA processing. A role for a 5′ → 3′ exoribonuclease in pre-rRNA processing was suggested by the purification by others of such an enzyme from nucleoli of mouse Ehrlich ascites tumor cells. It was proposed that the enzyme might be involved in 5′-trimming reactions to remove all or part of the external and internal transcribed spacers and/or hydrolysis of fragments of these spacers, generated by endoribonucleolytic cleavages. The rapid hydrolysis of the spacers of the pre-rRNAs following cleavage *in vivo* has made a study of the exact location of the cleavage sites for generation of the 5′ and 3′ termini of the mature rRNA molecules very difficult. With the yeast strain containing a disrupted *XRN1* gene, pre-rRNA processing has been analyzed to try to detect 5′-trimming errors or accumulation of spacer sequences. It has been found that large amounts of fragments of the internal transcribed spacer 1 found on 20S pre-rRNA, the direct precursor of the 18S mature rRNA species, accumulate. The largest fragment is 209 nucleotides, which is the length of the 20S pre-rRNA spacer. The fragment may accumulate in the cells lacking XRN1 because the enzyme is involved in its hydrolysis. The results show that an endonucleolytic cleavage at the exact 3′-terminus of the 18S rRNA can convert the 20S pre-rRNA to 18S rRNA.

Analysis of the role of XRN1 in mRNA turnover. A role for a 5′ → 3′ exoribonuclease in mRNA turnover was the function first suggested for this type of enzyme. It was found that mRNA containing a cap structure was not hydrolyzed by the 5′ → 3′ exoribonuclease activity of crude extracts or by the purified yeast enzyme, while decapped mRNA was. The status of mRNA

adenylation and turnover has been analyzed in the *XRN1* gene disruptants. The following results have been obtained. (1) The rate of total mRNA [poly(A)$^+$ RNA] synthesis is reduced to a level slightly less than the growth rate, and the half-life of *total* poly(A)$^+$ RNA as measured by both approach to steady-state labeling and inhibition of transcription with thiolutin is 1-1.4 × normal. (2) Analysis of the polyadenylation status of four mRNA species in a slow-growing disruptant shows that the strain has a high level (50-80%) of deadenylated mRNA; however, [^{3}H] pulse labeling shows that poly(A) chains are formed. The results show that the rate of deadenylation of mRNA is close to normal as measured by turnover of poly(A)$^+$ mRNA, but that poly(A)$^-$ mRNA accumulates. (3) Northern analysis of the half-lives of several specific mRNAs shows that they are lengthened, the short-lived mRNAs as much as 2-4×, even when a disrupted strain (in rich media) is growing at a rate similar to wild-type cells (in poor media). The results suggest strongly that XRN1 may play a key role in mRNA turnover.

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STRUCTURAL BIOLOGY

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This laboratory 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. This research will eventually provide atomic resolution information which is of fundamental importance to a detailed understanding of gene expression, transcription, DNA replication, and the structures of DNA and chromatin.

It is universally recognized that the three-dimensional structure of a molecule determines its biological function. Protein crystallography, in combination with cloning techniques and site directed mutagenesis, provides the best approach for determining the folding patterns of complex macromolecules and the three-dimensional architecture of catalytic sites of proteins. Such structural knowledge is necessary for the application of protein engineering and pharmaceutical design

research. The rules governing the folding pattern of amino acid sequences are largely unknown. With each new structure, protein crystallography contributes information that will help decipher the hierarchy of rules governing protein folding. This laboratory is studying several proteins in addition to the nucleosome core particle. The 2.3 Å resolution, low temperature, X-ray crystal structure of inorganic pyrophosphatase, an enzyme that drives nucleic acid and fatty acid biosynthesis reactions to completion, is undergoing crystallographic refinement by simulated annealing. Two other proteins, epidermal growth factor and O^6-methylguanine-DNA methyltransferase, are subjects of crystallization experiments. Experiments are being planned to define the crystallization conditions of 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.

The DNA in the nucleosome core particle bends much more substantially than any other DNA structure solved by crystallographic methods. The size and shape of the DNA grooves provide evidence of the distortions in the B-DNA structure which facilitate DNA bending. These measurements provide insight into the mechanisms of histone induced DNA bending, the sequence dependence of histone binding, and the helical parameters of nucleosomal DNA. Our research indicates that the bending of DNA is accomplished by considerable compression of the major grooves on the inner surface next to the protein core and expansion on the outside of the superhelix. We have also detected a structural motif in the DNA with a period of two B-DNA helical repeats, or about 20 base pairs. This feature results from the placement of AA and TT dinucleotides at specific positions within the 20 base pair repeat, and these dinucleotides modulate the DNA structure for optimum major and minor groove bending around the histone core. The level of detail in the histone portion of the structure at 8 Å resolution permits a more accurate assignment of histone domains, and clarification of the overall organization of the histone core. This information enables a detailed comparison to other related structures, and an evaluation of flexible regions of the histones. The histone structure suggests possible mechanisms of unfolding the nucleosome particle which could be related to control of gene expression. Analysis of the histone areas associated with contacts between nucleosomes in the crystal structure suggests possible modes of interaction between nucleosomes in chromatin higher order structure.

Nucleosomes reconstituted from homogeneous histone octamers and cloned specific-sequence DNA are necessary in order to determine a high

resolution (~ 3 Å) crystal structure. Several DNAs that form precisely positioned nucleosomes have been developed. These sequences are a repeat unit subcloned from human α-Satellite, a sequence from SV40 flanking the nucleosome free region, and a chicken t-RNA lysine gene complete with its polymerase promotor region. A 146 bp palindrome has been engineered based on one-half of the human α-Satellite sequence. Palindromic DNA is desirable to remove a source of disorder in the crystals. This occurs because there are two possible ways a nucleosome can incorporate itself into the crystal lattice. These orientations are related by a 180° rotation about the nucleosome's dyad axis. Non-palindromic DNA does not have true dyad symmetry at the base level about the nucleosome molecular dyad axis, resulting in two-fold crystal packing heterogeneity. The use of palindromic DNA makes the two halves of the nucleosome precisely equivalent both in the DNA and histone regions and therefore removes the two-fold disorder resulting in maximum resolution to resolve the details of histone-DNA interactions and DNA structure.

Milligram quantities of nucleosomes containing either the native α-Satellite DNA sequence or the α-Satellite palindrome sequence have been prepared by salt gradient dialysis reconstitution. Analytical amounts of nucleosomes containing the SV40 and tRNA gene sequences also have been produced by this reconstitution procedure.

Nucleosome cores containing the 145 base pair α-Satellite sequence DNA were used for surveying crystallization conditions. Crystals were grown by dialysis and direct addition of spermine or manganese. The crystals are birefringent, display sharp well formed edges, and show very clean crystal faces. Subsequent experiments using higher nucleosome concentrations and controlled step-wise retrograde manganese dialysis produced larger crystals with approximate dimensions of 2.0 x 0.75 x 0.15 mm.

Diffraction studies of crystals containing the native α-Satellite sequence show reflections past 3.5 Å, well past the 8 Å limit of the native chicken erythrocyte nucleosome structure. Reflections can be seen beyond 3.0 Å resolution near the horizontal axis. The ultimate resolution limit of the crystals remains to be determined. The nucleosomes used in the present experiments were not purified after reconstitution. There is always some free DNA, histones, and incorrectly formed nucleosomes present after the reconstitution. Purification of the nucleosomes is expected to improve the crystals considerably. Another area that requires additional exploration involves the composition of artificial mother liquor used to store and mount the crystals. Cooling the crystals during X-ray exposure will also improve their diffraction properties. The results presented here were obtained using a standard rotating anode X-ray source. The diffraction properties of the crystals should show marked improvement at a synchrotron source. This work demonstrates that nucleosomes reconstituted from purified histones and the

homogeneous specific sequence DNAs that we have selected will lead to a high resolution structure of the nucleosome.

The high resolution structure will be phased by using multiple energy anomalous dispersion data collected at the Brookhaven National Synchrotron Light Source. It will be possible to trace the peptide chains of the histone molecules and the phosphodiester backbone of the DNA, and locate the DNA bases in the resulting electron density map. This long term research will provide the structural knowledge to understand control of gene expression at the nucleosomal level.

Protein Structure Determination

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. Pyrophosphatase is therefore an essential enzyme in all life forms. The high resolution, low temperature structure of yeast inorganic pyrophosphatase is being refined in collaboration with D. Voet at the University of Pennsylvania.

PPase crystallizes in the monoclinic space group $P2_1$ with cell dimensions of a=69.96, b=95.27, c=51.77 Å, β=99.5° and a dimer in the asymmetric unit. Incomplete data up to 2.3Å resolution were previously collected at -50°C by rotation photography, optically scanned and processed. Coordinates of a 3Å resolution study of PPase at room temperature (280 residues only, Arutiunian *et al., Dolk. Akad. Nauk SSSR*, **258**: 1481, 1981) were taken from the Brookhaven Protein Data Bank and used to initiate refinement with a starting R-factor of 52%. The refinement proceeded smoothly by using the program, PROLSQ, installed on a Cray-2, and from $2F_o$-F_c maps the missing terminal amino acids were eventually fitted on a PS350 graphics system. Some corrections were made to the backbone and numerous side-chains were also readjusted. The R-factor was convergent at 28%. Refinement was continued by crystallographic simulated annealing using the program XPLOR installed on a Cray Y-MP. The R-factor stands at 24.4% with solvent water and amino acids with alternate side chain conformations presently being incorporated into the structure.

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.

The protein has been purified to homogeneity in large quantities (10-15 mg/l of culture) from a high expression plasmid. This protein is quite unstable. It generally requires 20-30% glycerol and approximately 200 mM NaCl to retain activity and remain soluble, once isolated. Much of the early work has been focussed on purifying the protein and finding ways to store it for later use. Two promising approaches for short term storage appear to be lyophilization from 0.2 M ammonium bicarbonate containing Tris-HCl, EDTA, and DTT or at -20° C in 50% glycerol containing Tris-HCl, EDTA, and DTT. Crystal survey experiments are being done to find conditions for crystal growth. Double-strand DNA fragments also have been prepared for co-crystallization experiments, which are in progress.

Epidermal Growth Factor (EGF)

The differentiation and growth of cells are regulated by a class of polypeptides known as growth factors. There is considerable interest in the study of growth factors because of their important role in normal and pathological cell division and growth. Epidermal growth factor (EGF) is a 6 kDa protein consisting of 53 amino acids with 3 internal disulfide bonds. The binding of EGF to its receptor initiates a series of biochemical reactions. Tyrosine kinase activity of the receptor is stimulated, leading to autophosphorylation and phosphorylation of endogenous proteins. These activities lead to increased glycolysis and protein synthesis, enhanced transcription of specific genes, and ultimately to increased DNA replication and cell division.

A sample of EGF taken to Germany by V. Zabel (Health and Safety Research Division) was crystallized by vapor diffusion vs. 22% PEG 1000 (polyethylene glycol). The crystallization solution contained EGF at 10 mg/ml along with NaCl, Ca acetate, Na acetate, 11% PEG 1000, and Tris-acetate buffer at a pH between 6.8-7.0. These experiments produced many small triangular plate-like crystals. Experiments using a rotating anode X-ray source demonstrated diffraction to about 2.2 Å resolution. The crystals deteriorated beyond usefulness while being transported to the synchrotron X-ray station at DESY. Crystallization experiments are in progress at ORNL to reproduce and improve on the previous results. Several milligrams of EGF, containing seleno-methionine in place of methionine, have been prepared for use as an intrinsic heavy atom derivative.

GENOME STRUCTURE AND ORGANIZATION

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Clones in a Crab Regenerating-Limb cDNA Library Contain Segments of the Repeat Unit of a Complex Satellite

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 (stDNA), which has the most complex satellite sequence known. The stDNA (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. Since this stDNA is so much more complex than most stDNAs, we are investigating whether it is transcribed.

A cDNA library has been constructed from poly(A)+ RNA from regenerating limbs of premolt crabs that are preparing to undergo ecdysis and grow, a time of intense biosynethesis in these animals. The library has been screened for clones containing cDNAs putatively copied from transcripts of the G+C-rich stDNA. We have obtained evidence that segments of two cDNA clones totaling >1.1 kb of a repeat unit are transcribed. The transcripts represent 77% of the conserved domains. The insert in one of the clones includes the highly conserved 3' end of the stDNA (as defined by a single EcoRI site/repeat) and extends into the 5' end of the adjacent repeat unit, also highly conserved. The insert in the other clone includes one copy of AMPL, a 142 bp segment that is amplified from 5 to 65 X in different variants of the stDNA; sequences upstream from the AMPL segment are also present. Since the library was constructed with oligo(dT) primers, the transcripts were presumably polyadenylated and transcription of the stDNA was probably by pol II. Since both clones contain non-stDNA plus stDNA, the transcribed segments of the stDNA may be those scattered in the crab's major component DNA rather than in the tandem repeats of the satellite. Evidence for this possibility includes the observation that, when cellular stDNA is cut with restriction enzymes and probed with radiolabeled segments of the stDNA, the sizes of the restriction fragments that light up differ from those of fragments released by digestion of cellular stDNA with the same restriction enzymes. In addition, the dispersed segments of the stDNA are much less methylated than tandem repeats of the stDNA. In general, transcription of under-methylated DNAs is much more extensive than transcription of methylated DNA segments.

The Molecular Biology of Crustacean Molting

Characteristics of exoskeletal layers of the land crab, *Gecarcinus lateralis*

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 % organics increase progressively in each deeper layer. As in insect cuticles, small M_r proteins predominate. The proteins of exocuticle and endocuticle are most similar to each other in M_rs and pI and are also very similar to proteins of the membranous layer. Epicuticle contains more unique proteins (on 2D gels) than the other layers and three times as many different proteins that bind Ca^{++} *in vitro*. A fifth layer, the ecdysial membrane, is of unknown origin; in insects and decapod crustacea, such as crabs and lobsters, it lines the exoskeleton shed at molting. Our data indicate that the ecdysial membrane is not a remnant of the membranous layer as has been presumed. We find: (1) Proteins from ecdysial membrane differ from those from membranous layer in M_r and pI. (2) Of three ecdysial membrane proteins that bind Ca^{++}, none correspond in M_r or pI to any of the Ca^{++}-binding proteins in membranous layer. Proteins similar to the major proteins in epicuticle, exocuticle, endocuticle, and membranous layer are also prominent in other architecturally important exoskeletal components (intersegmental membranes, apodemes to which the internal musculature is attached, regenerating limb bud sacs, and gastroliths, the latter being internal calcium-storage structures), suggesting that a relatively small complement of genes may code for many of the major proteins of the crustacean exoskeleton.

Epitope sharing among cuticular proteins of a crustacean (*Gecarcinus lateralis*) and two insects (*Manduca sexta* and *Drosophila melanogaster*)

Some major proteins occur in all four exoskeletal layers of *G. lateralis*; others in only one or two layers. Proteins with similar properties (based on M_r and pI) also occur in insect cuticles. Relationships of the cuticular proteins of these two arthropod classes (Crustacea and Insecta) have been explored by immunochemistry. Proteins from the four layers of the crab exoskeleton removed from animals at different stages of the intermolt cycle were analyzed on Western blots with polyclonal antibodies against cuticular proteins of two insects. The antibodies were against larval cuticular proteins (LCP) and pupal cuticular proteins (PCP) of the tobacco hornworm, *Manduca sexta* (the kind gifts of L. M. Riddiford), and against third instar larval cuticular proteins (L_3CP) 1+2 (both proteins are ~17.5 kDa; they differ in pI) or L_3CP 3 (9 kDa) + 4 (11 kDa) of *Drosophila melanogaster* (the kind gifts of W. J. Wolfgang). Anti-LCP of *M. sexta* reacts intensely with proteins from exoskeletons of intermolt and early premolt crabs.

Anti-LCP reacts only with higher M_r exoskeletal proteins from crabs in the late premolt stage, many lower M_r exoskeletal proteins having been degraded. Overall, anti-PCP of *M. sexta* reacted much less intensely with the crab exoskeletal proteins than did anti-LCP. This is of interest in that the acidic pIs of crab exoskeletal proteins are more similar to those of larval (so-called flexible or soft) insect cuticle proteins than to pupal (rigid) cuticle proteins. Antibody reaction was more intense to proteins extracted from exoskeletons of early premolt crabs than with proteins from intermolt crabs. The reactions of crab exoskeletal proteins with insect polyclonal antibodies suggests that some cuticular proteins may be evolutionarily conserved in members from these two arthropod classes.

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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. Four 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 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 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 ~10 μM, 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 kidney cells.

Protein Kinase C and the Regulation of Amino Acid Transport

Confluent LLC-PK_1 cells that have been depleted of endogenous amino acids respond to the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) with an upregulation of Na^+-dependent amino acid transport (A-system). The increases in transport and concomitant membrane-protein phosphorylation can both be inhibited by sphingosine, an inhibitor of protein kinase C. Several membrane-associated proteins show a rise in phosphorylation in response to TPA and sensitivities to sphingosine corresponding to the observed transport changes. Similar responses are elicited by diglycerides that also activate protein kinase C and stimulate the A-system. In confluent cultures sphingosine inhibits only the TPA-sensitive portion of A-system transport but not the basal rate. The results suggest that membrane-associated protein kinase C maintains the transporter or a transport regulator in a phosphorylated, active state in growing cells, but

dissociates from the membrane at confluence, after which time the regulators are dephosphorylated; TPA can reverse this differentiation-related distribution.

Firm conclusions from these results may be compromised by the long-term toxicity of sphingosine. We have therefore looked for other protein kinase C inhibitors that can permeate cells without toxic effects. Three diphenolic compounds, quercetin, phloretin, and diethylstilbestrol, meet these criteria. Their effects on TPA-stimulated upregulation of the A-system are the same as those of sphingosine and confirm our original interpretation. Addition of any of these compounds after 15 minutes (or later) following TPA results in no inhibition of the upregulation, suggesting that the signal for transport stimulation is completed in this interval. Antibodies that we developed against the catalytic moiety of protein kinase C, used in Western blot analysis, react with a protein of 67 kDa; this suggests that the kinase active in these cells is protein kinase C-zeta, a possibility that we are currently investigating in greater detail.

Cellular Responses to EGF Mutants

We have most recently been exploring the responses of Balb c/3T3, strain A31, cells and EGF-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 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 gaunidinium 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).

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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 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 extracellular matrix, no specific ligand has been identified for $\alpha 6\beta 4$.

Alpha6β4 molecules have been identified on four different human carcinoma cell lines (lung, breast, embryonal, and colon). Evidence indicates that the human proteins are analogous to, but distinct from, those on mouse cells. Two-site monoclonal antibody (MoAb) assays for $\alpha 6\beta 4$ in the mouse and the human have been developed which allow quantitation of low levels (ng/mg protein) of these proteins in normal and neoplastic tissue. The results of experiments in the mouse indicate that tumors have about 10 times more $\alpha 6\beta 4$ than do normal tissues. Benign lung adenomas have small amounts of this integrin and the amounts increase as the adenomas become larger or progress to adenocarcinomas. All murine lung carcinomas tested to date (including primary, spontaneous, or chemically induced from different strains of mice) contain high levels of $\alpha 6\beta 4$ as do BALB/c mammary carcinomas.

Analyses of human tissue show that $\alpha 6\beta 4$ is expressed at low levels in nearly all 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 is restricted to certain squamous epithelium, particularly of skin and colon, and some subsets of endothelium.

Molecular characterization of the $\alpha 6\beta 4$ complex has revealed five distinct glycoproteins on SDS-PAGE analysis. The bands 1-3 (Mr 204, 185, and 150 kDa) are different forms of the $\beta 4$ molecule while bands 4 (Mr 135) and 5 (Mr 116) represent precursor and mature $\alpha 6$, respectively. The band 1 protein of $\beta 4$ is phosphorylated in the cytoplasmic domain. Second generation MoAb to $\alpha 6\beta 4$ have allowed construction of 2-site assays specific for each of the protein bands. Expression in colon and lung tumors is restricted to band 3 $\beta 4$ protein.

Current work is focused on molecular characterization of $\alpha 6\beta 4$ and attempts to identify a specific receptor function for the complex. A cDNA library of Line 1 cells in λgt11 has been screened with a rabbit antiserum to $\alpha 6\beta 4$ and a consensus oligonucleotide from the N-terminal sequence of band 5. About one-half of the cDNA has been cloned and sequenced. This cDNA will be used to analyze mRNA sizes and synthesis as well as the gene's size and position in normal and neoplastic cells. The effects of known factors on the phosphorylation of $\beta 4$ and growth of Line 1 cells are being analyzed to establish a function for this complex.

The protein complex has been shown to be a component of hemidesmisomes in the skin and eye where it presumably functions in cell attachment to an unidentified extracellular matrix protein. We have shown that certain subsets of endothelial cells (which do not contain hemidesmisomes) also express $\alpha 6\beta 4$. The function of the molecule in these tissues is unknown.

Monoclonal Antibodies to Lung Endothelium

MoAb 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 MoAb 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 MoAb to this protein 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 MoAbs 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 a role in embryogenesis.

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RADIATION CARCINOGENESIS

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The external radiation carcinogenesis program has been consolidated into four programs including the *in vivo-in vitro* studies reported in this volume. The major effort has been devoted to an integrated study of myeloid leukemia in RFM mice at the molecular, chromosomal and whole-animal level.

In several strains of mice there is a non-random deletion of a segment of chromosome 2 in myeloid leukemia. The aim of the molecular studies is to define the deletion breakpoints which occur between regions B and C and to identify the genes located within the shortest deleted region on chromosome 2 and their potential role as a recessive oncogene or tumor suppressor gene. There are a number of possible approaches to the identification of the relevant sequences. Several telomeric breakpoints occur in different regions.

In this reporting period, a 628-bp DNA fragment corresponding to TCL-2 locus involved in chromosomal translocation in human acute lymphocytic leukemia (T-ALL) was cloned from the RFM mouse by the PCR technique and nucleotide sequences were determined. Although there is no evidence that TCL-2 locus is directly involved in the chromosome 2 deletion radiation-induced myeloid leukemia, it may be used as a molecular marker for subsequent cloning of deletion breakpoints.

Activation of oncogenes as a result of retroviral integration has been shown in virus-induced myeloid and lymphoid leukemia. A panel of probes has been

established and verified by DNA blotting and PCR techniques. Our preliminary results indicate that transposition of MuLV takes place in radiation-induced myeloid leukemia.

Chromosome analysis has been carried out by R. J. Preston and C. Gooch on a considerable number of histologically confirmed leukemias. A deletion of a segment of chromosome 2 has been found in almost all but, perhaps importantly, not in all the cases of myeloid leukemia. It is likely that the relevant deletion is small, so small, in fact, that it is not detectable with the light microscope in those cases in which no deletion is apparent. The natural incidence and susceptibility to induction of myeloid leukemia is markedly higher in males than in females in all of the strains studied. It has been found that loss of Y chromosome occurred in some cases of myeloid leukemia and in leukemic cells that have been transplanted and have grown in a host.

In collaboration with the Molecular Genetics of Cancer Group, retroviral expression vectors containing IL-3 or GM-SCF were constructed, and that Group has investigated the expression of the consequences of expression of IL-3 transgene. Transgenic mice expressing IL-3 may prove to be a model system in which to study the contribution of IL-3 in the development of myeloid leukemia.

The whole-animal studies are designed first, to test whether the linear-quadratic model describes adequately the dose-response relationship for the induction of myeloid leukemia by low-LET radiation. One important consequence of validating the model would be that we could obtain estimates of the initial slope in the low-dose region from data for relatively high doses given in small fractions or at a low dose rate. It is, of course, the effects of low doses that are important and are difficult to assess. Second, we plan to determine whether split-dose repair of the leukemogenic effect of low-LET radiation occurs, and, finally, we shall determine the effect of protraction on the induction of myeloid leukemia by neutron irradiation. These data are also used for the determination of the RBE for the induction of myeloid leukemia by neutrons and modeling of the neutron dose-response curve.

All the exposures to gamma rays and neutrons have been carried out, and about 45% of the animals have died or been sacrificed.

Skin Carcinogenesis

The study of the effects of fractionation of doses of soft X rays in the induction of skin cancer in SKH-hr-1 mice is nearing completion. It was shown that 64 fractions were much more effective than 16 fractions of the same total dose, but the question remained whether this somewhat surprising effect was dependent on the size of the dose per fraction. Current results suggest that this is the case.

Some of our other experiments indicate that the effect of fractionation with a relatively high dose per fraction (62.5 cGy) was on the expression and not the initiation of squamous cell carcinoma. The finding that the increased expression of initiated cells is dependent on the size of the dose per fraction must be reflected in the mechanism. The explanation may be as simple as disruption of the cell-cell interaction.

The other aspect of the studies of skin cancer has been concerned with the effects of ultraviolet radiation (UVR). The role of cyclobutane pyrimidine dimers in the induction of skin cancer by UVR has been the subject of considerable research and discussion. There is indirect evidence and there are some experimental data that implicate pyrimidine dimers as the pertinent DNA lesion. Some unequivocal evidence has been obtained in a collaborative study with R. D. Ley (Lovelace Medical Foundation) that cyclobutane pyrimidine dimers are involved in the induction of UVR of papillomas, squamous cell carcinomas of the skin, and fibrosarcomas of the cornea. These studies have been possible because the cells of the marsupial *Monodelphis domestica* reverse UVR-induced DNA lesions photoenzymatically. Repeated exposures of the opossums to UVR induced skin and eye tumors, but their incidence was decreased markedly by exposure to photoreactivating light after each UVR treatment. The fact that tumor induction by UVR was not eliminated completely may indicate that not all the DNA lesions are reversed photoenzymatically or that some other type of UVR-induced lesion, such as pyrimidine-pyrimidine (6-4) photoproduct, may also initiate neoplasia.

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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-LET radiation, and (3) the influence of cell-cell interactions mediated by diffusible growth factors, such as transforming growth factor type ß, on the evolution of respiratory neoplasias.

The following is a brief summary of specific ongoing projects:

Modulation of TGF-ß 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-ß 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-ß 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-ß 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.

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MOLECULAR GENETICS OF CANCER

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The purpose of this research project is to understand the molecular mechanisms of carcinogenesis that are induced by environmental agents. Our working hypothesis is that the germline-integrated retroviral and retrotransposable genes in the chromosomes are particularly responsive to genomic stresses that are caused by environmental carcinogenic insults to the cell; the activation of the insertion and the regulatory elements of these chromosomal retroviral genes may lead to genomic instability and to altered oncogene expression. We have previously found that different classes of chromosomal proviral genes, although generally silent, may show different tissue-specific expression in the mouse; for example, the ecotropic/xenotropic proviral class, the polytropic/modified polytropic and the MboI repeat proviral classes tends to be expressed, respectively, in the lympho-hematopoietic organs, in the liver and kidney, and in mature gonads. In addition to potentially active transcriptional promoters and enhancers, the long-terminal-repeats (LTR) of these endogenous retroviral genes were found to possess negative regulatory elements, similar to many cellular genes that are environmentally reactive. Our current research efforts have involved the application of gene transfer approaches to study the LTR functions. Both germline and somatic transgenic mice are examined as "minus-one-step" experimental models for studying the multistep process of carcinogenesis.

Development of Germline Transgenic Mice

Various LTR-oncogene and LTR-marker gene constructs were introduced by microinjection into fertilized mouse eggs to develop germline transgenic mice. Founder mice obtained include seven with *p26L-CAT*; seven with *pL2-CAT*; five each with *pBOR-Ha-v-ras*, *p16-Ha-v-ras*, and *pSV2-neo-Bendo*; and four with *pBOR-IL3*. Breeding of founder mice was performed to generate homozygous transgenic FVB/N mouse lines, which include three each for *p26L-CAT*, *pL2-CAT* and *pSV2-neo-Bendo* and two each for *pBOR-Ha-v-ras*, *p16-ha-v-ras* and *pBOR-IL3*.

Apparent Lack of LTR Transgene Expression

With the various procedures we used, we found no apparent expression of transgenes in the germline transgenic mice, developed from the six recombinant DNA constructs mentioned above. The negative experimental evidence includes: (a) no detectable activity of chloramphenicol acetyl transferase (CAT) in various tissues, i.e. liver, spleen, kidney, lung, intestine, skin, heart, brain, thymus, skeletal muscle, ovary and testis, of either *p26L-CAT* and *pL2-CAT* homozygous mice; (b) no detectable interleukin-3 (IL-3) transcripts in spleen and thymus of *pBOR-IL3* mice; (c) resistance to Smal restriction enzyme digestion at the LTR CCCGGG site of the transgene in *pBOR-Ha-v-ras* transgenic mice; and (d) inability of the embryo culture cells of the *pSV2-neo-Bendo* transgenic mice to survive in medium containing G418 antibiotics, despite the presence of the neomycin resistance gene.

Activation of Transgenes

Although the transgenes were largely silent in these mouse lines, a preliminary result suggests that they can be activated. When the primary culture cells of *pSV2-neo-Bendo* mouse embryos were treated with medium containing 0.75 μg/ml of azacytidine for 24 h and then serially subcultured *in vitro*, progeny cells resistant to G418 began to emerge after 5th or 7th passages. Treatment of the embryo cells with medium containing 5 μg/ml of benzo(a)pyrene appeared to have the similar effect of transgene activation, although to a lesser degree. Interestingly, many of the G418 resistant cell colonies that emerged showed the "pile-up" and criss-cross morphological features to suggest transformation of the cells. However, these cells were not tumorigenic when injected into congenic FVB/N mice. The G-418 resistant cell clones with the apparent "transformed" phenotypes also showed no detectable expression of the *B-endo* integrase gene.

Thymic Lymphoma Induction in *pBOR-IL3* Transgenic Mice

Experiments were therefore carried out to activate the transgenes in the animal. Two treatment procedures have been used. One involves three

azacytidine injections into baby mice at 3, 5, and 7 days of age; the other is a subcutaneous injection of pregnant mice at 17-18 days of gestation with 30 micrograms of dimethybenzanthracene (DMBA). In the *pBOR-IL3 (lines 12 & 15)* mice, both the neonatal azacytidine treatment and the prenatal DMBA treatment caused 30-40% mortality due to induction of thymic lymphomas within 3-5 months of age. The thymic lymphomas are highly malignant, as indicated by massive infiltration of the lymphoma cells in the lung, kidney, liver, and mesenteric lymph nodes in the animal. Some of the lymphoma cells could be grown in the cell culture as well as be transplantable in congenic FVB/N mice. Lung adenomas were also observed in the 3-5 month-old offspring of the transplacental DMBA-treated pBOR-IL3 line 12 pregnant mice.

Sporadic Occurrence of Tumors in Female Reproductive Organs of Homozygous p26L-CAT Mice

Progeny of the p26-CAT and the pL2-CAT founder mice were observed for the occurrence of spontaneous tumors within the first year of age. This was done with the consideration that, if introduction of a transgene into the mouse genome causes genetic alteration(s) that constitutes a step or steps in the carcinogenesis process, it may take less time for other genetic factors to be altered and hence complete the multistep mechanism of carcinogenesis. In other words, we are interested in knowing whether or not certain tumorigenesis process may be accelerated by incorporation of transgenes into the mouse genome. Indeed, three lines of p26L-CAT transgenic mice appeared to develop spontaneous tumors of reproductive organs, such as teratocarcinomas of the ovary and adenomas of mammary glands, at relatively young age; and this was particularly evident in the homozygous state. Germline incorporation of p26L-CAT construct into the mouse genome, if in an autosomal location, creates an aberrant situation in that the originally male-specific 26L LTR is now present in the female body. It remains to be determined whether or not and how the testis-specific transcriptional regulatory gene elements may respond to the female hormonal environment, thus predisposing the female animals to these reproductive tumors.

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CHROMOSOME CHEMISTRY

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The major goal of this laboratory 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 the Hypotrichous Ciliated Protozoa

All ciliated protozoa exhibit nuclear dimorphism, i.e., the existence of a transcriptionally-active macronucleus in the same cytoplasm with an inactive micronucleus. The hypotrichous ciliated protozoa possess two distinct nuclear features that distinguish them from other ciliates: (1) macronuclei consist of a "bag" of high polyploid (ca. 10^4-fold), short (ca. 2-3 kbp), linear DNA molecules of low sequence complexity — each fragment probably corresponding to an individual structural gene and its regulating flanking sequences; and (2) macronuclear DNA replication is localized exclusively in a Replication Band (RB) that migrates along the nucleus during S phase. Both of these features are unique in biological systems and offer considerable advantages in the study of nuclear structure and function compared to typical eukaryotic nuclei. During the past two years, we have continued to examine gene, chromatin and macronuclear structure. Several macronuclear genes have been cloned and sequenced. The total list now includes: 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. Several of the genes were chosen for subsequent studies of control of transcription; several were chosen for studies of gene and chromatin replication. The problem of organization of genes within a macronucleus prompted two additional studies. The first was on the nature of proteins bound to telomeric DNA sequences; the other was on a possible mechanism of organization based upon 10 nm nonchromatin (matrix) filaments. In addition, a study was completed on the spatial distribution of acetylated histone H4 and its possible relationship to macronuclear transcription and replication.

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. We have emphasized reconstruction of thick sections which cannot be visualized in a conventional, 100 KV, transmission electron microscope. To solve this problem, we have collected data on an energy filtering microscope (Zeiss 902). Employing a tilt series of these energy filtered images, we have reconstructed a large portion of a BR in a thick section (ca. 0.75 μm thick). Analysis of this large amount of data has presented a considerable challenge. One method for visualizing the data has employed "volume rendering," where each calculated voxel is assigned a brightness and transparency level prior to calculating stereo projections. When displayed in movie format, the resultant 3-D images are very instructive. Software tools are in development to edit and manipulate the 3-D space.

Considerable interest has resulted from our development of an improved DNA-specific stain for the electron microscope. We have now published our method for synthesis of the Osmium Ammine stain, and Polysciences, Inc., has decided to manufacture and market the stain. We are presently combining energy filtered imaging, electron microscope tomography and Osmium Ammine staining to examine the 3-D organization of transcription within sections of the BR.

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MOLECULAR BIOLOGY OF INSECTICIDE RESISTANCE

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The adverse socioeconomic effects of insect pests are well known. The capacity to control insect populations is seriously compromised by their ability to develop resistance to insecticides. Although the physiological and biochemical processes by which insects resist the toxic effects of insecticides are known, the molecular mechanisms by which these processes are regulated are not well understood. A better understanding of resistance at the molecular level could lead to development of strategies to prevent or reverse the development of resistance.

Metabolic detoxification of insecticides by cytochrome P450 mono-oxygenases is a major mechanism of resistance. The amounts of total P450 and/or specific isozymes are generally greater in resistant insects than in their susceptible counterparts. How these levels are regulated are not known. Unique features of its P450 makes *Drosophila melanogaster* a useful model system for studying the molecular biology of P450-dependent insecticide resistance. One Drosophila P450 subset, P450-B, is present in much greater amounts in resistant than in susceptible strains. Putative structural and regulatory genes involved in P450-B expression are located on chromosomes 2 and 3 at, or near, major insecticide resistance loci.

In the previous Progress Report, the use of a cDNA clone of P450-B1, an isozyme of the P450-B subset, to monitor P450-B1 gene expression and organization in the isogenic, susceptible, 91-C, and resistant, 91-R, Drosophila strains was described. Those studies indicated that a structurally altered, but not amplified, P450-B1 gene is responsible for the increased amounts of P450-B1 in resistant flies.

Subsequently, genomic clones of P450-B1 have been isolated and characterized. DNA sequence analysis revealed that the long terminal repeat (LTR) of transposable element *17.6* is present in the 3' end of the gene of 91-C and is absent in that of 91-R. Features of the LTR and its location in the P450-B1 gene of the susceptible fly suggest that a posttranscriptional mechanism involving mRNA stability could be involved in regulating P450-B1 gene expression. Future work will be directed toward elucidating the precise mechanism of P450-B1 gene

regulation and determining the generality of such a mechanism for gene regulation.

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FUNDAMENTAL AND APPLIED CRYOBIOLOGY

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Analysis of Equilibrium and Non-Equilibrium Freezing of Mouse Embryos

The first successful freezing of early embryos (Whittingham, Leibo, and Mazur, *Science* **178**, 1972) required that they be cooled slowly at ~1°C/min to about -70°C. Subsequent observations and physical-chemical analyses indicate that embryos cooled at that rate dehydrate sufficiently to maintain the chemical potential of their intracellular water close to that of the water in the partly-frozen extracellular solution. Consequently, such slow freezing is referred to as equilibrium freezing, and with equilibrium freezing, intracellular ice formation (which is usually lethal) does not occur.

We and a number of other investigators have studied the responses of embryos to departures from equilibrium freezing, some of which do not result in high mortality. One common element in these successful non-equilibrium procedures is that before the temperature has dropped to those that permit intracellular ice formation (nucleation zone), the embryo water content is reduced to the point where the subsequent rapid non-equilibrium cooling either results in the formation of small innocuous intracellular ice crystals or in the conversion of the intracellular solution into a glass (vitrification). In both cases, high survival requires that subsequent warming be rapid to prevent recrystallization or devitrification. Since cells that are sufficiently dehydrated as they enter the nucleation zone can survive small quantities of intracellular ice or limited devitrification by rapid warming, one refers to such cells as "rescuable." The physical-chemical analysis developed for initially non-dehydrated cells has now

been applied to those other non-equilibrium procedures, and appears generally to account for the observed results (Mazur, *Cell Biophys.* **17**: 53, 1990).

In the case of mouse embryos, the survivals obtained with equilibrium freezing procedures are as high as those obtained with the best non-equilibrium procedures. But there are other cell types and multi-cellular systems in which equilibrium freezing procedures are not successful (*e.g. Drosophila*, see below).

Factors Affecting the Intracellular Nucleation Zone

As just mentioned, if cells are to survive either equilibrium or non-equilibrium freezing, their water content has to be reduced to a "rescuable" value before they have cooled to the ice nucleation temperature zone. The specific subzero temperature that constitutes the upper boundary of that nucleation zone may thus be critical. Recent experiments have shown that the upper boundary of the nucleation zone is dramatically affected by the extent of hydration of the embryos prior to their being cooled to the nucleation zone. The nucleation temperature of fully hydrated embryos lies between -12° and -15°C. The nucleation temperature of partially dehydrated embryos is about -25°C.

Cryobiology of *Drosophila* Embryos

Paradoxically, whereas mammalian embryos normally exist in closely controlled environments while insect eggs are subject to large fluctuations of the environment, the former can now be frozen with high survival, whereas the latter in general cannot. With support from a grant from the National Science Foundation and in collaboration with A. P. Mahowald of the University of Chicago we are investigating this paradox with *Drosophila*, one reason being the importance to *Drosophila* geneticists of developing a method for the cryopreservation of some of the >10,000 mutant lines of this insect.

Permeabilization. One fundamental way in which the *Drosophila* egg differs from the mouse egg is in possessing a waxy layer in the vitelline membrane that allows vapor exchange but not that of liquid water or solutes. Since successful freezing requires protective solutes like glycerol inside the cell and generally the osmotic efflux of water during freezing, that waxy layer must be removed. Sporadic reports indicate that this can be done by a brief exposure to alkanes like octane. But in our hands the problem was vexing. Conditions that effectively permeabilized the eggs killed them. We have now found that the source of the problem is that minute amounts (>0.2%) of alcohol must be in the alkane to achieve effective permeabilization but that if more than minute amounts are present (>0.5%), the alkane-alcohol mixture becomes lethal. A manuscript defining the optimal conditions has been submitted to *Cryobiology*.

***Drosophila* Chilling Injury.** Another problem we found is that even in the absence of freezing, *Drosophila* eggs become increasingly sensitive to progressively lower subzero temperatures. Thus, at 0°, -9°, -15°, -20° and -25°C, half of 15-hr eggs are killed in 37 hrs, 4 hrs, 40 min, 15 min, and 6 min, respectively. Younger eggs are still more chill sensitive. This high chill sensitivity means that orthodox slow freezing procedures to remove intracellular freezable water before cooling to the egg ice nucleation temperature of -28° to -31°C will not succeed. Success will require preventing both the low temperature injury and intracellular freezing by cooling the eggs extremely rapidly to temperatures below which any adverse reactions can occur.

The killing kinetics at given subzero temperatures between 0° and -25°C have been found to be characterized by a shoulder followed by a more-or-less linear decrease in survival with time. The lower the temperature, the shorter the shoulder and the faster the post shoulder decline. The rate of both components follows Arrhenius kinetics, *i.e.* plots of log rate vs 1/absolute temperature are linear, the slopes being proportional to the activation energy. In both cases the activation energy is high and negative; namely, -46.5 kcal/mole for the shoulder length and -24.7 kcal/mole for the post-shoulder inactivation. Negative activation energies are rare, and according to absolute reaction rate theory, they exist only when there is a large entropy of activation.

At some sufficiently low subzero temperature, the chilling injury must cease. If we assume that temperature to be -65°C, the computations indicate that the cooling rate will have to be some 10,000°C/min if half the eggs are to avoid death from chilling. Even if the eggs are cooled rapidly enough to avoid chilling lethality, they will not survive if intracellular ice forms below the intraembryonic nucleation temperature at ~-30°C. However, cooling rates high enough to prevent chilling lethality may also be high enough to allow the intraembryonic cytoplasm to vitrify, provided that sufficient concentrations of glass-enhancing solutes are present.

Cryopreservation of *Drosophila*. This apparently is the case Steponkus *et al.* have recently reported (*Nature* **345**: 170-171, 1990) that some 55% of 12 hr permeabilized *Drosophila* embryos will survive 8 min exposure to 8.5 M ethylene glycol at 0°C, and that some 10-18% of such embryos survive subsequent cooling at ~30,000°C/min to -196°C, and presumably equally rapid warming.

We have now confirmed their findings. The clearest finding has been that the substitution of 6.5 M EG for 8.5 M yields 0% hatching. Survivals are higher if PVP or BSA is present in 8.5 M EG. Our best *average* survivals (11-13%) have been obtained when embryos in 8.5 M EG with 10 or 20% PVP30 or 6 or 12% BSA are cooled in nitrogen slush and warmed in D-20/sucrose. Survivals in the absence of polymers average about half that. But the striking feature of the survivals is their capriciousness. The four highest survivals obtained are 35, 29, 23,

and 21%. But the survivals for other supposedly repeat runs were often 0-7%. Injury is not all or none. Even when only 5-10% hatched some 40-50% were able to develop to the tracheal stage. We will shortly initiate experiments to determine whether these cooling and warming treatments truly avoid all ice crystal formation and experiments to determine the site of injury.

Fundamental Cryobiology of Human and Bovine Spermatozoa

Although sperm were among the first mammalian cells to be successfully frozen, and frozen bull sperm is a major component of the large artificial insemination (AI) industry, survivals are at best mediocre and surprisingly little is known about the fundamental cryobiology of these important cells. In collaboration with Dr. John Critser and Dr. Frederick Kleinhans of Methodist Hospital, Indianapolis, and with support from USDA and NIH grants, we have initiated a study of this matter in bovine and human spermatozoa, respectively.

Knowledge of the permeability of a cell to water and to cryoprotectants can be powerful tools 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 enough to preclude lethal intracellular freezing. Accordingly, the first step of our research is to determine the osmotic and permeability characteristics of bovine and human sperm to water and glycerol at temperatures between 0° and 20°C.

The first question is, do the sperm behave as ideal osmometers in the sense of their volume being a linear function of the reciprocal of the external osmolality of non-permeating solutes? The answer, contrary to the view of a number of authors, is that they do. Electron spin resonance (ESR) techniques provide a powerful tool for measuring the intracellular aqueous volume (Du *et al.*, 1990). To a suspension of sperm in an anisotonic solution of a non-permeating 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 remaining sharp intracellular signal amplitude with a standard and knowing the cell concentration, one can compute the average cell aqueous volume. Experiments using the technique on human and bovine sperm confirm ideal osmotic response both under 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, as with other cells, are placed in a solution that is hyperosmotic with respect to a permeating solute like glycerol but hypoosmotic with respect to non-permeating 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).

Our criterion of the attainment of the lytic volume is to determine with a fluorescence microscope or a laser cell sorter the time at which the fluorescence disappears from carboxyfluorescein labeled cells as a consequence of the development of membrane leakiness. Our recent experiments (Noiles *et al.*, 1990) show that the critical tonicity based on this procedure is about 0.05 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.* it will be 0.29/0.05 or about 5.8× isotonic. The reason that sperm can withstand swelling to 580% 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 much greater volume without increase in surface area.

With information on the critical tonicity and spermolytic volume in hand we are now in a position to determine the permeability coefficients of sperm to glycerol and to water. The indications are that these permeability coefficients are extraordinarily high. The values for both glycerol and water exceed the values for the human erythrocyte which heretofore have been among the highest values reported for any mammalian single cell. The permeabilities to water are so high and the temperature coefficient of that permeability so low (~3 kcal/mole) that the predicted cooling rates for the production of intracellular ice exceed 5000°C/min. Yet indirect evidence indicate that internal freezing occurs with cooling rates of 100-500°C/min. The anomaly needs to be resolved.

As an adjunct to these studies on the fundamental cryobiology of human and bovine sperm, we intend to seek funding from NIH or DOE for investigation of the feasibility of maintaining transgenic and other mutant lines by the cryopreservation of mouse sperm as an adjunct to their preservation as frozen embryos.

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 again in 1988); (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 descendents; and (4) to provide a method of maintaining genetic stocks that is cheaper than conventional breeding in terms of manpower, space, and facilities. In this reporting period we have been engaged in four mouse embryo banking projects funded by other agencies or institutions. The first, funded by the National Institute on Aging, involves the banking of embryos from special inbred lines maintained by NIA. The second one, funded by the National Institute of Child Health and Human Development, involved the freezing of some 2000 mouse embryos in the 8-cell precompaction state. The remaining two, funded by two grants from the National Institute on Alcohol and Alcohol Abuse to Dr. John Crabbe at Oregon Health Sciences University, involve the banking of mouse embryos at various stages in the genetic selection of lines exhibiting differences in response to alcohol. These projects constitute an example of the transfer of a technology (*i.e.*, mouse embryo freezing) first developed in this laboratory. The Mammalian Genetics and Development Section also has an ongoing program of the banking of embryos of many of the genetic lines maintained by L. B. Russell. We consult with and advise F. J. Stenglein, who is in charge of that freezing program. In addition, we expect to consult with R. P. Woychik and E. M. Rinchik on the cryopreservation of embryos derived from their insertional and chemical mutagenesis studies.

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Mammalian Genetics and Development Section

Section Overview - L. B. Russell

A major portion of the Section's research is devoted to studying the structure and function of genomic regions associated with heritable mutations, utilizing the genetic resources that have been, and continue to be, generated by various forms of mutagenesis, agent-induced and insertional. Because of the extensive homologies between mouse and human genomes, it is becoming increasingly feasible to exploit the mouse as a valuable experimental model system for the study of human genetics and human disease. A part of the Section's effort is therefore devoted to detailed phenotypic studies of selected mutations.

The Molecular Genetics Group has continued the molecular-genetic analysis of four regions *(c, p, b, and Sl)* of the mouse genome that are associated with overlapping deletion mutations generated in past and ongoing specific-locus mutagenesis experiments. In aggregate, these regions cover 2-3% of the genome. The mapping of DNA clones to, and within, these regions is greatly simplified by the availability of deletion and functional-unit maps generated in earlier and ongoing genetic studies. DNA restriction fragments that carry the breakpoints of several of the deletions have recently been cloned and have enabled molecular "jumps" into other regions of the complex, often megabases away. One such cloned fusion fragment has provided molecular access to the fumarylacetoacetase gene whose deficiency causes defined pathologies in humans. Several human clones have been found to map to another region on the same mouse chromosome. Particularly exciting among these are clones associated with the Prader-Willi/Angelman syndromes, the effects of which may now have become amenable to experimental study in the mouse.

The Molecular Genetics Group is also proceeding with experiments designed to saturate, with point mutations, regions defined by long deletions. These experiments are yielding estimates of the minimum number of genes within a defined-length region that are mutable to biologically significant phenotypes, and they will generate a fine-structure functional map that can subsequently be correlated with the molecular/physical map being developed for the same regions.

Molecular access to an additional specific-locus-associated region (*a*, agouti) was achieved by the Insertional Mutagenesis Group through exploitation of a complex radiation-induced chromosome rearrangement that altered the expression of two genes (agouti and limb deformity) normally separated by 20 cM. Utilizing molecular probes from an insertional limb-deformity mutation, the investigators eventually "jumped" 20 cM into the agouti locus.

Genetic analyses of regions surrounding a number of specific loci have in the past provided the essential skeletons for the current regional physical/functional mapping, and such genetic analyses continue to be performed, in close integration with molecular studies. The value of having preserved mutant stocks generated over a period of decades has become amply apparent, and the Genetic Analysis Group, in addition to performing research, maintains hundred of these mutant stocks (as well as inbred, congenic, and specially constructed strains, and the several very large stocks used for mutagenesis studies). Mutations that continue to arise in ongoing mutagenesis studies are tested genetically, analyzed cytogenetically, and checked with available DNA probes; many can then be added to the mutational resource available for high-intensity physical/functional mapping. In addition, knowledge gained from these analyses, especially that derived from achieving complete ablation of genes in certain complementing combinations, has allowed us to classify induced specific-locus mutations, merely on the basis of their phenotype, into large (multi-locus) and other lesions, thus adding qualitative to the quantitative capabilities of the specific-locus test.

Establishing structure/function relationships is a prime objective of the Insertional Mutagenesis Group, which has been producing 200 distinct lines of transgenic mice per year. In a recent innovation, the transgene now being used is a tyrosinase cDNA, which produces a coat-color trait and thus provides an easy means of following inheritance of the transgene. This facilitates the screening of the lines for a variety of phenotypes indicative of mutations. A large number of insertional mutations have been identified, and several of these are being studied in detail, both with respect to phenotypic characterization and molecular analysis. A new program has been initiated to locate transgene integration sites on metaphase chromosomes by means of fluorescence *in situ* hybridization (FISH). The Group has also taken on the task of developing a computerized database for the thousands of transgenic lines being generated worldwide.

Transgenic mutants, as well as the rich collection of agent-induced mutants, provide a valuable resource for the investigation of basic biological processes and as models for human developmental anomalies or genetic disorders. Godfrey's in-depth morphological and physiological analyses, as well as Selby's detailed skeletal studies have discovered pathological conditions in several transgenic-mutant lines that have strong resemblances to human disease syndromes. In addition, Godfrey's experimental studies on the spontaneous mutation *scurfy* have revealed a defect in the thymic microenvironment that shapes the developing immune system; such defects are postulated as the cause of several immune-system-mediated diseases in humans. The Chromosomal Damage Group utilizes certain types of agent-induced translocations, which produce relatively late-surviving unbalanced segregants, as dependable sources of specific malformations (models of human congenital anomalies). These are being analyzed

in collaborative studies by genetic, cytogenetic, anatomical and molecular means. Selby has added the study of genetic cataracts to his continuing interest in dominant skeletal mutations, several of which closely mimic specific human genetic disorders.

The Mouse Mutagenesis Group, in addition to enlarging the germ-cell mutagenicity database for different chemicals (in an attempt to reveal patterns of effect), is continuing to generate valuable genetic tools for molecular studies. The group also discovers mutagens and defines mutagenesis protocols that can manufacture high frequencies of deletions and are thus valuable for studies seeking to recover developmentally significant mutations, genome-wide, that are amenable to molecular access. Characterizations (performed by the Genetic Analysis and Molecular Genetics Groups) of the nature of the genetic lesions detected in specific-locus mutation-rate experiments have made it possible to analyze past and current data for patterns of mutagenesis and to discover that the germ-cell stage in which a mutation is induced is the strongest determinant of the type of lesion that is recovered. Chromosome aberration studies by the Chromosomal Damage Group have revealed several female-specific mutagens, and have led to the suggestion that the relatively diffuse state of oocyte chromosomes may permit attack by certain chemicals (e.g., intercalating agents) that are ineffective in the condensed chromatin of postmeiotic stages of the male. The Organismic Effects Group has carried out radiation mutagenesis studies on mouse oocytes exposed around the time of birth, when the state of the nucleus most resembles that of the human arrested oocyte; very low mutation rates were observed.

Studies by several groups have involved mutagen treatment of zygote and early cleavage stages. Treatment of early zygotes with ENU (ethylnitrosourea) was found by Russell to result in a very high rate of specific-locus mutations in the maternal genome, but a very much lower rate in the paternal genome. Most, if not all, mutations were mosaics. High frequencies of dominant skeletal mutations were recovered by Selby from ENU treatment of zygotes. Generoso had shown earlier that exposure of zygotes to certain chemicals (not including ENU) resulted in high frequencies of miscellaneous fetal malformations and of mid- and late-gestation death, and that these effects were not mediated by maternal damage nor caused by chromosome breakage or mis-segregation induced in the conceptus. More recently, he found similar effects from treating two-cell embryos with the inhibitor of C-methylase enzyme, 5-azacytidine. His current hypothesis is that certain chemical exposures at these stages cause disruptions in the finely tuned program of gene expression during early embryonic development, and that large classes of human congenital malformations may also be caused by such epigenetic mechanisms. In addition to sparking future basic investigations, this work obviously has implications for human hazards.

The assessment of dominant mutational damage – highly pertinent to genetic risk assessment – is the subject of research by the Organismic Effects Group. A new approach, which includes measurement of a number of endpoints, is being used in conjunction with mutagenic treatments known to induce primarily large, primarily small, or mixtures of large and small genomic lesions. One objective is to determine whether the ratio of specific-locus to dominant-mutation frequencies will be the same or different for the various treatments. An exciting result already obtained is the very high frequency of cataracts (about 5%) among offspring of males exposed to chlorambucil (CHL), an excellent deletion inducer.

CHL, unlike other chemical mutagens studied earlier, produces its peak effect in early spermatids. The Germ-Cell DNA/Proteins Group has explored the interactions of CHL with various molecular targets in germ cells. For other chemicals, which are characterized by peak mutagenicity in later spermatids or sperm, the molecular target was shown by Sega to be protamine (rather than DNA). By contrast, CHL may be damaging the modified histones present during germ-cell stages when the transition from histones to protamines is taking place. Alkaline-elution procedures that measure DNA strand breaks demonstrated that CHL causes DNA crosslinks, more of them in the stage that yields high mutation frequencies.

The Mammalian Biochemical Genetics Group has continued its research on abnormal hemoglobins. The hematological indices of murine and human beta-thalassemias were shown to be similar. Results of transplantation experiments involving normal and beta-thalassemic mice suggested that restoration of beta-thalassemic bone-marrow cells by gene therapy might provide a successful means for treatment of the disease in humans. The group is also attempting to develop a mouse model for sickle-cell anemia by breeding a human transgene into a genetic background of mice with high-oxygen-affinity hemoglobin. Results of experiments on virus-induced lymphocyte malignancies suggest that viral infections initially induce cellular hyperplasia, with each expanding clone containing a different retroviral insertion, and that, occasionally, a clone of T cells with rearranged *TcR* genes becomes malignant and overgrows the other cell lineages.

The DNA Sequencing Technology Group, which includes several investigators in other ORNL divisions and at other institutions, is attempting to develop faster and more accurate methods for DNA sequencing. Stable isotopes (e.g., of iron or tin) are used as DNA labels that are detected on electrophoresis gels by resonance ionization spectroscopy. Three other new sequencing methods that are under development eliminate the gel electrophoresis step.

While each group of the Mammalian Genetics and Development Section has its own active program, there are numerous interactions between groups. A prime example is the close interplay between the genetic characterization of mutations

in certain regions of the genome and the molecular analyses of these same regions (Genetic Analysis and Molecular Genetics Groups, Genetic Analysis and Insertional Mutagenesis Groups). Probes developed in insertional-mutagenesis studies are being used to analyze mutations generated in conventional mutagenesis experiments (Insertional Mutagenesis and Chromosomal Damage Groups). Functions associated with specific genomic regions are defined by in-depth studies in morphology and physiology (Godfrey interactions with Woychik and Russell). Methods developed for the detection of dominant skeletal and cataract mutations are being applied to search for mutations in transgenic lines of mice (Organismic Effects and Insertional Mutagenesis Groups). Patterns of adduct binding to DNA and/or proteins in germ cells are being correlated with patterns of induction of transmissible genetic damage (Germ-Cell DNA/Proteins, Chromosomal Damage, and Mouse Mutagenesis Groups). The examples could be greatly expanded. The numerous interactions enhance the output of individual groups and produce a fine spirit of cooperation in the Section. A large number of outside collaborations are also in place; many of these serve to build bridges between mouse and human developmental genetics.

MOLECULAR GENETICS AND MOUSE-GENOME STUDIES

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The major interests of the Molecular Genetics Group lie in exploiting spontaneous and induced heritable mutations of the mouse to learn more about the composition and organization of the mammalian genome, as well as to understand better the genetic control of normal and abnormal mammalian development. The continuing, underlying theme of the group's efforts combines mouse genetics and germline mutagenesis with the techniques of molecular biology and DNA analysis for the study of genomic regions associated with heritable mutations. Further, we are trying 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. 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, and it is these homologies that allow us to exploit the mouse for the study of human genetics and human genetic disease.

Germ-cell specific-locus mutagenesis experiments conducted at ORNL over the years have generated an impressive collection of various 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, which have occurred at 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 2-3% of the genome. This group's interests revolve around: (1) molecular mapping and genetic characterization of genomic regions associated with radiation-induced deletions; and (2) high-efficiency, saturation "point" mutagenesis of these same regions, as a model system for detailed, fine-structure functional mapping of large genomic segments.

Molecular Genetics of Radiation-Induced Mutations

Over the past two years, we have continued the molecular-genetic analysis of regions of the mouse genome associated with complexes of overlapping, radiation-induced deletion mutations. These regions are centered around the albino (*c*) and pink-eyed dilution (*p*) loci in chromosome 7, the brown (*b*) locus in chromosome 4, and the Steel (*Sl*) locus in chromosome 10.

The largest of the panel of *c* deletions is 6- to 11-cM in genetic length (perhaps 6-20 million bases of DNA). Genetic analysis of these deletions has resulted in a functional map that defines genes important for normal prenatal, neonatal, and juvenile development, for proper regulation of a host of metabolic enzymes and blood proteins, for male and female fertility, and for normal inner-ear development. Analysis of *p*-region and *b*-region deletions is yielding similar information on the phenotypes associated with genes in these regions (see also the section on Genetic Analysis).

We have used the panel of *c* deletions to map a number of DNA probes to this region of chromosome 7. These probes were derived from libraries created from flow-sorted or microdissected chromosomes or from the cloning of specific loci, such as the tyrosinase (*c*) gene itself or the integration sites of *c*-linked endogenous retroviral sequences. The extensive panel of overlapping deletions is simplifying the ordering of these clones both with respect to other clones and with respect to the gross functional map inferred from the genetic analysis of the deletions. Some of the clones discriminate between members of genetically defined complementation groups, and others have been used in large-fragment DNA analysis to identify distances to deletion breakpoints. We have recently been successful in identifying and cloning DNA restriction fragments that carry the breakpoints of several of the radiation-induced deletions. The molecular cloning of these fusion fragments enables molecular "jumps" into other regions of the physical/functional map, often megabases away. Recently, we identified and cloned such a breakpoint-fusion fragment in DNA derived from the c^{14CoS} deletion

chromosome, which provided molecular access both to the *msd* locus, known to be necessary for development of mesoderm in the gastrulating embryo, and to the *hsdr-1* locus, which is thought to encode a *trans*-acting regulatory protein that, during late prenatal life, regulates the induction of expression of certain liver enzymes and serum proteins. In fact, this cloned fusion fragment carries exons of the fumarylacetoacetase hydrolase (*Fah*) gene, whose deficiency is known to be intimately tied to hereditary tyrosinemia and liver failure in humans. This result has, therefore, raised the intriguing possibility that FAH deficiency, and not a *trans*-acting regulatory protein, may be responsible for at least part of the *hsdr-1* phenotype in mice.

Lethal *p*-locus mutations have been the targets of similar types of analyses. Recently, we (in collaboration with R. Nicholls at the University of Florida, Gainesville) have found that two loci defined by clones mapping to the region of human chromosome 15q associated with the Prader-Willi/Angelman (PWS/AS) syndromes are deleted in the majority of radiation-induced *p*-locus mutations. Likewise, at least four loci defined by clones mapping to human 11p are deleted in a large *p*-locus deletion. We have been quite excited by the PWS/AS-*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. There is a striking similarity between phenotypes observed in children with PWS/AS and those observed in mice homozygous for certain *p*-locus deletions. It will thus be of great interest to delve further into both the molecular and organismal phenotype(s) of the *p* deletions in the overall context of the human PWS/AS syndromes.

Analysis of a region of chromosome 4 associated with more than 25 lethal brown (*b*)-locus mutations has continued, with emphasis being placed on the overall genetic analysis of the mutations. These initial genetic analyses are forming the basis for a rudimentary functional/deletion map of the region. Several loci required for normal pre- and postnatal development, as well as a locus (depilated; *dep*), required for proper development of the epidermis, and a locus (whirler; *wi*), associated with defects in the inner ear, have now been located within the deletion complex. We have recently intensified experiments designed to provide molecular access to all regions of the *b*-deletion complex by initiating a collaboration with J. Friedman's group (Rockefeller) for mapping chromosome-microdissection clones derived from this region of chromosome 4.

Specific-locus mutagenesis tests designed to look for induction of mutations at recessive tester loci, such as those described above (see Section on Mouse Mutagenesis), routinely yield a number of dominant visible mutations at other loci. We have amassed a collection of such mutations at the Steel (*Sl*) locus in chromosome 10. Independent mutations at the *Sl* locus can have variable effects on hematopoeisis, pigment-cell migration, and gametogenesis.

The genetic analysis of *Sl* mutations is being carried out both by our group and by the Genetic Analysis Group. The Oak Ridge *Sl* mutations vary widely in their effects on each of the developmental pathways described above, and at least five of the mutations are associated with translocations involving chromosome 10. Recently, several laboratories have isolated cDNA clones corresponding to portions of the *Sl* transcription unit, providing probes for the molecular analysis of the gene and surrounding genomic regions. We are currently using the available probes, corresponding to approximately one-third of the 6-kb transcript, to identify the various sites and extents of mutations in our large collection of "steeloid" animals. We are also using these cDNA clones to examine patterns of *Sl*-gene expression in normal and mutant mice. Our eventual aim is to correlate positions of mutations and patterns of expression with specific steeloid phenotypes, and to use this information to derive a precise physical and functional map of the *Sl* gene itself as well as of the surrounding genomic regions. Our work thus far has identified a number of animals carrying deletions and other DNA rearrangements within this 3' third of the gene. However, the partial cDNA clones that are available allow the examination of only a small segment of the potentially large *Sl* locus. For example, all five known *Sl* translocations are not located within this limited segment of the gene. We are, therefore, concentrating on isolating full-length cDNA clones to open to detailed molecular analysis the as yet uncharted 5' end of this important gene.

High-Efficiency Mutagenesis of Deletion-Associated Regions

Over the past several 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 (the very regions that are the targets of our molecular-mapping analyses). 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 provide fundamental genetic, logistical, and statistical information on which to base strategies for subsequent large-scale expansion of the functional maps of mammalian genomes.

One experiment involves a large (6- to 11-cM) deletion of the *c* locus in chromosome 7. A two-generation-cross protocol places highly mutagenized (*N*-ethyl-*N*-nitrosourea-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 3300 such pedigrees has, so far, detected a total of 22 new mutations, defining at least seven loci. Five new loci are defined by lethal mutations; one locus (*fit-1*) is defined by a series of five alleles that specify a runting syndrome; and five mutations have been detected at the inner-ear-development locus shaker-1 (*sh-1*). Alleles at several of the loci differ with respect to the extent of their effect on the resultant phenotype. Each of these loci 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. For example, the *fit-1* locus has been positioned between the distal breakpoints of two distally extending, prenatally lethal *c* deletions. We have in hand molecular clones identifying the distal breakpoints of these two deletions, and, therefore, have bracketed the locus on the physical map. Identifying deletion breakpoints that bracket other 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, intragenic, developmentally significant mutation on a molecular map.

We have been applying a similar strategy to recover ENU-induced mutations within the region corresponding to a large (>4-cM) *p*-locus deletion. To date, screening of just over 300 pedigrees has already identified two lethal and two fitness mutations. The significance of the *p*-region saturation mutagenesis program has been magnified this year by the association of the mouse *p* region with the Prader-Willi/Angelman Syndrome region of human chromosome 15q (see above). Because the PWS/AS Syndrome is most often associated with either deletion or uniparental disomy of a region of human chromosome 15q, 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.

We anticipate that, in addition to contributing to 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, if technically feasible, will comprise an important strategy of gene identification for expressed DNA sequences derived from these regions. Furthermore, we hope that such high-efficiency mutagenesis strategies will allow the identification of developmentally crucial loci in the mouse genome that might also be found in homologous regions of the human genome (e.g., as with the PWS/AS syndrome 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

Our earlier genetic analyses of large sets of specific-locus mutations generated in Oak Ridge mutagenesis experiments have identified complexes of nested deletions that provide an invaluable resource for intensive physical and functional mapping of megabase-length regions of the mouse genome. In the case of the *c*-locus-centered deletion complex, for example, the genetic analyses resulted in the identification of new functional units and in an extensive complementation map that form the basis for the fine-structure work now being performed by our Molecular Genetics Group (see another section, this Report). Our genetic analyses of regions surrounding other loci have continued, in close integration with molecular studies. The value of having preserved mutant stocks generated over decades has become amply apparent.

(1) Pink-eye (*p*) region, Chromosome 7. Genetic studies on *p*-locus mutations provide the essential informational skeleton for the molecular studies summarized in the report by the Molecular Genetics Group. To determine the extent of radiation-induced pre- and postnatally lethal *p*-locus deletions, we have tested large numbers with the flanking markers *ru-2* (found deleted in one mutation, which is also deleted for *Ldh-1*) and *twt* (deleted in none, to date). Nearly 500 intercrosses among 43 independent *p*-locus mutations have been made and are providing survival data from which we have attempted to construct a preliminary complementation map. Our current analysis, which also incorporates the information that the DNA sequence TM15 is deleted in some of the mutations and not in others, indicates that there are at least 15 complementation groups, and probably 6 prenatal-lethal and 2 postnatal-lethal functional units surrounding the *p* locus. Several mutations cannot be fitted into a simple linear map of overlapping deletions, and these may be more complex rearrangements.

(2) Agouti (*a*) region, Chromosome 2. Genetic and cytogenetic analyses of available induced and spontaneous mutations in this region, including known rearrangements, are providing the background for the molecular studies carried out by Woychik's group (see another section of this Report). Recombination data in large, appropriately marked, progenies indicate the order in the region to be *Emv-15* - A^y - [*A*, a^t, *a*, a^x] - *bp*. A complementation grid, which is being completed for about 10 mutations, has revealed two functional units for prenatal survival, one between A^y and *a*, the other between *a* and *bp*. In addition, it was possible to

separate from a^{16H} a presumably independently induced, closely-linked, lethal mutation. As well as generating a first-cut genetic and functional map for the region, we also breed appropriate animals to provide the genetic constructs needed for the molecular studies

(3) Dilute-shortear (*d-se*) region, Chromosome 9. Our extensive genetic mapping of this region had earlier facilitated molecular analyses (with entry via the integrated provirus *Emv-3*) that found the structural (molecular) map to be concordant with the functional (complementation) map. Currently, our genetic studies are confined to (a) mapping newly-induced (e.g., chlorambucil) mutations relative to the already-characterized deletion complex, (b) defining the proximal extent of deletions by mapping first with *ash* and then with *sg* [we have already found one mutation to be Df(*sg ash d se*)], and (c) collaborating with Dr. David Kingsley and other members of the Jenkins/Copeland group for whom we have made special genetic constructs and supplied large numbers of stocks. Our cytogenetic analyses have localized the *d se* region to Giemsa bands 9D or 9E1.

(4) Steel (*Sl*) Region, Chromosome 10. The defect associated with *Sl* -locus mutations involves the microenvironment in which stem cells for the hematopoeitic, pigmentation, and germ-cell pathways reside. A partial cDNA for the *Sl* gene has recently been cloned by other institutions. Over 60 presumed *Sl* mutations that have arisen in our laboratory during the past 30 years are being genetically analyzed by us, and the results provide the framework for the molecular studies being carried out by Lisa Stubbs (see report by Molecular Genetics Group). The extensive genetic information has now been entered into a computer; the mutations were found to encompass a variety of classes with regard to viability, pigment phenotype, and fertility patterns. The incidence of reciprocal translocations among *Sl* mutations could be as high as 20%, suggesting that the locus might represents a mutational "hot spot" in the mouse genome. Cytogenetic (high-resolution G-banded) analysis of surviving translocation stocks has localized the *Sl* locus to band 10D1.32 (the second breakpoint is in a different chromosome in each case).

Genetic and Cytogenetic Analyses of Mutations Resulting from Mutagenesis Experiments

Analyses of heritable mutations, in addition to providing significant insights into the structural and functional composition of the mammalian genome, can also furnish information on the actions of specific mutagens or mutagenesis protocols. To our routine genetic tests on all recovered specific-locus mutations (allelism and homozygosity), we have more recently added cytogenetic studies in Giemsa-banded chromosomes and molecular analyses (for loci or regions for which DNA probes are available, see report by Molecular Genetics Group).

Genetic, cytogenetic, and/or molecular analyses were carried out for 18 and 24 mutations recovered in mutagenesis studies with chlorambucil (CHL) and melphalan (MLP), respectively (see report by Mouse Mutagenesis Group). Most of the mutations induced in postspermatogonial stages were shown to be associated with deletion of the marked locus; numerous translocations were also recovered, most of them independent of the specific-locus mutation. By contrast, almost none of the mutations recovered from CHL- or MLP-treated stem-cell spermatogonia were associated with deletions or translocations. Genetic analyses of over 230 mutations induced by ethylnitrosourea (ENU) or methylnitrosourea (MNU) showed a similar germ-cell-stage dependence.

Chromosome-Aberration Associated Sterility

Male sterility is a possible dominant effect of some mutagenic treatments that is observed considerably more frequently than are visible phenotypes. Over the past few years, we have established an ongoing program of histological and/or cytogenetic analysis of sterile sons of mutagenized and control animals (generated by the Chromosomal Damage Group), designed to build up a body of information on associations between the nature of the fertility impairment and the karyotype, and between chromosomal causes of infertility and the nature of the mutagenic treatments that produced them.

Translocations that are associated with male sterility have been found either to involve a sex chromosome or to be characterized by breakpoints located near a centromere and/or telomere of autosomes. The sterility associated with translocations results from blockage at different stages of the spermatogenic cycle; such males generally have small testes. To date, all sterile males with Y-autosome translocations have had a normal male phenotype. Sterile sons are rare among offspring of mutagenized spermatogonial stem cells, but, over the years, a considerable body of data has been accumulated. In contrast to sterile sons derived from treated postspermatogonial stages, these males do not carry translocation, have normal spermatogenesis, and fail to sire offspring because their spermatic ducts are occluded.

Genetic-Stock Maintenance and Management

In addition to performing research, the Genetic Analysis Group maintains large numbers of stocks that are employed for various purposes by Section investigators. Included are the large stocks used in mutagenesis studies; standard or specially constructed inbred or congenic strains, or stocks combining specific markers, utilized for genetic experiments or molecular studies; and hundreds of small stocks propagating mutations that have arisen in mutagenesis experiments over the past decades, and whose value for genome analysis is becoming increasingly recognized. Stocks are frequently shipped to collaborators or other

requesting investigators. A successful embryo-freezing program is in place for cryopreservation of selected stocks.

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

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The 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. In an attempt to identify numerous genes with specific developmental functions in mammals, we are conducting a large-scale insertional mutagenesis program in transgenic mice. A total of 200 distinct lines of transgenic mice are being produced each year, and each line is being screened in a systematic manner for a variety of phenotypes ranging from embryonic lethality to skeletal abnormalities. The tyrosinase cDNA is being used as the transgene in

these experiments; this confers a coat-color trait in the animals that carry the transgene and provides us with a simple means of following the inheritance of the transgene. To date, we have identified a large number of insertional mutations with phenotypes ranging from embryonic lethality to skeletal deformities.

Several of the insertional mutations we have generated are also being examined in detail. In one line, called Tg737, transgenic homozygotes express a complex phenotype associated with polydactyly on all four limbs, severe growth inhibition, scruffy fur during early development, markedly shortened life span, and tooth defects. Homozygous animals die early in development and develop bilateral polycystic kidneys (see separate report by Godfrey). In another line, called Tg370, homozygotes develop a characteristic skeletal defect involving an undulation of the distal vertebrae of the spine. In line Tg17B, heterozygous males are sterile, though they appear to be producing approximately normal numbers of sperm; however, very few of the sperm appear to be motile. The mutant locus in each of these lines is being cloned and characterized at the molecular level, and the phenotype of the mutant animals in each line is being further characterized.

Since the transgene in each line of transgenic mice can be an important molecular access point for many mutations throughout the mouse genome, we have initiated an effort to characterize transgene integration sites on metaphase chromosomes utilizing the fluorescence *in situ* hybridization (FISH) technology. We have now successfully assigned the chromosomal position of several of our insertional mutations by FISH, and are currently refining the methodology to establish the karyotype of individual animals by fluorescence banding techniques (involving hybridization with repetitive sequence elements).

We are also using probes derived from transgenic mice for the molecular characterization of other mutations in the mouse, particularly those involving radiation-induced major structural alterations. In one such mutation, Is1Gso, which arose in one of Generoso's radiation experiments, new alleles of limb deformity (*ld*) and agouti (*a*), two loci normally separated by 20 cM on Chromosome 2, arose simultaneously. Utilizing molecular and cytogenetic tools, we determined that a segment of chromosome 17 had been inserted interstitially into the distal end of chromosome 2, and that an interstitial inversion had occurred between the *ld* and *a* loci on chromosome 2. Utilizing molecular probes from an insertional mutation in a transgenic mouse (ld^{Hd}), we characterized the altered expression of a complex gene at the *ld* locus and then went on to ultimately clone the DNA breakpoint regions associated with most of the rearranged chromosomal regions in the mutant. Cloning the proximal and distal inversion breakpoint regions on the rearranged chromosome 2 allowed us to "jump" 20 cM from the *ld* locus into a region that we now predict is directly associated with a least one gene at the

a locus. Probes from this region at the *a* locus are being used to study the molecular biology of this developmentally important region of chromosome 2.

Finally, we are in the process of developing a computerized database to catalog the thousands of lines of transgenic mice that are being developed worldwide (including those developed here). We believe that this database will be a useful resource for all investigators interested in transgenic mice, particularly since we expect that much of the information in the database will not be available in written published form. Each line of transgenic mice will be entered as a separate record which will include: the method used to construct the line; the construct of the transgene that was inserted and its copy number; expression of the transgene in specific tissues as well as the phenotype associated with the transgene expression. Additionally, information on host insertional mutagenesis (genetics, cloning, characterization of the mutant locus, phenotype, etc.) will be included. Other categories of information relating to the availability of mice, investigators' names and addresses, and publications (including abstracts) will be in each record. We are developing software templates (running on IBM or Macintosh personal computers) which will allow researchers generating and studying transgenic mice to enter their lines of mice into the database. The database is being designed to be extremely user-friendly with a menu-driven interface and full-text search capabilities, and will be accessible through a toll-free connection to Tymenet.

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CHARACTERIZATION OF MUTANT MOUSE STOCKS

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The Mammalian Genetics Section possesses a rich collection of mutant mouse stocks. These animals provide a valuable resource for the investigation of basic biological processes and furnish animal models of human disease. Initial characterization of mutant stocks includes clinical observations, gross and microscopic morphology, hematology, clinical chemistry, and appropriate *in vitro* assays. Once the affected organs or organ systems are identified, the animals are scheduled for additional investigation at the Biology Division or made available to collaborators in the necessary fields. Two mutant stocks have been investigated by us in considerable detail.

The X-linked recessive mutation scurfy causes exfoliative dermatitis, anemia, cachexia, and early death (at 24 days of age, on average). The disease is associated with a lymphohistocytic proliferation in the liver, skin, and lymphoid organs. Scurfy disease requires an intact thymus, and can be transmitted to nude mice by thymic grafts or by T lymphocyte suspensions. Disease transmission by grafts of T lymphocytes is H-2 restricted. Scurfy disease is not transmitted by bone-marrow transplants from scurfy mice to lethally irradiated normal mice. These data suggest that scurfy is a defect in the thymic microenvironment that shapes the developing immune system. Such defects are postulated as the cause of many immune-system diseases in humans. Scurfy mice should provide a model system to test these hypothesis.

Initial evaluation of a transgenic mutant, Tg737, revealed polydactyly, polycystic kidneys, proliferation of hepatic periportal cells, skeletal abnormalities, and marked serum icterus. Similarities to human polycystic kidney diseases include bilateral renal involvement, and associated biliary lesions (congenital hepatic fibrosis). A number of other lines derived by insertional mutagenesis have also been examined.

Since insertional mutagenesis is carried out in the FVB strain, unusual features of normal FVB animals must be identified in order to differentiate them from potential mutant phenotypes. Examination of FVB mice has revealed lipoid degeneration of the adrenal-gland zona reticularis, hypertrophy of the

bulbourethral glands in males, and an increased susceptibility to infection by *Citrobacter freundii* which causes colonic mucosal hyperplasia.

Tissue-specific expression of integrin molecules was examined by histologic immunoperoxidase and immuno-autoradiography in collaboration with S. J. Kennel. Staining of particular tissues/organs with novel monoclonal antibodies was also examined.

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MOUSE MUTAGENESIS

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Enlarging the Germ-Line Mutagenicity Data Base

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 reports by Genetic Analysis and Molecular Genetics Groups) 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. Because of the very major dependence of effect on germ-cell stage that has been demonstrated (see below), it is obvious that somatic-cell or other standard short-term mutagenicity test systems would be of very limited utility in predicting overall germ-cell results. One objective of this program has been to enlarge the germ-cell 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 adriamycin, acrylamide monomer, chlorambucil, and melphalan, and initiated studies with glycidol. 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.

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 variants (at any locus throughout the genome) that are amenable to molecular access. We have clearly shown CHL to be such a mutagen (Russell *et al.*, 1989; Rinchik *et al.*, 1990) that can be of use to other laboratories; *e.g.*, in a cooperative study with L. Flaherty, Rinchik has used CHL mutagenesis successfully to induce miscellaneous viable recessives. In more recent work, we have demonstrated that the effects of MLP resemble those of CHL. Both chemicals are highly mutagenic in meiotic and postmeiotic stages, with the peak mutation yield derived from exposed early spermatids. The CHL yield for that stage was 7.8×10^{-4} per locus, about six times the yield for meiotic and postmeiotic stages overall. The pattern is very similar for MLP, and the overall rate is comparable to the CHL rate on an equimolar extrapolation. However, MLP is more toxic to the exposed animals than is CHL; for practical purposes only about one-half the dose can be used for the generation of mutations.

Extensive genetic, cytogenetic, and molecular analyses of the specific-locus mutations induced by the various chemicals studied (see reports by Genetic Analysis and Molecular Genetics Groups), indicated that, in the case of both CHL and MLP, most, if not all, mutations induced in postspermatogonial stages were deletions of various lengths; the incidence of translocations was also very high. In view of the utility of gross rearrangements – and particularly of deletions – for molecular-genetic studies, these two chemicals (which, at the most responsive stage, are more effective than X rays in inducing deletions) provide valuable research resources.

Because of logistic difficulties, few chemicals are routinely tested in females. CHL being of special interest, we undertook a study in which offspring of CHL-injected females were scored for mutations. If the relative effects in pertinent male and female germ-cell stages were the same for CHL as they are for acute irradiation, about three mutations would have been expected among offspring derived from exposed mature or maturing oocytes, and none among offspring derived from exposed immature oocytes. The numbers actually observed were zero and one, respectively. It thus appears that CHL is not highly mutagenic in oocytes. There is, of course, no cell stage in females that is comparable to the early spermatids of males, where CHL has shown by far the greatest mutagenicity.

Since the last report, we have augmented the Oak Ridge historical control rate by about 100,000 offspring, increasing sensitivity in statistical calculations of induced rates. The new historical control forward mutation rate for the 7 marked loci is 6.3×10^{-6} per locus per generation.

Factors that Affect the Frequency and Nature of Induced Mutations

As our mutagenicity studies have continued to expand the specific-locus-mutation data base, they have allowed us to make an examination of the effects of germ-cell stage on both quantity of mutation yield and nature of mutations. 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 majority of chemicals tested (including acrylamide monomer, studied during this reporting period) fall into Pattern 1; however, because only limited classes of chemicals have been tested to date, no meaning should probably be attached to the relative frequencies. Chemicals that are mutagenic both in stem-cell spermatogonia and in poststem-cell stages (the minority) do not preferentially exhibit any one of the three poststem-cell patterns.

The results of genetic and molecular analyses of genomic regions surrounding six of the specific-locus markers, and, particularly, what these have revealed about the phenotype associated with total ablation of the marker gene, allow us to conclude that any mutation that causes lethality of homozygotes (in the case of *d, pre*natal lethality, specifically) must involve one or more loci in addition to the marked one. Such mutations have been classified as "large lesions" (LL), the remainder as "other lesions" (OL). Analysis of our own data on a continuously expanding number of chemicals, as well as of data published by other laboratories, shows that, regardless of the nature of the chemical (Pattern-1, -2, or -3), (a) very few LL mutations are induced in either stem-cell or differentiating spermatogonia, and (b) very high frequencies of LLs are induced in postmeiotic stages. Particularly critical is the evidence from chemicals that are active in both pre- and postmeiotic stages; these were found to produce LL or OL mutations depending on what cell stage was exposed.

The constantly growing number of specific-locus mutations recovered from mutagenicity assays has also provided an increasingly valid picture of the distribution of mutations among the 7 marked loci. For mutations induced in stem-cell spermatogonia, this spectrum appears to be similar for all groups of chemicals. Though the numbers are smaller, the distribution for differentiating spermatogonia generally resembles that for stem cells. Since the frequency of LLs among spermatogonial mutations is very low, these spectra may be indicative of the relative sizes of the target loci. On the other hand, the spectrum for

postmeiotically-induced mutations most of which are LLs, departs in several ways from that for the other groups.

Differences Between the Maternal and Paternal Genomes of the Zygote in Mutagen Sensitivity

The two parental genomes lie separate within the zygote and may be differentially affected by environmental influences. We had shown earlier (Russell *et al.*, 1988) that the maternal genome within the mouse zygote is exquisitely sensitive to the induction of specific-locus mutations by *N*-ethyl-*N*-nitrosourea, ENU (which is known to induce primarily intragenic lesions). Two features of the results were striking: the very high mutation rate (considerably higher than in any germ-cell stage) and the high proportion of mosaics among mutants. Genetic tests indicated that the mosaics resulted from events affecting one DNA strand of the haploid maternal chromosome, and that these events were primarily intragenic lesions.

During this reporting period, we completed an experiment that measured specific-locus mutation induction in the *paternal* genome of zygotes. Observations at weaning age, yielded one possible mosaic among 2,113 classified offspring that had been treated with ENU as zygotes; this animal failed to transmit a mutation. By contrast, in the reciprocal cross (which tests the maternal genome), we had observed 8 specific-locus mutations (6 of them mosaics) among 1,555 offspring that had received the same dose of ENU at the time of sperm entry (and completion of oocyte meiosis-II). We conclude that at these stages, the paternal genome of the zygote is less sensitive than the maternal one to the induction of mutations by ENU.

Computer Management of Mutagenesis Data

For almost a decade, we have relied on a mainframe computer for database management. Since that mainframe will be phased out, we are in process of transferring altogether 800 files to a computer workstation in our Section. Programs are being developed by P. B. Selby that will permit direct entry into personal computers of data from ongoing specific-locus experiments. Use of our workstation will decrease the labor involved in entering data, and will make it easier to archive records and to access them once archived.

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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 Zygotes and Early Cleavage Stages

A recent discovery in our program points to the significance of nonconventional (perhaps epigenetic) mutational damage. It may also pave the way for molecular investigations into the causes of certain human developmental anomalies. We found that exposure of mouse zygotes to certain chemical mutagens, such as ethylene oxide or ethyl methanesulfonate, or of two-cell embryos to the inhibitor of C-methylase enzyme, 5-azacytidine, induces high frequencies of fetal anomalies. Remarkably, the types of anomalies produced in these experiments resemble the large class of defects in humans that are of unknown etiology. Our studies indicated that the mutagen-induced embryopathy in mice was caused by direct damage to the zygote, but was not attributable to gross chromosomal aberrations, small deficiencies, or gene mutations. The

efficacy of 5-azacytidine exposure of two-cell embryos in inducing malformations is speculated to be the result of derepression – thus, out-of-phase induction – of a set of developmental genes. We hypothesize that exposure of mouse zygotes or two-cell embryos to certain chemicals leads to fetal abnormalities by causing disruptions in the finely tuned program of gene expression during early embryonic development. Concomitantly, we hypothesize that many of those human malformations that are presently of unknown etiology are, in fact, caused by epigenetic changes that originate in the zygote and early cleavage stages.

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 to either intercalate between base pairs or to bind to specific sequences.

Creation of a Translocation Resource for Morphogenetic and Molecular Studies

Heritable chromosomal rearrangements, primarily reciprocal trans-locations, are produced in our mutagenesis experiments on a continuing basis. They are systematically analyzed for homozygous effects and for developmental anomalies in chromosomally unbalanced segregants. These rearrangements have been a dependable source of specific malformations that are used as models of human disorders and provide material for morphogenetic studies that could not be done in human subjects. Furthermore, as demonstrated by a rearrangement produced in this program that was instrumental in the recent cloning of the *a* locus, these rearrangements constitute an invaluable resource for studies of the relationship between underlying genomic structure and phenotype.

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ORGANISMIC EFFECTS

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Assessment of Dominant Damage for Radiation, Chlorambucil, and ENU

An important knowledge gap in 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 underway in experiments in our laboratory using the Assessment of Dominant Damage (ADD) approach (Selby 1990). First-generation progeny are being examined for several types of dominant organismic damage following exposure of their fathers to one of the following treatments: 10 mg/kg of chlorambucil (CHL, an inducer of deletions and other large DNA lesions), 4 weekly injections of 100 mg/kg of ethylnitrosourea (ENU, an inducer of small lesions), 600 R of 250 kVp X radiation delivered at 90 R/min, and 600 R of ^{137}Cs gamma radiation delivered at 4 mR/min, inducers of a mixture of both large and small DNA lesions. Except for the CHL experiment, in which offspring are derived only from early spermatids (the germ-cell stage that exhibits peak sensitivity to induction of specific-locus mutations), all other experiments deal exclusively with stem-cell spermatogonia. All groups have concurrent controls. The types of dominant organismic effects that are studied in the progeny are: skeletal malformations, cataracts and other eye abnormalities, stunted growth, dominant visibles, and survival to 11 weeks of age.

The cataract data collected to date have now been uncoded and have already yielded an exciting result. The frequency in the CHL group is statistically significantly higher than that in the CHL control or in the combined control ($P = 0.005$ and $P = 0.0002$, respectively, Fisher's exact test). The induced cataract-mutation frequency for CHL (i.e., experimental minus control) is 5.3%; thus, approximately 1 mouse in 20 has an induced cataract.

The cataract test, as developed and used at the mammalian genetics facility at Neuherberg, Germany, has either been negative or yielded very low estimates of mutation frequencies in experiments with several mutagens. Only cataracts that were transmitted in breeding tests were counted in those experiments. Our re-analysis of the German data (Selby 1990) indicated that they would have yielded significant results had all cataracts been counted and control frequencies subtracted from experimental ones. The rationale for this approach is that the breeding-test criterion fails to detect most cataracts caused by mutations with low penetrance or due to multiple factors, as well as those in animals that are sterile

or die before producing an adequate number of offspring. In the CHL studies summarized above, we have applied the approach used in our re-analysis of the German data and are gratified to find it to be so fruitful.

The cataracts included in our test are all distinct and easily seen in a quick (but careful) slit-lamp examination. Dr. Lee Smalley, an experienced ophthalmologist and eye surgeon (Oak Ridge), is collaborating with us by reexamining all cataracts and classifying them as to severity.

In its report of 1977, and in all three of its reports since then, UNSCEAR (United Nations Scientific Committee on the Effects of Atomic Radiation) has used the direct method of genetic risk estimation, as well as the doubling-dose approach, for estimating the genetic hazard to humans. The direct method is based, to a large extent, on data that we collected on skeletal malformations in the early 1970s. It seems certain that our large current experiment using gamma radiation, which will provide data for low dose rates (not available earlier) and which incorporates many advances over the earlier experiment, will provide the basis for a major improvement in genetic risk estimation for radiation.

Induction of Dominant Skeletal Mutations by ENU Exposure of Early Zygotes

Generoso *et al. (Mutat. Res.* **176**: 269-274, 1987; *Mutat Res.* **199**: 175-181, 1988) have reported remarkable increases in the incidences of embryonic and fetal deaths and of developmental abnormalities in surviving fetuses after early zygotic stages are exposed to any one of several chemicals, including ENU. L.B. Russell *et al. (PNAS* **85**: 9167-9170, 1988) found that ENU induces high frequencies of specific-locus mutations when it is administered to zygotes shortly after sperm entry (2.5-3 h after mating). Following up on these findings, we carried out a study on the induction of dominant skeletal mutations by 40 mg ENU/kg, injected i.p. into females at either 2.5 or 5 h after mating. Among mice surviving 3 weeks or longer after birth, the frequencies with possible dominant skeletal mutations were 4/108 (3.7%), 22/208 (10.6%), and 20/156 (12.8%) in the control, 2.5-h ENU, and 5-h ENU groups, respectively. The experimental frequencies were both significantly higher than the control, $P < 0.03$. The average induced frequency (i.e., experimental minus control) of presumed dominant skeletal mutations was 7.8%. First-generation offspring with stunted growth or externally visible abnormalities were mated before their skeletons were prepared to permit a breeding test for some of the presumed dominant skeletal mutations. The experiment is nearing completion, and at least 10 mutations will be confirmed by breeding tests. Some of the dominant mutations have complete penetrance; most have incomplete penetrance. Since such high frequencies of induced dominant mutations were found by postnatal examination of just one body system, and after only 40 mg/kg of exposure, it seems likely that the high frequency of abnormal

fetuses found by Generoso et al. following a 75 mg ENU/kg exposure also resulted from dominant mutation.

Organismic Effects of Insertional Mutations

In the offspring from transgenic lines, created in the program of R. P. Woychik, we have begun routine examinations 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 abnormalities, including extra molars, preaxial polydactyly in all four extremities, failure of the tibia and fibula to connect, and extra tarsals.

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

In additional work that does not deal with organismic effects, we completed this year an extensive analysis of specific-locus data that we had collected earlier.

Female mice were exposed to acute X radiation either as fetuses at 18.5 d post-conception (p.c.) or within 9 h after birth. The results from these two groups were similar, and in combination yielded a specific-locus mutation frequency of 9.4 x 10^{-} mutation/locus/R, which is statistically significantly higher than the historical-control mutation frequency, but much lower than the rate obtained by irradiating mature and maturing oocytes in adults. Other females, exposed at 18.5 days p.c. to the same dose of low-dose-rate gamma-radiation, yielded a mutation frequency that was statistically significantly lower than the frequency at high dose rates. The low-dose-rate group also had markedly higher fertility, showing that there is a dose-rate effect for oocyte killing as well as for mutation yield.

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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GERM-CELL DNA/PROTEINS

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The purpose of our research is to develop an understanding of the molecular processes that lead to the induction of mutations in mammalian germ cells, using the mouse (both males and females) as a model system. A better understanding of these mutational processes at the molecular level is important for establishing reasonable guidelines for estimating human genetic risk from exposure to chemicals and radiations in the environment. It is also a prerequisite for the development of molecular techniques to monitor human germ cells for potential genetic damage. The work can be divided into several interrelated areas of research: (1) *Molecular dosimetry* to measure the amounts and types of interaction between chemical agents and genetically significant targets within the germ cells. This dosimetry gives valuable information on the relationship between exposures of the whole animal to a mutagen and the amount of mutagen actually reaching the germ cells. (2) *In vivo DNA repair* studies that give important information, in a relatively short period of time, on the extent to which a chemical agent is able to reach the DNA of mammalian germ cells and produce "repairable" lesions. Differential DNA-repair capabilities of various germ-cell stages exposed to the same mutagen can also be measured by means of these studies. (3) *Measurement of DNA strand breaks or cross-links* in the germ cells following mutagen exposure, using alkaline elution techniques to recover broken pieces of DNA.

Molecular Dosimetry

Molecular dosimetry, using radioactively labeled chemical mutagens, has been a useful method to reveal the molecular events occurring within germ cells after treatment with mutagens. The procedure is very sensitive and can be used to

measure binding of chemical agents to germ cells and to germ-cell DNA at exposure levels that are orders of magnitude lower than those necessary to produce a readily measurable increase in heritable mutations. Typically, as little as 1 lesion in 10^8 deoxynucleotides can be detected with this method.

The chemotherapeutic agent chlorambucil (CHL) has been found to be highly mutagenic in mouse germ cells, with maximum yield occurring in early spermatids that are sampled in matings taking place during the third week posttreatment (L. B. Russell *et al.*, *PNAS* **86**: 3704, 1989). To study the relationship between the amount of CHL binding to various spermiogenic stages and the induced genetic damage, male mice were given a single i.p injection of ^{3}H-labeled CHL, and sperm, recovered at daily intervals over a three-week period, was assayed for CHL binding. No clear alkylation peaks were detectable, although binding levels were found to be somewhat elevated at intervals corresponding to exposed mature sperm and mid-spermatids.

This pattern of alkylation was quite different from that produced by chemicals such as acrylamide (see last Progress Report), which increase the level of binding by more than an order of magnitude in the second week posttreatment (late and mid-spermatids) over the level observed in the first week. With acrylamide and similar chemicals, the sharp increase in alkylations is accounted for by binding to protamine. The results for CHL suggest that there is little, if any, protamine binding.

The CHL-alkylation data also fail to provide an explanation for the very high mutation yield observed in dominant-lethal and specific-locus experiments in the third week after treatment (exposed early spermatids). CHL binding either to whole sperm or to sperm DNA did not increase during this period. For germ cells recovered in week 3, which (unlike those recovered in weeks 1 and 2) are capable of DNA repair, there is, however, the possibility that some DNA adducts could have been removed by repair prior to our assay for adducts.

Using another approach, we carried out CHL-binding measurements directly within various exposed germ-cell stages. At intervals of 1 hour, 1, 2, 3, and 4 days after a single i. p. injection of 10 mg [^{3}H] CHL/kg, pachytene spermatocytes, early-, elongating-, and late-spermatids were fractionated by centrifugal elutriation and assayed for bound CHL.

The level of CHL binding was found to be highest in the pachytene spermatocytes, averaging ~300 x 10^3 alkylations per cell over the 4-day time period, and there was a clear trend toward reduced alkylation with advancing germ-cell stage. Early spermatids, the stage most genetically sensitive to CHL, showed only about one-third of the chemical binding found in the much less sensitive pachytene spermatocytes. (However, some or all of the difference in

alkylation levels between these two stages may be a reflection of the fact that the cellular volume of pachytene spermatocytes is about 4 times that of early spermatids.)

When measurements were made in DNA extracted from the various germ-cell stages, no significant differences were observed in CHL binding levels (which were of the order of 1 adduct per 10^7 deoxynucleotides in all cases). Since histones are known to be phosphorylated and acetylated in early spermatids as a preparatory step for their replacement by transition proteins, and finally by protamine, it is possible that CHL may be particularly damaging to these modified histones. Work is now in progress to determine whether differences are detectable in the binding of CHL to histones recovered from different germ-cell stages.

As our molecular dosimetry data are combined with other genetic and cytogenetic data for various chemical agents, we will continue to learn more about the relationship between the extent of chemical damage in the germ cells and the amount of genetic damage expected at realistic exposure levels for humans. Furthermore, we will develop a better understanding of important molecular targets within mammalian germ cells.

DNA Repair

DNA repair can occur in a number of spermatogenic stages but not in the most mature ones. The DNA repair response of germ cells to a given chemical mutagen can vary several-fold among different mouse strains. Our observation of DNA repair in mammalian germ cells after mutagen exposure led us to develop a test for unscheduled DNA synthesis (UDS) that has been used for the screening of potential mutagens because it is fast, sensitive, and rather inexpensive.

Recent studies with bis-acrylamide (two acrylamide molecules joined back to back) have shown that this chemical is able to produce DNA lesions in early spermatids. Since this is a repair-competent stage in mammals, we looked for a UDS response; however, no significant incorporation of label was observed, regardless of whether the [^{3}H]dThd was administered immediately after the bis-acrylamide exposure or at different times after exposure (up to 24 h). In contrast, we have shown that acrylamide (monomer) is able to induce a very significant UDS response in early spermatids, although the maximum response does not occur until 6 h after chemical exposure. It appears that while acrylamide is metabolized to a more reactive chemical which then binds to the germ-cell DNA and elicits a delayed UDS response, bis-acrylamide may not be metabolized to a more reactive chemical. It is also possible that the DNA lesions produced by bis-acrylamide involve DNA crosslinks which may not be repaired.

Alkaline Elution

To further assess the damage produced in mammalian germ cells using model chemicals, we have developed an alkaline elution procedure to measure DNA strand breaks in germ cells. The procedure involves lysis of the cells after treatment with the test chemical, followed by separation of the DNA double helix using a strong base. Any small pieces of single-stranded DNA resulting from breakage by the test chemical will rapidly pass through a filter, while normal-sized DNA will take much longer to pass through the same filter. In the case of chemicals that may cause crosslinks in the DNA, or between DNA and chromosomal proteins, the chemically-treated cells are first exposed to X rays (600R) to induce a lot of DNA breakage and then placed on the filters. The eluted DNA is then analyzed as above. Retention of DNA on the filter (reduced DNA elution) is an indicator of the presence of DNA crosslinks.

Chlorambucil (CHL), a chemotherapeutic agent and a modified nitrogen mustard has two reactive chloroethyl arms that have the potential for forming crosslinks. As noted above, CHL is most effective in causing genetic damage in early spermatids. We therefore performed alkaline elution experiments to study the ability of CHL to induce DNA crosslinks (DNA:DNA or DNA:protein) in specific germ-cell stages isolated from mouse testes by centrifugal elutriation. The germ cells were exposed, in vitro, to 0.1 mg CHL/ml for 1 h. Specific stages were purified, exposed to 600R of X rays, and assayed for DNA crosslinks by alkaline elution. Controls were treated identically except for the absence of CHL. For mid- to early spermatids treated with CHL and X rays, there was ~50% retention of DNA on the filters compared to the controls. For pachytene spermatocytes this DNA retention was ~35%. In all of the stages studied, CHL was clearly producing DNA crosslinks that prevented elution of the X-ray-fragmented DNA through the filters. Further, there appeared to be somewhat less DNA crosslinkage in the genetically less sensitive pachytene spermatocytes than in the early spermatids. Future work will investigate the ability of the different germ-cell stages to remove the crosslinks over a period of time.

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2. Sega, G. A. Molecular targets, DNA breakage, DNA repair: Their roles in mutation induction in mammalian germ cells. In: *Banbury Report 34: Mutation Induction and Heritability in Mammalian Germ Cells*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1990, pp. 79-88.

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NEW METHODS FOR SEQUENCING THE MOUSE AND HUMAN GENOMES

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H. Arlinghaus[8]
G. M. Brown[5]
C. H. Chen[7]
M. W. England[7]
T. L. Ferrell[7]
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R. P. Woychik

DNA sequence provides detailed information about a genome. Faster and more accurate methods for sequence determination are under study. Four different approaches are being pursued. One 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. Methods have been developed to use stable isotopes of iron and tin; rare earth chemistry also is currently being explored.

The remaining new sequencing procedures eliminate the gel electrophoresis step. Sequencing-by-hybridization can analyze DNA of a wide variety of sizes by measuring its ability to hybridize to a complete set of oligonucleotides; for 8-mers there are 65,536 oligonucleotides in the complete set. Innovative chemical procedures to synthesize and arrange these oligonucleotides are being developed that will be convenient for this procedure. Another method is scanning tunneling microscopy in which DNA molecules of a wide variety of sizes can be analyzed. Several Nanoscopes of the most recent design are available and a variety of DNAs of different sizes are being used to develop procedures to image the DNA with nanometer resolution. The third non-electrophoretic procedure is high resolution mass spectroscopy. The DNA of interest will be replicated with DNA polymerase and specifically terminated fragments obtained by using dideoxynucleotides. Since

the four bases of DNA differ in molecular weight, the DNA fragments of which they are the terminal member can be distinguished if the time-of-flight mass spectrometer can measure >100,000 daltons with resolution of <9 daltons. It will be necessary to attach an easily ionizable moiety to the DNA fragments; the ferrocene tag developed in the stable-isotope project above is suitable and will be employed. The mass spectrometer with a tunable vacuum ultraviolet coherent beam is under construction, and simple oligonucleotides are being prepared to test this approach.

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MAMMALIAN BIOCHEMICAL GENETICS

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J. F. Gallager[4]
K. D. Russell[4]

Over the past two years our group has continued studies on animal models of human diseases, particularly those involving abnormal hemoglobins and virus-induced malignancies. Clinical biochemistry, immunological methods and

molecular biology are being used to characterize pathophysiological effects, induced mutations and altered gene products that cause health effects in mice. Our recent results are summarized below.

Hematopoiesis and Globin Gene Expression in Beta-Thalassemic Mice

We have completed a five-year project sponsored jointly by NIH and DOE on the "Regulation of the Human Beta-Globin Gene." Two congenic lines of beta-thalassemic mice were developed and used here and at many other research laboratories to study the pathophysiological effects of beta-thalassemia in mice. Human beta-thalassemia is a common hereditary disease of considerable social and economic consequence in many countries of the world, and the animal model is being used to find possible treatment through bone-marrow transplantation or gene therapy in humans.

The results of our studies established that the hematological indices of the murine and human beta-thalassemias are similar. The hypocellular, hypochromic, and microcytic anemia is caused by dysfunctional erythropoiesis, reduced red-cell lifespan, and deficient synthesis of beta-globin. To maintain homeostatic conditions, the daily red-cell production is three-fold higher in beta-thalassemic than in normal mice. Most of the additional erythropoiesis occurs in the spleen, which becomes six times normal size. The hematopoietic stem-cell compartments are not expanded in the bone marrow; however, in the spleen the BFU-E progenitor cell compartment is expanded five-fold and the CFU-E progenitor cell compartment is expanded 300-fold. The erythropoietin sensitive CFU-E progenitor cells respond to the elevated levels (3 to 5X) of erythropoietin in beta-thalassemic mice.

Transplantation of normal bone marrow into sublethally irradiated beta-thalassemic recipients restores normal hematopoiesis. Lethal irrradiation is required to transplant beta-thalassemic bone marrow successfully into normal mice. When mixtures of normal and beta-thalassemic bone marrow are grown in the same recipient, normal bone marrow repopulates and overgrows the co-injected beta-thalassemic bone marrow. These results suggest that restoration of beta-thalassemic bone marrow cells by gene therapy might provide a successful means for the treatment of beta-thalassemia in humans. To date, however, engraftment of genetically defective cells that have been "corrected by gene therapy" has not produced long-term grafts.

In beta-thalassemic mice the beta-d minor globin gene is upregulated more than three-fold. Upregulation results from both an increase in mRNA (1.5X) and an increased efficiency of translation stimulated by an undefined translation factor. Efforts to identify unique nucleotide sequences within, or adjacent to, structural genes that control the level of globin gene expression have been discontinued

because it is now established that locus control regions (LCR) 5 to 20 kb 5' to the compound hemoglobin loci contain the DNAse-I hypersensitivity sites that regulate the temporal and tissue-specific expression of the hemoglobin genes.

Development of a Mouse Model of Sickle Cell Anemia

We are in the third year of a five-year project sponsored jointly by NIH and DOE to develop mice with "Mutant Mouse Hemoglobins that Should Allow HbS to Sickle." We have identified mice in which ENU-induced germ-line mutations at *Hba* and *Hbb* encode hemoglobins that have a high affinity for oxygen and might provide an environment for gelation of sickling hemoglobins *in vivo*. In addition, $HbS^{Antilles}$ was used to make the founder transgenic mice because $HbS^{Antilles}$ has a lower solubility and lower affinity for oxygen than does HbS. Both of these properties should favor *in vivo* sickling of $HbS^{Antilles}$ because the mutant mouse hemoglobin has a higher affinity for oxygen, and a larger percentage of $HbS^{Antilles}$ will remain deoxygenated. Transgenic mice producing $HbS^{Antilles}$ were made by Dr. Ed Rubin, Lawrence Berkeley Laboratory. The human transgenes are being bred onto the genetic background of mice with the high oxygen affinity hemoglobin. The objective is to produce mice of Hba^{g2}/Hba^{g2};Hbb^{s2}/Hbb^{s2};Tghα, h$\beta^{SAntilles}$/Tghα,h$\beta^{SAntilles}$ genotype. We presently have several mice that express hematological characteristics found in humans with sickle cell disease although none of the mice are as yet homozygous at each of the four designated alleles. The experimental animal model of sickle cell anemia will be used to investigate the phenomenon of sickling *in vivo*, and to search for useful anti-sickling drugs.

Pathophysiological Effects of B10FV in B10.F Mice

A virus-free subline of mice genetically identical to B10.F was produced by mating congenic B10.F males with females of their inbred partner B10, then mating F1 progeny to produce F2 progeny that were tested to be homozygous for the $H\text{-}2^{n/n}$ genotype. These mice are virus-free but are genetically identical to B10.F mice; they are virus-free because the virus is not transmitted via the sperm and the B10FV virus does not replicate in B10 mice of $H\text{-}2^{b/b}$ genotype. B10FV isolated from cultures of SC1 cells infected with B10FV from B10.F spleen cells was injected into virus-free B10.F neonates to study the pathophysiological effects of the virus in susceptible mice. Neonates receiving an injection of the B10FV develop histological and immunological characteristics commonly found in B10.F mice that naturally acquire B10FV via their mother's milk. About 10 percent of the B10FV-injected neonates also develop lymphomas that are not found in virus-free control animals. Analysis of cell populations by fluorescent activated flow cytometry reveals that the incidence of Thy 1^+ T cells is reduced and the number of $Ly1^+$ B cells is increased in the spleen of B10FV-injected mice.

The germ-line DNA of the immunoglobulin (*Ig*) heavy-chain gene and of the beta-chain gene of the T-cell receptor (*TcR*) in T and B cells undergoes rearrangements during lymphocyte differentiation and maturation. DNA probes of *Ig* and *TcR* can be used to determine the time of clonal expansion and the cell lineage of cells comprising the hyperplastic responses and tumors that arise in lymph nodes, spleen and thymus of B10.F mice. A single, prominent proviral insertion was found in DNA prepared from malignant lymphomas, which indicates that each of these tumors arose by monoclonal expansion from a single cell in which one retrovirus had integrated. DNA from malignant lymphomas had rearranged *TcR* and germ-line *Ig* genes, so these are T-cell lymphomas induced during the time of thymocyte differentiation. DNA prepared from non-malignant tissues with moderate to severe hyperplasia commonly exhibit multiple proviral insertions and unrearranged, germ-line *Ig* and *TcR* genes. These observations suggest that viral infections initially induce cellular hyperplasia involving many clones of cells each containing a different retroviral insertion, and that the majority of these expanding clones contain undifferentiated cells. Occasionally, a clone of T cells with rearranged *TcR* genes becomes malignant and overgrows the multiple lineage of cells that exhibit clonal expansion during the initial phase of virus-induced cellular hyperplasia.

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2. Lewis, S. E., L. B. Barnett, and R. A. Popp. Mosaic mutants induced by ethylnitrosourea in late germ cells of mice. In: *Banbury Report 34: Biology of Mammalian Germ Cell Mutagenesis*, ed. by J. W. Allen, B. A. Bridges, M. F. Lyon and L. B. Russell. Cold Spring Harbor Laboratory Press, 1990, pp. 237-243.
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4. Popp, R. A., S. L. Bolch, S. G. Shinpock, and D. M. Popp. Expression of the globin genes and hematopoiesis in beta-thalassemic mice. In: *Molecular Biology of Erythropoiesis,* ed. by J. L. Ascensao, *et al.* Plenum Press, New York and London, 1990, pp. 161-176.

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 24 students and is a recognized center of quality education in the southeast. The University of Tennessee supports 16 students with research assistantships, 1 student is supported by an Oak Ridge Associated Universities Fellowship, 1 student is supported by a University of Tennessee Hilton Smith Fellowship, and 2 students are supported by National Institutes of Health predoctoral appointments. By September of 1991, a total of 136 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 three research professors (A. L. Olins, R. S. Foote, and E. C. Uberbacher) 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 from the Oak Ridge Associated Universities 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 14 coauthorships, 7 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 11 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 three undergraduate training programs: (i) Great Lakes Colleges Association/Associated Colleges of the Midwest (GLCA/ACM Science Semester), (ii) Southern Colleges University Union Science Semester (SCUU), and (iii) Oak Ridge Associated Universities Summer Student Trainee (ORAU). Under the auspices of these organizations and in cooperation with Oak Ridge National Laboratory, outstanding college juniors are offered opportunities for independent research in the life sciences. In the past 24 months, there were 21 students, possessing the educational qualifications and the potential for a successful scientific career, who spent 16 weeks (GLCA/ACM and SCUU) or 10 weeks (ORAU) 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 557 students have participated during the past 25 years, have received the enthusiastic endorsement of the students, their colleges, and the members of the Biology Division.

Appendices

Advisory Committee - FY 1990-1991

Dr. Irwin Fridovich
Department of Biochemistry
Duke University Medical Center
Durham, North Carolina 27710

Dr. H. Ronald Kaback
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University of California at Los Angeles
405 Hilgard Avenue
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Center for Demographic & Population Genetics
The University of Texas Health Science Center
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Dr. Shirley M. Tilghman
Howard A. Prior Professor of the Life Sciences
Department of Molecular Genetics
Princeton University
Princeton, New Jersey 08544

Dr. Joseph J. Villafranca
Professor, Department of Chemistry
152 Davey Laboratory
Pennsylvania State University
University Park, Pennsylvania 16802

Dr. Arthur Weissbach
Associate Director, Retired
Department of Cell Biology
Roche Institute of Molecular Biology
Post Office Box 168
Sanibel, Florida 33957

Extramural Activities

Society Committees

G. J. Bunick	-	Special Interest Committee on Small-Angle Scattering, American Crystallographic Association (1991—)
J. S. Cook	-	Publications Committee, American Physiological Society (1986—1989); Chairperson (1989—1992)
R. J. M. Fry	-	History Committee, Radiation Research Society (1982—)
F. C. Hartman	-	Symposium Director, The Protein Society (1990)
P. Mazur	-	Publications Committee (Chairman), Society for Cryobiology (1974—) Board of Governors, Society for Cryobiology (1967—)
A. L. Olins	-	Minorities Committee, American Society for Cell Biology (1985—1991) Organizer of workshop, "Careers in Biological Sciences: Advancement for Women and Minorities," April, 1990 (supported by National Science Foundation) University of Tennessee, Faculty Senate (1989—1992)
E. M. Rinchik	-	Chairman, Committee for Mouse Chromosome F, International Mammalian Genome Society (1990—)
L. B. Russell	-	Nominating Committee, Environmental Mutagen Society (1991—)
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—)

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—)
Special Study Sections, National Institutes of Health (1987—1989)
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—)
Council Member, National Council on Radiation Protection and Measurements (1980—)
Scientific Committee 75 (Chairman), National Council on Radiation Protection and Measurements (1983—)
Committee 1, International Commission on Radiological Protection (1985—)
Advisory Committee, Institute of Environmental Medicine, New York University Medical Center (1985—)
Committee on Space Biology and Medicine, Space Science Board, National Research Council, National Academy of Sciences (1986—1992)
Member, Aerospace Medicine Advisory Committee, National Aeronautical Space Administration (1988—1992)
Chairman, Radiation Discipline Working Group, National Aeronautical Space Administration (1988—1992)
Member, Bevalac Biomedical Program Advisory Committee, Lawrence Berkeley Laboratory (1988—1991)
Chairman, Subcommission on Radiation Biology, Committee on Space Research (COSPAR), (1990—1994)
Board of Directors, National Council on Radiation Protection and Measurements (1990—1994)
Visiting Committee, Medical Department, Brookhaven National Laboratory (1991—)

W. M. Generoso	-	Committee on Toxicology, National Research Council (National Academy of Sciences) (1989—1991) Member, Organizing Committee, First Latin-American Symposium on Environmental Mutagenesis, Laxambu, Brazil (1991)
F. C. Hartman	-	Executive Committee, Division of Biological Chemistry, American Chemical Society (1989—1991)
S. J. Kennel	-	Study Section on Health Effects Research, U.S. Environmental Protection Agency (1982—) Ad hoc member, National Cancer Institute Site Visit Committees (1989, 1990)
S. Mitra	-	Consultant, National Cancer Institute (1987—)
S. K. Niyogi	-	Scientific Expert, United Nations Industrial Development Organization, and External Advisor to Indian Jute Industries' Research Association, Government of India (1989—)
A. L. Olins	-	Member of the Corporation, Woods Hole Marine Biological Laboratory (1983—) Special Study Section, National Institutes of Health (1988—1989) National Science Foundation, Cell Biology Program, Review Panel (1990—1993)
D. E. Olins	-	Member of the Corporation, Woods Hole Marine Biological Laboratory (1983—) Advisory Committee, Helicon Foundation, La Jolla, California (1983—)
R. A. Popp	-	Mouse Hemoglobin Nomenclature (1984—)
E. M. Rinchik	-	Ad hoc Member, Genome Study Section, National Center for Human Genome Research (1990)
L. B. Russell	-	International Committee on Standardized Genetic Nomenclature for Mice (1977—1990) Board on Environmental Science and Toxicology, National Academy of Sciences (1986—1990) Environmental Health Institute, Fellow (1987—); Awards Nomination Committee (1991—)

D. M. Skinner	-	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 (1990)
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—)
W. K. Yang	-	Member, Cancer Biology and Immunology Contract Review Committee, National Cancer Institute (1987—1991)
	-	Outstanding Investigator Award Review Committee, National Cancer Institute (1989—1991)

Editorial Boards

J. S. Cook	-	*American Journal of Physiology*, Editorial Board (1987—1993) *Current Topics in Membranes and Transport*, Advisory Board (1983—) *New 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—1992) *Journal of Biological Chemistry* (1983—1995)
P. Mazur	-	*Cryobiology* (1967—)
D. E. Olins	-	*Chromosoma* (1990—)
L. B. Russell	-	*Mutation Research* (1976—) *Mouse Genome (formerly Mouse News Letter)* (1987—1990)
G. A. Sega	-	*Environmental and Molecular Mutagenesis* (1985—)
D. M. Skinner	-	*Gene* (1986—) *Physiological Zoology* (1989—)

Awards, Honors

G. J. Bunick	-	Martin Marietta Energy Systems, Inc., Technical Achievement Award (1991)
P. R. Hunsicker	-	Martin Marietta Energy Systems, Inc., Technical Support Award (1990)
S. J. Kennel	-	Martin Marietta Energy Systems, Inc., Publication Award (1990)
S. Mitra	-	Martin Marietta Energy Systems, Inc., Technical Achievement Award (1990)
D. M. Skinner	-	Martin Marietta Energy Systems, Inc., Technical Achievement Award (1990)

Invited Presentations at Major Conferences
October 1, 1989 - September 30, 1991

R. J. M. Fry

"The Effects of Space Radiation." NASA-NIA Workshop on Correlations of Aging and Space Effects on Biosystems, Chantilly, VA, October 30-November 1, 1989.

"Time-Dose Relationships on High-LET Radiation." Committee on Effects of Ionizing Radiation. Forum on the Effects of High-LET Radiation at Low Doses/Dose Rates, London, England, December 14, 1989.

"Space Radiation and Health Effects." European Symposium on Life Sciences Research, Trieste, Italy, May 29 - June 1, 1990.

"Review of Q for High-LET Radiation." Committee on Space Research XXVII Plenary Meeting and Associated Activities, The Hague, The Netherlands, June 25 - July 6, 1990.

"Radiation Carcinogenesis in the Whole-Body System: Symposium Summary." Japanese Radiation Research Society, Tokyo, Japan, December 5, 1990.

"Extrapolation of Risk Estimates Across Species." Workshop on Oncogenic Mechanisms in Radiation-Induced Cancer, Fort Collins, CO, January 16-19, 1991.

"Radiation Protection Guidelines for Humans in Space." 9th International Academy of Astronautics Man in Space Symposium, Cologne, Germany, June 17-21, 1991.

"Radiation Effects in Space: Research Needs." 9th International Congress of Radiation Research, Toronto, Canada, July 7-12, 1991.

"Radiation Environments in Space and Associated Health Effects." Health Physics Society Meeting, Washington, DC, July 21, 1991.

W. M. Generoso

"Developmental Anomalies: Mutational Consequence of Mouse Zygote Exposure." Banbury Conference on Biology of Mammalian Germ Cell Mutagenesis, Cold Spring Harbor, NY, November 12-15, 1989.

"Cellular and Molecular Processes in Early Mammalian Embryogenesis — Possible Role in the Pathogenesis of Developmental Defects." NAS/NRC Committee on Toxicology Workshop on Developmental Abnormalities, Washington, DC, January 9-10, 1991.

"Heritable Effects in Mice." First Latin American Symposium on Environmental Mutagenesis, Caxambu, Brazil, May 26-29, 1991.

F. C. Hartman

"Site-Directed Mutagenesis Without Benefit of Crystallographic Data: Boon or Boondoggle?" International Chemical Congress of Pacific Basin Societies. Honolulu, HI, December 17-22, 1989.

"Chemical Approaches for the Characterization of the Catalytic and Regulatory Sites of Phosphoribulokinase from Spinach." Jacques Monod Conference on Intracellular Redox Control in Animals, Plants, and Microorganisms by Thioredoxin and Glutaredoxin Systems. Roscoff, France, July 1-7, 1990.

"Rubisco Activity and Biochemistry of the Specificity Factor." Gordon Research Conference on CO_2 Fixation of Green Plants. New London, NH, July 23-27, 1990.

"Structure-Function Relationships of Ribulosebisphosphate Carboxylase/Oxygenase as Suggested by Site-Directed Mutagenesis." Annual Meeting of the American Society of Plant Physiologists. Indianapolis, IN, July 29 - August 2, 1990.

"Structure-Function Relationships of Ribulosebisphosphate Carboxylase/Oxygenase as Suggested by Site-Directed Mutagenesis." Fourth Symposium of the Protein Society. San Diego, CA, August 11-15, 1990.

"The Active Site of Ribulosebisphosphate Carboxylase/Oxygenase." 12th Enzyme Mechanism Conference. San Diego, CA, January 4-6, 1991.

"The Active Site of Ribulosebisphosphate Carboxylase/Oxygenase As Viewed By Site-Directed Mutagenesis." Nobel Symposium on CO_2-Fixation and CO_2-Reduction in Biological and Model Systems. Stockholm, Sweden, December 4-6, 1991.

K. B. Jacobson

"A Multiplex Method for DNA Sequencing Using Stable Isotopes." Genome Sequencing Conference II, Hilton Head, SC, September 30 - October 3, 1990.

"Labeling DNA with Stable Isotopes: Their Potential in Sequencing DNA." DOE Human Genome Program Workshop, Santa Fe, NM, February 17-20, 1991.

S. Mitra

"Cloning of cDNA for Human O^6-Methylguanine-DNA Methyltransferase." Brookhaven Symposia in Biology No. 36, DNA Damage and Repair in Human Tissues, Upton, Long Island, NY, October 1-4, 1989.

"Cloning of Human Genes for Alkylation Damage Repair." 15th International Cancer Congress, Hamburg, Germany, August 16-22, 1990.

"Molecular Cloning of Human O^6-Methylguanine-DNA Methyltransferase Gene." Preuss Foundation Symposium on Glioblastoma, Boston, MA, December 6-8, 1990.

"Expression of Mammalian Alkylation Repair Proteins in *E. coli*." Winter Gordon Conference on Mammalian DNA Repair, Oxnard, CA, February 4-8, 1991.

A. L. Olins

"Electron Microscope Tomography (EMT): Reconstruction Algorithms, Imaging and Mass Loss of Thick Sections." XIIth International Congress for Electron Microscopy, Seattle, WA, August 12-18, 1990.

"Methods for the Analysis of Thick Biological Specimens." Gordon Conference on 3-Dimensional Electron Microscopy of Macromolecules, New Hampton, New Hampshire, June 30 - July 5, 1991.

R. A. Popp

"Repopulation and Long-Term Function in Recipients of Hemopoietic Stem Cell from Normal, Beta-Thalassemic and Transgenic Beta-Thalassemic Mice." Sixth Cooley's Anemia Conference, New York, NY, March 13-15, 1990.

E. M. Rinchik

"Reverse Genetics in the Mouse and Its Application to the Study of Deafness." Genetics of Hearing Impairment Symposium, New York, NY, September 23-26, 1990.

L. B. Russell

"Factors Affecting the Nature of Induced Mutations." Banbury Conference on the Biology of Mammalian Germ Cell Mutagenesis, Cold Spring Harbor, NY, November 12-15, 1989.

"Utility of the Mouse Specific-Locus Test for Analyzing the Genetic and Molecular Nature of Germ-Line Mutations and for Studying Factors that Affect the Yield and Quality of Mutations." Environmental Mutagen Society Annual Meeting, Kissimmee, FL, April 7-11, 1991.

G. A. Sega

"Adducts in Sperm Protamine and DNA vs. Mutation Frequency." International Symposium on Trends in Biological Dosimetry, Lerici, Italy, October 23-27, 1990.

D. M. Skinner

"Molting: A Shell Shucking Experience." Symposium on the Compleat Crab, American Society of Zoologists, San Antonio, TX, December 27-30, 1990.

M. Terzaghi-Howe

"Expression of the Neoplastic Phenotype in Irradiated Rat Trachea is Regulated by Cell Environment." Radiation Research Society Workshop, Fort Collins, CO, January 17-19, 1991.

L. C. Waters

"Studies on the Expression of Insecticide Resistance-Associated Cytochrome P450 in Drosophila Using Cloned DNA." Seventh International Congress of Pesticide Chemistry, Hamburg, Germany, August 5-10, 1990.

"Regulation of Drosophila P450 Genes Involved in Insecticide Resistance." 202nd National Meeting of the American Chemical Society and the 4th Chemical Congress of North America, New York, NY, August 25-30, 1991.

R. P. Woychik

"Insertional Mutagenesis and the Molecular Analysis of Mouse Mutations." Banbury Conference on Mutation Induction and Heritability in Mammalian Germ Cells, Cold Spring Harbor, NY, November, 1989.

"Insertional Mutagenesis and the Molecular Analysis of Mouse Mutations." NIEHS Transgenic Methods Workshop, Research Triangle Park, NC, December, 1990.

"Insertional Mutagenesis and the Molecular Analysis of Mouse Mutations." Workshop on Mouse Development, National Resource Council, National Academy of Sciences, Washington, DC, January, 1991.

"Insertional Mutagenesis and the Molecular Analysis of Mouse Mutations." ICN-UCI Meeting on Models for Human Disease, Newport Beach, CA, March, 1991.

"Insertional Mutagenesis and the Molecular Analysis of Mouse Mutations." NIEHS Workshop on Transgenic Mice, Research Triangle Park, NC, April, 1991.

"Insertional Mutagenesis and the Molecular Analysis of Mouse Mutations." Symposium on Mouse Developmental Biology, Annual FASEB Meeting, Atlanta, GA, April, 1991.

"Insertional Mutagenesis and the Molecular Analysis of Mouse Mutations." Latin American Symposium on Environmental Mutagenesis, Caxambu, Brazil, May, 1991.

"Insertional Mutagenesis and the Molecular Analysis of Mouse Mutations." Wiley-Liss Keynote Symposium, Thirty-First Meeting of the Teratology Society, Boca Raton, FL, June 1991.

"Insertional Mutagenesis and the Molecular Analysis of Mouse Mutations." FASEB Conference on Transgenic Animals, Saxton River, VT, July, 1991.

W. K. Yang

"Tumorigenesis in Transgenic Mice Carrying Reconstructed Murine Endogenous Retroviral Long-Terminal-Repeat Sequences." NIEHS Conference on Transgenic Mice in Developmental Biology and Toxicology, Research Triangle Park, NC, April 1-2, 1991.

Abstracts For Technical Meetings Held
October 1, 1989 - September 30, 1991

Allison, D. P., J. R. Thompson, K. B. Jacobson, R. J. Warmack, W. L. Carrier, F. W. Larimer, R. P. Woychik, and T. L. Ferrell. Scanning tunneling microscopy of DNA. STM'90/NANO I Conference, Baltimore, MD, July 23-27, 1990.

Allison, D. P., J. R. Thompson, K. B. Jacobson, R. J. Warmack, and T. L. Ferrell. Scanning tunneling microscopy of macromolecules. DOE Contractor-Grantee Workshop, Santa Fe, NM, November 3-4, 1989.

Allison, D. P., J. R. Thompson, K. B. Jacobson, R. J. Warmack, and T. L. Ferrell. Scanning tunneling microscopy of macromolecules. XIIth International Congress for Electron Microscopy, Seattle, WA, August 12-18, 1990.

Allison, D. P., R. J. Warmack, and K. B. Jacobson. Scanning tunneling microscopy of nucleic acids. Scanning Microscopy Meeting, Bethesda, MD, May 4-9, 1991.

Arlinghaus, H. F., N. Thonnard, M. T. Spaar, and K. B. Jacobson. Use of RIS to significantly increase the speed of sequencing the human genome. Fifth International Symposium on Resonance Ionization Spectroscopy and Its Applications (RIS-90), Varese, Italy, September, 1990.

Arlinghaus, H. F., N. Thonnard, M. T. Spaar, and K. B. Jacobson. Comparison of sputter-initiated resonance ionization spectroscopy (SIRIS) and laser atomization RIS (LARIS) for trace element analysis in biological matrices. 37th Annual AVS Symposium and Topical Conferences, Toronto, Canada, October 8-12, 1990.

Arlinghaus, H. F., N. Thonnard, M. T. Spaar, A. W. McMahon, and K. B. Jacobson. Applications of resonance ionization spectroscopy for semiconductor, environmental sample and biomedical analysis, and for DNA sequencing. Optical Methods for Ultrasensitive Detection and Analysis: Techniques and Applications, Los Angeles, CA, January 20-25, 1991.

Bast, M. H., and J. S. Cook. Diphenolies inhibit TPA stimulation of A-system transport by inhibition of protein kinase C. 29th Annual Meeting of the American Society for Cell Biology, Houston, TX, November 5-9, 1989.

Blair, P., V. L. Godfrey, and J. E. Wilkinson. Does the scurfy mutation cause a defect in the thymic microenvironment? Federation of American Societies for Experimental Biology, Atlanta, GA, April 21-25, 1991.

Bolch, W. E., J. E. Turner, H. Yoshida, K. B. Jacobson, and R. N. Hamm. Calculated chemical yields from X-irradiation of glycylglycine in oxygen-free aqueous solution. I. Microsecond chemical yields. Radiation Research Society 38th Annual Meeting, New Orleans, LA, April 7-12, 1990.

Brandes, H. M., and F. C. Hartman. Crosslinking agents as active-site probes for phosphoribulokinase (PRK). American Society of Plant Physiologists, Albuquerque, NM, July 28 - August 1, 1991.

Brown, G. M., R. A. Sachleben, K. L. Burchett, K. B. Jacobson, F. W. Larimer, R. P. Woychik, R. S. Foote, F. V. Sloop, D. A. Jacobson, H. F. Arlinghaus, and N. Thonnard. Stable isotope labels for DNA sequencing and mapping. American Chemical Society, Boston, MA, April 22-27, 1990.

Bultman, S. J., W. M. Generoso, L. B. Russell, N. L. A. Cacheiro, and R. P. Woychik. Molecular and genetic characterization of a radiation-induced structural rearrangement in mouse chromosome 2 causing new mutations at the limb deformity (*ld*) and agouti (*a*) loci. Third Annual Meeting on Mouse Molecular Genetics, Cold Spring Harbor, NY, August 29 - September 2, 1990.

Bultman, S. J., L. B. Russell, G. Gutierrez-Espeleta, and R. P. Woychik. Molecular characterization of a region of DNA associated with mutations at the agouti locus in the mouse. NIEHS Conference on Transgenic Mice in Developmental Biology and Toxicology, Research Triangle Park, NC, April 1-2, 1991.

Cacheiro, N. L. A., K. T. Cain, C. V. Cornett, and W. M. Generoso. Chromosomal imbalance in malformed mouse fetuses. Environmental Mutagen Society Annual Meeting, Albuquerque, NM, March 25-29, 1990.

Campion, S. R., and S. K. Niyogi. The hydrophobic nature of epidermal growth factor receptor-ligand interactions. Joint Meeting of the American Society for Biochemistry and Molecular Biology and the American Association of Immunologists, New Orleans, LA, June 3-7, 1990.

Campion, S. R., and S. K. Niyogi. Models for the activation pathway of epidermal growth factor receptor protein-tyrosine kinase. American Society for Biochemists and Molecular Biologists, Atlanta, GA, April 21-25, 1991.

Carrier, W. L., and J. D. Regan. Variables affecting pyrimidine dimer production in DNA and in intact cells. American Society for Photobiology, Vancouver, B.C., Canada, June 16-20, 1990.

Ch'ang, L.-Y., P. R. Hoyt, T. H. Wang, R. Kanagala, D. C. Henley, D. M. Yang, and W. K. Yang. Structural and functional studies of murine Mbo I repeat LTR (MRL) retroviral genes on the Y chromosome. Federation of American Societies for Experimental Biology, Atlanta, GA, April 21-25, 1991.

Collier, J., S. Shinpock, and V. L. Godfrey. Pathogenesis of anemia in scurfy mice. Forty-First Annual Meeting of the American College of Veterinary Pathologists, Phoenix, AZ, December 11-14, 1990.

Coquerelle, T. M., G. Fritz, S. Mitra, and B. Kaina. Expression of the human 0^6-methylguanine-DNA methyltransferase gene in Chinese hamster cells protects them from genotoxic effects of alkylating agents. 9th International Congress of Radiation Research, Toronto, Canada, July 7-12, 1991.

Cosma, G. N., M. Zavada, A. C. Marchok, and S. J. Garte. Role of *ras* mutation in the neoplastic progression of cell lines derived from dimethylbenz[a]anthracene- exposed rat tracheas. American Association for Cancer Research, Houston, TX, May 15-18, 1991.

Curtis, S. B., L. W. Townsend, J. W. Wilson, P. Powers-Risius, E. L. Alpen, and R. J. M. Fry. Fluence-related risk coefficients using the Harderian gland data as an example. Committee on Space Research XXVIII Plenary Meeting and Associated Activities, The Hague, The Netherlands, June 25 - July 6, 1990.

D'Surney, S. J., and R. A. Popp. The nucleotide sequences of the two adult alpha globin genes of the Skive (*M. musculus musculus*) mouse. Joint Meeting of the American Society for Biochemistry and Molecular Biology and the American Association of Immunologists, New Orleans, LA, June 3-7, 1990.

Flaherty, L., E. M. Rinchik, A. Messer, and L. B. Russell. Chlorambucil induces viable recessive mutations. Fourth International Workshop on Mouse Genome Mapping, Annapolis, MD, November 4-8, 1990.

Ford, J. R., and M. Terzaghi-Howe. Transformation frequencies for alpha-particle exposed rat tracheas and isolated tracheal epithelial cells. Radiation Research Society - 38th Annual Meeting, North American Hyperthermia Group - 10th Annual Meeting, New Orleans, LA, April 7-12, 1990.

Fry, R. J. M. The effects of space radiation. Workshop on Correlations of Aging and Space Effects on Biosystems, Chantilly, VA, October 30 - November 1, 1989.

Fry, R. J. M. Time-dose relationships on high-LET radiation. Committee on Effects of Ionizing Radiation. Forum on the Effects of High-LET Radiation at Low Doses/Dose Rates, London, England, December 14, 1989.

Fry, R. J. M. Space radiation and health effects. European Symposium on Life Sciences Research, Trieste, Italy, May 29 - June 1, 1990.

Fry, R. J. M. Review of Q for high-LET radiation. Committee on Space Research XXVIII Plenary Meeting and Associated Activities, The Hague, The Netherlands, June 25 -July 6, 1990.

Fry, R. J. M. Extrapolation of risk estimates across species. Workshop on Oncogenic Mechanisms in Radiation-Induced Cancer, Fort Collins, CO, January 16-19, 1991.

Fry, R. J. M. Radiation effects in space: Research needs. 9th International Congress of Radiation Research, Toronto, Canada, July 7-12, 1991.

Fry, R. J. M. Radiation environments in space and associated health effects. Health Physics Society Meeting, Washington, DC, July 21, 1991.

Fry, R. J. M., and J. E. Turner. Neutron carcinogenesis: What do we know and what do we need to know? Armed Forces Radiobiology Research Institute Colloquium on Neutron Radiation Biology, Rockville, MD, November 5-7, 1990.

Fujimura, R. K., and W. M. Generoso. Chemical mutagen induced heritable retroviral integration in mice. Third Annual Meeting on Mouse Molecular Genetics, Cold Spring Harbor, NY, August 29 - September 2, 1990.

Gallager, J. F., D. M. Popp, and R. A. Popp. Isolation and characterization of an infectious ecotropic retrovirus from B10.F mice. Joint Meeting of the American Society for Biochemistry and Molecular Biology and the American Association of Immunologists, New Orleans, LA, June 3-7, 1990.

Galloway, P. J., and J. S. Cook. Distinct genes for Na^+-independent glucose transport in LLC-PK_1 cells. American Society for Cell Biology, San Diego, CA, December 9-13, 1990.

Godfrey, V. L., J. Collier, and J. E. Wilkinson. Thymus transplant transmission of scurfy mouse lymphoreticular disease is H-2 restricted. Federation of American Societies for Experimental Biology, Atlanta, GA, April 21-25, 1991.

Godfrey, V. L., J. E. Wilkinson, and L. B. Russell. The *scurfy* mouse: Potential model for thymic education. Joint Meeting of the American Society for Biochemistry and Molecular Biology and the American Association of Immunologists, New Orleans, LA, June 3-7, 1990.

Gomez-Espi, M., P. Olivares, R. Herranz, L. G. Littlefield, R. J. Preston, and M. Orera. Report of a whole-body accidental overexposure to ionizing radiation. 9th International Congress of Radiation Research, Toronto, Canada, July 7-12, 1991.

Greenfield, N. J., D. A. Engler, R. K. Matsunami, S. R. Campion, S. K. Niyogi, D. Rubenstein, E. Arnold, and G. T. Montelione. NMR studies of single-site mutants of human epidermal growth factor (hEGF). Annual Meeting of the Biophysical Society, San Francisco, CA, February 24-28, 1991.

Greer, G. A., and R. J. Preston. X-ray sensitive cells (ara-C resistant) are also hypersensitive to chromosome aberration induction by restriction enzymes. Environmental Mutagen Society, Albuquerque, NM, March 25-29, 1990.

Guan, X., E. Uberbacher, and R. Mural. Searching consensus patterns on hypercube. 6th Distributed Memory Computing Conference, Portland, OR, April 28 - May 2, 1991.

Hamm, R. N., J. E. Turner, T. D. Jones, and R. J. Preston. Calculations of DNA damage from photon and neutron irradiation. 9th International Congress of Radiation Research, Toronto, Canada, July 7-12, 1991.

Harpel, M. R., and F. C. Hartman. Chemical rescue of a catalytically-deficient mutant of *R. rubrum* ribulose bisphosphate (RuBP) carboxylase/oxygenase. Fourth Chemical Congress of North America, New York, NY (American Chemical Society), August 25-30, 1991.

Harpel, M. R., F. W. Larimer, and F. C. Hartman. Functionality of S368 and H287 of *R. rubrum* ribulose bisphosphate carboxylase/oxygenase. Fifth Symposium of the Protein Society, Baltimore, MD, June 22-26, 1991.

Harpel, M. R., F. W. Larimer, R. J. Mural, and F. C. Hartman. Evaluation of the roles of H321 and S368 of *R. rubrum* ribulose bisphosphate carboxylase/oxygenase by site-directed mutagenesis. Federation of American Societies for Experimental Biology, Atlanta, GA, April 21-25, 1991.

Hartman, F. C. Site-directed mutagenesis without benefit of crystallographic data: Boon or boondoggle? The 1989 International Chemical Congress of Pacific Basin Societies, Honolulu, HI, December 17-22, 1989.

Hartman, F. C., F. W. Larimer, E. H. Lee, R. J. Mural, H. B. Smith, and T. S. Soper. Structure-function relationships of ribulosebisphosphate carboxylase/oxygenase as suggested by site-directed mutagenesis. American Society of Plant Physiologists Annual Meeting, Indianapolis, IN, July 29 - August 3, 1990.

Hartman, F. C., F. W. Larimer, E. H. Lee, R. J. Mural, H. B. Smith, and T. S. Soper. Structure-function relationships of ribulosebisphosphate carboxylase/oxygenase as suggested by site-directed mutagenesis. Fourth Symposium of the Protein Society, San Diego, CA, August 11-15, 1990.

Hartman, F. C., and E. H. Lee. Assessment of the terminal protonation step catalyzed by a site-directed mutant of *R. rubrum* ribulosebisphosphate carboxylase/oxygenase. Federation of American Societies for Experimental Biology, Atlanta, GA, April 21-25, 1991.

Hauser, L., and D. E. Olins. 70kD heat shock gene family from *Euplotes eurystomus*: Unique gene structure and regulation. 29th Annual Meeting of the American Society for Cell Biology, Houston, TX, November 5-9, 1989.

Hauser, M. R., S. R. Campion, D. A. Engler, R. K. Matsunami, S. K. Niyogi, and J. S. Cook. Responses of A31 cells to site-specific mutants of human EGF. Federation of American Societies for Experimental Biology, Washington, DC, April 1-5, 1990.

Hauser, M. R., S. R. Campion, D. A. Engler, S. K. Niyogi, and J. S. Cook. Mitogenic responses of A31 cells to human EGF mutants derived by site-specific mutagenesis. 29th Annual Meeting of the American Society for Cell Biology, Houston, TX, November 5-9, 1989.

Hauser, M. R., D. A. Engler, S. K. Niyogi, and J. S. Cook. Aromaticity at position 37 in human EGF is not obligatory for activity. Federation of American Societies for Experimental Biology, Atlanta, GA, April 21-25, 1991.

Herman, A., A. Jaberaboansari, and S. Mitra. Comparison of X-ray-induced mutations in bacterial and mammalian cells. Eureka, The Fifth National Conference on Undergraduate Research, Pasadena, CA, March 21-23, 1991.

Hettich, R. L., and K. B. Jacobson. Laser desorption FTMS for the structural characterization of underivatized pteridines. American Society for Mass Spectrometry, Conference on Mass Spectrometry and Allied Topics, Tucson, AZ, June 2-8, 1990.

Horesovsky, G. J., and R. J. Preston. Molecular analysis of restriction enzyme-induced HPRT mutants. 22nd Annual Meeting of the Environmental Mutagen Society, Kissimmee, FL, April 6-11, 1991.

Hoyt, P. R., T. H. Wang, L. Y. Ch'ang, D. C. Henley, D. M. Yang, and W. K. Yang. Transcriptional regulatory mechanisms of murine chromosomal polytropic class retroviral genes in response to CCL_4 treatment in the mouse. American Association for Cancer Research, Houston, TX, May 15-18, 1991.

Isham, K. R., C. Cadilla, K.-L. Lee, and F. T. Kenney. Insulin increases gene 33 expression through cis-acting elements in 5'-flanking DNA. Joint Meeting of the American Society for Biochemistry and Molecular Biology and the American Association of Immunologists, New Orleans, LA, June 3-7, 1990.

Jaberaboansari, A., W. C. Dunn, R. J. Preston, S. Mitra, and L. C. Waters. A comparative analysis of mutations induced by low and high LET radiation in a human shuttle plasmid. Radiation Research Society - 38th Annual Meeting, New Orleans, LA, April 7-12, 1990.

Jaberaboansari, A., W. C. Dunn, L. C. Waters, R. J. Preston, and S. Mitra. A comparative analysis of point mutations induced after X-irradiation of a human shuttle plasmid under different free radical-scavenging conditions. 9th International Congress of Radiation Research, Toronto, Canada, July 7-12, 1991.

Jaberaboansari, A., A. M. Herman, and S. Mitra. X ray-induced point mutations in a plasmid replicated in *Escherichia coli* and human lymphoblasts: Is there a common denominator? 9th International Congress of Radiation Research, Toronto, Canada, July 7-12, 1991.

Jaberaboansari, A., S. Mitra, R. J. Preston, and L. C. Waters. Molecular analysis of ionizing radiation-induced mutations in a human shuttle plasmid. UCLA Symposium on Ionizing Radiation Damage to DNA: Molecular Aspects, Lake Tahoe, CA, January 16-21, 1990.

Jackson, I. J., D. Chambers, E. M. Rinchik, and D. Bennett. The mouse *b*-locus encodes a tyrosinase-related protein: Structure and expression of mutant alleles. Pan American Society for Pigment Cell Research, Bethesda, MD, April 23-26, 1990.

Jacobson, K. B. Uses of stable isotopes for DNA sequencing. DOE Contractor-Grantee Workshop, Sante Fe, NM, November 3-4, 1989.

Jacobson, K. B. Labeling DNA with stable isotopes: Their potential in sequencing DNA. DOE Human Genome Program Workshop, Santa Fe, NM, February 17-20, 1991.

Jacobson, K. B., F. W. Larimer, R. P. Woychik, R. S. Foote, G. M. Brown, K. L. Burchett, D. A. Jacobson, R. A. Sachleben, F. V. Sloop, H. F. Arlinghaus, and N. Thonnard. Use of stable isotopes in DNA sequencing. Human Genome I Conference, San Diego, CA, October 2-4, 1989.

Jacobson, K. B., F. W. Larimer, R. P. Woychik, R. S. Foote, G. M. Brown, K. L. Burchett, D. A. Jacobson, R. A. Sachleben, F. V. Sloop, H. F. Arlinghaus, and N. Thonnard. Sequencing DNA with stable isotopes. Genome Sequencing Conference, Rockville, MD, October 24-26, 1989.

Jacobson, K. B., H. F. Arlinghaus, N. Thonnard, R. S. Foote, F. W. Larimer, R. P. Woychik, R. A. Sachleben, G. M. Brown, F. V. Sloop, and M. W. England. A multiplex method for DNA sequencing using stable isotopes. Genome Sequencing Conference II, Hilton Head Island, SC, September 30 - October 3, 1990.

Jones, J., J. Peters, S. E. Lewis, and R. A. Popp. The molecular analysis of four mutations in globin genes induced by ethylnitrosourea. Cold Spring Harbor Symposium, Cold Spring Harbor, NY, August 29 - September 2, 1990.

Kalinowski, D., K. M. Mottus, M. J. Plewa, and F. W. Larimer. Molecular spectrum analysis of frameshift mutations in yeast. Fifth International Conference on Environmental Mutagens, Cleveland, OH, July 10-15, 1989.

Klebig, M. L., B. S. Kwon, R. Machanoff, and E. M. Rinchik. Molecular access to mouse chromosome-7 regions containing the HSDR-1 gene and gene(s) important for development in the implantation embryo. Third Annual Meeting on Mouse Molecular Genetics, Cold Spring Harbor, NY, August 29-September 2, 1990.

Kleinhans, F. W., J. Du, V. J. Spitzer, L. Horstman, P. Mazur, and J. K. Critser. ESR determined osmotic behavior of bull spermatozoa. Second International Conference on Boar Semen Preservation, Beltsville, MD, August 19-22, 1990.

Kumari, S., J. J. O'Brien, and D. M. Skinner. Epitope sharing among cuticular proteins of a crustacean *Gecarcinus lateralis* and two insects *Manduca sexta* and *Drosophila*. American Society of Zoologists, San Antonio, TX, December 27-30, 1990.

Larimer, F. W., and R. J. Mural. Megabase cloning in *E. coli*. Genome Mapping and Sequencing, Cold Spring Harbor, NY, May 2-6, 1990.

Larimer, F. W., and T. S. Soper. GroE heat shock protein is required for *in vivo* assembly of recombinant *Anabaena* ribulose bisphosphate ($Ru\text{-}P_2$) carboxylase/oxygenase. Federation of American Societies for Experimental Biology, Atlanta, GA, April 21-25, 1991.

Lee, E. H., and F. C. Hartman. Assessment of the terminal protonation step catalyzed by a site-directed mutant of *R. rubrum* ribulosebisphosphate carboxylase/oxygenase. Federation of American Societies for Experimental Biology, Atlanta, GA, April 21-25, 1991.

Lee, E. H., G. Schneider, C. -I. Braden, R. J. Mural, and F. C. Hartman. Significance of a *Cis* peptide bond at the active site of ribulosebisphosphate carboxylase/oxygenase. Fourth Symposium of the Protein Society, San Diego, CA, August 11-15, 1990.

Lee, K.-L., D.-M. Yang, A. Makkinje, and L.-Y. Ch'ang. Tyrosine aminotransferase mRNA turnover in CHO cells. American Society for Biochemists and Molecular Biologists, Atlanta, GA, April 21-25, 1991.

Lee-Tischler, M. J., and R. P. Woychik. Construction of an international database for transgenic mice. NIEHS Conference on Transgenic Mice in Developmental Biology and Toxicology, Research Triangle Park, NC, April 1-2, 1991.

Mazur, P. Equilibrium and non-equilibrium freezing of mammalian embryos. Annual Meeting of the Society for Cryobiology, Binghamton, NY, June 16-24, 1990.

Mazur, P. Kinetics of the killing of unfrozen intact Drosophila embryos at low temperatures. Annual Meeting of the Society for Cryobiology, Binghamton, NY, June 16-24, 1990.

Mazur, P., K. W. Cole, and A. P. Mahowald. Critical role of alcohol in the permeabilization of 12-hr Drosophila embryos by alkanes. Annual Meeting of the Society for Cryobiology, Leuven, Belgium, July 7-12, 1991.

Mazur, P., K. W. Cole, P. D. Schreuders, and A. P. Mahowald. Confirmation of the ability of permeabilization 12-hr Drosophila embryos to survive cooling to -200°C. Annual Meeting of the Society for Cryobiology, Leuven, Belgium, July 7-12, 1991.

Mazur, P., and J. W. Hall. Kinetics of ice nucleation of fully hydrated and partially dehydrated 8-cell mouse embryos at -15°, -20° and -25°. Annual Meeting of the Society for Cryobiology, Binghamton, NY, June 16-24, 1990.

Milanez, S., and R. J. Mural. Expression of spinach phosphoribulokinase in *Escherichia coli*. Joint Meeting of the American Society for Biochemistry and Molecular Biology and the American Association of Immunologists, New Orleans, LA, June 3-7, 1990.

Milanez, S., R. J. Mural, and F. C. Hartman. Examination of the roles of the cysteinyl residues of spinach phosphoribulokinase by site-directed mutagenesis. Federation of American Societies for Experimental Biology, Atlanta, GA, April 21-25, 1991.

Mitra, S. Cloning of human genes for alkylation damage repair. 15th International Cancer Congress, Hamburg, Germany, August 16-22, 1990.

Moyer, J. H., M. Lee-Tischler, V. L. Godfrey, P. B. Selby, M. E. Dickinson, B. L. M. Hogan, and R. P. Woychik. Characterization of a complex, pleiotropic mutation caused by insertional mutagenesis in one line of transgenic mice. NIEHS Conference on Transgenic Mice in Developmental Biology and Toxicology, Research Triangle Park, NC, April 1-2, 1991.

Mural, R. J., R. Einstein, X. Guan, R. Hand, R. Mann, and E. Uberbacher. An integrated intelligent system for DNA sequence pattern analysis and interpretation. Genome Mapping and Sequencing, Cold Spring Harbor, NY, May 8-12, 1991.

Mural, R. J., E. Uberbacher. Pattern recognition in DNA sequences: The intron-exon junction problem. Electrophoresis, Supercomputing, and Human Genome, Tallahassee, FL, April 10-13, 1990.

Noiles, E. E., N. A. Ruffing, F. W. Kleinhans, L. A. Mark, L. Horstman, P. Mazur, and J. K. Critser. Critical tonicity determination of mammalian sperm using dual fluorescent staining and flow cytometry. Second International Conference on Boar Semen Preservation, Beltsville, MD, August 19-22, 1990.

O'Brien, J. J., and D. M. Skinner. Proteins and proteinases in the exoskeleton of the land crab, *Gecarcinus lateralis*. American Society of Zoologists, Boston, MA, December 27-30, 1989.

O'Brien, J. J., S. S. Kumari, and D. M. Skinner. Characteristics of exoskeletal layers of the land crab, *Gecarcinus lateralis*. American Society of Zoologists, San Antonio, TX, December 27-30, 1990.

Olins, A. L., H. A. Levy, M. B. Shah, G. G. Lamprecht, and D. E. Olins. Electron microscope tomography (EMT): Reconstruction algorithms, imaging and mass loss of thick sections. XIIth International Congress for Electron Microscopy, Seattle, WA, August 12-18, 1990.

Popp, D. M., J. Gallager, and R. A. Popp. Retroviral insertion and rearrangement of IG and TCR genes in B10.F mice. Joint Meeting of the American Society for Biochemistry and Molecular Biology and the American Association of Immunologists, New Orleans, LA, June 3-7, 1990.

Popp, R. A., and S. J. D'Surney. A mouse model of sickle cell anemia. Joint Meeting of the American Society for Biochemistry and Molecular Biology and the American Association of Immunologists, New Orleans, LA, June 3-7, 1990.

Popp, R. A., and E. Rubin. Development of mice with high-oxygen-affinity hemoglobins that might enable HBS to sickle *in vivo*. Seventh Hemoglobin Switching Conference, Airlie, VA, September 8-11, 1990.

Popp, R. A., S. G. Shinpock, and D. M. Popp. Immunodeficiency of β-thalassemic mice. Sixth Cooley's Anemia Conference, New York, NY, March 13-15, 1990.

Popp, R. A., S. G. Shinpock, and D. M. Popp. Repopulation and long-term function in recipients of hemopoietic stem cell from normal, beta-thalassemic and transgenic beta-thalassemic mice. Sixth Cooley's Anemia Conference, New York, NY, March 13-15, 1990.

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Yang, W. K., P. R. Hoyt, T. H. Wang, L.-Y. Ch'ang, D. M. Yang, T. J. Savin, and H. I. Saavedra. Tumorigenesis in transgenic mice carrying reconstructed murine endogenous retroviral long-terminal-repeat sequences. NIEHS Conference on Transgenic Mice in Developmental Biology and Toxicology, Research Triangle Park, NC, April 1-2, 1991.

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Yoshida, H., R. L. Hettich, J. A. LaVerne, K. B. Jacobson, and J. E. Turner. Characterization of radiation-induced products in thymidine 3'-monophosphate by laser desorption FTMS. 9th International Congress of Radiation Research, Toronto, Canada, July 7-12, 1991.

Zabel, V., G. J. Bunick, P. A. Vanderhoff, E. C. Uberbacher, and D. Voet. Structure of yeast inorganic pyrophosphatase: Refinement. Federation of American Societies for Experimental Biology, Atlanta, GA, April 21-25, 1991.

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
P. Mazur	Fundamental Cryobiology of Bull and Ram Spermatozoa (USDA)	10/89 — 08/91
S. Mitra	DNA Repair (NIH)	09/82 — 07/96
	Structure and Regulation of DNA Repair (NIH)	02/91 — 01/96
S. K. Niyogi	Human Epidermal Growth Factor — Site Directed Mutagenesis (NIH)	08/89 — 07/92
A. L. Olins	Chromatin Structure (NIH)	11/90 — 10/93
D. E. Olins	Physical Properties of Isolated Nuclei and Chromosomes (NSF)	09/90 — 08/91
	Development of Electron Microscope Tomography (NSF)	05/91 — 10/93
	Structure and Function of the Replication Band (ACS)	01/89 — 12/91
R. A. Popp	Cloned Human Beta-Globin Gene (NIH)	07/86 — 06/91
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 — 09/92
A. L. Stevens	Yeast Enzymes (NIH)	08/88 — 07/91
M. Terzaghi-Howe	Cell Interactions: Expression of Preneoplastic Markers (NIH)	05/83 — 06/96
L. G. Waters	Molecular Biology Insecticide Resistance (USDA)	09/88 — 09/90

Grants (continued)

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 — 07/92
	Mutagen-Induced Fetal Anomalies Subsequent to Exposure of Mouse Zygotes (EPA)	03/89 — 03/92
P. Mazur	Establishment of a Bank of Frozen Embryos (NIH)	10/85 — 07/91
	Fundamental Cryobiology of Human Sperm (NIH)	09/89 — 09/92
	Frozen Embryos (NSF)	04/87 — 09/91
S. Mitra	Mechanism of Alkylation Mutagenesis (EPA)	12/89 — 12/91
R. A. Popp	Mutant Hemoglobins That Allow RBS to Sickle (NIH)	07/89 — 04/94
J. D. Regan	Oceanic Measurements (Caribbean Marine Research Center)	01/91 — 06/91
L. B. Russell/ R. P. Woychik	Chemical Mutagenicity Studies in Mice (NIEHS)	05/81 — 06/95
W. K. Yang	Mouse Endogenous Retroviral LTR Elements (NIEHS)	01/84 — 05/93

Financial Summary and Personnel Distribution

Total Biology Division
FY 1990

Funding Source	Funding in Thousands	Percent of Total Budget	Scientific/ Technical Person-Years*
Department of Energy	10,416	74.6	52.0
National Cancer Institute	598	4.3	3.5
National Heart, Lung and Blood Institute	109	0.8	0.8
National Institute of Child Health and Human Development	138	1.0	0.3
National Institute of Environmental Health Sciences	1,657	11.9	8.5
National Institute of General Medical Sciences	304	2.2	2.5
Department of Agriculture	117	0.8	0.8
Environmental Protection Agency	94	0.7	0.1
University of Tennessee	366	2.6	2.2
Oregon Health Sciences University	36	0.3	0.4
Methodist Hospital of Indiana	36	0.3	0.3
Department of Defense	94	0.7	0.9
	13,965	100.0	72.3

*Does not include ~58.0 person years: 49.5 PY Distributed - Administration & clerical, animal caretakers, histology, and kitchen; 8.5 PY supported by other divisions and ORNL seed money.

Financial Summary and Personnel Distribution

Total Biology Division
FY 1991

Funding Source	Funding in Thousands	Percent of Total Budget	Scientific/ Technical Person-Years*
Department of Energy	10,650	70.3	43.0
National Institute of Human Genome Research	240	1.6	1.2
National Cancer Institute	680	4.5	2.4
National Heart, Lung and Blood Institute	99	0.7	0.7
National Institute on Aging	92	0.6	0.1
National Institute of Child Health and Human Development	350	2.3	1.8
National Institute of Environmental Health Sciences	2,050	13.5	8.0
National Institute of General Medical Sciences	104	0.7	1.0
Department of Agriculture	150	1.0	0.2
Environmental Protection Agency	91	0.6	0.1
University of Tennessee	387	2.6	2.0
Oregon Health Sciences University	41	0.3	0.4
Methodist Hospital of Indiana	89	0.6	0.5
Department of Defense	75	0.5	0.5
Caribbean Marine Research Center	52	0.3	0.4
	15,150	100.0	62.3

*Does not include ~52.5 person years: 48.0 PY Distributed - Administration & clerical, animal caretakers, histology, and kitchen; 4.5 PY supported by other divisions and ORNL seed money.

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