

Genetics

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Unlike many toxicity endpoints, the deleterious effects of mutations are difficult or impossible to relieve and will persist into subsequent generations when mutations arise in germ cells. Thus, mutation prevention is a more hopeful strategy. To this end, our research is aimed at uncovering the mechanisms by which organisms generate mutations. Specifically, we use the bacterium *Escherichia coli* as a model system for a thorough dissection of the pathways of mutation production and mutation prevention.

For example, mutator mutants (with a higher mutation rate than the wild-type strain) provide detailed insights into mechanisms that organisms use to prevent mutations. These mechanisms include base selection by the DNA polymerase, exonucleolytic proofreading, and DNA mismatch correction, as well as numerous DNA repair systems not directly linked to DNA replication.

On the other hand, antimutator mutants (with a lower mutation rate than the wild-type) provide insights into the origins of mutations that normally escape correction, notably the background of spontaneous mutations. We are searching for antimutators that act through well defined pathways in order to understand the precise factors responsible for spontaneous mutations. As a first step, we have isolated antimutator strains that replicate their DNA with increased accuracy and we are using these strains to determine what fraction of spontaneous mutations is due to uncorrected replication errors.

Relevant Publications:

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The six billion nucleotides of the diploid human genome are replicated in only a few hours in a process generating so few errors that the spontaneous mutation rate may be much less than 1 mutation per genome per cell division. Three steps are responsible for this high replication fidelity: base selectivity and exonucleolytic proofreading of errors during DNA polymerization, and correction of errors afterwards.

The research in this laboratory is intended to further our understanding of these processes and how their failure or perturbation yields mutations. Our primary experimental approach is to study the fidelity of DNA synthesis *in vitro*. This includes analyzing reactions in which single-stranded DNA is replicated by DNA polymerases, especially those that replicate eukaryotic chromosomal DNA or the HIV-1 genome. Of particular interest is the relationship between replication fidelity and the structure of DNA polymerases as determined by X-ray crystallography. We are also investigating the fidelity of replication of double-stranded DNA catalyzed by the multiprotein replication apparatus of human cells, using either undamaged DNA or substrates containing adducts of known carcinogens.

Finally, we are examining the mechanisms and gene products that correct single-base mispairs and loops resulting from strand misalignments. Failure to perform mismatch repair accurately and efficiently leads to cell death and to various forms of genome instability, the consequences of which include cancer, heart disease, heritable birth defects, and perhaps aging.

Relevant Publications:

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Bebenek, K., Beard, W.A., Darden, T.A., Li, L., Prasad, R., Luxon, B.A., Gorenstein, D.G., Wilson, S.H. and Kunkel, T.A. (1997) A minor groove binding track in reverse transcriptase. *Nature Struct. Biol.* 4:194-197.

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We investigate the genetics and enzymology of DNA repair and mutation in the classical model system bacteriophage T4 which, together with *Escherichia coli*, has yielded most of our understanding of the molecular basis of mutation. We also investigate global aspects of spontaneous mutation rates.

The main determinant of the T4 mutation rate is its DNA polymerase and associated proofreading exonuclease. This enzyme maintains replication fidelity largely independently of its interactions with the other accessory proteins and enzymes of DNA replication. Using both T4 and the related phage RB69, we are probing the relationships between polymerase structure and replication fidelity both *in vivo* and *in vitro*.

Nonlethal mutations in genes encoding certain enzymes of DNA replication render T4 generally sensitive to DNA damage. We are investigating this "replication repair" process using both genetic and enzymological approaches.

Rates of spontaneous mutation fall into a few distinct categories that probably represent evolutionary balances between the deleterious consequences of most mutations and the investments required to further reduce mutation rates. All DNA-based microbes examined produce about one mutation per 300 chromosome replications. Higher eukaryotes have the same or a slightly higher rate, so that rates per sexual generation are close to the maximum compatible with life, about one mutation per gamete. RNA viruses have mutation rates of about one per genome per chromosome replication, and small increases in their mutation rates are lethal.

Relevant Publications:

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Smith, L.A., and Drake, J. W. (1998) Aspects of the ultraviolet photobiology of some T-even bacteriophages. *Genetics* 148:1611-1618.

Dressman, H.K., Wang, C.C., Karam, J.D., and Drake, J.W. (1997) Retention of replication fidelity by a DNA polymerase functioning in a distantly related environment. *Proc. Natl. Acad. Sci. USA* .94:8042-8046.

Epidemiology

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Environmental toxins are known to cause infertility, fetal loss and malformations in laboratory animals, but these effects have not been well studied in humans. Dr. Wilcox's work has been to extend the study of environmental exposures into the area of human reproduction.

Dr. Wilcox's work falls into three areas. The first is conception and early pregnancy. Dr. Wilcox and his research group have shown that at least one-quarter of human pregnancies end in loss before the woman is aware she is pregnant (1988). Their methods for measuring early pregnancy loss have been widely adapted by environmental researchers searching for subtle effects of reproductive toxins. They were the also the first to use biochemical markers of ovulation to determine a woman's fertile window. Among healthy women trying to conceive, there are an average of six fertile days in each menstrual cycle, ending on day of ovulation (1995a). Further, in pursuing causes of fertility and infertility they have shown that men prenatally exposed to high doses of estrogen (diethylstilbestrol) are not impaired in their own fertility (1995b).

His second area of interest is birthweight. Low birthweight is a convenient endpoint in studies of environmental toxins. However, low birthweight may not be on the causal pathway to perinatal risk, as most people assume. Dr. Wilcox developed this idea in a series of papers suggesting an alternative approach to the analysis of birthweight. He and his colleagues recently showed that the relatively high rate of infant mortality in the US compared to Norway is not due to the smaller size of US infants, but to the higher rates of preterm delivery in the US -- a public health problem that is only recently being recognized (1995c).

Wilcox's third area of research is the role of genetic susceptibility to environmental teratogens. Both genetic and environmental factors contribute to the risk of birth defects (1994). He and his colleagues have begun a case-control study of facial clefts in which they will test the hypothesis that the A2 allele of TGF-alpha (transforming growth factor) represents a susceptible genotype for the teratogenic effects of maternal smoking and perhaps other toxicants. A second hypothesis is that folic acid deficiency increases the risk of facial clefts, particularly within a genetically susceptible subgroup defined by an allele of the gene controlling the enzyme MTHFR.

Relevant Publications:

Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, Schlatterer JP, Canfield RE, Armstrong EG, Nisula BC: Incidence of early loss of pregnancy. *New Engl J Med* 319:189-94, 1988.

Lie RT, Wilcox AJ, Skjaerven R: A population-based study of risk of recurrence of birth defects. *New Engl J Med* 331:1-4; 1994.

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Dr. Sandler's research focuses on environmental causes of chronic disease in adults, in particular, studies of risk factors for leukemia and myelodysplasia, health effects of residential and occupational exposure to radon, and the health consequences of exposure to agricultural chemicals.

The failure of previous studies to identify strong risk factors for leukemia may be due, in part, to poor characterization of leukemia subtypes. The leukemias are heterogeneous with regard to tumor biology and prognosis and this heterogeneity may extend to risk factors. Dr. Sandler's studies have explored the role of clonal chromosome abnormalities detected in the bone marrow of patients, oncogenes, and polymorphisms in genes that affect the metabolism of potential carcinogens. They recently showed that persons with the null genotype for glutathione-S-transferase theta (GSTT1) are at greatly increased risk for myelodysplasia and that leukemia patients with ras-gene mutations are more likely to have been exposed to solvents. They further demonstrated links between smoking and other exposures and specific chromosome abnormalities in AML and ALL, although differences were not as great as expected. They also demonstrated that myelodysplasia may be a marker for chemical exposure in myeloid leukemia and are developing plans for a larger study to address this possibility and expand the ability to evaluate interactions between environmental exposures and genetic susceptibility.

The radon study was motivated by widespread interest in the possibility that indoor radon exposure is a major cause of lung cancer in the US. The study, based in Utah and Connecticut, involves 1,474 lung cancer patients and 1,811 population controls for whom were obtained detailed exposure histories and measured radon levels in current and past homes. Data collection is complete, and evaluation of statistical methods for estimating lifetime exposure in the face of inevitable missing data (some past homes could not be measured) and identifying factors that predict radon levels in homes is underway.

Another study is of cancer incidence in a cohort of 18,000 Czech uranium miners who are exposed to radon. Preliminary results suggest increased risk for cancers in addition to lung cancer, including cancer of the larynx, gastric cancer, and leukemia. On the other hand, in two small studies of childhood cancer we find no risk associated with either indoor exposure or parental occupational exposure to radon.

Finally, Dr. Sandler has been collaborating with the NCI and EPA on a prospective study of cancer risk in a cohort of nearly 75,000 licensed pesticide applicators and spouses. In this study, Dr. Sandler's efforts are focused on non-cancer outcomes including reproductive health, respiratory disease, immune function, and kidney disease. An important feature of this study is the comprehensive exposure and biologic monitoring for a sample of the cohort and the opportunity for long-term follow-up of the cohort.

Relevant Publications:

Sandler DP, Smith JC, Weinberg CR, Buckalew VM, Dennis V, Blythe W, Burgess WP: Analgesic use and chronic renal disease. *New Engl J Med* 320: 1238-43, 1989.

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The development of most cancers and chronic diseases is a multifactorial process involving issues of susceptibility (from genetic or nutritional factors) and environmental exposures. Males and females may respond differently to some environmental exposures, and this variability may reflect hormonal influences on disease susceptibility and etiology. Dr. Cooper's research has focuses on these complex interactions.

Ovarian function has direct and indirect consequences for women's health since, in women, the production of estrogen and progesterone is controlled by the ovary. Menopause (or "ovarian failure") represents a normal aspect of aging, but it also influences risk for a wide variety of diseases. Understanding the factors that influence follicular atresia may provide insights into apoptosis, the aging process, and disease risk. Osteoporosis and some autoimmune conditions are examples of hormonally-mediated diseases which occur in either gender, but are more common in women.

Ovarian function may be viewed as an outcome in itself, and as a factor that interacts with a broad array of environmental exposures and genetic characteristics to influence the development of chronic diseases. This broad view of the multiple factors involved in disease pathogenesis is necessary in order to fully understand the mechanisms through which hormones affect disease risk, and to develop approaches to decrease the incidence of diseases which differentially affect women. Exposures that either mimic or modulate hormones are particularly relevant to the mission of NIEHS, and Dr. Cooper's work incorporates this interest.

Relevant Publications:

Cooper GS, Hulka BS, Baird DD, Savitz DA, Hughes CL, Weinberg CR, Coleman RA, Shields JM. Galactose consumption, metabolism, and gonadotropin levels in women of late reproductive age. *Fertil Steril* 62:1168-75; 1994.

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Neurosciences

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Neuronal communication between cells in the nervous system occurs at the synapse, where the release of neurotransmitter (NT) (e.g. serotonin [5-HT], acetylcholine [ACh], glutamate, or GABA) by the presynaptic terminal diffuses across the synaptic cleft and binds to and activates various ligand-gated ion channels on the postsynaptic membrane. Therefore ligand-gated ion channels mediate rapid (e.g. on the order of milliseconds) synaptic transmission, and changes in the function of these channels will have profound effects on neuronal excitability. As these channels are the targets of various environmental toxins and signal transduction cascades, which can regulate their function on a much longer time scale (e.g. seconds, minutes, or even hours), these pathways can regulate to a large extent synaptic efficacy (i.e. the regulation of the strength of the synaptic connections between neurons) and plasticity. To better understand the basic mechanisms and regulation of synaptic transmission in the CNS, the lab is focusing on the function and regulation of the ligand-gated ion channels gated by acetylcholine (ACh; this is referred to as the nicotinic receptor as it is activated by nicotine) and serotonin (i.e., the 5-HT₃ receptor) in the hippocampus. Hippocampal interneurons contain functional somato-dendritic nicotinic and 5-HT₃ receptors, both receptors of which may be involved in cognitive processes. Hippocampal interneurons are inhibitory as they are known to release the inhibitory neurotransmitter GABA. Although there are many fewer interneurons than the numbers of principal excitatory cells, a single interneuron can innervate and regulate the activity of hundreds of excitatory cells in the hippocampus. Although the nicotinic and 5-HT₃ receptor channels are known to be involved in a variety of physiological processes, the precise nature of these actions are not currently known, and is the major focus of investigation in the lab.

Relevant Publications:

Kriegler, S., Sudweeks, S, & Yakel, J.L. (1999) MTSEA potentiates 5-HT₃ receptors containing the nicotinic $\alpha 4$ subunit. *Neuropharmacology*. In Press.

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Pathology

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Research interests have focused on the biology and pathogenesis of rodent hepatic and pulmonary toxicity and carcinogenesis and on the application of new technologies to these areas. There is a strong emphasis on immunohistochemistry and image analysis and recent efforts have utilized magnetic resonance microscopy as a research tool. Techniques utilized include quantitative stereology of altered hepatic foci, various measures of cell proliferation in tissues of treated rodents, and nonisotopic in situ hybridization. Collaborative research includes study of the role of oncogenes and suppressor genes in rodent experimental carcinogenesis and investigation of the utility of transgenic mice in hazard identification.

Relevant Publications:

Maronpot RR, Fox T, Malarkey DE, and Goldsworthy TL, 1995. Mutations in the ras Proto-oncogene: Clues to Etiology and Molecular Pathogenesis of Mouse Liver Tumors. *Toxicology* 101: 125-156.

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The Female Reproductive Pathology Group provides support for NTP reproductive toxicity studies and conducts research to study the pathophysiology of chemically mediated ovarian dysfunction and ovarian cancer in women and rodents.

Exposures to environmental chemicals have the potential to cause ovarian dysfunction and ovarian cancer in women, and disruption of ovarian function greatly impacts the reproductive and endocrine health and, thus, the general health of women. The overall goals of our research are to identify the ovarian target cell and biochemical and molecular mechanisms by which synthetic or naturally-occurring environmental chemicals cause ovarian dysfunction or ovarian cancer in in vivo and in vitro models; determine the role of key genes and signaling molecules in ovarian cell growth, differentiation, and physiology, and understand how to modify these pathways to ameliorate ovarian dysfunction and cancer.

Prostaglandins are critical signaling molecules in ovarian function, ovulation, and luteal function; chemical disruption of these pathways would result in marked impairment of reproductive functions. Mice genetically deficient in either cyclooxygenase (COX)-1 or COX-2 have greatly reduced prostaglandin levels however, because some cells and tissues contain both enzymes, prostaglandin production is not completely eliminated. During the past year studies conducted in this laboratory help establish the roles of the isoforms of cyclooxygenase enzymes (COX) and related prostaglandins in ovarian function and reproductive function using the COX-1 and COX-2 deficient mice. First, these studies determine that COX-2 and not COX-1 is required for the gonadotropin surge increase in ovarian prostaglandin levels and that COX-2 is necessary for cumulus expansion and the release of oocytes. These are the first studies to show that ovulation can be restored in COX-2 (-/-) mice by simultaneous treatment with gonadotropins and PGE₂ or IL1b, and that IL1b can function independently of COX-2. Second, these studies have identified aberrant estradiol production in a model of COX-1 deficiency, and show that both prostaglandins and estradiol are necessary for normal parturition. These studies suggest the final pathway for parturition is that COX-1-related prostaglandins are necessary to support an enhanced production of ovarian estradiol, and estradiol is necessary and required to up-regulate COX-2-related prostaglandins. The novelty of these observations is that in most studies examining the relation of the COX isoforms and estrogens, it is generally thought that the primary interactions are through COX-2 related prostaglandin induction of estrogen pathways. Studies for the next year continue to evaluate specific chemicals and their effect on ovarian function and interaction with prostaglandin pathways.

Biometry

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The Genetic Risk Group's (GRG) primary goal is to characterize the adverse health risk for human populations associated with exposure to chemicals, and also with genetic variability in the metabolism of these chemicals. Thus, GRG seeks to determine the risk associated with human exposure to carcinogens by investigating the link between environmental exposures, the resulting biological effects of the exposure (biomarkers), and the susceptibility factors that modulate this process. Specifically we are engaged in population-based molecular epidemiologic studies that investigate: 1) the relationship between exposure to chemicals and intermediate biomarkers of genetic damage, 2) the relationship between phenotypic polymorphism and genotype, 3) the role of metabolism polymorphism in modulating exposure-related genetic damage, 4) the role of genetic polymorphism in modulating exposure-related risk of cancer. A major interest is in developing new molecular markers of risk, using these markers in multiendpoint molecular epidemiology studies, and incorporating data from our studies into risk assessment models.

We have recently observed that individuals with "at risk" N-acetyltransferase 1 genotypes (NAT1*10) have 2-fold higher levels of DNA adducts in bladder tissue, are at 2.8 to 26 fold increased risk of bladder cancer, and have increased risk for gastric and colorectal cancer. These data suggest that NAT1 genotype may be an important new genetic risk factor in cancers associated with aromatic amine exposure. Glutathione S-transferase theta 1 (GSTT1), which is expressed in hematopoietic cells, is polymorphic and the lack of GSTT1 enzyme activity is due to an inherited deletion of the GSTT1 gene (null genotype). We have found that smokers with the GSTT1 null genotype have significantly higher levels of smoking-induced ethylene oxide-hemoglobin adducts and a higher frequency of smoking-induced mutations at the glycophorin A (GPA) locus in erythrocytes. Consistent with these observations that GSTT1 modulates exposure-related damage in blood cells, we find that the null genotype for GSTT1 is a significant genetic risk factor in myelodysplastic syndrome (MDS), a proliferative disease of the bone marrow that has been linked with chemical exposures. MDS patients were 4.3-fold more likely to carry the inherited GSTT1 null genotype ($p < 0.0001$).

Relevant Publications:

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The Statistical Modeling and Risk Assessment Group conducts and coordinates research into the development and use of biologically-based mechanistic models for characterizing and quantifying human health risks associated with exposure to environmental agents. This involves an active research program in computer-based mathematical modeling, ranging from efforts at the cellular and molecular levels to whole animals and focused on describing and evaluating chemical structures, biological response mechanisms and their perturbations by potentially hazardous environmental agents. This group's research activities cover four broadly-based areas: risk assessment, stochastic modeling of carcinogenesis, physiological/pharmacological/biochemical modeling, and predictive toxicology.

Relevant Publications:

Kohn, M. and Portier, C. "Effects of the mechanism of receptor-mediated gene expression on the shape of the dose-response curve." *Risk Anal.* 13: 565-572, 1993.

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Kohn, M.C., Sewall, C.H., Lucier, G.W., and Portier, C.J. "A mechanistic model of effects of dioxin on thyroid hormones in the rat." *Toxicol. Appl. Pharmacol.* 136:29-48, 1996.

Portier, C.J., Kohn, M.C., Kopp-Schneider, A., Sherman, C.D., Maronpot, R., and Lucier, G.W. "Modeling the number and size of hepatic focal lesions following exposure to 2,3,7,8-TCDD." *Toxicol. Appl. Pharmacol.* 138: 20-30, 1996.

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My research is directed toward understanding the interaction between genes and environmental exposures in human carcinogenesis. There are two main elements to this work, one directed at investigating the role of environmental exposure in critical target gene mutation and one directed at investigating the role of genetic susceptibility and environmental exposure in cancer risk.

The research on critical target genes addresses the hypothesis that different environmental exposures cause different patterns of mutation in genes that are important in carcinogenesis. My initial focus has been on mutational activation of oncogenes and deactivation of tumor suppressor genes. Most of my work has been on lung and bladder cancer, two tumors that have strong environmental determinants. In a recent study we showed that roughly one third of large and squamous cell lung tumors from uranium miners had an identical mutation in the tumor suppressor gene p53. This is one of only four known examples of an exposure-specific pattern of critical target gene mutation in human tumors. Such patterns can be used both to identify novel critical target genes and to suggest mutational mechanisms by which an environmental agent causes cancer. If specific carcinogens produce characteristic patterns of gene mutation in tumors, the detection of those patterns would be a powerful tool in studies of environmental risk and for use in prevention and early diagnosis.

My research on genetic susceptibility tests the hypothesis that commonly inherited allelic variants of selected candidate genes, in conjunction with environmental exposures, affect a person's risk of developing cancer. We are studying inherited polymorphisms in selected genes that have potential links to bladder cancer risk: genes involved in carcinogen metabolism, proto- oncogenes, tumor suppressor genes, and genes involved in DNA synthesis and repair. Working with genetically susceptible subgroups may allow us to identify the environmental exposures that cause disease and the true risks associated with exposure. It could also lead to public health programs for protecting susceptible populations, and for targeted screening of groups at higher risk of disease.

Relevant Publications:

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Chen, H., Sandler, D., Taylor, J.A., Watson, M., Shore, D.L., Liu, E., and Bell, D.A.: Increased risk for myelodysplastic syndromes in individuals with glutathione transferase theta 1 (GSTT1) gene defect. *Lancet* 347: 295-297, 1996.

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Cell and Developmental Biology

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Studies in polypeptide hormone action.

Our laboratory has been interested in several aspects of signal transduction resulting from binding of polypeptide hormones to their surface receptors on cells. One major topic under study is the role of direct substrates for protein kinase C (PKC) in mediating the many cellular effects resulting from activation of this family of kinases by hormones and other agonists. We have been studying a small family of PKC substrates consisting of MARCKS and its smaller homologue, the MARCKS-like protein or MLP. Ongoing projects include structure-function studies of the protein and its mutant derivatives in two major systems, development of the mouse central nervous system, and early embryogenesis in *Xenopus laevis*. We are also studying gene promoter elements in these two species to determine which elements are important for the developmentally regulated, tissue-specific and cytokine-induced expression of these genes. We are investigating potential interactions of these proteins with a protease that specifically cleaves MARCKS, cathepsin B, and potential roles of these interactions in growth and metastasis of certain tumors, especially human breast cancer. Finally, we are investigating the possibility that mutations in the MARCKS and MLP genes are involved in human neural tube defects, particularly at the level of increasing a genetic predisposition to environmental causes of these defects.

A second major area of study in the laboratory began with the cloning of a gene that was rapidly and massively induced by insulin. The protein encoded by this gene, known as TTP, is the prototype of a novel class of CCCH zinc finger proteins; however, no function has yet been proven for any member of this class of proteins. We have shown in the past that TTP is rapidly induced, translocated from the nucleus to the cytosol, and phosphorylated on serine residues by insulin and by many other mitogens and growth factors. In addition, mice deficient in this protein develop a complex syndrome consisting of arthritis, wasting, dermatitis, and early death; more recent work has identified an excess of circulating tumor necrosis factor (TNF) as the cause of most if not all aspects of the syndrome. We have shown that TNF is over-produced by macrophages derived from these knockout mice, and that this is secondary to enhanced stability of TNF mRNA in these cells. More recently, we have found that TTP can bind to specific regions in this mRNA, and stimulate both the removal of the polyA tail of the mRNA and promote its rapid turnover. Current studies are attempting to elucidate the molecular details of this

interaction; to identify other important mRNAs whose stability is regulated by TTP in normal physiology; to identify TTP binding proteins, which might modulate its activity; to knock-out the two known mammalian relatives of TTP in the hope of producing other informative phenotypes; and to investigate possible abnormalities in human autoimmune disease.

Relevant Publications:

Shi, Y, Sullivan, SK, Pitterle DM, Kennington, EA, Graff, JM and Blackshear, PJ (1997) Mechanisms of MARCKS gene activation during *Xenopus* development. *J. Biol. Chem.* 272:29290-29300.

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Kim HS, Swierczynski SL, Tuttle JS, Lai WS and Blackshear PJ.(1998) Transgenic complementation of MARCKS deficiency with a non-myristoylatable, pseudo-phosphorylated form of MARCKS: Evidence for simultaneous positive and dominant-negative effects on central nervous system development. *Devel. Biol.* 200:146-157.

Carballo E, Lai, WS and Blackshear PJ. (1998) Feedback inhibition of macrophage tumor necrosis factor (production by tristetraprolin. *Science* 281:1001-1005

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Many genes are expressed in male germ cells that: 1) encode unique proteins, 2) are regulated developmentally, 3) and are either transcribed only in male germ cells or produce mRNAs specific to these cells. The underlying hypothesis of our research is that such genes encode proteins with key structural or functional roles in the successive mitotic, meiotic, and post-meiotic phases of male germ cell development. Some of these proteins are germ cell-specific members of protein families, some are products of variant transcripts, and yet others are products of unique genes. In some cases, the gene encoding a protein isoform expressed in somatic cells is down-regulated, while a gene encoding a germ cell-specific isoform is up-regulated. The new protein presumably has properties that are advantageous to germ cells. However, the presence of unique proteins may also render male germ cells more susceptible than other cells to environmental chemicals.

The major aims of our studies are to determine if germ cell-specific proteins are responsible for the unique and essential events of germ cell development, resulting in the production of sperm that contain an intact haploid genome and are structurally and functionally competent to fertilize the egg. The strategy chosen has been to identify genes expressed in different phases of germ cell development, to define the roles of the proteins encoded by some of these genes and of genes characterized by collaborators, and to determine how selected genes are regulated developmentally. Regulation may occur directly through the intrinsic developmental program of germ cells, or through extrinsic endocrine or paracrine signals that indirectly influence gene expression.

Relevant Publications:

Dix DJ, Rosario-Herrle MO, Gotoh H, Mori C, Goulding EH, Voelker CR, Eddy EM. Developmentally regulated expression of Hsp70-2 and Hsp70-2/LacZ transgene during spermatogenesis. *Dev Biol* 174: 310-321, 1996.

Dix DJ, Allen JW, Collins BW, Mori C, Nakamura N, Poorman-Allen P, Goulding EH, Eddy EM. Targeted gene disruption of Hsp70-2 results in failed meiosis, germ cell apoptosis, and male infertility. *Proc Natl Acad Sci USA* 93: 3294-3268, 1996.

Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB, Korach KS. Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* 137:4796-4805, 1996.

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The research in the Cell Biology Group focuses on two aspects of gene regulation: the molecular mechanisms that control squamous differentiation and the mechanisms by which nuclear receptors regulate gene expression. Squamous differentiation is a major pathway of terminal differentiation that can occur in many different tissues. Irreversible growth arrest is an early event in this multi-step process of differentiation and precedes the expression of squamous-specific genes. Alterations in the control of this differentiation process and the expression of specific genes have been linked to multiple diseases, including cancer, ichthyosis and psoriasis. In particular defects in the commitment to irreversible growth arrest is assumed to play a major role in the development of cancer. The effect of several differentiation-inducers on the expression and activity of cell-cycle control genes (e.g., Rb, cdk-inhibitors) and the mechanism of the transcriptional regulation of several squamous-specific genes (e.g., transglutaminase type I and cornifins) are investigated.

Members of the nuclear receptor superfamily consist of ligand-dependent transcriptional factors that regulate development, cell growth, apoptosis and differentiation, and include steroid hormone and retinoid receptors, and orphan receptors for which the ligand has yet to be discovered. Alterations in receptor signaling pathways have been linked to several diseases, including cancer. Retinoids play an important role in the regulation of squamous cell differentiation. The function of retinoid receptor signaling pathways in the control of this differentiation process by retinoids and the molecular defects in retinoid signaling pathways observed in carcinoma cells, are being studied. Three novel nuclear orphan receptors, RORg, TAK1 and RTR have been identified by our group. The interaction of these receptors with hormone response elements, their transactivation activity and interaction with other nuclear proteins is investigated. In addition, the role that these receptors play in development and the control of differentiation are studied.

Relevant Publications:

Harvat, B., and Jetten, A.M. (1996) Interferon gamma induces an irreversible growth arrest in mid-G1 in mammary epithelial cells which correlates with a block in hyperphosphorylation of RB. *Cell Growth Diff.* 7: 289-300.

Austin, S.J., Fujimoto, W., Marvin, K.W., Vollberg, T.M., Lorand, L., and Jetten, A.M. (1996) Cloning and regulation of cornifin b, a new member of the cornifin/spr family. Suppression by retinoids. *J. Biol. Chem.* 271: 3737-3742.

Marvin, K.W., Fujimoto, W., and Jetten, A.M. (1995) Identification and characterization of a novel squamous cell-associated gene related to PMP22. *J. Biol. Chem.* 270: 28910-28916.

Zhang, L-X., Mills, K.J., Dawson, M.I., Collins, S.J., and Jetten, A.M. (1995) Evidence for the involvement of an RAR α -dependent signaling pathway in the induction of tissue transglutaminase and apoptosis by retinoids. *J. Biol. Chem.* 270: 6022-6029.

Hirose, T., O'Brien, D., and Jetten, A.M. (1995) RTR: A new member of the nuclear receptor superfamily that is highly expressed in the testis. *Gene* 152:247-251.

Hirose, T., Apfel, R., Pfahl, M., and Jetten, A.M. (1995) The orphan receptor TAK1 acts as a repressor of RAR-, RXR-, and T3R-mediated signaling pathways. *Biochem. Biophys. Res. Comm.* 211:83-91.

Hirose, T., Fujimoto, W., Yamaai, T., Kim, K.H., Matsuura, H., and Jetten, A.M. (1994) TAK1: Molecular cloning and characterization of a new member of the nuclear receptor family. *Mol. Endocrinol.* 8: 1667-1680.

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Environmental agents produce a variety of effects on the reproductive tract, some of which result in infertility and toxicity. In many cases such effects are direct and produced by agents having estrogenic hormonal activity, but with little structural resemblance to the natural ligand. Mechanistically, hormonal activity is believed to be mediated through an intracellular receptor protein, although the tissues and specific responses vary, a primary response is increased gene transcription. The receptor demonstrates specific stereochemistry for endogenous compounds but appears to interact less selectively with exogenous chemicals. In order to better understand such differences, studies are underway to determine a structural and chemical basis for the stimulation of estrogenic responses and the involvement of the estrogen receptor in this process. Two major tissue systems are investigated including the reproductive tract and bone. Reproductive tract tissue is a principal target site of estrogen action related to hormone responsiveness being investigated at the biochemical and molecular level. Bone tissue has been known to be susceptible and sensitive to estrogen withdrawal and treatment. Until recently the effect was believed to be indirect, however, the description of estrogen receptors in bone tissue indicates estrogen can act directly. Investigations are involved to identifying the estrogenic responses in bone cells and evaluate the different cellular mechanisms involved in their activation.

Research in the Receptor Biology Section involves three major interrelated approaches. First, the structural basis of ligand interactions with the estrogen receptor is investigated in order to more fully understand the importance of the ligand binding to nuclear estrogen receptor interactions and its role in stimulating estrogenic responses. Gene transfection studies are being used to examine the influence of different ligand structures and receptor forms on the regulation of exogenous hormonally responsive reporter gene constructs. Second, the biochemical and molecular properties of the estrogen receptor protein are analyzed to determine what processes are involved in its activation and role in mediating biological responses. Steroid hormone receptor protein modifications may function to modulate the activity and specificity of responsiveness as proposed for other signaling systems. Finally, the group is investigating the expression of uterine estrogen responses and evaluating the possible coupling of other signal transduction mechanisms such as growth factors and their involvement in the mechanism of uterine stimulation to aid in determining an overall understanding of estrogen

hormonal tissue responses. Experimental approaches involve tissue culture; transgenic animal models including insertional and knock-out transgenics; nucleic acid biochemistry and gene cloning; and protein biochemistry, purification, and characterization.

Relevant Publications:

Ignar-Trowbridge, D.M., Nelson, K.G., Bidwell, M.C., Curtis, S.W., Washburn, T.F., McLachlan, J.A., Korach, K.S. (1992) Coupling of dual signaling pathways: Epidermal growth factor action involves the estrogen receptor. *Proc. Natl. Acad. Sci. USA* 89:4658-4662.

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Korach, K.S. (1994) Insights from the study of animals lacking functional estrogen receptor. *Science* 266:1524-1527.

Smith, E.P., Boyd, J., Frank, G.R., Takahashi, H., Cohen, R.M., Specker, B., Williams, T.C., Lubahn, D.B., Korach, K.S. (1994) Estrogen insensitivity syndrome in an adult man caused by homozygous nonsense mutation of the estrogen receptor gene. *N. Engl. J. Med.* 331:1056-1061.

Couse, J.F., Curtis, S.W., Washburn, T.F., Linzey, J., Golding, T.S., Lubahn, D.B., Smithies, O., and Korach, K.S. (1995) Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene. *Mol. Endocrinol.* 9:1441-1454

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The overall goal of the Cell Biology Section is to develop an understanding of the mechanisms involved in cell injury and death. Particular attention has been devoted to the role of ionic and biochemical alterations which are involved in cell injury. We hypothesize that environmental stress initiates a cascade which, depending on the size and duration of the stress, as well as the cell type, can lead to either the development of cell protection, apoptosis or necrosis. The Cell Biology Section is elucidating the signaling pathways responsible for protective adaptation and apoptosis. To accomplish this goal two model systems are studied: 1) signaling pathways involved in cardioprotection and, 2) signaling pathways involved in apoptosis. We find that stressing cardiac tissue activates protein kinase C, which our preliminary data suggest activates lipoxygenase and epoxygenase pathways of eicosanoid metabolism, leading to cardioprotection. We are currently investigating the role of lipoxygenase and epoxygenase metabolites in altering sarcoplasmic reticulum (SR) calcium and cytosolic calcium as well as altering K and Ca channel activity. We have recently used a new NMR indicator to measure in situ SR ionized calcium. We report a basal SR ionized Ca²⁺ concentration of 1.5 mM.

Another area of investigation involves understanding signaling pathways involved in apoptosis. Evidence is emerging that a decrease in endoplasmic reticulum (ER) calcium is involved in signaling apoptosis. Our current research is directed to define the mechanism(s) responsible for the decrease in ER Ca²⁺ and apoptosis.

Relevant Publications:

Chen, W., Steenbergen, C., Levy, L., Vance, J., London, R.E., and Murphy, E: Measurement of free Ca²⁺ in sarcoplasmic reticulum in perfused rabbit heart loaded with TF-BAPTA. *J. Biol. Chem.* 271: 7398-7403, 1996.

Preston, G.A., Barrett, J.C., Biermann, J.A., and Murphy, E.: Effects of alterations in calcium homeostasis on apoptosis during neoplastic progression. *Cancer Res.* 57: 537-542, 1997.

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Cytochrome P450 enzymes and sulfotransferases (STs) represent two large groups of enzymes which play key roles in the First Phase and the Second Phase metabolism of xenobiotics and steroid, respectively. Characteristically, these enzymes display remarkably diverse substrate specificities to metabolize numerous endogenous substrates including steroids, lipids and neurotransmitters, as well as exogenous chemicals such as drugs, environmental pollutants and procarcinogens. In addition, P450s and STs are induced by various xenobiotic chemicals as well as endocrine signals such as growth hormone. As a cellular defense mechanism against the toxicity and carcinogenicity, the induction of and metabolism by P450s and STs usually lead to increased detoxification and elimination of xenobiotics. Paradoxically, however, they can often result in the bioactivation of toxic and carcinogenic xenobiotics. Thus, understanding the molecular mechanisms of the substrate specificity and induction process is critical to delineate the roles of the P450s and STs in human susceptibility to environmental toxins and carcinogens. Site-directed mutagenesis studies have identified that few key amino acid residues regulate the substrate specificity of the P450s. The high resolution diffraction data are collected from crystals of STs. A mouse primary hepatocyte system has been established and used to determine a phenobarbital-responsive enhancer module (PBREM) of a P450 gene. A PRBM-binding nuclear protein is purified and its cDNA is being cloned. The sex-specific P450 genes are found to exhibit the sexually dimorphic demethylations. Various nuclear and cytoplasmic proteins have been investigated in respect to their capacities to transduce growth hormone signals and to recognize demethylated P450 promoters.

Relevant Publications:

Structural flexibility and functional versatility of mammalian P450 enzymes. Negishi, M., Uno, T., Darden, T.A., Sueyoshi, T., and Pedersen, L.G. *FASEB J.* 10: 683-689, 1996

Characterization of phenobarbital-inducible mouse Cyp2b10 gene transcription in primary hepatocytes. Honkakoski, P., Moore, R., Gynther, J., and Negishi, M. *J. Biol. Chem.* 271: 9746-9753, 1996.

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A nuclear factor (NF2d9) that binds to the male-specific P450 (Cyp 2d-9) in mouse liver. Sueyoshi, T., Kobayashi, R., Nishio, K., Aida, K., Moore, R., Wada, T., Handa, H., and Negishi, M. *Mol. Cell. Biol.* 15: 4158-4166, 1995.

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Alteration of high and low spin equilibrium by a single mutation of amino acid 209 in mouse cytochromes P450. Iwasaki, M., Juvonen, R., Lindberg, R., and Negishi, M. *J. Biol. Chem.* 266: 3380-3382, 1991.

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Alteration of the substrate specificity of mouse cytochrome P450coh by the mutation of a single amino-acid residue. Lindberg, R.L.P. and Negishi, M. *Nature (London)* 339: 632-634, 1989.

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Research in this group is concerned with trying to understand, at the cellular and molecular level, how cells regulate calcium and how hormones and neurotransmitters utilize calcium as a cellular signal. An early event following the activation of receptors in this class is the hydrolysis of a membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, to yield two putative second messenger molecules, diacylglycerol and inositol 1,4,5-trisphosphate. Diacylglycerol activates protein kinase C, and inositol 1,4,5-trisphosphate releases calcium from an intracellular organelle. In addition to the release of intracellular calcium, receptor activation also leads to an increased entry of calcium into the cell across the plasma membrane. This calcium entry is activated by a signal generated by depletion of intracellular calcium stores. In single cells, the pattern of calcium signalling often takes the form of discrete calcium spikes or oscillations which traverse the cytoplasm as calcium waves. We are currently attempting to investigate the mechanisms underlying the phenomena of intracellular calcium release and entry, as well as the mechanisms by which calcium oscillations arise, by combining the techniques of cellular microinjection, whole cell and single channel patch-clamp, single cell calcium analysis, calcium imaging, and molecular biology.

Relevant Publications:

Bird, G.St.J. and Putney, J.W., Jr. (1996) Effect of inositol 1,3,4,5-tetrakisphosphate on inositol trisphosphate-activated Ca^{2+} signaling in mouse lacrimal acinar cells. *J. Biol. Chem.* 271: 6766-6770.

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*Characterization and Functional Significance of Cytochrome P450
Arachidonic Acid Epoxygenases.*

Work from our laboratory has demonstrated that cytochromes P450 metabolize arachidonic acid (AA) to a novel group of fatty acid epoxides called epoxyeicosatrienoic acids (EETs). These compounds have been shown to possess potent biological activities including effects on vascular and airway smooth muscle tone, stimulation of peptide hormone release, and modulation of ion transport. We hypothesize that the EETs play important roles in cell/organ physiology and that aberrant expression of P450 epoxygenase genes leads to cell/organ dysfunction. To address these hypotheses, we propose the following specific aims: (1) to characterize the catalytic properties of the P450 AA epoxygenases; (2) to investigate the functional roles of P450 epoxygenase products in cell/organ physiology; and (3) to study the regulation of P450 epoxygenase gene expression. Our goal is to understand the importance of epoxygenase genes and their products with respect to normal physiology and pathophysiology.

To study the catalytic and regulatory properties of P450 AA epoxygenases, we have cloned several novel P450 epoxygenase cDNAs of the CYP2 family and expressed the recombinant proteins in insect cells using the baculovirus expression system. Recombinant P450 catalytic activities were reconstituted with AA and the product profiles were analyzed using HPLC/GC/MS. Polyclonal antibodies raised against the purified, recombinant proteins were used for immunoblotting studies to determine the relative organ abundance of the P450 epoxygenases and immunohistochemical studies to examine the cellular localization of P450 epoxygenase expression. The *in vivo* production and concentration of epoxygenase products were evaluated by detecting EET regio- and stereoisomers in various tissues using HPLC/GC/MS. Protein immunoblotting and Northern analysis were used to study changes in epoxygenase gene expression in response to pharmacologic and physiologic manipulations. Synthetic EETs were prepared and utilized to examine the function of these eicosanoids in cell and organ physiology.

CYP2J subfamily P450s: physiologically relevant cardiac hemoproteins.

A new human P450 cDNA (CYP2J2) and the corresponding rat homologue (CYP2J3) were cloned and expressed in insect cells using baculovirus. Both recombinant proteins catalyzed the regio- and stereoselective metabolism of AA to EETs. Northern analysis and protein immunoblotting revealed that CYP2J2 and CYP2J3

were highly expressed in the heart. The *in vivo* significance of the epoxygenase pathway was documented by detecting, for the first time, the presence of EETs in the heart. Immunohistochemistry of heart tissue sections showed that CYP2J2 and CYP2J3 were highly enriched in atrial and ventricular myocytes and present at lower levels in cardiac endothelial cells. The cellular localization of CYP2J proteins to cardiac myocytes suggested a potential functional role of the EETs in normal cardiac muscle cell physiology and in the myocardial response to ischemia. In this regard, we showed that synthetic 11,12-EET significantly improved recovery of heart contractile function following prolonged, global cardiac ischemia. Importantly, brief intermittent periods of ischemia and reperfusion (preconditioning), which have been shown to protect against the functional consequences of a subsequent prolonged period of ischemia, resulted in a significant increase in rat heart 11,12-EET without an associated increase in cardiac expression of CYP2J3 protein. These data suggested that AA epoxygenase metabolites may be partly responsible for the cardioprotective effects of preconditioning and that the increase in epoxygenase metabolites were either due to increased enzyme activity or increased AA substrate availability. Future studies are aimed at: (a) delineating the mechanisms of the cardioprotective effects of the EETs; and (b) developing transgenic animal models which facilitate studies on the effects of increased or reduced EETs on cardiac cell function and on the response of the heart to ischemia and reperfusion.

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Steroid hormones regulate tissue-specific gene expression in animals via receptor dependent intracellular signal transduction pathways. These receptors, when activated by the appropriate ligands, both activate and repress the transcription of subsets of genes in target cells which results in altered gene expression and altered function. We are particularly interested in glucocorticoid receptors and their actions because they reflect the primary response to environmental stress. Current research projects are examining the following aspects of glucocorticoid hormone action: (1) mutual interference of signaling between the glucocorticoid receptor and NFB; (2) the role of receptor phosphorylation in signal transduction; (3) the regulation of glucocorticoid receptor gene expression; (4) the involvement of the form glucocorticoid receptor in the generation of steroid resistance.

A second major interest of the laboratory focuses on evaluating the mechanisms involved in the regulation of apoptosis in normal and neoplastic cells. Research is aimed at the identification and cloning of genes that are responsible for the initiation and execution of apoptosis. Current projects include: (1) identification of nucleases that cleave chromatin during apoptosis; (2) evaluation of the role of ribosomal RNA degradation in apoptosis; (3) cloning of inhibitors of apoptosis; (4) the role of cell volume regulation and ion fluxes in the activation of apoptosis; (5) apoptosis in yeast; (6) evaluation of the role of NFB in apoptosis.

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Dr. Mishina focuses on the characterization of signal transduction pathways important in mammalian development. His research focuses on the role of bone morphogenic proteins (BMPs) and BMP receptors in murine development. Dr. Mishina's research has linked BMP2/4 signaling to a critical inductive role in mesoderm formation during gastrulation. Currently, his research focuses on using BMP2/4 knockout mice and the BMP-receptor knockout mice to characterize BMP signaling pathways and associated downstream targets and gene responses. These studies are being extended to produce tissue specific BMP-receptor knockouts in chondrocytes and bone tissue. These transgenic and knockout mouse lines will be used to do comparative studies on Mullerian-inhibiting substance and BMP signaling pathways to determine their respective roles in mammalian development and to evaluate the activity of environmental chemicals in perturbing BMP signaling that results in developmental toxicity and teratogenesis.

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The focus of our group centers around the role of adaptor proteins in the integration of signal transduction cascades. In particular, we are interested in proteins which modulate the function of receptor tyrosine kinases (RTKs). A major target of numerous RTKs is the Shc family of adaptor proteins. This family of proteins lacks an enzymatic domain but consists entirely of modular protein:protein recognition motifs including Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains. Specifically, we have identified a novel Shc protein, ShcC, which is highly restricted in its pattern of expression to the central nervous system. Expression of ShcC is induced in differentiating neurons as well as in regions of the developing mouse brain undergoing differentiation. We are examining the role of ShcC in neural development and signaling using a combination of dominant negative proteins as well as activated forms of the protein. Although the members of the Shc family are well conserved in sequence, current evidence from our group, as well as others, suggests distinct functions of the various Shc family members and as such we are interested in understanding these activities.

In addition, our group is also studying a novel adaptor protein, intersectin, which consists of two amino-terminal Eps homology (EH) domains and 5 Src homology 3 (SH3) domains and is thought to regulate numerous signaling cascades including endocytosis. Recent data from a number of groups suggests that regulation of endocytosis is important for proper signaling by RTK as well as GPCR. In collaboration with two other groups, our lab has shown that intersectin co-localizes with clathrin and that expression of intersectin can inhibit endocytosis of the transferrin receptor. Furthermore, we have demonstrated that intersectin over-expression is able to activate transcriptional pathways suggesting that intersectin also regulates gene expression. Interestingly, there is a larger isoform of intersectin which is restricted to the brain. This larger version is identical to the more widely expressed form except for a carboxy-terminal extension which encodes a classic Dbl homology and pleckstrin homology domain (DH/PH domain). This larger isoform of intersectin is thus thought to regulate Rho/Rac GTPases and may also play an important role in neural development. Further work by our group will help to elucidate the mechanism of intersectin function.

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Transcription in Breast Cancer Cells

Summary--- Steroid hormones, such as estrogen and progesterone, play an important role in the development and treatment of breast cancer. In my laboratory, the scientists have undertaken detailed analysis of the mechanism of action of the steroid receptors and clinically important steroid receptor antagonists that are used to block their action. It is hoped that these studies will provide new insight into the role that steroid hormone receptors play in breast cancer and the possible development of novel and effective treatments. With the recent arrival of our research group at the NIEHS we will expand our research focus to encompass a mechanistic examination of environmental agents that act as endocrine disruptors in the reproductive process and in mammary cells.

Current Research Projects --- Breast cancer will constitute greater than 30% of all new cancers diagnosed in women this year. Despite intensive efforts, the mortality resulting from this disease has not decreased significantly over the last decade. Of the treatments currently available, hormone therapy remains one of the most effective means of clinical intervention. However, the mechanisms by which these agents achieve their effect remains to be elucidated. Steroid hormones act via a group of high affinity receptors that regulate cellular growth and development by binding to response elements located within the promoters of hormone inducible genes. In higher animals, including humans, DNA is organized in a characteristic structure with nuclear proteins to form chromatin which plays an important role in controlling gene expression. A thorough understanding of steroid hormone action has to accommodate the fact that their receptors function in concert with other transcription factors in the context of chromatin. These considerations will become increasingly important as we seek a molecular definition of the possible ways steroid hormone control of gene transcription may influence cancer initiation and progression.

Transcriptional Regulation by Steroid Hormones --- The mouse mammary tumour virus (MMTV) promoter provides an excellent model for studies of hormone-regulated transcriptional control in the context of chromatin. It is hormone-inducible and reproducibly assembles into a phased array of nucleosomes when stably introduced into cells. The research in my laboratory is designed to further define the mechanisms responsible for steroid regulation of transcription from the MMTV promoter in vivo. In recent experiments we have shown that the MMTV promoter assembled in chromatin was refractory to progesterone stimulation, but

was highly responsive to a glucocorticoid hormone. In contrast, both steroids activate transcription from the same reporter cassette when transiently introduced into cells. Results with human breast cancer cells that contain only the progesterone receptor demonstrate that the chromatin adopts an "open" structure that allows nuclear factor 1 binding in the absence of hormone. This novel finding has provided a new level of understanding of how steroid hormones regulate gene transcription and how chromatin influences this fundamental biological process. Hormone antagonists, such as RU486, are important tools in hormone therapy for breast cancer, and have proved to be extremely useful in experimental model systems aimed at elucidating the mechanisms of action of steroid hormone receptors. We have taken advantage of our novel human and mouse breast cancer cell lines to describe the mode of action of these hormone antagonists. Our studies demonstrate that antagonists can be divided into at least two functional groups: antagonists that permit receptor interactions with genetic material and those that do not allow it. It is expected that further studies of these important events should improve our knowledge of hormone action and reveal novel ways of controlling steroid-dependent disease processes. Our studies with these specific antagonists will also provide a framework for our expanded investigations of environmentally important endocrine disrupters.

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Cancer Biology

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Cell adhesion and migration contribute to normal processes such as cellular differentiation and embryonic development, as well as to the progression of diseases and pathological conditions, such as cancer and inflammation of the airway epithelium, that can result from either acute or chronic exposure to environmental toxicants. Fibronectin is an extracellular glycoprotein that functions in a variety of cell adhesive processes. Cell interactions with fibronectin occur via specific receptors, the best characterized of which are members of the integrin family. Integrins are all non-covalent, heterodimeric complexes consisting of an α subunit and a β subunit. The $\alpha_5\beta_1$ integrin is the major fibronectin receptor on most cells that functions in adhesion, migration, and invasion of tumor cells as well as in signal transduction processes that result in cellular responses to fibronectin substrates. Our major research focus is to characterize the molecular mechanisms of the fibronectin-integrin interaction and the resulting downstream processes that are important for the control of proliferation, adhesion, and migration of a variety of cells, especially focusing on human tumor cells. The primary approaches use monoclonal antibodies, protein and peptide biochemistry, physical biochemistry, and cell and molecular biology to investigate both integrin receptors and fibronectin itself. The central cell adhesive region of fibronectin binds to the $\alpha_5\beta_1$ integrin in an interaction that requires at least two distinct sequences: the Arg-Gly-Asp (RGD) site and a Pro-His-Ser-Arg-Asn (PHSRN) sequence that acts in synergy with the RGD site. The crucial sequences are necessary, but not sufficient, to account for the cell adhesive and migration-promoting activities of fibronectin. One current goal of our work is to elucidate the structural elements that play a role in the mechanism of fibronectin-mediated cell adhesion. We expect that reagents developed for the manipulation of cell adhesive processes will lead to the discovery of novel therapeutic agents for the treatment of human disease.

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Neoplastic development of cancers is a multistep process requiring multiple genetic changes. Significant advances have been made in the elucidation of the genes involved in genetic predisposition to cancer but less is known about the genes involved in the later stages of malignant progression. Identification of the target genes for different steps in the cancer process is important in understanding the environmental causes of cancer as well as the endogenous causes of cancer, which include spontaneous mutations, aging, and hormones. The role of aging in cancer is studied by cloning and characterizing genes involved in cellular aging. Unlike tumor cells, normal cells have a finite lifespan and enter a state of irreversible growth arrest, termed cellular senescence, at the end of their lifespan. We have shown that cellular senescence is genetically controlled and that multiple senescence genes are altered in immortal cancer cells. Only a few senescence genes have been identified and efforts to clone new genes are actively being pursued. Studies of the regulation of these genes by environmental factors may elucidate the causes of aging and cancer.

Another area of active investigation is the mechanisms of metastatic progression. The malignant phenotype of a cancer cell is under both positive and negative controls but little is known about the genes that control metastasis. We have recently cloned a novel metastasis suppressor gene, KAI1, which may be important in prostate, breast, lung, and possibly other cancers. Further studies of this and related genes may yield important new insights into cancer diagnosis and treatment. In addition, molecular markers for the later stages of cancer progression may help define the environmental factors that influence malignant development.

Hormones are major factors in human cancers and the Cancer and Aging Section is actively involved in studying multiple aspects of hormonal carcinogenesis, including molecular alterations of hormonally associated cancers (breast, prostate, and endometrial), mechanisms of estrogen-induced chromosomal changes, and the role of BRCA1 in regulating growth and tumorigenicity of breast cancer.

Oxidative stress is a major form of endogenous and exogenous damage to cells. The role of oxidative stress in cell senescence and cell death (apoptosis) is under study. Interestingly, the same genes (e.g., p53, Rb) are involved in cell senescence and cell death. A better understanding of the molecular mechanism of signal transduction leading to cell senescence or cell death through divergent pathways is required to understand cellular responses to environmental stresses, particularly oxidative damage.

As cells progress to cancer, their responses to apoptotic signals change. Interestingly, cancer cells die at a higher rate than normal cells, suggesting that environmental modulators of apoptosis can influence the rate of tumor growth. One example of this is dietary restriction of animals, which reduces cancer progression by stimulating apoptosis of precancerous cells. We have shown that this is in part due to modulation by dietary restriction of circulating IGF1 levels, which blocks apoptosis of cancer cells. Further studies on the genetic controls of apoptosis may help elucidate the role of diet and other environmental factors in cancer.

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Metastasis is the principal cause of morbidity and mortality in cancer patients. We are exploring characteristics of metastatic cells, such as adhesion and motility, that are critical steps in the metastatic cascade. Current work focuses on the role of 21 integrin complex in the adhesion of human breast carcinoma cells to basement membrane proteins. We have shown that this adhesion is stimulated by cis-polyunsaturated fatty acids, such as arachidonic acid, and that the stimulation is dependent on protein kinase C and tyrosine kinase activities. Our goal is to define the molecules involved in this adhesion and to elucidate specific signal transduction pathways that regulate the stimulation of adhesion by physiological agents. A second area of emphasis is the role of protein glycosylation in tumor cell biology. We have previously shown that an inhibitor of protein glycosylation, swainsonine, reduces tumor growth and metastasis in a murine model system, enhances the host immune response and stimulates bone marrow progenitor cells. We are pursuing the pathways by which this compound affects cells, such as adherence to basement membrane proteins, cytokine production, protein kinase C activity, and Ca⁺⁺ transport. We are also examining the ability of swainsonine to protect bone marrow stem cells from cytotoxic agents. We are seeking to understand the underlying processes of cell growth and death (apoptosis) in these hematopoietic cells. The ability to alter the growth characteristics of such stem cells could have multiple applications in the areas of chemotherapy and bone marrow transplantation.

Relevant Publications:

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This group conducts research in animals and in vitro systems to understand the role of specific genetic and epigenetic events in the induction and development of cancers. Efforts are also directed to derive insights into relationships between chemical structure and carcinogenic mechanisms. A major goal of these efforts is to improve our understanding and ability to identify and classify potential carcinogens.

The first major objective of the Cancer Biology Group's research program is to understand the mechanisms of environmental carcinogenesis. This is a goal shared by a number of other laboratories throughout the world. Our studies, however, have unique aspects that are related to, or are derivatives of, the problem of identifying carcinogens. There has been remarkable progress over the past decade in identifying specific mutated genes that occur in human cancers such as retinoblastoma, Wilms tumor, and adenocarcinoma of the colon. These accomplishments have strengthened the concept that multiple, time-dependent genetic changes are involved in the development of malignancies. However, since the genetic changes are difficult to resolve into causal versus consequential events, and to distinguish them from those that are the result of increasing genomic instability. Thus, despite advances in molecular methodologies that allow human cancers to be studied directly, researchers must still depend upon animal models to study the processes by which neoplasias arise.

Two stage carcinogenesis models have provided important tools for the study of neoplastic processes. Skin models in particular have played a significant role in establishing the concepts of initiation, promotion and progression in tumor development. Studies on the processes of malignant conversion underlying the development of squamous cell carcinomas from papillomas have provided an important system for the identification of genetic changes. They have also provided insights into the regulation of epidermal growth and differentiation, and the identification of chemicals with the capacity to promote tumor development.

In the past decade, technical advances have given us the capacity to genetically manipulate the mammalian genome. A product of this technology is the development of transgenic mice with altered expression of specific genes. Transgenic mouse models are particularly important in defining cellular and molecular processes involved in chemical carcinogenesis since they have the inherent capability for tissue distribution and metabolism of chemicals which are deficiencies of in vitro systems. The research program of the Cancer Biology Group has extensively utilized transgenic models and a particular interest has been

the Tg.AC skin carcinogenesis model. Mutation of the endogenous c-Ha-ras gene has been observed frequently in mouse skin carcinogenesis and in in vitro models. Evidence supports a role for the ras gene in papilloma induction and in malignant progression but its role is complex and involves the action of other genes. A transgenic Tg.AC mouse line with unique properties was produced by the introduction of an activated v-Ha-ras transgene zygote pronuclear injection into FVB/N mice (Leder et al., 1990). We have recently made some significant observations related to papilloma induction in Tg.AC mice and the use of transgenic models as bioassays for carcinogens. This work is described in the manuscripts listed below.

Relevant Publications:

Tennant, R.W., French, J.E. and Spalding, J.W. Identifying Chemical Carcinogens and Assessing Potential Risk in Short-term Bioassays Using Transgenic Mouse Models. *Environ. Health Perspect.* 1995; 103(10):942-950.

Hansen L, Tennant RW. Follicular origin of epidermal papillomas in v-Ha-ras transgenic TG.AC mouse skin. *Proc. Na'l. Acad. Sci. USA* 1994; 91:7822-7826.

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Biophysics/Structural Biology

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The Nucleic Acid Enzymology Section conducts physical and biochemical studies of mammalian DNA polymerases and in particular, studies gap-filling DNA synthesis during DNA repair. To define the biological role of DNA polymerase β , the group and collaborators recently constructed DNA polymerase "knock-out" cell lines from a transgenic mouse model. These cell lines are devoid of all DNA polymerase β mRNA and protein, and cell extracts lack base excision repair capacity, thus establishing the requirement of this particular DNA polymerase for the short gap-filling DNA synthesis required in uracil-initiated base excision repair.

The group and collaborators have reported many crystal structures of complexes of rat and human DNA polymerases with the two substrates (DNA and dNTP), and the group and collaborators have solved the NMR structure of the enzyme's 8 kDa domain. This work has improved our understanding of the fundamental mechanism of DNA synthesis and of the phenomenon of templating. The group also studies gene expression control for DNA polymerase β , since base excision repair capacity is a tightly regulated process in mammalian cells.

Finally, the research program also includes studies of structure-function relationships of the HIV-1 Reverse Transcriptase. The group and collaborators have conducted extensive kinetic studies of RT-nucleic acid interactions. This work provides a framework for drug design and for biochemical analysis of the relationship between the structure of the reverse transcriptase and its functions. The recombinant expression system for this enzyme developed by the group and studies of frameshift mutagenesis in collaboration with Dr. Kunkel and colleagues have facilitated understanding of this important reverse transcriptase.

Relevant Publications:

Sobol, R.W., Horton, J.K., Singhal, R., Prasad, R., Rajewsky, K., and Wilson, S.H.: Requirement of DNA Polymerase β in Mammalian Base Excision Repair. *Nature* 379: 183-186, 1996.

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Prasad, R., Singhal, R.K., Srivastava, D.K., Tomkinson, A.E., and Wilson, S.H.: Specific Interaction of DNA Polymerase β and DNA Ligase I in a Multiprotein Base Excision Repair Complex from Bovine Testis. *J. Biol. Chem.* 271: 16000-16007, 1996.

Narayan, S., He, F. and Wilson, S.H.: Activation of the Human DNA Polymerase β Promoter by a DNA-alkylating Agent Through Induced Phosphorylation of CREB-1. *J. Biol. Chem.* 271: 18508-18513, 1996.

Pelletier, H., Sawaya, M.R., Wolfle, W., Wilson, S.H., and Kraut, J.: Crystal Structures of Human DNA Polymerase β Complexed with DNA, Implications for Catalytic Mechanism, Processivity, and Fidelity. *Biochemistry* 35: 12742-12761, 1996.

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The Macromolecular Structure Group uses the tools of structural biology, primarily x-ray crystallography, to study the overlapping areas of embryonic development, cell signaling, and RNA-protein interactions. Many macromolecules involved in the processes of development, signaling, and RNA transactions are regulators of cell growth and differentiation. Thus fundamental knowledge about the structure and function of these macromolecules will contribute to our understanding of diseases such as cancer where environmental influences have resulted in aberrant growth and signaling and will also provide a structural framework for the design of therapeutic compounds that induce or inhibit specific signaling pathways.

The current projects in the Macromolecular Structure Group include structural studies of two types of proteins involved in post-transcriptional gene regulation and a model system for ribonucleoprotein machines. The first two projects focus on two types of proteins that are involved in regulating messenger RNA (mRNA) stability by binding to adenosine-uridine (AU)-rich elements (AREs) in the 3' untranslated regions of some mRNAs. These AREs have been shown to confer instability on the transcripts that contain them and are important players in regulating gene expression. Crystal structures of these proteins bound to AREs and follow up functional studies will identify residues important for sequence-specific RNA recognition and suggest how these proteins participate in regulating mRNA stability.

The third project focuses on a one protein-one RNA ribonucleoprotein catalyst to provide insight into complex, multicomponent ribonucleoprotein machines such as the ribosome or spliceosome. The system is the fifth intron of yeast mitochondrial cytochrome b pre-mRNA (bI5) and the protein CBP2 (cytochrome b pre-mRNA processing protein 2). The bI5 intron is a group I self-splicing intron. It contains the active site for the splicing reaction, but the protein co-factor, CBP2, assists in the reaction by holding the RNA in its active conformation. Determining the crystal structure of this simpler model system will allow the correlation of structure and function and should produce some general principles that can be applied to more complex protein-RNA systems involved in chromosome maintenance, mRNA splicing, and protein synthesis.

Relevant Publications:

Crystal Structure of a Hedgehog Autoprocessing Domain: Homology between Hedgehog and Self-Splicing Proteins. (1997) Traci M. Tanaka Hall, Jeffery A. Porter, Keith E. Young, Eugene V. Koonin, Philip A. Beachy, and Daniel J. Leahy. *Cell*, 91:85-97.

Multiple roles of cholesterol in hedgehog protein biogenesis and signaling. (1997) Philip A. Beachy, Michael K. Cooper, Keith E. Young, Doris P. von Kessler, Woo-Jin Park, Traci M.T. Hall, Daniel J. Leahy, Jeffery A. Porter. *Cold Spring Harb Symp Quant Biol* , 62:191-204.

A potential catalytic site revealed by the 1.7 Å crystal structure of the amino-terminal signalling domain of Sonic hedgehog. (1995) Traci M. Tanaka Hall, Jeffery A. Porter, Philip A. Beachy, and Daniel J. Leahy. *Nature*, 378:212-216.

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The NMR group uses magnetic resonance spectroscopy to study at the molecular level the structural and metabolic perturbations which can result from exposure to agents of environmental concern. These studies include structural characterizations of adducts which form between chemicals of environmental or pharmacological interest, and macromolecular targets or model systems. Recent studies have involved adducts formed from carbon-13 labeled aspirin and haloacetic acids, with the proteins ubiquitin and hemoglobin, as well as the labile boronate adducts formed with boric and boronic acids. The group is also interested in the structure of *E. coli* DNA polymerase III, in order to better understand how structural and chemical factors influence replication fidelity. Finally, studies of AIDS related proteins such as HIV protease are also in progress.

NMR methods are developed in concert with the programmatic work outlined above. Recent efforts have focused on developing dynamic shift multiplet perturbations as a tool for evaluating molecular dynamics, extension of the transferred NOE experiment, and the development of NMR methods for the detection of radical protein adducts. Methods involving fluorine-19 labeling for NMR studies of larger systems are also under evaluation.

In addition to structural NMR work, studies of intracellular ions, and the development of methods to determine ion levels in cells are also done by the group. Recent work has focused on intracellular magnesium and its measurement.

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London RE and Gabel SA: Fluorine-19 NMR studies of fluorobenzeneboronic acids 2. Kinetic Characterization of the Interaction with Subtilisin Carlsberg and Model Ligands. *J. Am. Chem. Soc.* 116: 2570-2575, 1994.

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Chen, W., Steenbergen, C., Levy, L. A., Vance, J., London, R. E., and Murphy, E.. Measurement of free Ca²⁺ in sarcoplasmic reticulum in perfused rabbit heart loaded with 1,2-Bis(2-amino-5,6-difluorophenoxy)ethane-N,N,N',N'-tetraacetic acid by ¹⁹F NMR. *J. Biol. Chem.* 271: 7398-7403; 1996.

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Vance, J. E., LeBlanc, D. A., and London, R. E. Cleavage of the X-Pro peptide bond by Pepsin is Specific for the trans isomer, *Biochemistry* 36: 13232-13240; 1997.

Macdonald J.M., LeBlanc, D. A., Haas, A. L. and London, R. E. An NMR Analysis of the Reaction of Ubiquitin with [Acetyl-1-¹³C]Aspirin, *Biochem. Pharmacol.*, in press.

Xu, A. S. L., Macdonald, J. M., Labotka, R. J., and London, R. E. NMR Study of the Acetylation Sites of Human Hemoglobin by Aspirin, *Biochim. Biophys. Acta*, in press.

Li, D., Allen, D., Harvey, S., Perrino, F. W., Schaaper, R. M., and London, R. E., A Preliminary CD and NMR Study of the E. coli DNA Polymerase III Theta Subunit, *Proteins: Structure, Function, and Genetics*, in press.

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Dr. Ronald P. Mason is the workgroup leader of the Free Radical Metabolites Workgroup, which uses electron spin resonance to detect and identify free radical metabolites of toxic chemicals and drugs. The chemical reactions of free radical metabolites have biochemical and toxicological consequences that cause cellular damage and death. The group has pioneered the application of the electron spin resonance spin trapping technique to biochemical, pharmacological and toxicological problems with particular emphasis on the use of spin traps *in vivo*. The *in vivo* experiments are critical because, unless free radical metabolites can be demonstrated with a whole animal model, there will always be some question as to their actual existence in biology. With the aid of this technique the group has demonstrated the formation of free radicals from rancid unsaturated fatty acids, established the role of hydroxyl radicals in iron and copper toxicity, and implicated the involvement of an ethanol-derived free radical in alcohol-induced cirrhosis of the liver.

Currently the Free Radical Metabolite Workgroup is active in four areas of research. 1) The mechanism of free radical generation by the reaction of hemoproteins with hydroperoxides. The central unanswered question in these reactions is whether the alkoxyl radical or the peroxy radical is the true product of reaction. We have been able to demonstrate that in every case examined thus far (i.e., cytochrome c, hematin, chloroperoxidase, and cytochrome P-450) that the alkoxyl radical is the species initially produced and that all other detected free radical are the result of the free radical chemistry of this species. 2) The detection, identification, and reactivity of myoglobin-derived radicals. The structures of the reactive free radicals formed by the reaction of metmyoglobin with hydrogen peroxide have been unknown since their discovery nearly forty years ago. With the use of isotopic labeling, spin trapping, and molecular biology the oxygen-reactive free radical has been identified as tryptophan. 3) *In vivo* detection of metal-mediated free radical formation. Many investigations of acute metal toxicity have detected the *in vivo* formation of either the hydroxyl radical or alkyl radical products of lipid peroxidation. Recently, we successfully detected hydroxyl radical generation in rats with chronic dietary iron supplementation in the absence of liver toxicity and with only modest serum ferritin increases of the magnitude thought to lead to a greater risk of myocardial infarction in man. 4) The role of nitric oxide in the metabolism of toxic chemicals and drugs. *In vivo* nitric oxide production and its role in the synergistic carbon tetrachloride/endotoxin toxicity have been investigated. The *in vivo* metabolism of hydroxyurea to nitric oxide has been discovered.

Relevant Publications:

Barr, D.P., Martin, M.V., Guengerich, F.P., and Mason, R.P.: Reaction of cytochrome P450 with cumene hydroperoxide: ESR spin trapping evidence for the homolytic scission of the peroxide O-O bond by ferric cytochrome P450 1A2. *Chem. Res. Toxicol.* 9: 318-325, 1996.

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