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Abstracts of Poster Presentations

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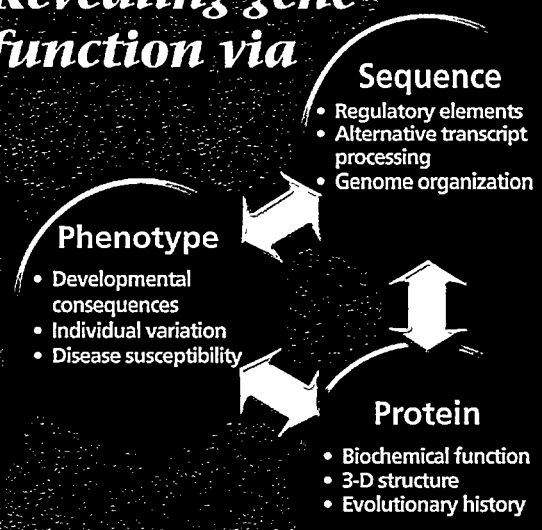
Objective

To present a leading research resource for uncovering gene function on a genome-wide scale through the combined capabilities of the ORNL Laboratory for Comparative and Functional Genomics and other special ORNL and University of Tennessee competencies in genetics, molecular biology, and computational and engineering sciences.

Approaches include

- Mouse mutagenesis and genomics
- Phenotype screening
- Gene expression analysis
- DNA analysis technology development
- Bioinformatics
- Comparative analyses of mouse, human, and yeast sequences
- Pilot projects to evaluate methodologies

Revealing gene function via



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A Glimpse at the PAI-1: Vitronectin Complex--Unexpected Properties and Implications for Regulating Plasmin Activation and Receptor Interactions

C. B. Peterson, University of Tennessee

Agouti-Induced C_a^{2+} Signaling as a Therapeutic Target for Obesity and Insulin Resistance/Hyperinsulinemia

M. B. Zemel, W. O. Wilkison, N. Moustaid-Moussa, and B. Xue, University of Tennessee

Immunolocalization of Proteins During Meiotic G2/M Cell Cycle Transition

John Cobb, Shannon Eaker, and Mary Ann Handel, University of Tennessee

Interaction of Retinoblastoma Gene Product With Transcription Factors ATF-a and ATF-2

H. Li and W. D. Wicks, University of Tennessee

In Vitro and In Vivo Models of Amyloidosis: Potential for Screening Anti-Amyloid Compounds

Jonathan Wall, Maria Schell, Charles Murphy, Rudi Hrncic, and Alan Solomon, University of Tennessee Medical Center

In Vitro Modulation of the Mammalian Circadian Clock by Gaba, Serotonin, and Neuropeptide Y Agonists

R. A. Prosser and K. R. Biggs, University of Tennessee

Mechanisms of Agouti-Induced Obesity: Role of Adipocyte Metabolism

N. Moustaid-Moussa, K. Claycombe, B. Jones, B. Xue, M. Zemel, W. Wilkison, and R. Mynatt, University of Tennessee, Oak Ridge National Laboratory, Zen-Bio, Inc., and Pennington Biomedical Research Center

Molecular and Cellular Responses of Normal Human Trophoblast to VEGF/PIGF

J. Desai, V. Shore, Z. Wang, M. Caudle, and D. S. Torry, University of Tennessee Medical Center

Regulation of Cell Cycle Proteins and Signal Transduction Molecules in Breast and Ovarian Cancer Cells and Placental Cells

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A Research Program on the Biochemical Mechanisms of Alzheimer's Disease

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MOUSE GENETICS AND MUTAGENESIS AT ORNL

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As sequence from the human genome is generated, the mouse will play an ever-increasing role in assigning organismal functions to specific human DNA sequences. ORNL will focus on utilizing the mouse for enriching DNA-sequence information with functional information based on the genetic analysis of induced mouse mutations. This endeavor will lead naturally into a parallel dissection of the complex interactions of biomolecules in mammals, and of the role of individual genetic variation in susceptibility to disease and/or environmental influences.

Other institutions are proposing to induce mouse mutations to elucidate gene function. ORNL's unique strength lies in the integration of mouse mutagenesis into strong institutional programs in computional biology/bioinformatics and structural biology, employing analytical technologies including mass spectrometry, laser photonics, and neutron crystallography.

We rely on large-scale mutagenesis, primarily by the use of *N*-ethyl-*N*-nitrosourea (ENU), and several different types of genetic screens, to recover phenotypically significant mutations that map to any chromosomal region, genome-wide, or that disrupt or modify complex biological pathways. We are also developing a significantly improved, user-friendly system of embryonic stem (ES)-cell-based mutagenesis to facilitate genetic screens. Following all mutagenesis, we employ broad-based phenotype-screening, for both obvious and subtle mutant phenotypes, in biochemical, behavioral, and morphological parameters. ORNL collaborators are developing instrumentation to expand and automate our screening capabilities.

We have learned that many genes assume new and different functional roles as the organism grows and develops or changes environments. Nulls frequently result in embryonic lethality, so that only the earliest-acting phenotype is seen as an indicator of gene function. In the search for structure/function relationships, the more useful mutations simply alter and don't eliminate the gene product; the knock-out mutation leaves the structural biologist no material to study. By making subtle, domain-by-domain alterations in a gene product, we will enable the study of how variations in protein structure or quantity affect the whole-organism phenotype. These are the kinds of differences most relevant to human genetics.

We also have established capabilities in germ-cell biology and mutagenesis for the study of the genetics of individual variation in susceptibility to mutations induced by exposure to mutagens. The Mouse Genetics Program at the Oak Ridge National Laboratory has a 50-year history of research on germ-cell biology. We have made significant contributions to the understanding of the mutagenic potential of numerous environmental agents on germ cells, the stages in germ-cell development most susceptible to different agent-induced mutations, and the types of DNA damage induced in germ cells throughout their development by various environmental agents.

BEHAVIOR TESTING RESULTS FOR THE 30*PUb* AND 723SJ AND 1060SJ MUTANTS AT OAK RIDGE NATIONAL LABORATORY

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Testing and analysis of two mutant stocks housed at the Oak Ridge Mouse House have demonstrated the effectiveness of the first level behavioral screen developed in the Behavior Laboratory at Oak Ridge National Laboratory. Until recently little attention has been given to behavioral test batteries for mice, with most researchers restricting their investigations to one or two items like learning and memory or balance and coordination through the use of equipment of limited sophistication such as the Morris Water Maze or Radial Arm Maze. The quantitative multilevel screen developed at Oak Ridge was structured to give detailed information on diverse behaviors to differentiate possible phenotypic variations in an extensive and comprehensive manner. The screen utilizes sophisticated equipment not only to gain an in-depth understanding of the obvious aberrant behaviors of certain mutants, but also to help identify more subtle phenotypes and provide a data base of pertinent information on endogenous behavior traits of background and parental stocks/strains used for development of mutants. A vital key to the proficiency and accuracy of the screening of the 30*PUb* and the 723 and 1060 SJ stocks has been the fact that the behavioral assessment is performed in a quantitative manner though the use of state-of-the-art computer-driven equipment.

Extensive data from testing of the 30*PUb* mutant was collected. There were 83 animals tested at 30 days, 76 at 60 days and 26 at 120 days. Certain behaviors were found to be significantly different between groups in three of the primary level screening areas. Three genotypes were tested: the maternally-inherited deletion (pxpl M), the paternally-inherited deletion (pxpl-P) and the wild-type littermates of mice with the deleted region (pxpx). After administering behavioral screening tests at 30, 60 and 120 days, statistically significant functional and performance differences were found between mutants and wild-type animals for the Rotor Rod, immobility in the Porsolt swim test, weight, and exploration in the open field between mutants that inherited the 30*PUb* deletion paternally vs. those that inherited the deletion maternally and wild type littermates.

The 723SJ and 1060SJ mutants resulted from a chemically induced point mutation. Animals that are homozygous for this catatonic mutation have seizures from birth and die around 14-21 days post-natally. The point mutation occurs on chromosome 7 near the pink-eyed dilution locus. An analysis of primary screening data again demonstrated the effectiveness and robustness of our behavioral test battery. Data was collected from heterozygous mutant gene carriers, deletion mutants and wild-type littermates. The BJR background stock was also tested as an additional control for possible contributions of background genes. The animals were tested at 20,40, 60 and 120 days of age. BJR mice were tested only at 60 and 120 days. The carrier and deletion groups were significantly more active than both the wild-type group and background stock for horizontal activity measures. Although carriers were consistently more active than the deletion group across activity measures, mean differences

were not statistically significant for total path distance and total activity. However, carrier mice reared significantly more often than deletion mice. Activity levels for carrier and deletion groups increased with age, but wild-type and background mice maintained a constant level of activity with increasing age. Carrier and deletion mice were progressively less aversive to center exploration, whereas wild-type and BJR mice heightened their avoidance of central exploration with increasing age.

These studies confirm that a comprehensive behavioral screening tool can be used to identify subtle differences in mice with genetic mutations and can play a key role in the elucidation and function of genes that contribute to or assist in behavior function or regulation.

BEHAVIORAL AND FUNCTIONAL ANALYSIS OF BACKGROUND STOCKS AND MUTANT MICE AT OAK RIDGE NATIONAL LABORATORY

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Over the past 4-5 years molecular genetic methods for generating targeted deletions and insertions in mice have become increasingly effective for creating mouse models of human disease. In order to completely understand the aberrant biology seen in mouse mutations and to identify subtle behavior variations a complete characterization of phenotypic and functional differences is required. This is especially true for mouse models of certain disorders such as schizophrenia, Alzheimer, alcoholism, Down Syndrome, and attention deficit disorder. Of equal importance to molecular genetic investigators is an inclusive data base that provides pertinent information on endogenous behavior traits from background and parental stocks/strains. Oak Ridge National Behavioral Laboratory has developed a comprehensive multi-level behavior and functional screening profile that is both qualitative and quantitative in nature. This profile is used for screening background stocks/strains as well as mutants. First level screen tests have been designed to access physical traits such as weight, limb deformities, and any aberrant behavior such as excessive grooming, licking, biting, head-bobbing, tremoring or circling and visual and auditory acuity. Through the use of specialty designed computer driven equipment available in the Oak Ridge Behavior Laboratory, information pertaining to locomotor activity, exploratory bias and activity, learning and memory, balance and equilibrium, swimming performance and distress and fear can be qualitatively and quantitatively evaluated. First level screening tests are generally administered to mice at 30, 60 or 120 days of age or may be administered at only one selected time period. Second level tests are implemented only if there is evidence of a performance or behavioral abnormality after the first level test is given. Second level tests include more definitive learning and memory tasks, evaluation of startle response and latencies and magnitude of those responses, ability to habituate to noise and sensory input, and circadian rhythms.
Specialized equipment that is currently available at the Oak Ridge Behavioral Laboratory and is used for first level screening includes:

- Poly -Track Video Tracking System
- Open Field Photobeam Activity System
- Gemini Avoidance System for Cue and Contextual Memory
- Roto-Rod
- ICR Click box
- A.C.T. test of swim performance (Porsolt)

Specialized equipment that is available at the Oak Ridge Behavioral Lab and is used for second level screening includes:

- Data Col 3 Activity System
- Responder X
- Elevated Plus Maze
- T-Maze

By using this screening profile and comparing behavior results of mutants to background stocks/strains and wild type litter mates a number of behavior and functional differences have been identified in mutants that can be directly attributed to genetic modifications. Collaborations between behavioral neuroscientists and molecular geneticists at Oak Ridge are proving to be a powerful tool in understanding genetic disorders which result in functional and behavior variations and differences.

A SUBDERMAL PHYSIOLOGICAL MONITORING SYSTEM FOR AUTOMATED SCREENING OF MICE IN GENE EXPRESSION STUDIES

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ABSTRACT

Researchers at the Oak Ridge National Laboratory are developing a highly automated integrated-circuit based research tool for subdermal monitoring of physiological parameters in mice used for gene expression studies. Application of this new instrumentation capability to genome studies will accelerate mass specimen screening by providing automated detailed observation and reporting of multiple key physiological parameters of interest. Body temperature, heart rate, physical activity level and movement trajectory will be measured by an implanted integrated-circuit based instrument containing multiple integrated sensors. Measured data will be transmitted periodically via wireless techniques for subsequent data processing, visualization, fusion, and storage. The integrated sensor/telemetry package will be low-cost, and reusable. The system will provide detailed parameter measurement and analysis capabilities not presently available to genomics researchers. Advanced multi-parametric data presentation will permit improved detail and accuracy in high-volume phenotype screening and increased detectability of subtle genetic defects.

This paper will present preliminary information on parameter measurement methods, sensor selection, instrument and system architectures, instrument miniaturization techniques, and data processing methods. Project status and preliminary measurement data using implanted sensors will be discussed.

A HIGH-RESOLUTION COMPUTED TOMOGRAPHY SYSTEM FOR MOUSE PHENOTYPE IMAGING

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ABSTRACT

A novel high-resolution x-ray computed-tomography imaging system, the ORNL MicroCAT (Figure 1), is being developed for high-throughput mouse-phenotype screening. Unlike standard medical CT systems, which have resolutions of approximately 1 mm [1], the MicroCAT currently has a spatial resolution of 0.25 mm and will soon have a spatial resolution of <0.05 mm. The higher resolution is required to accurately image the small organs and skeletal structures of the mice. Also, unlike standard CT systems that measure only the average x-ray flux passing through the subject, the MicroCAT detects and measures the energy of each individual x-ray photon using a novel semiconductor detector. The inclusion of the x-ray energy in the image formation provides more detailed information about the composition of the subject and reduces some artifacts found in traditional systems.

The first reconstructed images obtained using the MicroCAT are shown in Figure 2. The subject is a three-week old normal mouse for which images of the skull, thorax and abdomen were acquired. A 30 kVp x-ray tube and a collimated (0.16 mm) cadmium zinc telluride detector were used to acquire the data sets and a standard filtered back-projection algorithm was used to reconstruct the images.

After the images were acquired and reconstructed, an image segmentation algorithm was applied to define boundaries between organs. The segmentation algorithm successfully segmented the heart, lungs, skeleton, and soft tissue in a thorax scan (Figure 3).

This research is sponsored by the U.S. Department of Energy and performed at Oak Ridge National Laboratory, managed by Lockheed Martin Energy Research Corporation for the U.S. Department of Energy under Contract No. DE-AC05-96OR22464.

Reference

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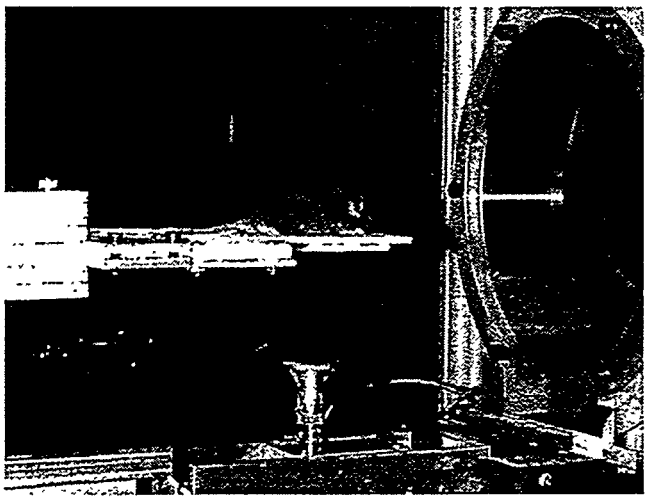

Figure 1. The ORNL MicroCAT x-ray CT scanner.

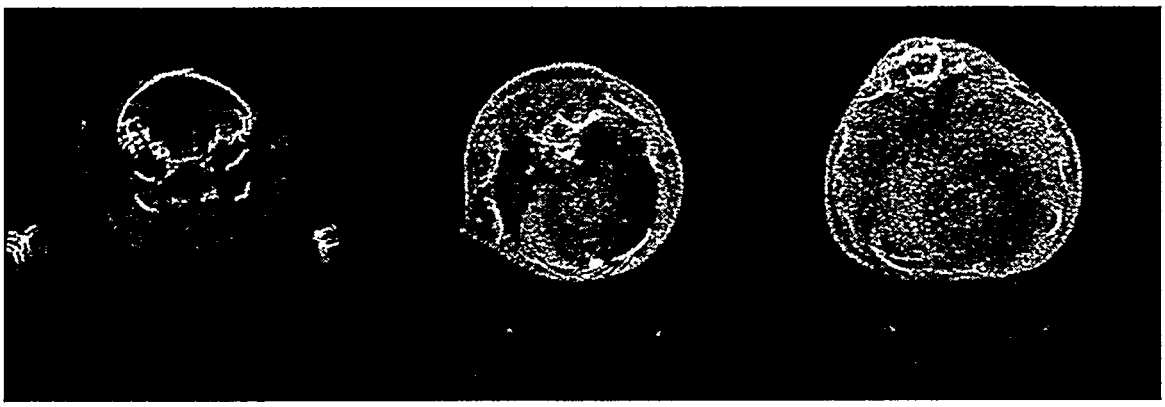

Figure 2. First reconstructed images from the ORNL MicroCAT showing a three-week old normal mouse (a) skull, (b) thorax, and (c) abdomen.

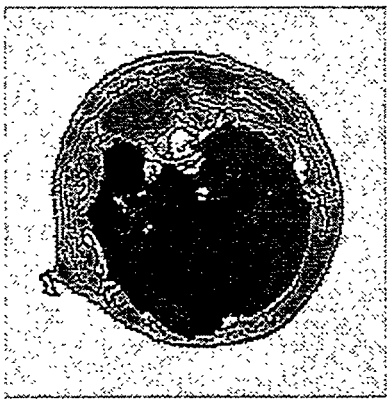

Figure 3. Output of an automatic segmentation algorithm showing the boundaries between organs in the normal mouse thorax image (Figure 2b).

CURRENT AUTOMATED DNA SEQUENCING AND GENOTYPING CAPACITY AT ORNL

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High-throughput, automated DNA sequencing and genotyping has become an essential component of any genetics/molecular biology research program. Because of its ease, sequencing a few hundred kilobases of DNA in order to identify disease gene candidates in positional cloning projects has become the method of choice. Automated PCR-based genotyping is replacing older methods due to its speed, reproducibility and reduced starting material needs. Gene expression studies now depend on data obtained from the sequencing of large numbers of random cDNAs from tissues of interest

We currently have an ABI373 automated DNA sequencer (upgraded to 64 lane capacity) and an ABI377 automated DNA sequencer. If both of these machines were dedicated to full time DNA sequencing we could produce, conservatively, 1-2 million finished bases per year. The sorts of biological objects that are reasonable targets for this capacity are BACs (Bacterial Artificial Chromosomes) which are generally 100 to 150 kb in length and/or large bacterial plasmids which are object of about the same size. Seqeuncing well-chosen BACs and plasmids can provide a large amount of biologically useful information. BACs of mouse genomic DNA selected from regions which are being sequenced from the human genome provide not only a look at the structure of the mouse syntenic region, but allow at direct comparison of the mouse and human sequences. Large plasmids in bacteria often encode multiple factors for drug resistance or pathways for the biochemical degradation of various environmental pollutants. Having the DNA sequence of such plasmids is of great utility for understanding these processes and for engineering biochemical pathways which maybe useful in bioremediation.

Producing this much sequence requires templates for approximately 20-25,000 sequencing reactions. To do this efficiently requires some degree of laboratory automation. At ORNL we have obtained a Qiagen Template robot, a MJR Quad PCR machine and a Robbins Hydra pipettor. These instruments can prepare templates and assemble and run sequencing reactions at a rate about 3 times our current gel running capacity.

Dedicating the ABI373 for fluorescence based genotyping, and making some modest assumptions about multiplexing, we should be able to process about 40,000 markers per year. If a complete genome screen consists of 100 markers on 100 animals, we should be able to complete 4 such screens per year. Again our instrumentation, namely the MJR PCR machine and the Robbins Hydra pipettor will greatly facilitate the genotyping process.

Event-Based Flexible Automated DNA Sequencing

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The ability of the DOE or other organizations to carry out cutting edge biological research and to move toward the more complex realm of gene function study requires the capacity to quickly generate and analyze large amounts of DNA sequence. This ability is vital because DNA sequence is becoming the most basic unit of information exchange in biology. A few hundred bases of DNA sequence can be a unique signature of a DNA fragment, can identify a gene, or, when translated into a protein sequence, can provide a clue to gene function. Compared to complex and technically demanding techniques such as exon-trapping or cDNA selection, sequencing a few hundred kilobases of DNA is often the easiest method to locate a gene of interest. This has been clearly demonstrated in a number of successful positional cloning projects where genomic sequencing was used for the final technical step. Random sequencing of cDNAs is becoming a viable and powerful technique to establish patterns of gene expression. In short, DNA sequencing is a basic, central technique of modern molecular biology, and without a significant capacity to carry out DNA sequencing it is not possible to have a world class, cutting edge program in the basic biological sciences.

In the beginning stages of DNA sequencing, the preparation, sequencing, and analysis were done by "hand" using standard, yet labor-intensive laboratory procedures. Because of the amount of sequencing required (e.g. the human genome has ~3.0 Billion base pairs) selected steps in the sequencing process have been automated. The steps automated so far are those that: 1) had to be automated to be viable (e.g. PCR), 2) were easily automated (e.g. multi-well pipetters), or 3) were very tedious (e.g. sample preparation). In the realm of manufacturing, automation moves from "hand" operation to automation of selected steps and culminates in a continuous flow process (or at least as close as possible to continuous flow). DNA sequencing and analysis on the scale demanded by today's research and development programs is very similar to other manufacturing processes. DNA sequencing and analysis must become a fully automated process to achieve its maximum throughput, thereby having the greatest possible impact upon research. The ideal system must be flexible and contain specific components to allow for continuous optimization. Seamless interfacing of hardware and software components, running multiple steps in parallel, batch and sample tracking, smooth startup and shutdown, and error recovery are all essential features in any automated system. Achieving long term flexibility is possible only with accommodations for the incorporation of new hardware (e.g. new sequencing machines and new PCR machines), scaling for yet-to-be-developed capillary or micro-scale systems, the incorporation of new reaction and preparation hardware, and the integration of new analysis software as it is developed.

ORNL is presently automating their DNA sequencing system for higher throughput and improved quality. The objective of this effort is to move automated DNA sequencing from a sequential, batch-mode process to a continuous-mode, hands-off process including steps from picking and growing bacteria colonies to sequencing and analysis. Parallel and flexible automation will be accomplished in a two-step approach. ORNL researchers are developing an event-based hardware and control system which will fully integrate

existing automated sequencing equipment and provide for rapid and seamless integration of new state-of-the-art hardware. Existing programs aimed at automating DNA sequencing are complemented by this development, but the proposed program makes additional vital contributions in the areas of complete, hands-off sequencing automation, true plug-and-play integration of new hardware, and continuous processing for extra high throughput.

Large-Scale Framework for Analysis and Annotation of Genomic Sequences

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Human and model organism sequencing projects will soon be producing data at a rate which will require new methods and infrastructure for users to be able to effectively view and understand the data. A multi-institutional project was recently funded to provide large-scale analytical processing and annotation capabilities and a pilot system to annotate the human genome has been constructed. The Genome Channel provides a graphical user interface to comprehensively browse and query assembled sequence from the Human Genome Project and from model organisms. It is a JAVA interface tool which relies on a number of underlying data resources, analysis tools and data retrieval agents to provide up-to-date view of genomic sequences, as well as computational and experimental annotation. Navigation from a whole chromosome view to contigs provided by sequencing centers allows one to zoom in on regions of interest to see information about clones, markers, ESTs, computationally and experimentally determined genes, the sequence and sequence source information, related homology and functional information, and hyperlinks to numerous underlying primary data resources. More than 60Mb of human genomic sequence are currently annotated in the system prototype for CpG islands, simple and complex repeats, protein coding exons and potential genes. The conceptual translation of all predicted genes (Genscan and GRAIL exp) are searched using BEAUTY and links to underlying data are available through the browser. In addition, annotation presented in GenBank is visualized and mapping links to GDB are maintained. The data warehouse supporting the Genome Channel view is updated daily by automated Internet agents and event triggers which facilitate analysis procedures. The Genome Channel browser, the underlying warehouse, and analysis tools framework is being constructed by the Genome Annotation Consortium. This project is jointly sponsored by the Computational Grand Challenge Program of the Office of Computational and Technology Research and the Human Genome Program of the Office of Biological and Environmental Research for the Department of Energy.

A GLIMPSE AT THE PAI-1:VITRONECTIN COMPLEX--UNEXPECTED PROPERTIES AND IMPLICATIONS FOR REGULATING PLASMIN ACTIVATION AND RECEPTOR INTERACTIONS

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ABSTRACT

Plasminogen activator inhibitor-type 1 (PAI-1) is the primary inhibitor of endogenous plasminogen activators that generate plasmin in the vicinity of a thrombus to initiate thrombolysis, or in the pericellular region of cells to facilitate migration and/or tissue remodeling. It has been shown that the physiologically relevant form of PAI-1 is in a complex with the abundant plasma glycoprotein, vitronectin. The interaction between vitronectin and PAI-1 is important for stabilizing the inhibitor in a reactive conformation. Although the complex is clearly significant, information has been vague regarding the composition of the complex and consequences of its formation on the distribution and activity of vitronectin *in vivo*. A major objective of this work was the determination of the size and stoichiometry of PAI-1:vitronectin complexes. These experiments are of high importance for several reasons: 1) more than one binding site for PAI-1 has been reported on vitronectin, 2) only poor evaluations of binding stoichiometry have been performed previously, and 3) a proposal has been made that PAI-1 binding induces self-association of vitronectin. In this study, analytical ultracentrifugation was used to directly observe complexes that form in solution; the sedimentation equilibrium method was used in large part to provide accurate determinations of molecular weights for the PAI-1:vitronectin complexes. Mixtures of vitronectin and PAI-1 were evaluated, and a chromophore was specifically incorporated into PAI-1 to allow for the labeled PAI-1 and vitronectin to be distinguished spectrally in the ultracentrifuge. The predominant associating species present in the mixtures exhibits a high molecular weight (M~320,000) corresponding to a 4:2 mixture of PAI-1 and vitronectin. Thus, self-association of vitronectin is indeed observed subsequent to the interaction with PAI-1, supporting the idea that interaction with PAI-1 is the physiological stimulus for self-association that is characteristic of tissue-associated forms of vitronectin. Other biophysical methods including circular dichroism measurements and surface plasmon resonance were also used to characterize complexes formed from purified vitronectin and PAI-1. Together the data demonstrate that *two* molecules of PAI-1 bind per vitronectin molecule, presenting the intriguing idea that both proposed PAI-1-binding sites are functional. Sites for PAI-1 binding on vitronectin have been controversial, previously localized to either the N-terminal 44 amino acids (the Somatomedin B domain) or amino acids 345-379 (the known heparin-binding domain.) Coincidentally, both of these regions have been suggested as the binding sites on vitronectin for the urokinase receptor. The association of vitronectin and PAI-1 into the observed higher order complexes is proposed to facilitate interaction with cell surfaces and the extracellular matrix.

AGOUTI-INDUCED Ca^{2+} SIGNALING AS A THERAPEUTIC TARGET FOR OBESITY AND INSULIN RESISTANCE/HYPERINSULINEMIA

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Overexpression of the murine *agouti* gene results in a syndrome of obesity and hyperinsulinemia/insulin resistance as well as yellow coat color. Although the role of the *agouti* gene in human obesity is unknown, it is expressed in human adipocytes, and we have recently demonstrated expression in human pancreatic islets as well. Effects of agouti protein on coat color are regulated via competitive antagonism of α-MSH at the melanocortin-1 receptor (MC1-R). Since agouti also antagonizes other classes of MCRs, competitive antagonism of MCR binding has served as a paradigm for agouti's action in obesity. In support of this notion, central antagonism of MC4-R recapitulates agouti-induced hyperphagia. However, we found daily injection of a potent melanocortin agonist (NDP-MSH) agonist against all MCRs to affect coat color but not the obese/diabetic phenotype. In contrast, agouti exerts peripheral effects which are independent of MCR antagonism and which are likely to affect the obese/diabetic phenotype.

We have found agouti to increase intracellular Ca^{2+} ($[Ca^{2+}]i$ in several cell types, including both human and murine adipocytes, with an EC_{50} of 18-62 nM. This effect is dependent upon the presence of MCRs, but is not mediated by competitive MCR antagonism. Agouti-induced Ca^{2+} signaling results in a two-fold increase in fatty acid synthase expression and activity in human and murine adipocytes ($p<0.005$) which is mimicked by depolarization with 40 mM KCl ($p<0.03$) and prevented by Ca^{2+} channel antagonism with nitrendipine. Since recent data indicates that increasing $[Ca^{2+}]i$ may also inhibit lipolysis, we investigated the role of agouti in regulating human adipocyte lipolysis. Long-term (24 hr) agouti treatment caused a 60% decrease in basal lipolysis ($p<0.0001$), although short-term (1 hr) agouti treatment was without effect. However, 1-hr agouti treatment totally inhibited ACTH-induced lipolysis ($p<0.05$); similarly, agouti inhibited forskolin-induced lipolysis, demonstrating that the antilipolytic effect of agouti is distal to the ACTH receptor. In contrast, this antilipolytic effect was blocked by nitrendipine and mimicked by stimulation of voltage- and receptor-mediated Ca^{2+} influx with KCl and vasopressin, respectively ($p<0.005$), demonstrating this effect to be Ca^{2+}-dependent. Thus, agouti exerts a Ca^{2+}-mediated coordinate control on both lipogenesis and lipolysis in human adipocytes. These data suggest that adipocyte-specific $Ca^{2=}$ channels may be targets for the development of anti-obesity therapeutic strategies.

However, adipocyte-specific transgenic mice do not become obese unless hyperinsulinemia is induced. Consequently, we sought to determine the role of agouti in Ca^{2+}-mediated insulin release in freshly isolated human pancreatic islets. Agouti induced increases in $[Ca^{2+}]i$ and potentiated the effects of KCl on $[Ca^{2+}]i$, resulting in a 2.4 fold increase in insulin release over a two-hour period. This suggests a potential role for agouti in the development of hyperinsulinemia. This increase in insulin secretion may act in concert with the lipogenic and antilipolytic effects of agouti in adipocytes, thereby contributing to obesity.

IMMUNOLOCALIZATION OF PROTEINS DURING MEIOTIC G2/M CELL CYCLE TRANSITION

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Meiosis, a defining event in the formation of gametes, is a specialized cell division resulting in the reduction of chromosome number. Accuracy of this process is essential and errors in meiosis cause aberrant chromosome division, resulting in aneuploidy, such as human Trisomy 21, or Down syndrome. The meiotic division phase is thus of critical importance in determining the success of gametogenesis.

In our laboratory, we study meiosis during the formation of sperm cells in mice, a suitable model for human spermatogenesis. In particular, we focus on regulation of the transition out of the G2 phase of the cell cycle, or meiotic prophase when chromosome pairing and genetic recombination occur, to the division phase, or metaphase (M), when chromosomes are poised for separation. In order to get out of prophase and into the meiotic division phase, cells must disassemble the synaptonemal complex (SC), the specialized structure that holds paired and recombining meiotic chromosomes together. They also must assemble the spindle apparatus for segregation of chromosomes and disentangle and condense intertwined chromosomes for positioning them on the spindle apparatus. Finally they must properly localize all the components needed to mediate the separation of chromosomes in the division phase. Here we report on the use of techniques for immunofluorescence to localize proteins thought to be important for the G2/M
transition.

We used two different specific inhibitors to demonstrate a requirement in this process for function of topoisomerase II (TOP2), an enzyme that decatenates DNA. We find a marked change in localization of the TOP2 protein as cells progress from early prophase (G2) to the division phase (M). We also have shown, by using an antibody against mitotic protein phosphoepitopes, the MPM2 antibody, that dramatic increases in protein phosphorylation occur during the G2/M meiotic cell cycle transition. A major goal now in the laboratory is the identification and localization of both the kinases and their target proteins that become newly phosphorylated in the meiotic division phase. One such candidate protein is BUB1, the murine homolog of a yeast protein kinase known to be essential for ensuring accurate segregation of chromosomes. Recently it has been shown that mutations in the human *BUB1* gene are associated with the chromosome instability phenotype of some colon cancers. We used an antibody against the murine BUB1 protein for immunolocalization in meiotic spermatocytes. Interestingly, it co-localizes in centromeric heterochromatin with the TOP2 protein, a localization consistent with a role of BUB1 in ensuring successful segregation of meiotic chromosomes in the division phase.

Taken together, these results show dramatic reorganization of protein localization and phosphorylation as meiotic cells enter the division phase, implicate an important role for TOP2, and show structural co-localization of BUB1 protein with TOP2 in meiotic chromosomal dynamics.

INTERACTION OF RETINOBLASTOMA GENE PRODUCT WITH TRANSCRIPTION FACTORS ATF-a AND ATF-2

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The retinoblastoma gene product (pRb) plays an important role in constraining cellular proliferation and in regulating the cell cycle. pRb's regulation of the expression of transforming growth factor β2 (TGF-β2) was suggested by Kim et. al. to be mediated by Activating Transcription Factor 2 (ATF-2). Our lab has confirmed this and also shown that a closely related factor, ATF-a, has a similar effect. When added separately ATF-2, ATF-a and pRb all enhance TGF-β2 expression to a similar extent. However, when pRb is co-transfected with each of the two ATF's, a striking differential response is observed: ATF-2's activity is increased while ATF-a's is decreased. We have explored the functional interaction sites for both ATF-2 and ATF-a on pRb by co-transfecting CHO cells with a series of truncated pRb mutants ± ATF-2 or ATF-a.

Our results demonstrate that, despite the opposing effects of pRb on their actions, ATF-2 and ATF-a share the same interaction sites on pRb *in vivo*; primarily in the so-called A/B pocket of pRb, since deletion of amino acid sequences in these domains causes dramatic decreases in the ability of pRb to affect the TGF-β2 promoter by itself as well as the actions of the two ATF's. The levels of expression of transfected wt and mutant pRb's have been monitored using an antibody against the HA tag located on the C-terminus of transfected pRb's. All mutant forms of pRb have been shown to be expressed at levels similar to wt pRb and overexpression of ATF-2 and ATF-a does not affect the levels of expression of wt and mutant pRb's.

A similar pattern of regulation by pRb ± ATF-2 and ATF-a is observed in the SKUT-1 cell line. These cells lack endogenous pRb and introduction of foreign pRb does not cause cellular apoptosis.

In an effort to begin probing the basis for the differential effects of pRb on the actions of these two ATF's, we have co-transfected a dominant negative mutant of cJun (TAM 67). TAM 67 reduces basal and ATF-2-stimulated expression of TGF-β2, but has no significant effect on the actions of ATF-a. These results raise the possibility that differential heterodimerization of ATF-2 and ATF-a with Jun family members may be involved in the differential effects of pRb.

In vitro and *in vivo* Models of Amyloidosis: Potential for Screening Anti-Amyloid Compounds.

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Immunoglobulin light chain amyloidosis (AL) results from the aggregation and deposition of partially cleaved light-chains in association with a variety of accessory molecules. The factors which govern the aggregation of normally innocuous precursor proteins into amyloid fibrils *in vivo* are unknown, however, it is widely accepted that in vitro fibrillogenesis is a nucleation-dependent polymerization, akin to crystallization. We have developed two models to study the formation and deposition of amyloid and to use as tools in testing potentially therapeutic compounds.

The first, is a human-IL6 transgenic mouse which, as a result of the increased and systemic production of IL-6, produces high titers of apolipoprotein A, the precursor protein of inflammation associated amyloid (sAA). Heterozygous individuals develop amyloid deposits in the liver, spleen and kidney as young as 3 months old, mortality usually occurs at 9 months. The pathology is unequivocal providing an excellent system to screen inhibitors of fibril formation and deposition as well as compounds for amyloid removal.

The second, is an *in vitro* model of fibrillogenesis using recombinant V_L light chains. A comparison of two λ6 V_L proteins, one form a patient with amyloid the other with a more benign aggregation disease has demonstrated that differences in the thermodynamic stability of the proteins correlates with the propensity to form fibrils. A system was developed during this work in which these precursor proteins can be reproducibly induced to form fibril aggregates. Analysis of the data provides parameters which can be used to determine the efficacy of potential therapeutics on each stage of the polymerization process.

In summary, we have developed 2 models of amyloidosis, one of which, the *in vitro* λ6, lends itself to the rapid screening of a large number of potential therapeutics, and the other provides the opportunity to test candidate compounds for their *in vivo* efficacy in a murine sAA system.

IN VITRO MODULATION OF THE MAMMALIAN CIRCADIAN CLOCK BY GABA, SEROTONIN, AND NEUROPEPTIDE Y AGONISTS

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All organisms exhibit daily rhythms in their behavior and physiology that are controlled by endogenous, self-sustaining clocks. These 'circadian' clocks remain synchronized to the external environment through a variety of neuronal and hormonal signals. Research in our laboratory explores the cellular basis of the mammalian circadian pacemaker, located in the suprachiasmatic nuclei (SCN), and how the phase of the SCN pacemaker is modulated by synchronizing signals.

The SCN clock generates 24 h rhythms in spontaneous neuronal activity when isolated in a brain slice preparation. This unique characteristic allows us to experimentally manipulate the circadian clock outside the animal, and therefore determine what stimuli act directly on the pacemaker. For these experiments, 500 um coronal brain slices containing the SCN were prepared from adult, male Sprague-Dawley rats, and maintained under constant perfusion conditions. Drugs were bath-applied for 1 h during the first day in vitro. Extracellular recordings of spontaneous neuronal activity were made during the second day in vitro. Firing rates of individual neurons were used to calculate 2 hr running averages (± SEM) of neuronal activity. These data were then used to determine the time of peak neuronal activity, a reliable measure of the phase of the underlying circadian pacemaker.

The SCN pacemaker displays distinct periods of sensitivity to different modulatory signals. By determining when each signal is capable of resetting the pacemaker, the specific receptors associated with those effects, and the cellular processes underlying their actions, we can begin to piece together some of the mechanisms involved with clock functioning. Currently we are investigating the effects of three neurotransmitters that are associated with SCN neuronal inputs: γ-aminobutyric acid (GABA), serotonin (5-HT) and neuropeptide Y (NPY). We will present data showing that each neurotransmitter affects the SCN clock in a distinct fashion: GABA, acting on $GABA_B$ receptors, advances the clock when applied during the middle of the day, and delays the clock when applied during late night; 5-HT, acting on $5HT_7$ receptors, advances the clock only when applied during the middle of the day; and NPY, acting on Y_2 receptors, advances the clock when applied during late day. In addition to these actions, some of these neurotransmitters modulate each other's actions on the SCN clock. For example, NPY blocks 5-HT-induced phase shifts, while 5-HT does not affect NPY-induced shifts. For further details, see:

Prosser,R.A., Dean R.R., Edgar D.M., Heller, H.C., Miller, J.D. (1993) Serotonin and the mammalian circadian system: I. In vitro phase shifts by serotonergic agonists and antagonists. J.Biol.Rhythms 8:1-16.
Prosser,R.A., Heller, H.C., Miller J.D. (1994) Serotonergic phase advances of the mammalian circadian clock involve protein kinase A and K^+ channel opening. Brain Res.644:67-73

MECHANISMS OF AGOUTI-INDUCED OBESITY: ROLE OF ADIPOCYTE METABOLISM.

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Several dominant mutations at the *agouti* locus such as viable yellow (A^{vy}) and lethal yellow (A^{y}) cause a syndrome of marked obesity and diabetes as well as yellow coat color. Both central and peripheral actions of agouti have been linked to its role in obesity. The purpose of this work was to determine the mechanisms by which agouti's action in adipocytes may contribute to obesity. More specifically, we determined in vivo and in vitro effects of agouti on 1. adipocyte fatty acid synthase (FAS) gene, a key lipogenic gene, and 2. adipocyte leptin, a recently cloned obesity gene (*ob*) involved in body weight regulation.

Our first objective was to investigate regulation of the FAS gene by agouti. In agreement with increased lipogenesis and triglyceride stores in the obese yellow mouse compared to lean controls, we have shown that agouti, like insulin, increased adipocyte FAS gene expression in a Ca^{2+}-dependent manner. Furthermore, agouti and insulin exerted additive effects on adipocyte lipogenesis. We have demonstrated that agouti's effect on FAS gene was mediated by specific cis-acting elements in the FAS promoter, distinct from the insulin response sequences. Future studies will determine the adipocyte trans-acting factors involved in agouti regulation of FAS and their role in obesity and agouti action.

The second objective of this work was to investigate agouti's effect on adipocyte leptin. We demonstrated that like obese yellow mice (A^{vy} mice or mice ubiquitously expressing agouti), all transgenic mice overexpressing agouti in adipose tissue (aP2 transgenic mice), whether hyperinsulinimic or not, express significantly higher levels of leptin both in plasma and adipose tissue compared to controls. This suggests that agouti upregulates leptin content in adipocytes, independent of obesity. We did not detect a significant effect of insulin on leptin in this model. In agreement with these in vivo studies, we demonstrated that agouti directly increases leptin content in 3T3-L1 adipocytes as well as in culture media from these cells.

In conclusion, these studies indicate that agouti increases lipogenesis and triglyceride storage, leading to adipocyte hypertrophy and; consequently increased secretion of leptin. This leptin response may then serve to limit agouti-induced obesity.

MOLECULAR AND CELLULAR RESPONSES OF NORMAL HUMAN TROPHOBLAST TO VEGF/PlGF.

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Successful pregnancy is dependent upon adequate vascular growth, permeability and remolding in the placenta and endometrium. Vascular and placental insufficiencies are associated with many obstetrical complications, most notably preeclampsia and IUGR (idiopathic intrauterine retardation). However, little is known regarding the regulation of angiogenic growth factor production by trophoblast in placenta. We have characterized the expression of two angiogenic growth factors, VEGF (vascular endothelial growth factor) and PlGF (placenta growth factor) in isolated normal trophoblast. Both growth factors are known to be mitogenic to endothelial cells *in vitro* and are able to induce all aspects of angiogenesis *in vivo*. Trophoblast express high levels of PlGF under normoxic culture conditions, however, the same cells express relatively little VEGF. In contrast, trophoblast expression of PlGF is abrogated by hypoxia *in vitro*, while VEGF expression is strongly upregulated. These results show that trophoblast expression of PlGF/VEGF is modulated by oxygen tension and that trophoblast-derived VEGF/PlGF could act in a paracrine fashion to regulate endometrial blood vessel growth and stability during pregnancy.

Two receptors for VEGF/PlGF have been characterized, flt-1 and KDR. Trophoblast were found to express only flt-1 receptors. The presence of flt-1 receptor on trophoblast suggest that VEGF/PlGF may have active autocrine roles in regulating trophoblast function in addition to their angiogenic roles in placenta and endometrium. Binding of VEGF/PlGF to flt-1 on normal trophoblast resulted in activation of c-jun-N-terminal kinase/Stress activated protein kinase (JNK/SAPK) and $_{p}$38 kinase, with little to no induction of the mitogen activated protein kinase (MAPK) pathways, ERK1&2. Activation of the JNK/SAPK pathway functioned to protect cultured trophoblast from apoptosis induced by growth factor withdrawal but not by pro-inflammatory cytokines. These results provide the first evidence of a functional role for VEGF/PlGF/flt-1 in mediating normal trophoblast function. Collectively, our studies suggest that aberrant trophoblast expression of VEGF/PlGF would contribute to both the vascular and placental pathologies commonly noted in perfusion compromised pregnancies.

REGULATION OF CELL CYCLE PROTEINS AND SIGNAL TRANSDUCTION MOLECULES IN BREAST AND OVARIAN CANCER CELLS AND PLACENTAL CELLS

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During the past few years, we have studied signaling proteins mediating growth promoting actions of estrogens in breast cancner cells, microtubule disrupting agents in ovarian cancer cells and those which may play a role in trophoblast differentiation in the human placenta. Our studies demonstrate that estrogens (both natural and man-made) increase synthesis of G_1 phase cyclins and activate cyclin-dependent kinases (Cdks) and decrease the activity of Cdk inhibitors. The regulation of these critical cell cycle proteins is likely to underlie the growth promoting action of estrogens in breast cancer. Our studies in human trophoblast differentiation have shown that G_1 cyclins, Cdks, Cdk inhibitors may play a significant role in the events which lead to the terminal differentiation of cytotrophoblasts into syncytiotrophoblast, cells critical for normal fetal development. Microtubule disrupting agents have been successfully used in treating both breast and ovarian cancer. Our studies of such agents, e.g. paclitaxel, have lead us to propose a novel signaling pathway which may be important in how ovarian cancer cells signal disruption of microtubules that initiate a cascade of events culminating in apoptosis.
Supported by NIH

A RESEARCH PROGRAM ON THE BIOCHEMICAL MECHANISMS OF ALZHEIMER'S DISEASE

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The molecular and cellular events leading to the behavioral, cognitive, and functional abnormalities that constitute Alzheimer's disease (AD) are still not understood. A number of hypotheses have been framed, each of which has accumulated vast amounts of supporting data. Perhaps the most compelling data is our knowledge of four genes, mutations in which are linked to varying degrees of susceptibility to AD. The study of the products of these genes, even those associated with rare familial forms of AD, will provide important clues to the mechanisms of AD.

The protein products of each of these four genes are metabolically linked to a peptide called Aβ; different mutations in these genes seem to influence, directly or indirectly, the accumulation of insoluble Aβ deposits, called amyloid plaques, in the brain. Because these deposits turn out to be a characteristic feature of Alzheimer's brain pathology, many researchers in this field would like to believe that it is the ability of the gene products to influence plaque amounts that in turn influences the rate of development of AD in an individual.

The Alzheimer's Disease Research Program at the UT Medical Center in Knoxville is being developed around the central idea that amyloid deposition plays a key mechanistic role in the development of Alzheimer's disease. The main focal points of the research program will be: (1) the structure of Aβ amyloid fibrils and their mechanism of formation, (2) the search for other molecules which inhibit the formation of amyloid fibrils, as candidates for therapy, and (3) the elucidation of the mechanism(s) by which amyloid fibrils cause neuronal death and/or dystrophy. These studies are being pursued, in part, via collaborations with researchers and clinicians at the main campus of the University of Tennessee, at the Oak Ridge National Laboratories, at the Cole Neurosciences Center, and with other groups within the Medical Center.

Saccharomyces cerevisiae (BAKER'S YEAST): MODEL ORGANISM FOR FUNCTIONAL GENOMICS STUDIES

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ABSTRACT

The yeast *S. cerevisiae* is the first eukaryotic organism whose genome has been totally sequenced thus providing a wealth of information for use in functional genomics studies including the ability to use genosensor array to probe expression of every gene of this organism. *S. cerevisiae* is also an ideal organism for functional complementation studies using human genes or genes from other eukaryotic organisms.

We are pursuing three lines of investigation with approaches that may be considered universal in their application to eukaryotic cells: 1) the interaction between small peptide ligands and a G protein-coupled receptor as a model for activation of signal transduction pathways; 2) the mechanism and regulation of small peptide uptake by membrane transport proteins; and 3) the identification of virulence factors in *Candida albicans*, an important human pathogenic yeast.

An understanding of the interaction between a hormone and its receptor is necessary for comprehending signal transduction/intercellular communication and for designing therapeutically useful analogs. Our studies combine chemistry and molecular biology to investigate the biologically relevant conformation of small peptide ligands and their interaction with a seven-transmembrane-domain, G protein-coupled receptor representing a major target for pharmacological agents used in human medicine.

We have succeeded in cloning, sequencing and characterizing peptide transport genes from *Saccharomyces cerevisiae*, *Candida albicans*, and the plant *Arabidopsis thaliana*. Analysis of the peptide transport proteins encoded by these genes indicates that we have found two new families of eukaryotic membrane transport proteins. In addition, we have discovered genes that are involved in the regulation of peptide transport including a regulatory gene encoding a component of the ubiquitin-mediated proteolytic pathway.

Fungi are increasingly important as opportunistic pathogens in humans immuno-compromised by infection with HIV, by treatment with immunosuppressants during organ transplantation, or by administration of anticancer drugs. We are interested in identifying the fungal genes encoding "virulence factors" that allow fungi to manifest their pathogenicity. We have identified several such genes by expressing them in *S. cerevisiae,* deleting those genes in *Candida albicans,* and then determining the pathogenicity of the strain carrying the deletion.

ADVANCED TECHNOLOGIES FOR BIOMARKER MONITORING AND GENE DIAGNOSTICS

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Abstract

For the past few years, several advanced techniques and instrumentation have been developed in our laboratory for the measurement of biomarkers of exposure and health effects. Chemicals, such as polycyclic aromatic compounds (PACs) or vinyl chloride, which are active as carcinogens and mutagens, have electrophilic properties or are metabolically converted into electrophiles. These reactions result in the formation of covalent adducts. Our research focus has been on DNA adducts, since these species are believed to represent initiating events leading to cell mutation and/or malignant transformation. Protein adducts have also been investigated as biomarkers for exposure. We have also developed several advanced technologies for use in gene diagnostics, genetic susceptibiltiy monitoring and functional genomics research.

Synchronous Luminescence. A significant improvement in fluorescence detection of DNA adducts is the synchronous luminescence (SL) technique. Conventional luminescence spectrometry uses either a fixed excitation or fixed emission wavelength. With SL, both excitation and emission wavelength are scanned synchronously, producing a spectrum with a more resolved structure and more readily identified peaks. The combination of tunable laser excitation with synchronous scanning has shown to further improve the sensitivity to zeptomole (10-21 mole). The SL technique has been used to detect low levels of carcinogen-DNA adducts in human populations (e.g., coke oven workers) due to environmental exposure to PACs.

Surface-enhanced Raman scattering (SERS). The application of Raman spectroscopy for the study of biological species is rapidly expanding because of the specificity of this analytical technique for chemical identification. The SERS effect, whereby an enhancement factor of up to 108 in Raman signals from molecules adsorbed on rough metallic surfaces, has generated increasing interest in the Raman technique. BPDE-DNA adducts have been analyzed by SERS. For biological samples, conventional Raman spectroscopy has the disadvantage of requiring large samples (usually 10-100 mg of the bulk pure specimens). The increased sensitivity provided by the SERS effect has eliminated this major limitation. Our study also indicates that other types of adducts can also be identified in the DNA sample without requiring chromatographic separation of the adduct species from the DNA products. The SERS technique has recently been used for multiplex gene mapping.

Antibody-based fluoroimmunosensors (FISs) have been developed for the carcinogen benzo[a]pyrene (BaP) and related adducts such as benzopyrene tetrol (BPT). Polyclonal or monoclonal antibodies produced against BaP and BPT are immobilized at the terminus of a fiberoptics probe or contained in a microsensing cavity within the FIS for use both in *in vitro* and

in-vivo fluorescence assays. High sensitivity is provided by laser excitation and optical detection. The FIS device utilizes the back-scattering of light emitted at the remote sensor probe. A single fiber is used to transmit the excitation radiation into the sample and collect the fluorescence emission from the antigen. The laser radiation reaches the sensor probe and excites the BaP bound to the antibodies immobilized at the fiberoptics probe. The excellent sensitivity of this device illustrates that it has considerable potential to perform trace analyses of chemical and biological samples in complex matrices. Measurements are simple and rapid (~ 5 min), and the technique is applicable to other compounds provided appropriate antibodies are used. The FIS instrument can detect 1 femtomole of BaP and 40 attomoles of BP-tetrol.

Biochips. We have recently investigated a new generation of biosensors using DNA probes (DNA Biochip) Probe recognition is based on the molecular hybridization process, which involves the joining of a strand of nucleic acid with a complementary sequence. Biologically active DNA probes are directly immobilized on optical transducers which allow detection of Raman, SERS, or fluorescent probe labels. DNA biosensors could have useful applications in areas where nucleic acid identification is involved. The DNA probes could be used to diagnose genetic susceptibility and diseases. The Biochip using antibody probes has recently been developed to detect the p53 protein system.

LASER DESORPTION MASS SPECTROMETRY FOR DNA ANALYSIS AND SEQUENCING

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During the past 7 years, the Photophysics group in Life Sciences Division at Oak Ridge National Laboratory has been developing new Laser Desorption Mass Spectrometry (LDMS) technologies for DNA analysis and sequencing. The potential applications include (1) rapid DNA sequencing for Human Genome Project (2) fast clinical diagnosis and population screening for genetic diseases, (3) microbial genome sequencing for bioremediation programs (4) mutation detection for environmental impact analysis by selected contaminants and (5) quick DNA typing for forensic applications.

Historically, DNA analysis and/or sequencing has replied heavily on gel electrophoresis which is a somewhat time-consuming and labor-intensive process. LDMS reduces the analysis and/or sequencing time to seconds compared to hours required by the electrophoretic approach. In addition, no radioactive or dye tagging is required. Thus, LDMS for sequencing/analysis can not only save time and cost but also reduce the production of secondary contaminants.

LDMS utilizes laser desorption to produce gas phase DNA ions which are subsequently analyzed by a mass spectrometer (MS). Since some of the chemical bonds in DNA are "fragile" and easily broken by direct laser ablation, we have tried different laser desorption processes to obtain intact DNA ions for mass spectrometric analysis. One approach is to use matrix-assisted laser desorption/ionization (MALDI) which was developed by Hillenkamp and his colleagues for protein analysis. With MALDI, DNA molecules are mixed with a much larger amount of small organic compounds such as 3-hydroxypicolinic acid and placed inside a mass spectrometer for analysis. The wavelength of the laser is selected to be strongly absorbed by the matrix compound which is subsequently vaporized and gently carries the large DNA molecules into space without breakup. Some DNA ions are produced by proton transfer, electrostatically accelerated, and detected by a time-of-flight mass spectrometer (TOF-MS).

During the past few years, we have been successful in developing MALDI for a number of DNA sequencing applications. One approach is to produce DNA ladders by Sanger's enzymatic method and then measure these ladders with MALDI-TOF-MS to obtain the sequence information. Figure 1 shows DNA sequencing of ss-DNA of 130 nucleotides by MALDI-TOF-MS. Double-stranded DNA up to 200 bp was also sequenced by MALDI. Furthermore, we developed matrix-assisted ultra-violet laser desorption/ionization with selected fragmentation (MALDIF) for direct DNA sequencing without the need of the preparation of DNA ladders. Figure 2 shows the direct DNA sequencing of 28 nucleotide ss-DNA by MALDIF. In addition to sequencing, MALDI has been used to achieve disease diagnosis for cystic fibrosis, Huntington's disease and dentatorubral-pallidoluysian atrophy (DRPLA). Measurements of trinucleotide repeats for dynamic mutation analysis can also be

applied to DNA fingerprinting for forensic applications. Preliminary results indicate a high feasibility of using MALDI-TOF-MS for rapid forensic sample analysis.

Currently, DNA analysis/sequencing by MALDI is somewhat limited by poor mass resolution of large DNAs. A new approach using laser induced acoustic desorption will be pursued to improve mass resolution. If mass resolution can be significantly improved, LDMS is expected to emerge as valuable biotechnology for DNA analysis.

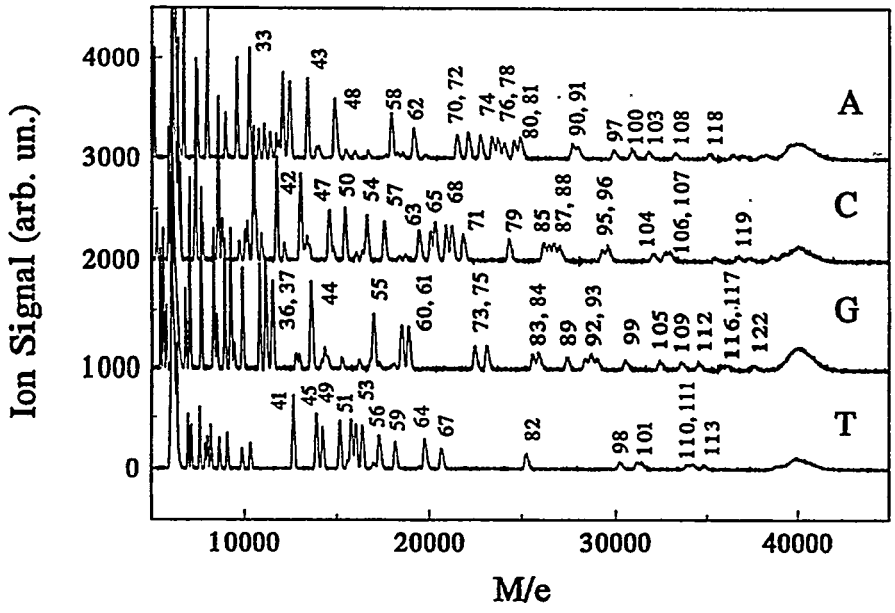

Fig. 1

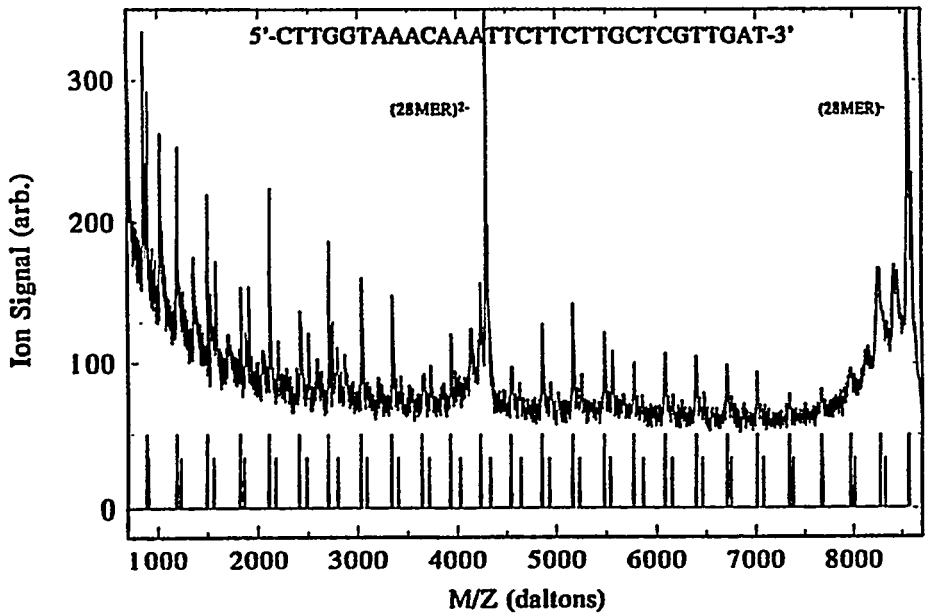

Fig. 2

Research sponsored in part by the Office of Biological and Environmental Research, U. S. Department of Energy under contract DE-AC05-96OR22464 with Lockheed Martin Energy Research Corporation, and the National Institute of Justice.

IMPROVED MASS SPECTROMETRIC RESOLUTION FOR PCR PRODUCT SIZE MEASUREMENT

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While a wealth of biological and genetic information can be gleaned from properly-designed polymerase chain reaction (PCR) assays, currently-used technologies for analysis of the resulting oligonucleotides all suffer from limitations in speed, accuracy, or convenience. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) offers considerable potential for rapid and accurate molecular mass determination of biopolymers, such as proteins and DNA. In order to achieve this potential, we are working to improve the utility of MALDI-MS for measurement of PCR product size.

The resolution (ability to distinguish products of similar molecular mass) of MALDI-MS is determined by both chemical and instrumental factors. The presence of reaction components necessary for polymerase activity, particularly metal ions, causes broadening of the observed peaks in MALDI mass spectra of PCR products. Post-PCR removal of these metal ions and other interferences can be performed efficiently using reverse-phase cartridges in syringe-mounted or microtiter plate formats. The wide energy distribution imparted to biomolecules by the laser desorption process also broadens mass spectrometric peaks. Delayed ion extraction has greatly improved the resolution of MALDI-MS by compensating for this energy spread. Combining these techniques, PCR products differing in length by a single base can be resolved up to a total length of 60 bases or more, and larger oligonucleotides can be detected if single-base resolution is not required. The design of PCR products that are shorter than typically used for gel electrophoresis is thus a high priority in improving the applicability of MALDI-MS.

Other important practical considerations are the reproducibility and throughput of MALDI-MS. Commercially-available instrumentation allows robotic loading onto multiple-sample plates followed by automated analysis. However, samples outside narrow constraints of concentration, size, and purity still require human intervention because of the inhomogeneity of the dried matrix/sample mixture. We are currently developing methods for combining oligonucleotides with the MALDI matrix to yield a homogenous preparation resulting in a uniform signal at any point interrogated by the desorption laser.

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INVESTIGATING PROTEIN-DNA INTERACTIONS BY AFM

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ABSTRACT

Deoxyribonucleic acid (DNA), is a 2-nanometer-wide double helical biopolymer whose nucleotide sequence serves as the genetic code for most living organisms. The ultimate products of the genetic code are proteins most of which have structural or metabolic functions. Cellular processes are controlled by proteins which interact with DNA at specific sites for a variety of purposes. Restriction enzymes recognize and cleave DNA molecules at specific nucleotide sequences and have been exploited by scientists for physical mapping. Transcription factor proteins recognize specific nucleotide sequences in gene promoter regions and along with other sequence specific regulatory enzymes initiate transcription and modulate gene expression. Other proteins recognize and repair sites of uv damage, small deletions, and even point mutations in DNA molecules.

The genetic sequence of several complete genomes exist and the ongoing Human Genome Project will eventually provide detailed information on all proteins comprising the human. This information will greatly complement investigation of DNA-protein interactions to better understand development, metabolism, and structure/function relationships. Our laboratory has demonstrated that the native activity of site-specific proteins, interacting with DNA, can be documented by atomic force microscopy (AFM).

Scanning probe microscopes, initially developed and used extensively by the physical science community, are now finding varied applications in life sciences research. The first was the scanning tunneling microscope (STM), invented in 1982 [1], and proved to be a stunning success in being able to visualize the positions of atoms on a variety of surfaces. The atomic force microscope (AFM) was developed in 1986 [2] and has been more frequently used to image biomolecules, because it does not require conducting samples. The AFM is a contact stylus profilometer that has been refined to allow topographic measurements of surfaces with sufficient sensitivity to nondestructively resolve the atomic lattice. The scanning mechanism is similar to that of other SPMs but with mechanical sensing provided by the precise monitoring of a tiny cantilever in contact with the surface. AFM research has documented atomic periodicity and sometimes even single atomic defects on flat surfaces such as crystal faces [3] . For rough surfaces the resolution is limited by the radius of curvature of the probe tip, which is typically 10-50 nm for commercially produced products. Nevertheless, images of a variety of biomolecules including DNA and proteins are routinely possible and are not compromised by having to stain or chemically treat the molecules prior to imaging.

Our AFM laboratory at ORNL is known world wide for biological imaging and has pioneered immobilization of genetically functional molecules on surfaces [4]. We have studied a variety of biomolecules including tobacco mosaic virus (TMV), DNA, RNA, various proteins, and protein complexes. For example, high-resolution AFM imaging and site-specific mapping of the protein *Eco*RI endonuclease bound to plasmid [5] and to cosmid-sized genomic clones [6] has been

accomplished in our laboratory. These studies have been expanded through commercial collaborations to include imaging of the site-specific binding of a mutant *Bam*HI endonuclease protein to target DNA molecules. These results have prompted us to look at other site-specific DNA-protein interactions pertinent to genomic function. Recently, we have used the AFM to image transcription factor binding to defined DNA clones. Both human and yeast TFIID : TFIIB complexes have been imaged interacting with gene promoters. By increasing the number of specific proteins that define the minimal transcription initiation complex, the number and position of genes on large DNA clones (BAC or Cosmid) may be identified. Eventually, the structures of specific transcription complexes, and long-range transcriptional control mechanisms, may be elucidated. DNA repair proteins which bind to specific lesions, rather than sequences are also being investigated in our laboratory to identify the positions of small damaged sites. Under proper conditions these proteins will migrate to lesions with very high specificity enabling the AFM to position mutation sites on large DNA clones. These experiments will have applications in the technically challenging areas of identifying point mutations, and sequence polymorphisms on unsequenced individual DNA molecules.

Additionally, informatic approaches are being developed to facilitate and interpret image data. These programs will automate quantitation and position measurements as well as improve throughput by allowing mixed samples to be concurrently analyzed. Genetically engineered DNA sequences are also being constructed for the precise positioning of sequence specific proteins along DNA molecules. In this way the conserved structure of DNA and the sequence specific binding properties of proteins can be exploited for nanopositioning of proteins and other structures. Our demonstrated results are creating opportunities to study interaction at the level of the single molecule, with previously unattainable potential for future applications.

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FLOWTHROUGH GENOSENSOR CHIPS: DEVICE DEVELOPMENT AND APPLICATIONS

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A flowthrough genosensor instrument for ultrahigh throughput analysis of nucleic acids is under development at Oak Ridge National Laboratory. The core of this microscale instrumentation is a microchannel hybridization array, containing a library of thousands of specific DNA sequences, immobilized within individual microporous cells in a thin layer of silicon or glass. When a labeled nucleic acid sample is passed through the microchannel genosensor at precisely controlled temperature and flow rate, the target strands bind to positions within the array containing complementary probe sequences. The quantitative binding pattern reflects the base sequence of the nucleic acid strands present in the analyte and reveals the relative abundance of different sequences. The porous glass configuration offers several important advantages over flat surface DNA chips being developed by others: greatly improved hybridization kinetics, superior detection sensitivity and dynamic range, the ability to analyze dilute solutions of nucleic acids, and direct detection of heat-denatured PCR fragments without prior isolation of single strands.

The prototype genosensor system includes an extensively modified Hamilton MicroLab fluidic dispensing robot for arraying of probes onto the genosensor chip; a fluidics module for flowing the sample through the chip at precisely controlled temperature and flow rate; and a CCD imaging system for quantitation of hybridized fluorescent or chemiluminescent strands at each position in the microarray. A key objective in the project is to develop important applications of the flowthrough genosensor for nucleic acid sequence analysis and gene mapping. Feasibility studies for several genosensor applications are being pursued. In one project, a series of miniature "genochips" containing arrays of genomic DNA fragments are being prepared for use in gene discovery and mapping and comparative genomic hybridization. Another project employs flowthrough arrays of DNA probes for transcriptional profiling, facilitating the discovery of genes that function in specific biological processes. A third application of the flowthrough genosensor involves model hybridization studies with defined nucleic acid sequences, aimed at providing a more complete understanding of the specificity of oligonucleotide hybridization, which will facilitate appropriate selection of probes and valid interpretation of hybridization patterns. Another project, being pursued in collaboration with Dr. James Weber at the Marshfield Medical Research Foundation, is high throughput genotyping. In this work miniature flowthrough genosensors are fabricated to simultaneously analyze thousands of biallelic single nucleotide polymorphisms (SNPs) and short insertion-deletion polymorphisms. Finally, a collaboration with the Army Waterways Experiment Station and the Naval Research Laboratory has been initiated to apply the flowthrough genosensor for ecotoxicity response assessment. In this work genosensor arrays are being developed for detection of gene expression responses associated with exposure of soil and water organisms to toxic chemicals and radiation.

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MICROFABRICATED DEVICES FOR GENOME ANALYSIS

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The ability to perform all of the steps of a biological assay on a single microdevice promises significant advantages to biochemists and molecular biologists in terms of speed, cost and automation. Microfabricated devices are being developed in our laboratory for integrated processing and analysis of DNA. These devices employ electrokinetic manipulation of fluid samples and reagents in microfabricated channels, and fluorescence labeling and detection of analytes. The first integrated microfluidic biochip performed the steps of on-chip mixing and incubation of DNA with restriction enzyme in a nanoliter reaction chamber, followed by electrophoretic separation of the restriction fragments in a channel containing sieving matrix. Recently we have integrated PCR amplification and electrophoretic sizing of amplicons in a monolithic device. The steps of thermal cell lysis to release DNA, simultaneous amplification of multiple gene loci, and electrophoretic analysis of amplified products were performed on plasmid-containing *E. coli* cells in a single microchip (Figure 1).

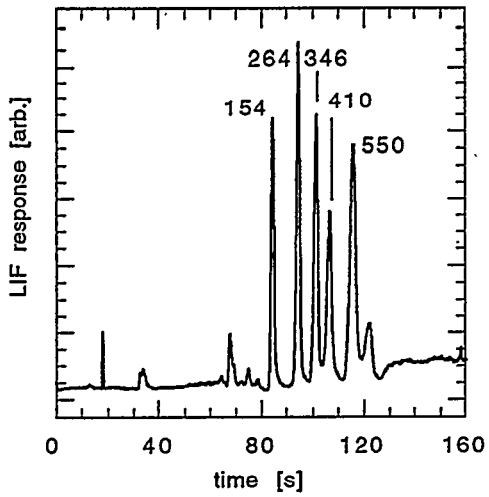

Figure 1. Separation of amplicons from on-chip multiplex PCR of *E. coli* cells containing cloned plant gene *agNt84* in pcDNAII plasmid. The 154, 264 and 346 bp fragments were amplified from *E. coli* genomic DNA and the 410 and 550 fragments were from the cloned gene. Fragments were labeled with intercalating dye (TO-PRO).

Parallel assays can be performed in microchips containing multiple reaction chambers coupled to a common separation channel. Appropriate manipulation of electrical potentials allows the analysis of samples from individual reaction mixtures or simultaneous injection and analysis from multiple reactions (Figure 2). Such chips may be particularly useful in situations where the incompatibility of PCR primers prevents their being used together in single multiplex reactions. Microchip architectures containing multiple parallel separation channels, each coupled to multiple reaction wells, will allow large format microassay devices, e.g. 96-well chips. Current work in our laboratory has demonstated the ability to thermally cycle microchip devices at rates comparable to or faster than those attainable in conventional PCR in tubes, i.e. ≤1 min/cycle.

The ability to isolate and concentrate DNA products from on-chip reactions prior to electrophoretic analysis would greatly enhance the speed and reliability of assays. We have demonstrated >1000-fold preconcentration of DNA fragments in microchips followed by size separation. This technique will allow analysis of PCR products at low cycle numbers as well as enhancing the analysis of on-chip cycle sequencing reactions and other reaction mixtures containing low DNA levels.

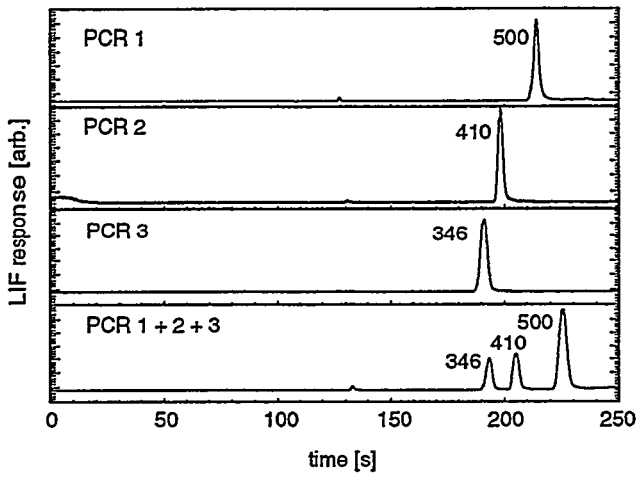

Figure 2. PCR analysis in a multireactor microchip. PCRs 1 (500 bp), 2 (410 bp) and 3 (346 bp) were performed in separate reactors and then analyzed individually or together (bottom panel) in the same separation channel.

Future efforts will be directed toward expanding the number of different DNA samples that can be analyzed in this format. The goal is to adapt this microchip technology to the analysis of mammalian DNAs for research (functional genomics), forensics, and medical diagnostics.

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MICROFLUIDIC DEVICES COUPLED TO MASS SPECTROMETRY FOR PROTEOME MAPPING

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Miniaturized chemical instruments, "Lab-on-a-Chip" technologies, are being developed for rapid, comprehensive analysis of cellular proteins, as an alternative to the slow and labor-intensive 2D gel methods currently used for protein mapping. The microfabricated devices integrate on a single structure, multiple elements that enable multidimensional separations of protein mixtures and electrospray ionization of the analytes for direct, on-line interfacing with mass spectrometry. The platform exploits the many advantages of Lab-on-a-Chip devices, including small size, inexpensive fabrication, high speed, low volume materials consumption, high throughput, and automated operation. Potential applications of the technology include quantification of gene product levels in specific cell types, comparative analysis of patterns of gene expression in different tissues at different stages of development, analysis of structural and/or expression level changes resulting from mutagenesis or genetic disease, and identification of specific protein markers of disease.

Microchips that incorporate liquid-phase separations such as electrophoresis and electrochromatography are being increasingly recognized as a convenient means for manipulating small quantities of material.[1,2,3] The devices are fabricated on glass or quartz substrates using standard micromachining techniques such as photolithography, wet chemical etching, and thin film deposition. The products are planar devices with micron-sized channels through which materials are directed using electrokinetic forces. Structures with various interconnecting channels are easily fabricated allowing separations and reactions to be performed at relatively high speed. The ability to design and construct systems with the necessary architecture to perform diverse and complex chemical and analytical functions on a given sample is a major advantage. Recent advances that have been made with the Lab-on-a-Chip devices make these microsystems good candidates for high-throughput protein separations and analysis.

The conventional method for resolving cellular proteins is 2D polyacrylamide gel electrophoresis, a technique that is labor intensive, slow, and has poor reproducibility, sensitivity, and sample recovery. Individual spots may be identified off-line using mass spectrometry (MS) but sample extraction and transfer processes are problematic. Column liquid chromatography or capillary electrophoresis (CE), which are more easily coupled with MS using electrospray ionization (ES), in general, lack the resolution required for the analysis of complex biological samples. Two dimensional separations greatly increase the resolving power, provided the individual methods are orthogonal, and when combined with MS result in a powerful technique, given the multiplicative effect of joining different separation mechanisms. Microchip structures that integrate capillary electrochromatography (CEC), CE, and ES have been designed for this program. Devices that allow rapid, on-chip adjustments of the elution strength of buffers

by electrokinetic fluid control have been developed for CEC.[4,5] Gradients of various shapes can be generated in fractions of a minute to enable rapid and efficient optimization of run conditions and reduce analysis times. High speed electrophoretic separations, which are necessary to effectively sample and analyze the effluent from the first dimension, have also been demonstrated.[6] We have also recently shown that ES may be generated from microchip devices using electroosmotic pumping.[7] A diagram of the microchip used for this purpose is shown in the adjacent figure. Sample is introduced at one of the uppermost reservoirs, traverses down the main center channel by electrokinetic forces, and is transported to the opening by electroosmotic induced pressures. The fluid, as it exits the channel, is electrostatically sprayed by applying a potential of sufficient magnitude between the microchip and the front aperture of the mass spectrometer. The device is now being interfaced to an electrospray/time-of-flight analyzer that has the acquisition rates necessary to capture mass spectra from rapidly eluting components from the microchips. As a final element, structures that incorporate multiple sample ports for rapid, sequential analysis will be developed. Increases in sample throughput are anticipated to be greater than two orders of magnitude as compared to 2D gels.

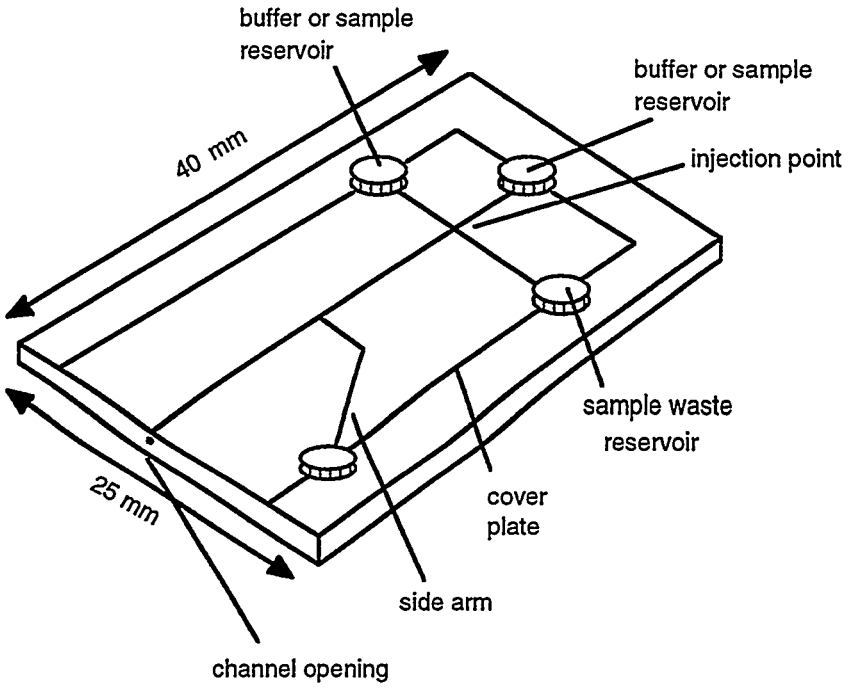

Fig.1: Microchip device used to generate electrospray

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