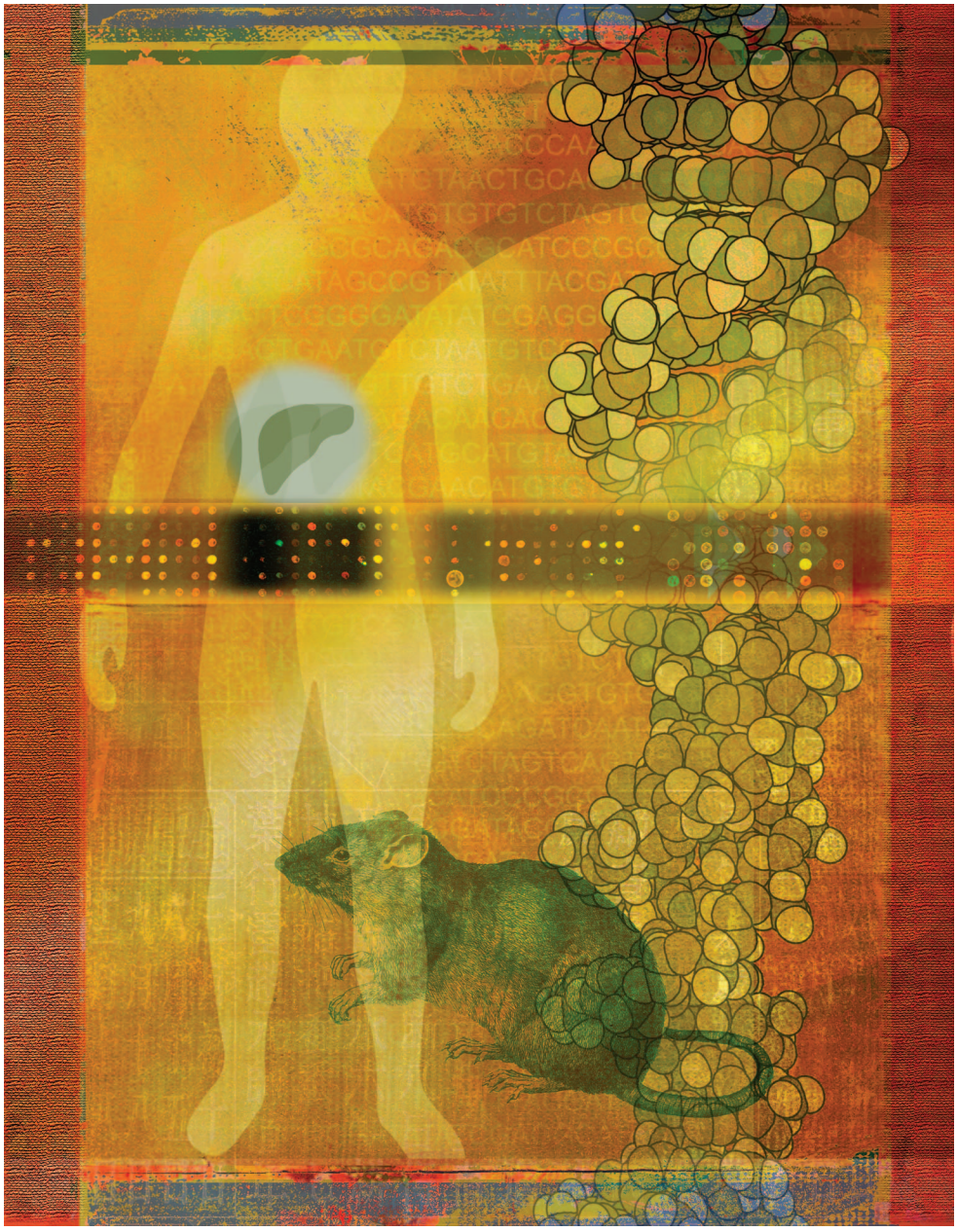
Liver Library

Creating a Microarray for Hepatotoxicants

Mechanical improvements in high-throughput applications continue to increase the utility of the microarray approach for investigating toxic effects on genes. But improvements in the content of arrays may be the key to maximizing the value of these technologies, according to a paper in this month's issue [***EHP* 111:863–870**]. In the report, researchers at Abbott Laboratories and Rosetta Inpharmatics, led by senior research scientist Jeffrey Waring, lay out the development of a microarray specifically constructed for studying the effects of hepatotoxicants.



Array of hope. Scientists have a new tool for understanding liver responses to toxic exposures.

Work in toxicogenomics has so far focused primarily on hepatotoxicity because of the importance of the liver as a site of toxic response. Whereas earlier toxicology-focused arrays were put together using DNA libraries from normal or diseased tissues, building a library from toxicant-challenged animals is a new approach. Because these animals were specifically expressing genes regulated in response to toxic exposures, it was possible for the Abbott–Rosetta team to enrich for genes regulated by toxic compounds, making their array a highly specific tool for understanding the function of rat liver undergoing toxic exposure. Understanding how gene expression changes when animals face different toxicants is especially important in light of growing evidence suggesting that even dissimilar toxicants can elicit similar response mechanisms calling similar groups of genes into play.

The array was made from cDNA derived from RNA from male Sprague-Dawley rats exposed to 52 different compounds at two levels during three-day toxicity studies. Applying the compounds orally, interperitoneally, or intravenously (depending on the compound), the scientists exposed three rats to both levels of each toxicant. They formed the pool of RNAs used to make the array from a total of 312 exposed rats. The exposure compounds induce a variety of toxic mechanisms including DNA damage, cirrhosis, oxidative stress, steatosis (accumulation of fat in the liver), and necrosis.

The scientists enriched their library for genes induced by exposure to the study toxicants by using a subtractive hybridization approach that allowed them to eliminate transcripts that were also present in nonexposed animals. Using animals exposed for three days allowed induction of gene-level responses in the liver, but avoided capturing genes involved in the later processes of secondary inflammation or fibrosis. Sequencing clones from the library allowed identification of more than 2,700 expressed putative genes. About 20% of these genes, the scientists indicate, do not appear to have been previously described.

Genes from this library make up about 25% of the array, which contains 25,000 probes. The other 75% includes rat genes with known human orthologs (which help compare gene expression patterns between species), genes allowing comparisons between specific and nonspecific hybridization, hybridization targets to allow comparisons of hybridization intensity, and other controls.

The researchers say these gene expression profiles can be used to build a predictive database encapsulating biological responses to toxic insult. If the concept of "guilt by association" is to prove accurate, they write—if compounds are considered to have toxic liabilities when they closely associate with a known toxicant—it is extremely important to equip the array with the correct genes to distinguish the mechanism of toxicity.

–Victoria McGovern

Matt Ray/EHP; Joseph Tarr/EHP; Artville, EyeWire

Monitoring Estrogenic Effects

A Genomics Approach

Genomics, the revolutionary field that promises to one day reveal the genetic code of every living organism, is opening up unforeseen opportunities for advances in many areas of the life sciences. In this issue, a team led by Patrick Larkin of the University of Florida in Gainesville and EcoArray LLC describes a genomics approach to monitoring toxic chemicals in the environment and uncovering their effects on organisms at the molecular level [***EHP* 111:839–846**]. Larkin and colleagues hope to produce an easy-to-use biomarker test capable of detecting metabolic pathways affected by environmental chemicals, and ultimately to formulate specific gene profiles that will permit identification of particular chemical contaminant exposures. In this article, the team describes an expression profiling model system for endocrine-disrupting compounds (EDCs) that mimic estrogens.

Natural and synthetic estrogens are found in pharmaceuticals, industrial by-products, and pesticides, and can cause human health effects including vaginal cancer and reproductive tract abnormalities. Because estrogen is a female reproductive hormone, genes in the estrogen pathway are normally not highly expressed in males. However, when male fish are exposed to natural or synthetic estrogens, the result is an increase in the expression of female-specific genes. The estrogen pathway has been highly conserved during vertebrate evolution—it is shared by many different organisms—so changes due to exposure in fish may presage effects in other animals, including humans.

The team created a gene array by cloning 30 genes—some involved in the estrogen pathway and some controls—from sheepshead minnows. The genes had been previously identified by differential display reverse transcriptase–polymerase chain reaction, a method that screens thousands of RNA messages to identify genes that are turned on or off by specific treatments. The team used microarray analysis to discover which of the 30 preselected genes were significantly changed by exposure of the fish to estrogenic compounds. They also measured changes in levels of gene expression when the fish were exposed to different concentrations of 17α-ethinyl estradiol, a synthetic estrogen found in birth control pills (which can end up in waterways via sewer systems).

Once they had their array in place, the team exposed male sheepshead minnows to a constant concentration of either strong or weak environmental estrogens. The strong estrogens included 17β-estradiol (the normal estrogen found in vertebrates), 17α-ethinyl estradiol, and diethylstilbestrol (a synthetic estrogen formerly used to prevent miscarriage that caused cancer, reproductive tract abnormalities, and infertility in the children of women who took it). The weak environmental estrogens included *p*-nonylphenol (a breakdown product of alkylphenol ethoxylates, which are used in various products such as washing and cleaning agents, emulsifiers, wetting agents, and foaming and foam-reducing agents) and the organochlorine pesticides methoxychlor and endosulfan. Single-stranded DNA for the 30 genes was bound to multiple membranes.

To analyze genes that were differentially expressed in the livers of control and treated fish, the team extracted mRNA and converted it to cDNA, which during this process was labeled by the addition of a tracer amount of radiolabeled nucleotides. The cDNA was then incubated with the membranes and bound proportionately to the 30 genes present thereon. The intensity of the radioactivity in the spots was directly related to the amount of mRNA present in the sample and, when compared to controls, was used to determine whether the expression of a gene was elevated or decreased as a result of exposure to the EDC.

There was an increase in expression of certain genes as a result of exposure. One endocrine receptor (ERα) was upregulated by every test compound. Four genes involved in the formation of egg cells were upregulated by every compound except endosulfan. A gene that plays an important role in blood clotting also was upregulated by the same five compounds. Interestingly, the gene for ubiquitin-conjugating enzyme 9, whose metabolic role is to tag enzymes that have completed their cellular functions and defective proteins for removal from the cell, was upregulated by *p*-nonylphenol. The expression of three genes involved in other processes was downregulated by the five compounds. Exposure to different concentrations of 17α-ethinyl estradiol also revealed that the microarray method is dose-sensitive, and that exposure thresholds vary for different genes. These findings could enable calculation of gene-dependent dose–response curves for evaluating the seriousness of chemical contamination in environmental cleanup efforts.

Monitoring mimics. A new model system profiles the expression of genes affected by exposure to environmental chemicals—such as those in birth control pills—that may disrupt the endocrine system.

The scientists plan to expand the expression profiling method to compounds that mimic other reproductive hormones such as androgen and progesterone. They are also going to make microarrays for different game fish species used for food as well as other fish species that are used as standards for monitoring environmental chemicals.

One hurdle for this technology is obtaining reproducible results. Successful replication depends on the accuracy of the DNA amplification of each gene, the correct identification of which genes are bound to the membrane and where, and the RNA extraction efficiency, because RNA degrades rapidly and can become contaminated with DNA. These technical steps also require careful laboratory techniques and multiple replicate experiments to ensure consistent results.

As the methodology expands to include more genes, chemicals, and organisms, the management and analysis of huge volumes of data will become another hurdle. Bioinformatics will become increasingly important as these EDC expression profiling data sets expand. This genetic biomarker assay is an exciting application of genomics tools for toxicology with promise for finding genes that are affected by EDCs, for understanding mechanisms that lead to disease, for applying that knowledge to environmental monitoring and cleanup, and for the rational design of new compounds that will be safer for human health and the environment. **–Mary Eubanks**

PhotoDisc

Metals Leave Their Mark

Fingerprints of Low-Dose Exposure

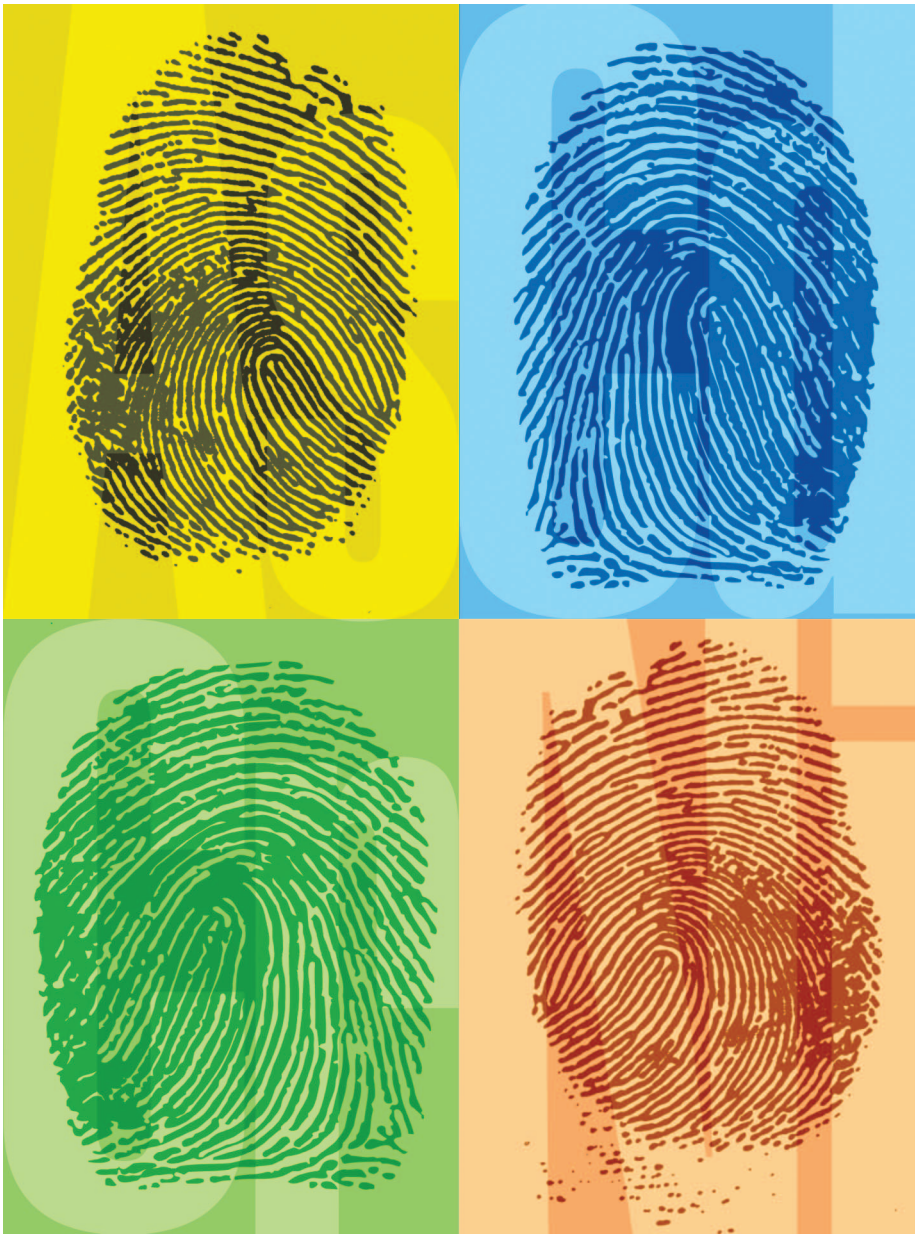
As the emerging field of toxicogenomics continues to progress, the search for biologically relevant biomarkers of exposure, effect, and susceptibility is in full swing. Much of the current work focuses on the genomic effects of potentially toxic metals. In this issue, Angeline Andrew and her colleagues at Dartmouth College report the results of their study of four metals—arsenic, cadmium, chromium, and nickel—that have been associated with a variety of adverse health effects [***EHP* 111:825–837**]. They identified "fingerprints" of early changes in gene and protein expression in response to each metal that may someday serve as biomarkers of exposure to these toxicants.

The team used cDNA microarrays to compare the effects of each metal on the expression of 1,200 human genes in human bronchial BEAS-2B cells. These cells were chosen because inhalation is a common route of exposure for the metals currently under study.

In order to ensure that the effects seen were not nonspecific responses to toxic high doses, the cells were exposed to low, relatively nontoxic doses of the metal compounds sodium arsenite, cadmium chloride, sodium dichromate, and nickel subsulfide for 4 hours. They also administered a much higher, cytotoxic dose of sodium arsenite to explore the effects of dose.

Although the results showed that each of the exposures modified expression of only a small subset of the 1,200 genes, the data suggest that each metal modifies expression of a largely unique set of genes that may be characteristic of each substance. This supports the potential for the development of metal-specific biomarkers.

There was some overlap in which genes were modified, but none were affected by all five chemical treatments, and only one, heat shock protein 90A, was modified by four of the five. Conversely, the authors found it remarkable that the genes that were altered by more than one treatment were all modified in the same direction, with either increased or decreased expression. They say this lends support to the idea that these represent biologically relevant responses to these treatments.



Fingerprint findings. New research shows signature effects of four metals on gene expression at low-dose exposures. This information could lead to molecular biomarkers of metal exposure.

Comparison of the effects of the low and high doses of arsenic also yielded some unexpected insights. Of a total of 158 genes modified, only 16 were altered at both doses, and substantially more genes were modified by the lower dose than by the higher one.

The lower dose modified expression of a wide variety of genes representing a diverse range of protein classes, such as transcription factors, inflammatory cytokines, kinases, and DNA repair proteins. The higher dose showed what the authors call a "striking shift" in the profile, modifying a variety of heat shock proteins and other genes involved in stress response pathways.

The researchers suggest that this dramatic contrast in gene expression profiles represents a switch from a survival-based biological response at the lower dose to a cell death–inducing apoptotic response at the higher dose. Whereas the high dose of arsenic clearly induced a stress response, it was a more universal, less toxicant-specific response; the lower doses of the four metals produced "a more subtle modification of cell signaling pathways," implying a unique, identifiable signature in the gene expression profile generated by each chemical.

The authors conclude that these metal response patterns may shed new light on the mechanisms of human diseases caused by toxic metal exposures, and may also be useful for developing molecular biomarkers of exposure and effect in future mechanistic, epidemiologic, and risk assessment studies. **–Ernie Hood**

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