Development of a DNA Microarray for Toxicology Based on Hepatotoxin-Regulated Sequences

Jeffrey F. Waring,[1] Guy Cavet,[2] Robert A. Jolly,[1] Jeff McDowell,[1] Hongye Dai,[2] Rita Ciurlionis,[1] Chunsheng Zhang,[2] Roland Stoughton,[2] Pek Lum,[2] Allan Ferguson,[2] Christopher J. Roberts,[2] and Roger G. Ulrich[1]

[1]Abbott Laboratories, Abbott Park, Ilinois, USA; [2]Rosetta Inpharmatics, Kirkland, Washington, USA

Toxicogenomics is an emerging field combining genomics and bioinformatics to identify and characterize mechanisms of toxicity of compounds. One of the main tools used in toxicogenomics is DNA microarrays. We have used a novel strategy to create a library highly enriched for genes expressed in the liver under hepatotoxic conditions. Using this library, we have created a new oligonucleotide microarray dedicated to the study of rat liver function. Oligonucleotide probes for these genes were designed and used in experimental hybridization studies to deduce the correct sequence orientation and to determine those sequences exhibiting differential regulation under a variety of toxicity-related treatments and conditions. The final array was benchmarked on treatments with 3-methylcholanthrene, Aroclor 1254, and cyclopropane carboxylic acid. Our results showed that the subtractive hybridization greatly enriched for genes regulated during a hepatotoxic response. Overall, our strategy for design of a new rat toxicology microarray can be applied to other systems as well and should aid greatly in the development of microarrays targeted for specific scientific areas. *Key words:* **Aroclor, cyclopropane carboxylic acid, hybridization kinetics, microarray, toxicogenomics, toxicology, 3-methylcholanthrene.** *Environ Health Perspect* **111:863–870 (2003). doi:10.1289/txg.5998 available via** *http://dx.doi.org/* **[Online 18 November 2002]**

High-density microarrays offer a powerful approach to detecting changes in gene expression. Such analysis allows one to identify transcriptional changes in thousands of genes simultaneously. The application of microarrays as well as bioinformatics toward toxicology has resulted in the creation of a new science termed toxicogenomics. The field of toxicogenomics uses gene expression changes to identify mechanisms of toxicity and to predict potential toxicity on the basis of similarities of expression profiles to known toxicants. Several recent publications have described the use of microarray analysis to identify discrete gene changes associated with a toxic response (Gerhold et al. 1999, Holden et al. 2000, Reilly et al. 2001). In addition, our research, as well as the research of others, has shown that compounds associated with a particular mechanism of toxicity yield similar gene expression profiles (Bulera et al. 2001; Hamadeh et al. 2002a, 2002b; Thomas et al. 2001; Waring et al. 2001a, 2001b). Furthermore, these gene expression profiles can be used to build a predictive database encapsulating biological responses to toxic insult. To date, the field of toxicogenomics has largely concentrated on hepatotoxicity, since the liver is the primary target of most toxic responses and liver injury is the principle reason for new drugs being withdrawn from the market.

The question of which genes to analyze in order to build an expression database for hepatotoxicity is of paramount importance. There are commercially available microarrays specifically designed for use in toxicology, yet the gene sequences on these microarrays are derived mainly from DNA libraries from normal or disease tissues and not from tissues that have been challenged with xenobiotics. Ideally, one would like to analyze all genes that have the potential to be transcriptionally regulated during a hepatotoxic response. Although there have been some genes identified that are linked with hepatotoxicity, the identification of all genes regulated by the liver in response to toxic insult has yet to be resolved. This issue becomes more complex in the rat, which is the model often used in traditional toxicology because much of the rat genome remains unsequenced to date. To address this issue, we have constructed a microarray containing sequences from a rat library highly enriched for genes regulated by toxic compounds in the liver. We demonstrate the utility of this microarray by testing it against three hepatotoxins: 3-methylcholanthrene (3-MC), Aroclor 1254, and cyclopropane carboxylic acid (CPCA).

Materials and Methods

Construction of subtractive hybridization library. All rat liver RNA samples used in sequence screening experiments were prepared at Abbott Laboratories from 3-day toxicity studies. Rats were treated with 52 different compounds previously associated with hepatotoxicity (Table 1). Male Sprague-Dawley rats (Charles River Laboratories, Portage, MI, USA) approximately 6–12 weeks of age and weighing 225–275 g were treated with vehicle (corn oil or saline) or with compound. There were three rats per treatment group. The dosing methods used were oral, interperitoneal, or intravenous, depending on the preferred method of delivery for the compound. Two dose levels were chosen for each compound. Rats were dosed daily for 3 days and sacrificed on day 4. Blood samples drawn from the animals at necropsy were used to measure serum concentrations or activities of blood urea nitrogen, creatinine, alanine amino transferase, aspartate aminotransferase, gamma glutamyltransferase, alkaline phosphatase (Abbott Laboratories Diagnostic Division), cholesterol, triglycerides, bilirubin, glucose, total protein, albumin, and globulins using an Abbott Aeroset clinical chemistry analyzer (Abbott Laboratories Diagnostic Division, Abbott Park, IL, USA). The terminal body weights and liver weights were recorded. The left lateral lobe of the liver was processed for histopathologic evaluation. Approximately 100 mg from each liver was placed into TRIzolâ reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and immediately homogenized using a Turrax tissue grinder (Diagger, Vernon Hills, IL, USA). The remaining portion of the liver was retained frozen for future study.

Subtractive hybridization was performed using the method of suppressive subtractive hybridization (Diatchenko et al. 1996). A pool of liver RNA containing 10 mg from all rats treated with hepatotoxin was made and subtracted against a pool of liver RNA containing 150 mg from three naïve rats. Subtraction was performed in the reverse direction as well. Subtraction efficiency was measured by quantitating the levels of D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) before and after subtraction.

The two libraries were cloned into the vector pCR2.1 (Invitrogen). The libraries

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Address correspondence to J.F. Waring, Dept. of Cellular and Molecular Toxicology, Abbott Laboratories, D463, Abbott Park, IL 60064-6104 USA. Telephone: (847) 935-4124. Fax: (847) 935-7845. E-mail: jeff.waring@abbott.com

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were sequenced by Incyte Genomics (Palo Alto, CA, USA). A total of 16,128 reads were sequenced, corresponding to 8,064 attempted reads for each library. Each library was sequenced with M13 reverse ET-terminator in one direction with a sequencing efficiency of 92%. The average length read was 350 bp.

Sequence similarity searching. Individual sequence reads from the subtractive hybridization libraries were compared to the National Center for Biotechnology Information nr and nt collections (Altschul et al. 1994) using BLAST 2 (Altschul et al. 1997). Hits with E < 1e-8 were considered significant. Contiguous sequences (contigs) were compared to a comprehensive collection of 364,170 nucleotide sequences for *Rattus norvegicus* taken from GenBank (*http://www.ncbi.nlm.nih.gov*), dbEST (database of expressed sequence tags), and RefSeq, also using BLAST 2. The threshold for considering an alignment significant required that it be at least 100 bp in length, have at least 95% identity, and cover at least 20% of the length of the shorter of query and subject sequences.

Oligonucleotide probe design. A nonredundant set of rat gene transcripts was determined by clustering and assembly of cDNA sequences from the subtractive hybridization libraries and publicly available sequences. Sequences were assigned into clusters and assembled into contigs using a derivative of the Smith-Waterman algorithm (Smith and Waterman 1981). From this,

Table 1. Hepatotoxins and dose levels used in the subtractive hybridization study.

Compound	Low dose (mg/kg/day)	High dose (mg/kg/day)	Vehicle	Method of delivery	Reference for dose	Type of lesion
3-Methylcholanthrene	10	100	Corn oil	ip	Kleeberg et al. 1975	Hypertrophy
Acetominophen	70	700	Saline	Oral	Smith et al. 1998	Necrosis
Actinomycin D	0.04	0.4	Saline	iv	Bader et al. 1974	Necrosis
Adriamycin	1	10	Saline	iv		Necrosis
Aflatoxin B1	0.05	0.5	Saline	Oral	Root et al. 1997	Necrosis
Allopurinol	10	100	Saline	Oral		Phospholipidosis
Allyl alcohol	10	40	Corn oil	ip	Ganey and Schultze 1995	Necrosis
Amiodarone	10	100	Saline	ip	Reasor et al. 1996	Phospholipidosis
ANIT	10	40	Saline	Oral	Chisholm and Dolphin 1996	Cholestasis
Aroclor 1254	100	400	Corn oil	ip	Wolfgang et al. 1990	Hypertrophy
Arsenic	2	20	Saline	ip	Mahaffey et al. 1981	Cirrhosis
Aspirin	15	150	Saline	Oral		Steatosis
Bezafibrate	20	200	Saline	Oral	Beier et al. 1988	Cholestasis
Carbamazepine	50	250	Saline	ip	Regnaud et al. 1988	Necrosis
Carbon tetrachloride	100	1,000	Corn oil	ip	Wolfgang et al. 1990	Necrosis
Chlorpromazine	25	100	Saline	Oral		Cholestasis
Cycloheximide	0.1	1.0	Saline	iv		Steatosis
Cyclophosphamide	10	100	Saline	ip	Brown et al. 1985	Necrosis
Dexamethasone	1	10	Saline	iv		Varying
Diclofenac	10	40	Saline	Oral		Cirrhosis
Diethylnitrosamine	10	100	Saline	ip	Williams et al. 1996	Necrosis
Diethylstilbestrol	2.5	25	Corn oil	ip		Tumor formation
Dimethyl formamide	100	1,000	Saline	ip	Mathew et al. 1980	Necrosis
Dinitrophenol	5	25	Saline	Oral		Steatosis
Diquat	17.2	68.8[a]	Saline	ip	Wolfgang et al. 1991	Necrosis
Erythromycin	80	800	Saline	Oral	Kaltiala et al. 1967	Cholestasis
Estradiol glucuronide	1	10	Saline	iv		Cholestasis
Ethanol	ND	3,000	Saline	Oral	Speisky et al. 1985	Steatosis
Etoposide	5	50	Saline	ip	Linden 1989	Necrosis
Galactosamine	50	500	Saline	iv		Steatosis
Ibuprofen	20	200	Saline	Oral	Adams et al. 1969	Cholestasis
Indomethacin	2	20	Saline	ip	Fracasso et al. 1987	Cholestasis
Iodoacetic acid	5	50	Saline	iv		Varying
Iron dextran	50	500	Saline	ip	Younes et al. 1989	Necrosis
Ketoconazole	30	120	Saline	Oral	Heel et al. 1982	Hepititis
L-Ethionine	5	50	Saline	ip		Steatosis
Methapyrilene	5	50	Saline	ip	Graichen et al. 1985	Steatosis
Methotrexate	50	250	Saline	ip	Custer et al. 1977	Steatosis
Microcystin	0.005	0.05	Saline	iv		Necrosis
Monocrotaline	5	50	Saline	ip	Perazzo et al. 1999	Necrosis
Nicotinic acid	200	2,000	Saline	Oral		Cholestasis
Oligomycin	0.1	1.0	Saline	iv	Kramar et al. 1984	Steatosis
Perhexilene	32	320	Saline	Oral		Phospholipidosis
Phenytoin	45	450	Saline	Oral	Garzon De la Mora et al. 1990	Cholestasis
Quinidine	7.5	75	Saline	iv		Varying
Retinol	50	500	Saline	Oral	Lettinga et al. 1996	Steatosis
Tamoxifen	5	50	Saline	Oral	Wogan 1997	Cholestasis
Tetracycline	50	500	Saline	Oral		Steatosis
Tumor necrosis factor-α	0.001	0.010	Saline	iv		Hepititis
Trovafloxacin	150	400	Saline	Oral		Necrosis
Valproic acid	50	500	Saline	Oral	Kesterson et al. 1984	Steatosis
Verapamil	40	200	Saline	Oral	Landon et al. 1986	Cholestasis

Abbreviations: ip, intraperitoneal; iv, intravenous; ND, not done. [a]All animals in this treatment group died before day 3 and were not used. Where no reference is provided, dose levels were selected from unpublished results.

60-mer oligonucleotides were selected for the microarray. The process of probe selection is described in "Results."

Expression profiling experiments. Rats were treated with Aroclor 1254 (Monsanto, St. Louis, MO, USA), 3-MC (Sigma Chemicals, St. Louis, MO, USA), or CPCA (Sigma) at levels previously shown to be hepatotoxic but not lethal. Male Sprague-Dawley rats approximately 6–12 weeks of age and weighing 225–275 g were injected intraperitoneally with vehicle (corn oil or saline) or with Aroclor 1254 (400 mg/kg/day) (Wolfgang et al. 1990), 3-MC (100 mg/kg/day) (Kleeberg et al. 1975), or CPCA (500 mg/kg/day) (Ulrich et al. 1998) for 3 days, and rats were sacrificed on day 4. Preparation of RNA was as described above. Preparation of labeled cDNA from the RNA, hybridization and scanning of the microarray were performed at Rosetta Inpharmatics (Kirkland, WA, USA) as described previously (Marton et al. 1998). For microarray analysis, tRNA from individual treated animals was hybridized against RNA from a pool of three vehicle-treated animals. Data analysis was performed using the Rosetta Resolver system (Rosetta). The fold change and *p*-value calculations have been previously described (Hughes et al. 2000).

Results

Subtractive hybridization. All rat liver RNA samples used in sequence screening experiments were prepared at Abbott Laboratories from 3-day toxicity studies. Rats were treated with 52 different compounds that have been previously associated with hepatotoxicity (Table 1). The selected compounds induced a hepatotoxic response by a variety of observed mechanisms, including necrosis, steatosis, DNA damage, cirrhosis, hypertrophy, phospholipidosis, and hepatic carcinoma. A 3-day time point was chosen to accommodate induction of liver genes without complication of a significant secondary inflammatory response (cellular infiltration) or fibrosis likely to be encountered over longer periods. Also, an earlier time point would likely have included immediate early response genes.

Using suppressive subtractive hybridization (Diatchenko et al. 1996), we performed a subtractive hybridization in both directions using pooled RNA from all hepatotoxin-treated rats against liver RNA from naïve rats to enrich for genes regulated during a hepatotoxic response. By performing the subtractive hybridization in both directions, genes that are both upregulated (higher in treated animals) and downregulated (higher in naïve animals) should be enriched. We used polymerase chain reaction to analyze the efficiency of the subtractive hybridization by measuring the levels of GAPDH before and after subtraction. The results indicated that we obtained between 10- and 15-fold subtraction efficiency (unpublished data). Sequences were obtained for 14,523 clones. Of these sequences, 16% had no significant similarity to known public domain sequences from any organism.

Oligonucleotide probe design. The first step in microarray design was to select a nonredundant set of rat gene transcripts by clustering and assembly of cDNA sequences. The input to clustering included 14,523 sequences from subtractive hybridization of hepatotoxin-treated rats (described above) and 92,319 public domain sequences. Sequences were assigned into clusters and assembled into contigs on the basis of sequence similarities identified by a derivative of the Smith–Waterman algorithm (Smith and Waterman 1981). This process resulted in 43,954 clusters and 54,437 contigs. For purposes of probe design, each cluster was represented by its longest contig sequence. Some genes of interest, notably those encoding cytochrome P450 enzymes (*CYP* genes), are difficult to cluster accurately with automated methods because of high levels of homology between distinct family members. *CYP* sequences were curated and clustered manually on the basis of their annotation and sequence similarity. Sequences were prioritized and ordered in terms of their biological interest and quality of annotation.

The 14,523 sequences from the subtractive hybridization libraries fell into 2,728 clusters, suggesting that they represent approximately this number of genes. Comparison of representative contig sequences for these clusters against public domain rat sequences showed no significant hits for 555 sequences (20%). This suggests that around 20% of the genes represented in the subtractive hybridization libraries have not previously been described in the rat.

A single 60-mer oligonucleotide probe was selected to represent each input sequence. All possible probes within 900 bp of the 3′ end of each sequence were considered candidates for selection. Probes closer to the 3′ end were favored because the polyA-dependent sample preparation results in higher hybridization intensities closer to the 3′ end of each transcript. Up to 12 probes (6 from each strand) were selected for each of 1,342 sequences for purposes of sequence orientation and empirical selection of the best probes. Of these sequences, 1,227 were of biological interest (e.g., because they arose from the subtractive hybridization libraries) but were of unknown orientation. A further 115 sequences of known orientation were included to provide training data for the orientation algorithms. For the selection of multiple probes, all probes within 900 bp of both ends were considered. Candidate probes were passed through a series of filters. First, probes containing repeat elements, vector contamination, or low-complexity sequences were eliminated. The best probes were then selected on the basis of a combination of sequence complexity, base composition, predicted thermodynamic properties, predicted cross-hybridization, and position within the transcript (Hughes et al. 2001). In total, 49,554 probes were selected and printed on microarrays. These microarrays were used in screening experiments to determine which probes should be included on the final 25,000-probe microarray.

Empirical probe evaluation. Empirical selection of probes on the basis of hybridization data is useful for refining microarray design in a number of ways. It enables the identification of genes of interest for a specific area of biological study, and it can lead to improved annotation of those genes (e.g., by revealing their orientation). It can also provide enhanced selection of optimal probes from a short list designed by computational methods, for example, by identifying probes subject to cross-hybridization. These advantages are particularly relevant when, as in this case, a microarray needs to be focused on a particular area of biology and when many input sequences are incomplete and poorly annotated.

The microarrays designed as described above were hybridized with samples relevant to hepatotoxicity. A total of 52 drugs and environmental toxins with cited liver toxicities were assembled and used in dedicated rat studies (Table 1). In these studies, RNA samples derived from compound treatments were pooled into nine groups, each comprising three to seven compound-treated rats. In total, 22 competitive hybridization experiments with fluor reversal were performed: naïve liver versus each sample pool for compound-treated liver (including differing hybridization times) and naïve liver versus isolated hepatocytes. To aid subsequent analysis of kinetic differences in hybridization intensity, one sample pool was also hybridized at two time points: 4 hr and 48 hr.

An important aspect of empirical probe selection was to orient sequences for which direction could not be determined reliably by computation alone. The direction of the sequences was thus inferred experimentally by consideration of a combination of three factors: hybridization intensity, differential expression, and kinetics of hybridization

(48-hr compared with 4-hr intensity). These criteria were used because probes detecting the transcribed strand should *a*) give brighter signal intensity, *b*) be more likely to show differential regulation between the labeled samples, and *c*) show a brighter hybridization signal at 48 hr compared with 4 hr (Dai et al. 2002). A total of 1,227 unoriented sequences were screened in this way by testing 12 oligonucleotide probes, 6 in the forward direction and 6 in the reverse (relative to the input sequence). A further 115 training set gene sequences with known orientation were also tested to assess accuracy and train parameters.

Regarding hybridization kinetics for determining the orientation of the sequences, the significance of specific hybridization for each probe was calculated by comparing the intensity differences from two time points (4 hr and 48 hr) to their errors. For each sequence, six probes from each direction were grouped together, and the direction was predicted based on significance of forward versus reverse direction hybridization (Figure 1). Using parameters optimized for the training set, orientation was predicted correctly for 74 of 115 (64%) of training sequences, with 2 incorrect (2.7% of predictions). The orientations of 67% of the unoriented sequences were predicted using the same parameters.

Motivation for using differential regulation to determine sequence orientation is dictated by two considerations. First, differential regulation is evidence of sequence-specific hybridization since cross-hybridization of DNA sequences to oligonucleotide probes in most cases tends to suppress differential regulation. Second, significant regulation leads to the selection of oligonucleotide probes with intensity above background levels. In this study, six probes in each direction were averaged together to get an estimation of the significance of regulation over 10 experiments. The correct orientation was determined by the strand that gave lower *p*-values for differential regulation. Using parameters optimized for the training set, orientation was predicted correctly for 53 of 115 (46%) of training sequences, with 4 incorrect (7.5% of predictions). The orientations of 44% of the unoriented sequences were predicted using the same parameters.

Regarding intensity, it is clear that probes in the correct orientation should exhibit higher intensity for those sequences expressed. In this study the average intensity of forward strand probes for each sequence was compared with that of the reverse strand probes. The parameters for predicting the correct direction were determined again by optimizing for the training set sequences. The resulting parameters predicted orientation correctly for 62 of 115 (53%) training sequences, with 1 incorrect (1.6% of predictions). They yielded orientations for 76% of the unoriented sequences.

The error rates for all three types of evidence were reduced by applying filters on the basis of the observed trend in intensity along the length of a sequence. The polyT-primed RNA amplification protocol used in these experiments generally results in higher intensities closer to the 3′ end of a transcript. A correctly oriented sequence should show a trend of increasing intensity toward the 3′ end. A small subset of sequences for which predictions were initially made did not fit the expected pattern, and their predictions were discarded. The majority of these predictions were from the intensity-based analysis alone.

A final step in empirical orientation of sequences combined the predictions from the above analyses. Kinetics predictions, when available, took priority. Orientation for the remaining sequences was predicted by intensity or regulation, except that contradictory predictions from these two sources were discarded. With these criteria, 90 of 115 (78%) training sequences were correctly predicted, with just 3 sequences incorrect (3.3% of predictions). This translated into confident prediction for 78.5% of unoriented sequences.

The use of a large-format screening array made it possible to use 12 probes per sequence of unknown orientation. Using fewer probes would have yielded many of the same orientations, but any reduction would affect sensitivity. Simulations suggest that reducing the number of probes per sequence to 4 would have resulted in 50–90% of the sensitivity achieved with 12.

Selection of oligonucleotides for 25K array. The second data analysis step was to select approximately 25,000 oligonucleotides of those screened to fit the final 25K rat liver microarray. All genes on the 25K microarray were represented by a single probe. Single oligonucleotide probes on inkjet arrays have been shown to reliably report expression ratios in yeast and mammalian cells (Hughes et al. 2001).

Probes for genes of special interest were set aside for inclusion without any further preselection or analysis. In particular, 60 sequences representing cytochrome P450 genes and 2,475 sequences derived from subtracted hybridization libraries were automatically included in the selection for the 25K microarray. An additional 1,142 sequences were selected on the basis that they have known human orthologs (Makalowski et al. 1998). These are particularly useful in comparing and translating gene expression patterns between organisms. The remaining probes were prioritized in terms of their differential regulation compared with vehicle, by hybridization kinetics (as an indication of specific hybridization), and last, by their brightness ranking. These criteria produced mainly overlapping sets of probes. Application of a significance threshold in differential regulation ($p < 0.2$ in any experiments) produced 17,508 probes for potential inclusion on the final array. Kinetic comparisons, which help separate

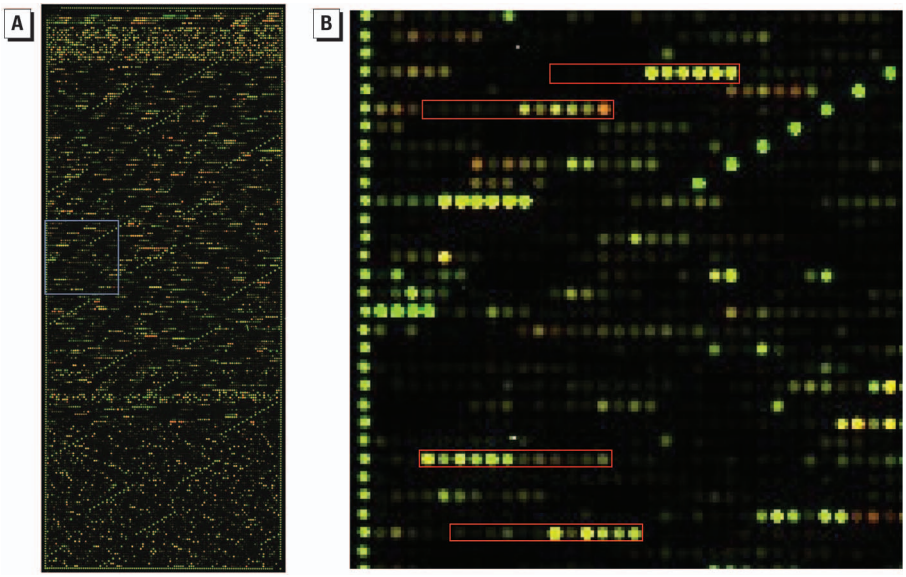

Figure 1. (*A*) Image from 50K microarray. (*B*) Zoom in from *A* showing probe sets to determine orientation. Red rectangles show examples of probe sets that allowed for correct determination of orientation. Each rectangle contains six probes derived from the forward strand of a sequence followed by 6 probes derived from the reverse complement strand of the sequence.

specific and nonspecific hybridization, resulted in 11,746 candidate probes. Combining these two criteria and eliminating probes already selected for inclusion enabled us to select 14,216 probes. Figure 2 summarizes the distribution of intensities for all candidate probes with known orientation and shows the average intensity distributions for the applied criteria.

Final specifications for microarray. Experimental screening of the approximate 50K probes provided valuable data to help prioritize sequences for representation on a 25K probe microarray. The majority of oligonucleotide probes (~14,000) selected for the microarray were derived from combination of significant differential regulation ($p < 0.2$ in any experiment) and favorable hybridization kinetics (lower probabilities of cross-hybridization) and biological interest. A smaller proportion of probes (~6,000) exhibiting lower (but significant) signal intensity in screening experiments were also included. The final breakdown for probe selection is shown in Table 2.

Transcriptional regulation by hepatotoxins. To determine the utility of the microarray toxicology, we examined gene changes in the liver induced by two aromatic hydrocarbons, Aroclor 1254 and 3-MC, and a short-chain fatty acid, CPCA. All these agents are well-described hepatotoxins, and previous research has identified some genes that are regulated by Aroclor 1254 and 3-MC (Borlak and Thum 2001; Jauregui et al. 1991). We wanted to determine if the subtractive hybridization enriched for genes that are regulated during a hepatotoxic response. Both Aroclor 1254 and 3-MC were used in the subtractive hybridization experiment, whereas CPCA was not. Rats were treated with Aroclor 1254 at 400 mg/kg, 3-MC at 100 mg/kg, or CPCA at 500 mg/kg for 3 days (Table 1). Significant gene regulation was defined as fold change >1.5-fold over control with $p \leq 0.01$.

Figure 3 shows a K-means clustering of genes regulated by treatment with CPCA, Aroclor 1254, 3-MC, or vehicle. When expression profiles from individual animals treated with vehicle control were compared with a pool, the results show that relatively few genes were significantly regulated (Figure 3). In contrast, treatment with the three hepatotoxins resulted in numerous gene changes that were highly reproducible across the three replicate animals for each different treatment. Rats treated with Aroclor 1254 and 3-MC yield similar patterns of gene expression; the two compounds significantly regulated 786 genes in common. Many of these genes have previously been shown to be regulated by aromatic hydrocarbons, including cytochromes P450 1A1, and 1B1, malic enzyme,

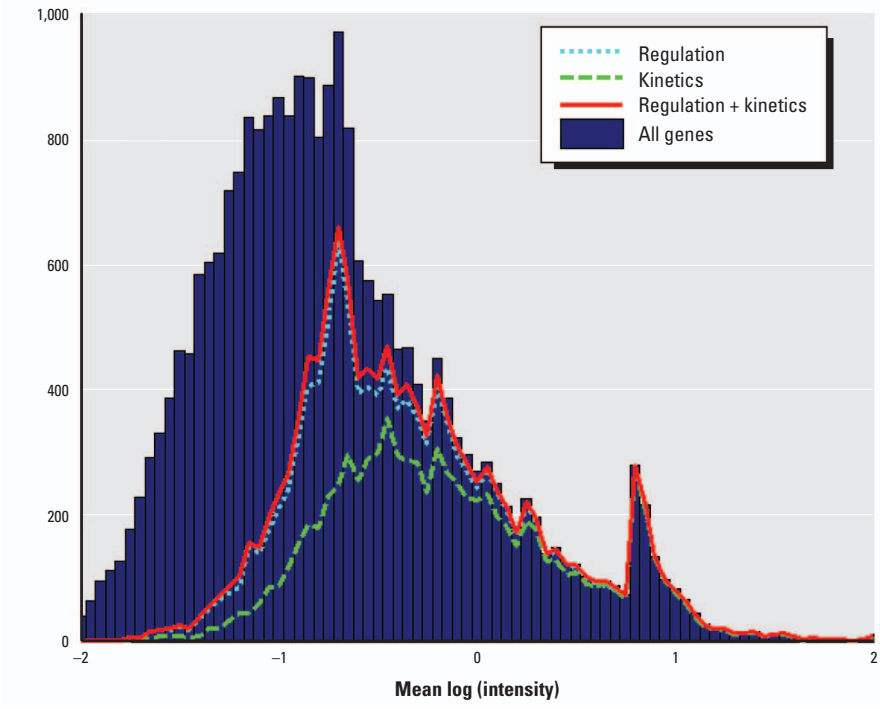

Figure 2. Intensity distributions for probes selected for inclusion on the 25K array. Distribution of intensities for all probes on the 50K array is plotted as blue bars. The cyan line describes probes selected through differential regulation, the green line describes probes selected through kinetics, and the red line describes probes selected by both methods.

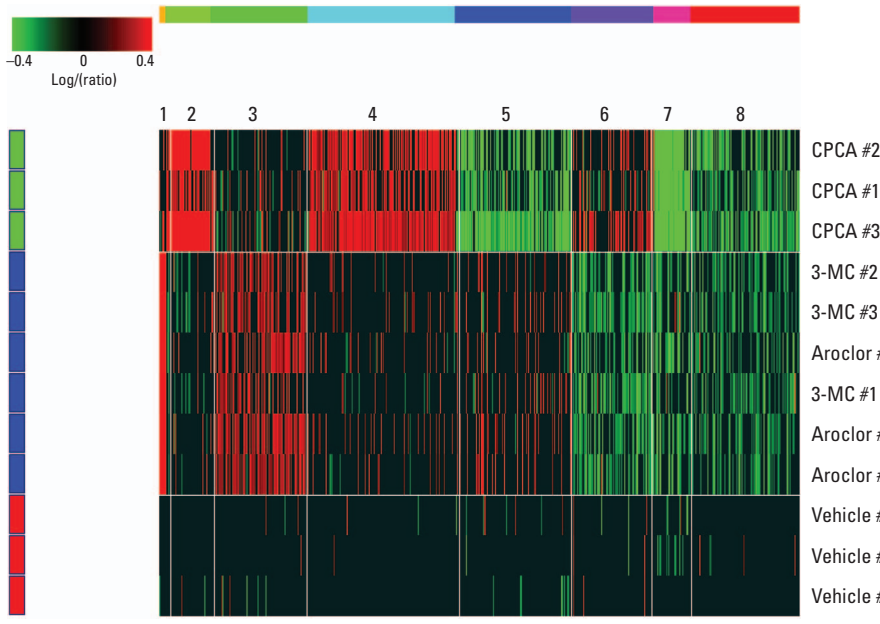

Figure 3. K-means cluster showing the genes regulated by CPCA, 3-MC, Aroclor 1254, or vehicle control. The results show the similarity in gene expression profiles between 3-MC and Aroclor 1254. The regulated genes have been assigned to eight treatment groups.

Table 2. Breakdown of probe selection for 25K chip.

Subtractive hybridization	
Unoriented	927
Oriented	1,548
CYP gene sequences	60
RefSeq sequences[a]	55
Sequences with human orthologs	1,144
Sequences selected by regulation and/or kinetics	14,216
Sequences selected by intensity	6,449
Total	24,399

[a]Used in training set for sequence orientation.

glutathione-*S*-transferase, and UDP-glucuronosyltransferase 1 (Figure 3, group 1) (Borlak and Thum 2001; Jauregui et al. 1991; Lubet et al. 1991; Mehlman et al. 1975; Saarikoski et al. 1998). Other genes regulated by 3-MC and Aroclor 1254 were *Erp29*, liver tricarboxylate carrier, and cholesterol hydroxylase 7α (Table 3).

CPCA is an inhibitor of β-oxidation and has been shown to result in microvesicular steatosis (Ulrich et al. 1998). Therefore, it would be expected to result in regulation of mitochondrial and peroxisomal genes involved in β-oxidation. Table 3 shows that genes involved in mitochondrial or peroxisomal β-oxidation such as acyl–coenzyme A (acyl–CoA) dehydrogenase and enoyl–CoA hydratase were inhibited by treatment with CPCA. Treatment with CPCA also resulted in regulation of a number of genes in the cholesterol and fatty acid pathways, including an upregulation of adipocyte lipid binding protein, squalene epoxidase, 7-dehydrocholesterol reductase, and downregulation of cholesterol hydroxylase 7α, lecithin-cholesterol acyltransferase, fatty acid desaturase, fatty acid amide hydrolase, and fatty acid binding proteins 1 and 7 (Table 3).

Table 4 summarizes the gene regulation for the three compounds. The results show that the sequences from the subtractive hybridization contributed significantly to the expression profiles for the three compounds. While these sequences represented approximately 9% of the sequences on the chip, they represented approximately 17% of the signature profile for all three compounds. This is true for all three compounds despite the fact that CPCA was not included in the set of compounds used in the subtractive hybridization. This suggests that the generation of the subtractive hybridization library enriched for many genes involved in responses to a wide range of hepatotoxic compounds, not only those treatments that were used. In addition, roughly 5% of the regulated sequences were identified in the subtractive hybridization and have not been previously described.

Discussion

We have constructed a new rat toxicology microarray using a strategy to specifically represent genes that are regulated during a hepatotoxic response. The strategy we employed in constructing this array provided several advantages over other commercially available toxicology arrays. First, we attempted to represent every gene that can be regulated during a hepatotoxic response. Toward this end, we dosed rats with 52 known hepatotoxins using a combination of drugs and chemicals that have been associated with different mechanisms of hepatotoxicity including DNA damage, oxidative stress, peroxisomal proliferation, necrosis, cirrhosis, steatosis, and protein synthesis inhibitors. We sequenced approximately 16,000 clones, which collapsed down to roughly 2,700 contigs. Although oligonucleotides representing the subtractive hybridization clones made up only 9% of the final 25K array, these sequences represented approximately 17% of the signature profile for three different hepatotoxins. In addition, many of the sequences from the subtractive hybridization had not been described previously. Some of these sequences were regulated by the hepatotoxins tested and may be critical genes involved in the mechanism of toxicity. Further evidence of this is provided by experiments performed with two compounds considered to be nonhepatotoxic, spectinomycin and penicillin. Microarray results showed that these sequences were regulated by the nonhepatotoxins to a much lesser extent than by the three hepatotoxic compounds, suggesting that these sequences are relatively specific for a hepatotoxic reaction (unpublished data).

The strategy we employed for the subtractive hybridization does have inherent limitations. Because the RNA from all the treatment groups was pooled, it is possible a gene that may have been up- or downregulated by only one hepatotoxin may not be represented in the sequenced library. In addition, if a gene was upregulated by half the hepatotoxins and downregulated by the other half, this gene also would likely be missed. An alternative strategy would have been to perform subtractive hybridization on pooled RNA from compounds with similar mechanisms of toxicity rather than pooling all of the hepatotoxins. However, we believe that the strategy we employed identified sufficient sequences to allow for the classification of compounds into different mechanistic classes.

Our strategy for chip construction is similar to that employed by Thomas et al. (2001). In this study, the authors constructed an environmental database for gene expression (EDGE) composed of libraries from mice treated with various toxins, including dioxin and phenobarbitol. These libraries were than used to construct microarrays, which were used to identify gene changes in mice treated with compounds representing peroxisome

Table 3. Some of the genes regulated by Aroclor 1254, 3-MC, or CPCA.

Accession number[a]	Gene	Aroclor 1254	3-MC	CPCA	Cluster (Fig. 3)
E00717	*CYP1A1*	30.06	36.65	NC	1
NM_012940	*CYP1B1*	63.13	100	NC	1
NM_012600	Malic enzyme	5.16	3.45	1.61	1
NM_017014	Glutathione-*S*-transferase	5.91	5.72	2.06	3
D83796	UDP-Glucuronosyltransferase 1	2.88	2.95	NC	3
U36482	*Erp29*	1.6	1.67	NC	3
AA964229	Tricarboxylate carrier	–2.14	–2.68	NC	6
J05030	Acyl–CoA dehydrogenase	NC	NC	–1.94	NS
D16478	Enoyl–CoA hydratase	NC	NC	–1.35	NS
K03249	Peroxisomal bifunctional enzyme	–1.84	–1.97	–5.41	7
J02749	Acetyl–CoA acyltransferase, 3-Oxo-acyl–CoA thiolase A, peroxisomal	4.23	2.93	–1.82	1
AI105060	Adipocyte lipid binding protein	NC	NC	4.66	2
D37920	Squalene epoxidase	NC	NC	10.21	2
AB016800	7-Dehydrocholesterol reductase	NC	NC	1.83	NS
J05509	Cholesterol hydroxylase 7α	–7.81	–1.87	–5.52	7
NM_017024	Lecithin–cholesterol acyltransferase	NC	NC	–2.45	5
AB021980	Fatty acid desaturase	1.22	2.11	–3.4	5
NM_024132	Fatty acid amide hydrolase	NC	NC	–2.11	5
NM_012556	Fatty acid binding protein 1	NC	NC	–3.92	7
NM_030832	Fatty acid binding protein 7	NC	NC	–3.79	7
AW534367	Prohepcidin	–7.57	–3.42	10.0	2

Abbreviations: NC, no change in expression; NS, gene was not shown in Figure 3 cluster.
[a]Accession numbers are from GenBank (*http://www.ncbi.nlm.nih.gov*).

Table 4. Summary of regulated genes for Aroclor 1254, 3-MC, and CPCA.

Compound	Regulated genes	Identified in Sub. hyb.	Previously unknown sequences
Aroclor 1254	4,266	817	252
3-MC	5,005	892	251
CPCA	5,471	953	253

Sub. hyb., subtractive hybridization library.

proliferators, aryl hydrocarbon receptor agonists, and noncoplanar polychlorinated biphenyls. Similar to our results, the authors found the arrays they constructed were able to classify the toxicants into different categories on the basis of mechanism of toxicity. While our current microarray has 25,000 sequences represented, almost certainly this number can be reduced once a large number of structurally and mechanistically distinct hepatotoxins have been tested. However, having a large number of sequences represented has obvious advantages. For instance, on the basis of our results, it is quite clear that the aromatic hydrocarbons and CPCA have vastly different effects on the liver. CPCA regulated over 1,000 genes in the liver that were not regulated by either Aroclor 1254 or 3-MC (Figure 3). However, the compounds did regulate approximately 800 genes in common; the genes regulated in common were both high- and low-level expression genes. With a smaller array that might have only a subset of these genes present, the potential exists that it would be more difficult to distinguish between an aromatic hydrocarbon and an inhibitor of mitochondrial β-oxidation. Clearly, if the concept of guilt by association is to prove accurate whereby compounds are considered to have toxic liabilities if they closely associate with a known toxin, it is extremely important to have the correct genes present that distinguish the mechanism of toxicity. This also applies toward understanding the mechanism behind the manifested toxicity. For instance, quite possibly the genes important for understanding the mechanism of toxicity of CPCA are represented in the unique set of genes for this compound. A smaller array, which might not have these genes present, would prove ineffective in aiding our understanding of the mechanism of toxicity of this compound.

With the 25K microarray, we observed regulation of genes that have been previously shown to be regulated by aromatic hydrocarbons and CPCA. This indicates that our strategy for optimizing oligonucleotide probe selection, using hybridization kinetics, differential regulation, and hybridization intensity, was a viable approach and should prove useful in future oligonucleotide probe design.

In addition, other genes were shown to be regulated that have not been previously associated with the hepatotoxins we tested. For instance, endoplasmic reticulum protein p29 (Erp29) was upregulated by both 3-MC and Aroclor 1254 treatment. *Erp29* is a recently cloned gene shown to be a member of the endoplasmic reticulum stress-response machinery (Mkrtchian et al. 1998). Both 3-MC and Aroclor 1254 cause hypertrophy in the liver as a result of proliferation of the endoplasmic reticulum (Borlakoglu et al. 1991). Both 3-MC and Aroclor 1254 also downregulated the liver tricarboxylate carrier gene. The protein product of the liver tricarboxylate carrier gene functions to transport citrate and malate from the rat liver mitochondria. Previous research has shown that the function of the protein is reduced in conjuction with altered lipid accumulation in the liver, hepatocyte apoptosis, and hepatic hyperplasia, all of which occurred because of treatment with 3-MC and Aroclor 1254 (Dini et al. 1999, Waring et al. 2001b).

Although it has been shown that CPCA inhibits mitochondrial β-oxidation, the mechanism by which this occurs is not well understood. Interestingly, peroxisomal proliferators, which also inhibit mitochondrial β-oxidation, result in an increase in peroxisomal β-oxidation and an upregulation of fatty acid binding protein (Kaikaus et al. 1993). Our results with CPCA show that it inhibits peroxisomal β-oxidation and downregulates fatty acid binding protein, suggesting that CPCA inhibits mitochondrial β-oxidation by a mechanism other than the peroxisomal proliferators. In addition, CPCA upregulated prohepcidin, which is a liver-specific gene that is overexpressed with iron overload and may be also upregulated during oxidative stress (Pigeon et al. 2001).

Overall, we believe our strategy for designing a rat toxicology microarray has great advantages. This type of strategy can be applied toward other systems as well and should prove beneficial in the design of new microarrays.

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