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Aryl Hydrocarbon Receptor Regulates Distinct Dioxin-Dependent and Dioxin-Independent Gene Batteries[S]

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ABSTRACT

Conventional biochemical and molecular techniques identified previously several genes whose expression is regulated by the aryl hydrocarbon receptor (AHR). We sought to map the complete spectrum of AHR-dependent genes in male adult liver using expression arrays to contrast mRNA profiles in *Ahr*-null mice ($Ahr^{-/-}$) with those in mice with wild-type AHR ($Ahr^{+/+}$). Transcript profiles were determined both in untreated mice and in mice treated 19 h earlier with 1000 μg/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Expression of 456 ProbeSets was significantly altered by TCDD in an AHR-dependent manner, including members of the classic AHRE-I gene battery, such as *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, and Nqo1. In the absence of exogenous ligand, AHR status alone affected expression of 392 ProbeSets, suggesting that the AHR has multiple functions in normal physiology. In $Ahr^{-/-}$ mice, only 32 ProbeSets exhibited responses to TCDD, indicating that the AHR is required for virtually all transcriptional responses to dioxin exposure in liver. The flavin-containing monooxygenases, Fmo2 and Fmo3, considered previously to be uninducible, were highly induced by TCDD in an AHR-dependent manner. The estrogen receptor α as well as two estrogen-receptor-related genes (α and γ) exhibit AHR-dependent expression, thereby extending cross-talk opportunities between the intensively studied AHR and estrogen receptor pathways. p53 binding sites are over-represented in genes down-regulated by TCDD, suggesting that TCDD inhibits p53 transcriptional activity. Overall, our study identifies a wide range of genes that depend on the AHR, either for constitutive expression or for response to TCDD.

Initial studies of the aryl hydrocarbon receptor (AHR) focused on its roles in regulating the induction of CYP1 enzymes (Nebert et al., 2004) and mediating toxicity of dioxin-like chemicals (Okey et al., 2005). More recently, the creation of mice with altered AHR signaling revealed phenotypic changes that implicate the AHR in multiple aspects of growth, development, differentiation, and physiology, irrespective of exposure to toxic environmental chemicals (Fernandez-Salguero et al., 1995; Lahvis et al., 2000; Bunger et

al., 2003; Walisser et al., 2004a,b). The AHR is a member of the basic helix-loop-helix PAS superfamily and is located in the cytoplasm in association with chaperone proteins such as heat shock protein 90 and XAP2. The AHR is activated by binding to TCDD, translocates to the nucleus, and dimerizes with another basic helix-loop-helix protein, ARNT. The activated AHR/ARNT heterodimer complex interacts with AH-responsive elements and activates the expression of AHR target genes (Nebert et al., 2004).

Mice in which the *Ahr* gene has been knocked out ($Ahr^{-/-}$) are extraordinarily resistant to TCDD toxicity (Mimura et al., 1997; Peters et al., 1999; Bunger et al., 2003). Major toxic effects in mice such as hepatic toxicity, thymic atrophy, and cleft palate formation (Bunger et al., 2003) require that the AHR have an intact nuclear translocation/transactivation domain. Rats with a large deletion in the AHR transactivation domain also are highly resistant to lethality from TCDD

ABBREVIATIONS: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; AHRE-I, aryl hydrocarbon response element (also known as DRE or XRE-I); AHRE-II, aryl hydrocarbon response element-II (also known as XRE-II); GO, gene ontology; TFBS, transcription factor binding site; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TSS, transcription start site; bp, base pair; Mt1, metallothionein 1; ER, estrogen receptor; MMP, metalloproteinase; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

(Okey et al., 2005). Moreover, mice that are hypomorphic for the AHR's essential dimerization partner, ARNT, are highly resistant to TCDD toxicity (Walisser et al., 2004b). This composite evidence strongly points to AHR-mediated transcriptional effects as the mechanism of dioxin toxicity.

The AHR acts as a ligand-dependent transcription factor, but the spectrum of genes regulated by the AHR is incompletely defined, and the specific genes whose AHR-mediated dysregulation by dioxins leads to major forms of dioxin toxicity are largely unknown. Expression arrays can be highly effective tools for identifying the suite of genes whose expression is altered by xenobiotic chemicals. Several previous array studies examined the responses of various cells or tissues to TCDD or to other xenobiotic chemicals that act as AHR ligands (Puga et al., 2000; Martinez et al., 2002; Guo et al., 2004; Karyala et al., 2004; Boverhof et al., 2005; Fletcher et al., 2005). The majority of these studies were performed in cell culture rather than in whole animals.

Very few of the expression changes in previous studies have been shown to be direct AHR-mediated responses, either by promoter analysis or by determination of AHR-dependence. The possibility remains that some changes in gene expression might be mediated by pathways other than the AHR, or that they might represent a cascade of secondary effects attendant to the onset of dioxin toxicity after long exposure to TCDD. One strategy to assess AHR-dependence is to compare gene expression profiles of cells or tissues that have the wild-type AHR with those of *Ahr*-null cells in a matched genetic background. This has been done in vitro in two array experiments on aortic smooth muscle cells, derived from $Ahr^{-/-}$ versus $Ahr^{+/+}$ mice, treated in culture with TCDD (Guo et al., 2004) or benzo[a]pyrene (Karyala et al., 2004) but not previously in tissues harvested from *Ahr*-null animals treated in vivo. We used expression arrays to identify the batteries of genes whose expression in vivo is affected by the AHR status only, by TCDD only, or by the combination of TCDD and the AHR. This is the first transcriptomic analysis of tissue from $Ahr^{-/-}$ animals.

Materials and Methods

TCDD. TCDD was purchased from the UFA-Oil Institute (Ufa, Russia) and was >99% pure as determined by gas chromatography-mass spectrometry.

Animals and In Vivo Treatment. Male $Ahr^{-/-}$ mice (10 weeks old) in a C57BL/6J background were obtained from The Jackson Laboratory (Bar Harbor, ME). Wild-type ($Ahr^{+/+}$) male C57BL/6J mice (15 weeks old) were bred at the National Public Health Institute (Kuopio, Finland) from stock originally obtained from The Jackson Laboratory. Mice were given a single dose of 1000 μg/kg TCDD or corn oil vehicle by gavage. TCDD initially was dissolved in ether and added to corn oil; the ether subsequently was evaporated off. Liver was harvested 19 h after treatment, sliced, snap-frozen, and stored in liquid nitrogen until homogenization. There were three TCDD-treated and three control mice in the $Ahr^{-/-}$ groups and six TCDD-treated and five control mice in the $Ahr^{+/+}$ groups (Fig. 1).

RNA Isolation and Expression Array Studies. Total RNA was extracted using RNeasy kits (QIAGEN, Valencia, CA) according to the manufacturer's instructions. DNase (QIAGEN) was added to the RNeasy elution column as recommended by the manufacturer. RNA yield was quantified by UV spectrophotometry, and RNA integrity was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The isolated RNA was then assayed on Affymetrix MOE430-2 arrays (Affymetrix, Santa Clara, CA) at The

Centre for Applied Genomics at The Hospital for Sick Children (Toronto, Canada) following standard manufacturer's protocols.

Preprocessing of Array Data. Array data were loaded into the R statistical package (version 2.0.0) using the affy package (version 1.5.8; http://www.bioconductor.org) (Gautier et al., 2004) of the BioConductor open-source project (Gentleman et al., 2004). Data were investigated for spatial and distributional homogeneity, then preprocessed with a sequence-specific version of the RMA algorithm (Irizarry et al., 2003) termed GCRMA, as implemented in BioConductor (version 1.1.3). The data then were written to disk and parsed with Perl scripts into a custom-built Oracle database partially derived from the MAGE-OM-compliant RAD schema (Stoeckert et al., 2001). The normalized data were then associated with updated sequence annotation as detailed elsewhere (P. C. Boutros, N. Tijet, R. Pohjanvirta, and A. B. Okey, manuuscript in preparation). In brief, the annotation process involved comprehensive alignment of each target sequence to species-specific expressed sequence tag databases, followed by a poll-based cluster-assignment algorithm to determine the best matching to UniGene clusters (Wheeler et al., 2004).

Significance Testing of Array Data. Normalized, annotated data were written out from the Oracle database and significance-tested with a general-linear model using the limma package (version 1.8.14) in BioConductor. The following linear model was fit to each individual ProbeSet on the MOE430-2 array: Y = Basal + AHR + TCDD + AHR-TCDD + Batch. Here, *Y* refers to the expression level of a single ProbeSet; *Basal* refers to the underlying basal expression level across all animals; *AHR* captures "AHR-dependent, TCDD-independent" expression changes; *TCDD* captures "TCDD-dependent, AHR-independent" expression changes; *AHR-TCDD* captures "AHR-dependent, TCDD-dependent" expression changes; and *Batch* captures the effects of the hybridization batch. After fitting the linear model, we used empirical Bayes moderation of the standard error (Smyth et al., 2003), followed by a false-discovery rate correction for multiple testing (Efron and Tibshirani, 2002). To identify differentially expressed genes, we used a nested *F* test as implemented in limma on the AHR, TCDD, and AHR-TCDD effects. This test considers first whether a ProbeSet displays any differential expression and then assesses which contrasts contribute to that differential expression. ProbeSets were deemed differentially expressed at the $p = 10^{-3}$ significance level. For each effect, we extracted estimates of differential expression ("M") for all ProbeSets
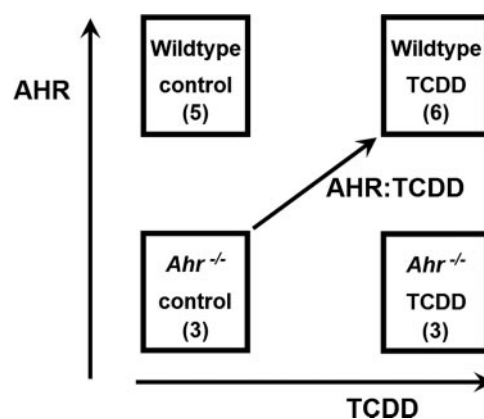
Fig. 1. Experimental design for high-throughput transcriptional profiling of the effects of the AHR, of TCDD, and their interaction. Wild-type C57BL/6 and $Ahr^{-/-}$ mice in a C57BL/6 genetic background were treated either with 1000 μg/kg TCDD or corn-oil vehicle (control). The number of independent animals studied, each on a separate array, is given in brackets for each group. The three effects fitted to a general linear model are illustrated. The AHR-effect refers to "AHR-dependent, TCDD-independent" changes in expression profiles. The TCDD-effect refers to "AHR-independent, TCDD-dependent" changes in expression profiles. The AHR-TCDD interaction captures "AHR-dependent, TCDD-dependent" changes in expression profiles.

and parsed these values back into the Oracle database for storage and downstream processing. Complete output files are available in Supplemental Table S2.

Transcription-Factor Binding Site Analysis. To understand the regulatory networks underlying AHR-dependent expression, we performed two separate transcription-factor binding-site (TFBS) analyses. Both analyses used the University of California at Santa Cruz's Genome Browser Database build mm5 of the mouse genome, from which regulatory sequences were extracted using BioPerl-based scripts (Stajich et al., 2002). Transcriptional start sites (TSSs) were taken from the University of California at Santa Cruz's Genome Browser Database (Karolchik et al., 2003). For some genes, the annotated TSSs are uncertain or in error, including *Cyp1a1* (Sun et al., 2004); for *Cyp1a1,* we used the manually annotated TSS of Chr9:57,902,425, courtesy of Dr. Patricia Harper. To identify the genomic region encoding a specific ProbeSet, we mapped the Uni-Gene clusters annotated for each ProbeSet to the RefSeq annotation using the LocusLink-UniGene mapping made available by the National Center for Biotechnology Information (Wheeler et al., 2004).

First, to identify the known AHR binding sites, we scanned the region from -5000 to $+1000$ bp relative to each TSS for AHRE-I and AHRE-II binding sites, as described previously (Boutros et al., 2004).

Second, to characterize the patterns of transcription factor binding sites across the set of putative AHR-regulated genes, we used a library-based TFBS search. These library-based searches compare a set of known TFBSs with the promoter regions of a set of coregulated genes and then apply statistical tests to identify TFBSs that are found more often (enriched) or less often (depleted) than expected by chance in the set of coregulated genes. Using the Clover software (Frith et al., 2004), we tested separately the sets of genes up- and down-regulated in both the AHR and AHR-TCDD contrasts. Statistical testing used both mononucleotide and dinucleotide randomizations of the parental sequences (1000 iterations), randomization of scoring matrices, and two separate background sequence sets (murine CpG islands and human chromosome 21). Two separate libraries of TFBSs were scanned: the JASPAR database (Sandelin et al., 2004), and a custom-designed database that included three variants of the AHRE-I consensus sequence, the AHRE-II sequence, the AnoC element, and a *Cyp1a1* negative regulatory element. This search scanned the region from -4000 to $+1000$ bp relative to the annotated TSS for each gene. The sequences are the following (Boutros et al., 2004): AHRE-1(Core) = GCTCG; AHRE-I(Extended) = TNGCGTG; AHRE-I(Full) = [T|G]NGCGTG[A|C][G|C]A; AHRE-II = CATG{N6}C[T|A]TG; AnoC = [C|T]GCG[C|T]GCGC[C|A]GC; and *Cyp1a1* negative regulatory element = G[G|T]GCTCTG[G|C][G|C][G|A][G|A]-[T|A]CA[G|A][G|A][G|T]C[C|A][C|A].

Real-Time qRT-PCR. mRNA levels for selected genes were quantified by real-time qRT-PCR. See Supplemental Table S1 for primer and probe sequences and details of the procedure.

Functional Analysis of Differentially Expressed Genes. Ontological analysis used build 140 of the GO-Miner software package (Zeeberg et al., 2003), which uses the Fisher's exact test to identify significantly enriched functional categories (GO terms) in the lists of differentially expressed genes.

Chromosomal Location of Differentially Expressed Genes. To identify spatial patterns of gene expression across the genome, we developed a novel spatial-search algorithm. For each chromosome, a 400,000-bp region of genomic sequence was checked for two criteria: 1) at least four genes in this region were present on the array and 2) there was at least one differentially expressed gene in the effect being considered. If these criteria were met, then the probability of statistically significant enrichment in this region was calculated from the hypergeometric distribution. The window was then stepped in 200,000-bp increments across the chromosome. At the end of the process, p values were adjusted with a Bonferroni correction for multiple-testing, and significant spatial clustering was reported at the $p < 0.05$ level. To help visualize these patterns, we plotted the genomic locations of all genes that were responsive to the AHR or

AHR-TCDD effects using the Geneplotter package of the BioConductor open-source project (Gentleman et al., 2004).

Results

Genotype Confirmation. The $Ahr^{-/-}$ mice we used originally were generated by replacing exon 2 with the neomycin-resistance gene (Schmidt et al., 1996). Before array studies, we confirmed the *Ahr* genotypes of all mice using primers localized in exon 1 and exon 4. As expected, the amplified product from mRNA of each $Ahr^{-/-}$ mouse differed in size from that in the $Ahr^{+/+}$ wild-type mice (Supplemental Fig. S1).

Overall Patterns of Gene Expression Detected by Arrays. To obtain the maximum information and usefulness from our array experiments, we used a two-factor, two-level design: the factors were genotype ($Ahr^{+/+}$ or $Ahr^{-/-}$) and treatment (TCDD-treated or control). As shown in Fig. 1, we characterized three distinct sets of genes: 1) those affected by *Ahr* genotype independent of TCDD; 2) those responsive to TCDD in an AHR-dependent manner; and 3) those responsive to TCDD in an AHR-independent manner. The linear modeling approach we used increases the statistical power of the study by using all 17 animals in the estimation of each of the three contrasts rather than using multiple pairwise comparisons, each involving fewer animals. From the Venn diagram (Fig. 2), it is clear that the AHR is the critical factor governing changes in gene expression. More than 90% of ProbeSets that exhibited differential expression do so solely in response to either the AHR genotype (244/706 = 35%) or the AHR genotype in combination with TCDD administration (430/706 = 61%). Furthermore, there is a strong directionality to the effect of TCDD. Fully 75% of TCDD-responsive ProbeSets are up-regulated (Fig. 3).

AHR Effects Independent of TCDD. Most studies of the AHR's role in gene expression have focused on AHR-mediated transcriptional enhancement in response to foreign agonists such as TCDD. However, there is a large suite of genes whose hepatic expression is clearly affected by the AHR status independent of exposure to any exogenous AHR li-
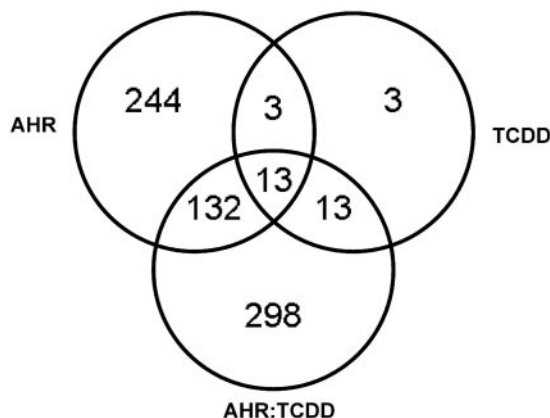
Fig. 2. Overlap of effects. ProbeSets were identified as differentially expressed by first normalizing the raw expression data, then fitting a linear model to the three effects, followed by Bayesian moderation of standard error and a false-discovery rate correction. ProbeSets were deemed significantly differentially expressed by a nested F test at the $p < 0.001$ level, which identified 706 ProbeSets from among the 45,101 on the array. The Venn diagram shows the overlap between effects in terms of the number of ProbeSets.

gand. Table 1 and Fig. 2 reveal a total of 392 ProbeSets whose expression levels differ significantly between $Ahr^{-/-}$ and $Ahr^{+/+}$ mice independent of TCDD treatment. The AHR seems to influence hepatic expression of nearly as many ProbeSets in the absence of exogenous ligands (392) as it does after TCDD administration (456) (Table 1). Although not all of these ProbeSets are necessarily regulated directly by the AHR, the impact of AHR status on constitutive expression of numerous genes suggests multiple and diverse roles for the AHR in normal physiology in addition to the AHR's ability to mediate gene expression in response to xenobiotic ligands. We harvested tissue 19 h after TCDD administration; thus, some changes in gene expression could be AHR-dependent but be secondary, downstream responses to other AHR-dependent changes rather than being primary responses directly regulated by the AHR.

The magnitude of the AHR effect on some genes was very large. For example, in untreated mice, the mRNA for Serpina12, a proteinase inhibitor, was 220-fold more abundant in liver from $Ahr^{+/+}$ mice than from $Ahr^{-/-}$ mice (Table 2 and Fig. 4). Constitutive expression of numerous other genes also was substantially higher in $Ahr^{+/+}$ mice than in $Ahr^{-/-}$ mice (Table 2).

Cyp1a2 represents a gene whose expression is well known to be inducible by TCDD via the AHR (Nebert et al., 2004). In addition to its AHR-regulated inducibility by TCDD, the *Cyp1a2* gene also displayed significantly higher expression (4-fold) in $Ahr^{+/+}$ mice than in $Ahr^{-/-}$ mice, independent of TCDD administration (Table 2); higher constitutive hepatic CYP1A2 mRNA expression in $Ahr^{+/+}$ mice than in $Ahr^{-/-}$ mice was previously observed (Shimada et al., 2002). It is possible that some endogenous ligand interacts with the AHR to support "constitutive" expression of *Cyp1a2* in mice bearing the wild-type AHR. However, if such an endogenous ligand exists, it provokes a selective response, because in the absence of an exogenous ligand, the expression of other prototypical AHR-regulated genes such as *Cyp1a1* and *Cyp1b1* was not higher in $Ahr^{+/+}$ mice than in $Ahr^{-/-}$ mice. Hectd2, a gene recently reported to be induced by AHR ligands (Hayes et al., 2005), behaved very much like *Cyp1a2*; that is, in addition to being inducible by TCDD in an AHR-dependent fashion, constitutive Hectd2 expression also was substan-

tially higher in mice with wild-type AHR than in $Ahr^{-/-}$ mice (Table 2).

Effects of the AHR on hepatic expression in the absence of exogenous ligand are not confined to up-regulation (i.e., higher expression in $Ahr^{+/+}$ mice than in $Ahr^{-/-}$ mice). In fact, presence of the AHR is associated with lowered expression of significantly more genes than it "up-regulates" (Table 1 and Fig. 3). Two cytochrome P450 mRNAs—CYP17A1 and CYP2B20—seem to be dramatically suppressed by the presence of the AHR in the absence of any exogenous ligand (Table 2 and Fig. 4). In the absence of treatment, mRNA levels for metallothionein 1 and metallothionein 2 are 40- to 64-fold lower in $Ahr^{+/+}$ mice than in $Ahr^{-/-}$ mice (Table 2 and Fig. 4); mechanistically, this may not be caused by direct suppression of metallothionein gene expression by the AHR (see *Discussion*).

AHR-Dependent Effects of TCDD. The expression levels of 456 ProbeSets were altered by TCDD in an AHR-dependent manner; that is, these ProbeSets showed differential responses to TCDD between $Ahr^{+/+}$ versus $Ahr^{-/-}$ mice (Table 1 and Fig. 3). There is substantial overlap between the TCDD-dependent and the -independent effects of the AHR (145/703 ProbeSets differentially expressed in both effects). These effects usually are in accord (125/145 common ProbeSets); it is particularly rare for the AHR to control the up-regulation of a gene's basal levels but the down-regulation of its response to TCDD (3/145 common probe sets). Basal down-regulation combined with TCDD-induced up-regulation is somewhat more common (17/145 common ProbeSets)

TABLE 1

Number of ProbeSets whose expression is significantly affected by the AHR, by TCDD, or by the AHR-TCDD interaction

Wild-type $Ahr^{+/+}$ mice and $Ahr^{-/-}$ mice were treated, and array experiments were performed as described under *Materials and Methods*. Cells in the table indicate the number ProbeSets whose expression was affected at a significance level of $p < 0.001$.

	Total		
	Responsive ProbeSets	Up	Down
AHR effect	392	174	218
TCDD effect	32	24	8
AHR-TCDD interaction	456	338	118

A Down-Regulated

B Up-Regulated
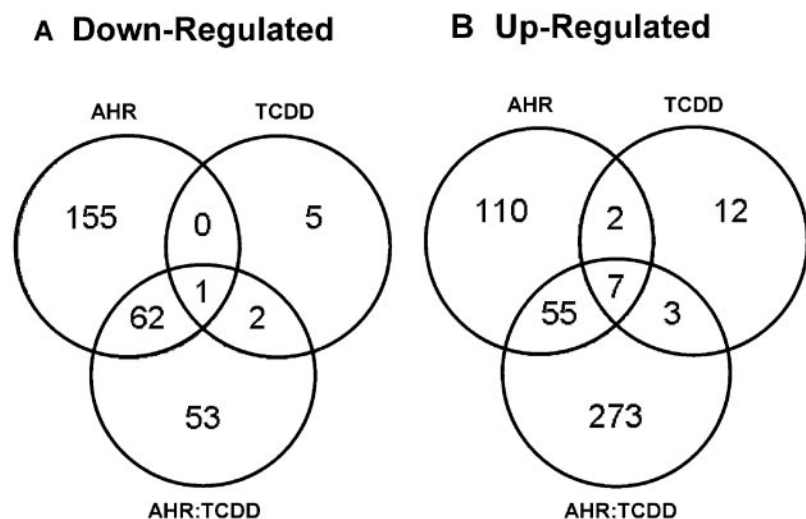
Fig. 3. Directionality of effect overlap. Differentially expressed ProbeSets were classified by the direction of change and show the profound directionality of the three effects. The AHR effect is evenly distributed between up- and down-regulation (44% of ProbeSets up-regulated). In contrast, the TCDD effect is skewed toward up-regulation (75% of ProbeSets up-regulated) as is the AHR-TCDD interaction (74% up-regulated). Note: ProbeSets regulated in opposite directions will not be represented in an intersection of the Venn diagrams; hence, the values in Figs. 2 and 3 are not additive on a subset-by-subset basis.

TABLE 2

AHR effects independent of TCDD

The 25 ProbeSets that are most up-regulated and the 25 most down-regulated are shown. Both transient (UniGene) and stable (LocusLink) gene identifiers are given. ProbeSets that carry no LocusLink could be mapped to a UniGene cluster, but that cluster did not correspond to a RefSeq gene. ProbeSets that carry no name and no UniGene cluster identifier could not be mapped to any cluster of transcribed sequences and may represent repetitive or nonspecific sequences. In some cases, the array contains multiple ProbeSets that map to the same gene; thus, the same gene name can appear at multiple places in the table. In cases in which genomic sequence is available, the number of AHRE-I and AHRE-II elements found in the region -5000 to $+1000$ bp relative to the TSS are given; see *Materials and Methods* for the sequence of each element. "M" values indicate the magnitude in $\log_2$-space of each contrast. For example, an effect of -3 refers to an 8-fold reduction in expression, whereas an effect of $+2$ refers to a 4-fold increase in mRNA levels. The abbreviated names of genes that are discussed in the text are shown in boldface type in parentheses. A full list of all responsive genes is available in Supplemental Table S2.

ProbeSet	UniGene Cluster	Locus-Link	AHRE-I Core	AHRE-I Extended	AHRE-I Full	AHRE-II	Cluster Name	M AHR	M AHR-TCDD	M TCDD
Up-regulated										
1421092_at	Mm.20286	68054	6				Serine (or cysteine) proteinase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 12 (**Serpina12**)	7.8*	−2.3	2.2
1438758_at								5.7*	−0.8	1.1
1453220_at	Mm.31626	78252	4	2		1	RIKEN cDNA 4432416J03 gene (**RIKEN**)	4.0*	−1.2	0.3
1451460_a_at	Mm.100765	108114	6	1		1	Solute carrier family 22 (organic anion transporter), member 7	3.2*	−0.2	0.5
1420531_at	Mm.17910	15496	1	1			Hydroxysteroid dehydrogenase-5, $\delta <5>$-3-β	3.0*	−0.7	0.3
1454758_a_at	Mm.153272	21807	11	3	1	1	RIKEN cDNA D430022A14 gene	3.0*	−0.1	0.7
1425742_a_at	Mm.153272	21807	11	3	1	1	RIKEN cDNA D430022A14 gene	2.6*	−0.1	0.7
1454971_x_at	Mm.153272	21807	11	3	1	1	RIKEN cDNA D430022A14 gene	2.5*	−0.2	0.7
1438596_at	Mm.61107	226098	7				HECT domain-containing 2 (**Hectd2**)	2.4*	2.8*	0.1
1438759_x_at								2.4*	−0.2	0.3
1429549_at	Mm.194116	373864	5			3	Procollagen, type XXVII, α1	2.4*	−0.8	0.3
1430785_at	Mm.158320	70061	7	2		1	Orphan short chain dehydrogenase/reductase	2.4*	−0.7	0.5
1424811_at	Mm.347969	69049	3	1			Camello-like 5	2.3*	0.9*	0.7*
1449844_at	Mm.103665	28248	2	1		1	Solute carrier organic anion transporter family, member 1a1	2.2*	−0.7	0.1
1456074_at	Mm.158320	70061	7	2		1	Orphan short chain dehydrogenase/reductase	2.1*	−0.5	0.3
1426263_at	Mm.178322	260299	15	5	1		Immunoglobulin superfamily, member 4C	2.1*	0.2	−0.3
1450715_at	Mm.15537	13077	1			1	P450, family 1, subfamily a polypeptide 2 (**Cyp1a2**)	2.0*	1.4*	0.8*
1431817_at	Mm.158750	71749					Alcohol dehydrogenase 6 (class V), pseudogene 1	2.0*	−0.9	−0.4
1420379_at	Mm.103665	28248	2	1		1	Solute carrier organic anion transporter family, member 1a1	1.9*	−0.5	0.0
1455663_at	Mm.77425	244198	3	1			Olfactomedin-like 1	1.8*	0.6	−0.2
1421258_a_at	Mm.8359	18770	7				Pyruvate kinase liver and red blood cell	1.8*	−0.9	−0.2
1423693_at	Mm.2131	109901	12	2		1	Elastase 1, pancreatic	1.7*	−1.1	0.2
1417991_at	Mm.148342	13370	11	1	1	2	Deiodinase, iodothyronine, type I	1.7*	−0.7	0.7
1424245_at	Mm.28191	234671	2	1			Carboxylesterase 2	1.7*	0.1	0.4
1438711_at	Mm.8359	18770	7				Pyruvate kinase liver and red blood cell	1.6*	−0.6	−0.4
Down-regulated										
1422557_s_at	Mm.192991	17748	11	2		1	Metallothionein 1 (**Mt1**)	−6.0†	0.0	0.2
1428942_at	Mm.147226	17750	13		1	1	Metallothionein 2 (**Mt2**)	−5.4†	0.6	−0.6
1417017_at	Mm.1262	13074	6	2		1	P450, family 17, subfamily a, polypeptide 1 (**Cyp17a1**)	−5.1†	0.8	0.2
1417370_at	Mm.4641	21786					Trefoil factor 3, intestinal	−4.9†	−1.3	0.6
1425645_s_at	Mm.218749	13088	7				P450, family 2, subfamily b, polypeptide 20 (**Cyp2b20**)	−4.8†	−0.4	0.4
1424534_at	Mm.48712	75104	11			2	Monocyte to macrophage differentiation-associated 2	−4.5†	0.2	−0.4
1438654_x_at	Mm.48712	75104	11			2	Monocyte to macrophage differentiation-associated 2	−4.5†	0.0	−0.2
1422257_s_at	Mm.218749	13088	7				P450, family 2, subfamily b, polypeptide 20 (**Cyp2b20**)	−4.4†	−0.5	0.5
1421286_a_at	Mm.12821	11944	14	2		1	ATPase, H+/K+-transporting, α-polypeptide	−3.9†	−0.3	0.1
1439816_at	Mm.350954	433816					Hypothetical gene supported by AK087915	−3.8†	−0.5	0.1
1427425_at	Mm.260210	77670					Mitochondrial carrier triple repeat 1	−3.2†	0.1	−0.3
1434473_at	Mm.25773	217316					Solute carrier family 16 (monocarboxylic acid transporters), member 5	−3.2†	1.1	−1.1
1451787_at	Mm.218749	13088	7				P450, family 2, subfamily b, polypeptide 20 (**Cyp2b20**)	−2.9†	0.6	−0.2
1451260_at	Mm.331583	72535	8	3	1		Aldehyde dehydrogenase 1 family, member B1	−2.9†	0.2	−0.2
1420405_at	Mm.255586	28250	2				Solute carrier organic anion transporter family, member 1a4	−2.8†	−0.6	-0.7
1432517_a_at	Mm.8362	18113	1			1	Nicotinamide N-methyltransferase	−2.7†	−0.7	0.0
1416250_at	Mm.239605	12227	7				B-cell translocation gene 2, antiproliferative	−2.7†	−0.7	−0.1
1419518_at	Mm.32884	53857	7	3	1	5	Tubulin, α8 (**Tuba8**)	−2.6†	5.0*	−0.1
1429204_at	Mm.31199	73047					RIKEN cDNA 2900075A18 gene	−2.5†	0.5	−0.5
1424029_at	Mm.21485	72480	10	1	1		DNA segment, Chr 10, Brigham & Women's Genetics 0791-expressed	−2.5†	−0.1	−0.1
1450970_at	Mm.19039	14718	9	2			Glutamate oxaloacetate transaminase 1, soluble	−2.5†	−0.4	−0.2
1424744_at	Mm.28685	231691	15	3			Serine dehydratase	−2.4†	−1.0†	−0.2
1442406_at	Mm.260210	77670					Mitochondrial carrier triple repeat 1	−2.3†	0.4	−0.6
1441430_at	Mm.365912						Transcribed locus	−2.3†	−0.6	0.0
1448754_at	Mm.279741	19659	6				Retinol binding protein 1, cellular	−2.3†	−0.3	−0.1

P450, cytochrome P450.

* Effects showing statistically significant up-regulation ($p < 10^{-3}$ in the nested F test).

† Effects showing statistically significant down-regulation.

and includes genes such as *Cyp2a4* and the known TCDD-responsive Gstm3.

To assess the validity of the array data and the overall treatment and sample preparation, we examined the expression of genes that are well known to be up-regulated by TCDD in animals that have wild-type AHR. Classic AHR-regulated genes such as *Cyp1a1*, *Cyp1b1* (Nebert et al., 2004), Nqo1, and Tiparp displayed robust AHR-dependent induction by TCDD (Table 3), confirming that the experimental protocol was sound. However, outside of the prototypical AH gene battery, there is only a moderate overlap in responsive genes detected in our high-throughput study compared with several other array studies of response to AHR ligands in varied tissues and cell types. There also is little consistency among the sets of genes identified across the previous array studies themselves (data not shown).

Several genes that were not known previously to respond to AHR ligands demonstrated AHR-dependent induction by TCDD of 20-fold or more. The genes whose expression was most highly up-regulated (Table 4) represent a variety of functional categories, including the flavin-containing monooxygenases Fmo2 (30-fold) and Fmo3 (80-fold), two neuronal

TABLE 3
Responses of ProbeSets that represent genes in the classic AH gene battery to AHR status or to AHR-TCDD interaction

Data are derived from array experiments as described under *Materials and Methods*. "AHR" represents the effect of having wild-type AHR (versus $Ahr^{-/-}$), whereas "AHR-TCDD" represents the effect of TCDD treatment in mice with wild-type AHR ($Ahr^{+/+}$mice). As in Table 2, "M" represents the magnitude in log_2-space for each contrast. There are multiple entries in the table for some genes because the array contains multiple ProbeSets for these genes.

ProbeSet	LocusLink	Gene	M	
			AHR	AHR-TCDD
1422217_a_at	13076	*Cyp1a1*	−0.5	7.7*
1450715_at	13077	*Cyp1a2*	2.0*	1.4*
1416612_at	13078	*Cyp1b1*	0.1	7.9*
1416613_at	13078	*Cyp1b1*	0.1	6.0*
1448330_at	14862	*GST, μ1*	−0.2	0.6*
1425627_x_at	14862	*GST, μ1*	−0.3[†]	0.5*
1416416_x_at	14862	*GST, μ1*	−0.2[†]	0.4*
1423627_at	18104	*Nqo1*	−0.8	4.5*
1452161_at	99929	*Tiparp*	0.0	5.5*
1452160_at	99929	*Tiparp*	0.0	5.3*
1426721_s_at	99929	*Tiparp*	0.1	5.3*
1426261_s_at	94284	*Ugt1a6*	0.1*	0.6*
1424783_a_at	84284	*Ugt1a6*	0.0	0.5*

P450, cytochrome P450.
* Effects showing statistically significant up-regulation ($p < 10^{-3}$ in the nested F test).
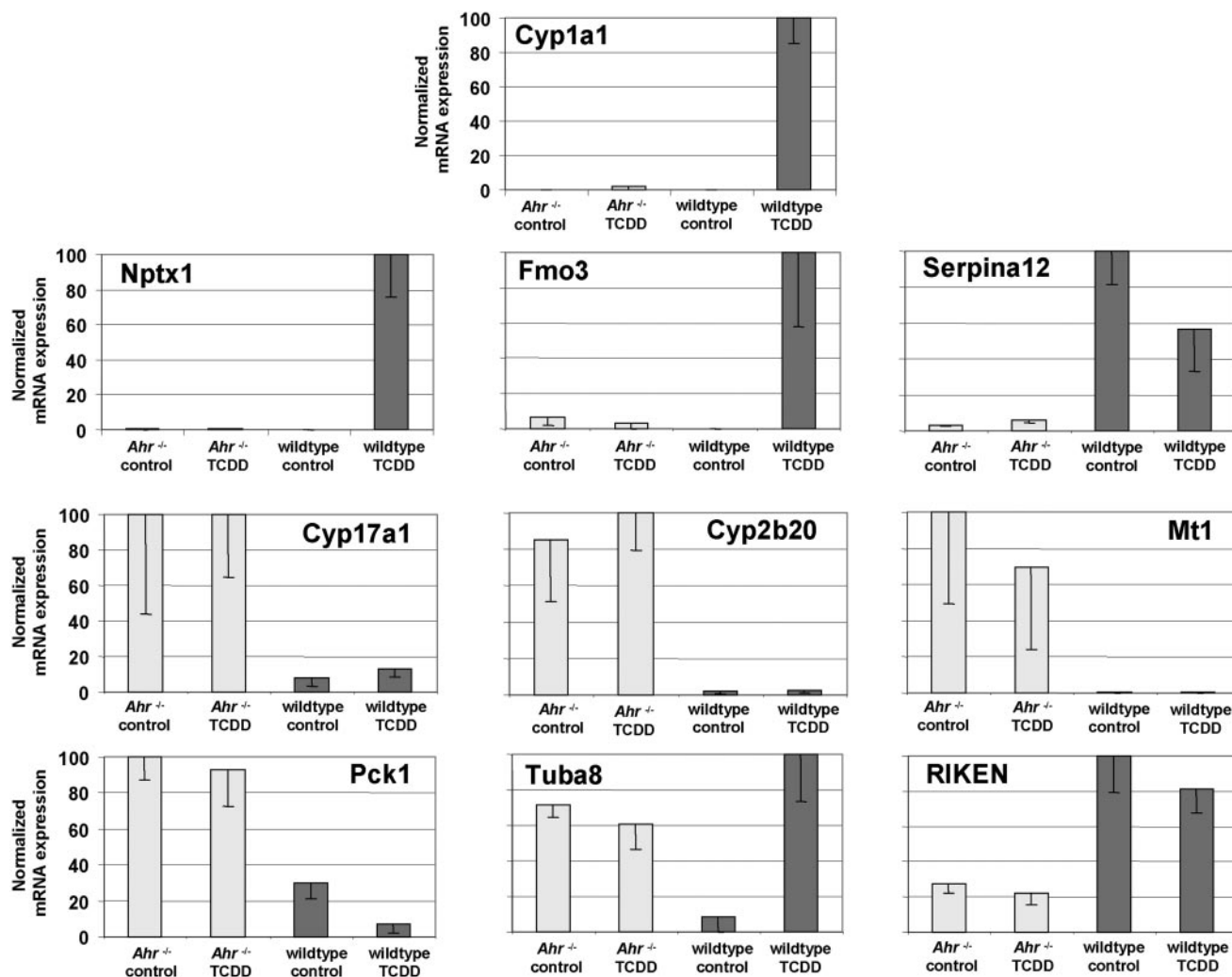† Effects showing statistically significant down-regulation.

Fig. 4. Confirmation of mRNA expression levels by real-time qRT-PCR. RNA used in the array studies was assayed for levels of mRNA for a selected set of the most responsive genes by real-time qRT-PCR as described in Supplemental Data. Bars represent the mean of three animals for $Ahr^{-/-}$ mice and of five or six animals for mice with wild-type AHR. Data are normalized so that for each gene, the treatment group with the highest expression is set at 100%, and expression in other groups is relative to this. Error bars represent S.D. from the mean. RIKEN represents RIKEN gene 4432416J03Rik.

TABLE 4

AHR-dependent effects of TCDD

The 25 ProbeSets that are most up-regulated and the 24 most down-regulated are shown. Both transient (UniGene) and stable(LocusLink) gene identifiers are given. ProbeSets that carry no LocusLink could be mapped to a UniGene cluster, but that cluster did not correspond to a RefSeq gene. ProbeSets that carry no name and no UniGene cluster identifier could not be mapped to any cluster of transcribed sequences and may represent repetitive or nonspecific sequences. In some cases, the array contains multiple ProbeSets that map to the same gene; thus, the same gene name can appear at multiple places in the table. In cases in which genomic sequence is available, the number of AHRE-I and AHRE-II elements found in the region -5000 to $+1000$ bp relative to the TSS are given; see *Materials and Methods* for the sequence of each element. "M" values indicate the magnitude in $\log_2$-space of each contrast. For example, an effect of -3 refers to an 8-fold reduction in expression, whereas an effect of $+2$ refers to a 4-fold increase in mRNA levels. The abbreviated names of genes that are discussed in the text are shown in boldface type in parentheses. A full list of all responsive genes is available in Supplemental Table S2.

ProbeSet	UniGene Cluster	Locus-Link	AHRE-I			AHRE-II	Cluster Name	M		
			Core	Extended	Full			AHR	AHR-TCDD	TCDD
Up-regulated										
1416612_at	Mm.214016	13078	16	3	1		P450, family 1, subfamily b, polypeptide 1 (**Cyp1b1**)	0.1	7.9*	0.2
1422217_a_at	Mm.14089	13076	16	8	5	1	P450, family 1, subfamily a, polypeptide 1 (**Cyp1a1**)	−0.5	7.7*	1.4
1449525_at	Mm.2900	14262	5	1		2	Flavin-containing monooxygenase 3 (**Fmo3**)	−0.3	6.3*	−0.4
1452913_at	Mm.273087	66425					Purkinje cell protein 4-like 1 (**Pcp4l1**)	0.2	6.2*	0.1
1434877_at	Mm.5142	18164	19	4	1	1	Neuronal pentraxin 1 (**Nptx1**)	0.1	6.2*	0.1
1416613_at	Mm.214016	13078	16	3	1	3	P450, family 1, subfamily b, polypeptide 1 (**Cyp1b1**)	0.1	6.0*	0.1
1452161_at	Mm.246398	99929	16		1	2	TCDD-inducible poly(ADP-ribose) polymerase	0.0	5.5*	1.0
1452160_at	Mm.246398	99929	16		1	2	TCDD-inducible poly(ADP-ribose) polymerase	0.0	5.3*	0.8
1426721_s_at	Mm.246398	99929	16		1	2	TCDD-inducible poly(ADP-ribose) polymerase	0.1	5.3*	0.5
1419518_at	Mm.32884	53857	7	3	1	5	Tubulin, α8 (**Tuba8**)	−2.6†	5.0*	−0.1
1419149_at	Mm.250422	18787	11	3			Serine (or cysteine) proteinase inhibitor, clade E, member 1 (**Serpine1**)	0.3	4.9*	0.4
1435459_at	Mm.10929	55990	7			1	Flavin-containing monooxygenase 2 (**Fmo2**)	−0.7	4.9*	−0.3
1446771_at	Mm.32884	53857	7	3	1	5	Tubulin, α8 (**Tuba8**)	−0.8	4.8*	−0.2
1424167_a_at	Mm.18939	29858	11	4	2		Phosphomannomutase 1	−0.4	4.6*	−0.3
1423627_at	Mm.252	18104	8				NAD(P)H dehydrogenase, quinone 1	−0.8	4.5*	−0.1
1452026_a_at	Mm.151951	66350	10	1		1	Phospholipase A2, group XIIA	−1.4	4.4*	−0.7
1430780_a_at	Mm.18939	29858	11	4	2		Phosphomannomutase 1	−0.1	4.4*	0.1
1449273_at	Mm.154358	76884	9	2			Hypothetical A830091E24	0.1	4.2*	0.2
1448147_at	Mm.281356	29820	10	1		2	Tumor necrosis factor receptor superfamily, member 19	0.2	4.2*	0.1
1431788_at	Mm.47052	75497	11	2		1	RIKEN cDNA 1700008G05 gene	0.0	4.1*	0.1
1453435_s_at	Mm.10929	55990	7			1	Flavin-containing monooxygenase 2 (**Fmo2**)	−0.4	3.9*	0.1
1428347_at	Mm.154358	76884	9	2			Hypothetical A830091E24	0.0	3.8*	0.3
1427912_at	Mm.4512	109857	20	5	1	2	Carbonyl reductase 3	0.0	3.6*	0.1
1427385_s_at	Mm.253564	20904	6	2		2	Actinin, α1	0.1	3.5*	0.1
1455267_at	Mm.89989	26381	17	2			Estrogen-receptor-related γ (**Esrrg**)	0.1	3.5*	0.0
Down-regulated										
1415944_at	Mm.2580	20969	10			1	Syndecan 1	−1.5†	−1.2†	0.0
1423267_s_at	Mm.16234	16402	9				Integrin α5 (fibronectin receptor α)	−0.1	−1.2†	0.4
1460197_a_at	Mm.31403	117167	2	1			Tumor necrosis factor, α-induced protein 9	0.3	−1.2†	0.1
1459141_at	Mm.175096	73625					RIKEN cDNA 1810008I18 gene	−0.5	−1.3†	−0.5
1456609_at	Mm.41603	66259					RIKEN cDNA 1810006K23 gene	−0.4	−1.3†	0.2
1437929_at	Mm.79993	240025	22	2		2	Dapper homolog 2, antagonist of β-catenin (*Xenopus laevis*)	−0.5	−1.3†	0.5
1428936_at	Mm.166944	67972					RIKEN cDNA 2810442I22 gene	−0.9†	−1.3†	0.2
1425033_at	Mm.24030	218103	4				Solute carrier family 17 (sodium phosphate), member 2	0.4	−1.3†	0.0
1427963_s_at	Mm.347374	103142	3	1			Retinol dehydrogenase 9	0.6	−1.4†	−0.4
1451753_at	Mm.2251	18845	9	2		1	Plexin A2	−0.1	−1.4†	0.1
1434153_at	Mm.251716	230126					Src homology 2 domain-containing transforming protein B	−0.5	−1.4†	0.5
1425829_a_at	Mm.31403	117167	2	1			Tumor necrosis factor, α-induced protein 9	0.9	−1.4†	0.2
1448158_at	Mm.2580	20969	10			1	Syndecan 1	−1.1†	−1.5†	0.1
1456500_at	Mm.41291	69695					Anterior pharynx defective 1c homolog (*Caenorhabditis elegans*)	−0.5	−1.5†	1.1*
1417982_at	Mm.27136	72999	12			2	Insulin-induced gene 2	0.7	−1.5†	0.5
1422473_at	Mm.20181	18578	4				Phosphodiesterase 4B, cAMP-specific	−1.2†	−1.6†	−0.2
1434674_at	Mm.342337	17101	1	1			Lysosomal-trafficking regulator	0.1	−1.7†	0.4
1428739_at	Mm.79127	68919					RIKEN cDNA 2310040A07 gene	−0.1	−1.8†	0.4
1434592_at	Mm.186778	52015					Solute carrier family 16 (monocarboxylic acid transporters), member 10	0.3	−1.9†	0.3
1416258_at	Mm.2661	21877	13	2	1	1	Thymidine kinase 1	−0.7†	−2.1†	0.1
1452893_s_at	Mm0.79127	68919					RIKEN cDNA 2310040A07 gene	−0.3	−2.2†	0.4
1439617_s_at	Mm.266867	18534	7	2		1	Phosphoenolpyruvate carboxykinase 1, cytosolic (**Pck1**)	−0.9	−2.4†	0.2
1448080_at	Mm.240566	108961					RIKEN cDNA 4432406C08 gene	0.9	−3.5†	0.3
1438617_at	Mm.211704	331535				1	Serine (or cysteine) proteinase inhibitor, clade A (α−1 antiproteinase, antitrypsin), member 7 (**Serpina7**)	−0.5	−4.3†	−0.4

P450, cytochrome P450.

* Effects showing statistically significant up-regulation ($p < 10^{-3}$ in the nested F test).

† Effects showing statistically significant down-regulation.

proteins; and Purkinje cell protein 4-like (Pcp4l1, 80-fold), neuronal pentraxin 1 (Nptx1; 80-fold), and tubulin α8 (Tuba8; 30-fold).

As shown in Table 1, the AHR-TCDD interaction encompasses approximately 3-fold more ProbeSets that are induced than are repressed. Nevertheless, the expression of many genes was strongly suppressed by TCDD in an AHR-dependent manner (Table 4). The mRNA whose expression was down-regulated to the greatest extent (20-fold) in an AHR-dependent fashion is the proteinase inhibitor Serpina7. Down-regulation of Serpina7 by TCDD is in sharp contrast to the response of Serpina1, which is highly induced by TCDD (Table 4), and Serpina12, whose constitutive expression is dramatically higher in $Ahr^{+/+}$ mice than in $Ahr^{-/-}$ mice (Table 2) and which is moderately down-

regulated by TCDD in an AHR-dependent fashion (Table 4 and Fig. 4).

TCDD Has Few AHR-Independent Effects. One of the most striking findings of our study is that very few genes respond to TCDD in the absence of the AHR. Only 32 genes showed statistically significant AHR-independent responses to TCDD, and the maximum difference in expression between TCDD-treated and untreated mice in the absence of the AHR was approximately 2-fold (Table 5). It was anticipated that TCDD would mediate gene expression primarily through the AHR. Our broad interrogation of the mouse transcriptome confirms that almost all transcriptomic effects of TCDD do indeed require the AHR.

Confirmation of mRNA Levels by Real-Time Quantitative RT-PCR. Array methods for measuring mRNA

TABLE 5
Complete list of all ProbeSets that displayed an AHR-independent response to TCDD

The right-hand column indicates the AHR-independent effect of TCDD. As shown in the other two columns, some of the genes that exhibited an AHR-independent effect of TCDD also displayed a TCDD-independent AHR-effect (left-hand column) or an AHR-TCDD interaction effect (middle column). ProbeSets that carry no LocusLink could be mapped to a UniGene cluster, but that cluster did not correspond to a RefSeq gene. ProbeSets that carry no name and no UniGene cluster identifier could not be mapped to any cluster of transcribed sequences and may represent repetitive or nonspecific sequences. In some cases, the array contains multiple ProbeSets that map to the same gene; thus, the same gene name can appear at multiple places in the table. In cases in which genomic sequence is available, the number of AHRE-I and AHRE-II elements found in the region −5000 to +1000 bp relative to the TSS are given; see *Materials and Methods* for the sequence of each element. "M" values indicate the magnitude in $\log_2$-space of each contrast. For example, an effect of −3 refers to an 8-fold reduction in expression, whereas an effect of +2 refers to a 4-fold increase in mRNA levels. The abbreviated names of genes that are discussed in the text are shown in boldface type in parentheses. A full list of all responsive genes is available in Supplemental Table S2.

ProbeSet	UniGene Cluster	LocusLink	AHRE-I Core	AHRE-I Extended	AHRE-I Full	AHRE-II	Cluster Name	M AHR	M AHR-TCDD	M TCDD
1433992_at	Mm.40796	110380					Apical protein, *Xenopus laevis*-like	0.1	1.0*	−0.6†
1438430_at	Mm.87639	73389	3	1			High mobility group box transcription factor 1	0.3	0.6*	−0.5†
1438359_at	Mm.46323	75606					RIKEN cDNA 2010003K15 gene	0.3	0.5*	−0.4†
1419267_at	Mm.245998	18045	7	1		1	Nuclear transcription factor-Y β	0.0	−0.5†	−0.4†
1448649_at	Mm.1193	13809	9	4			Glutamyl aminopeptidase	−0.5†	−0.4†	−0.3†
1425844_a_at	Mm.240024	24018	5				RNA guanylyltransferase and 5′-phosphatase	0.1	0.3*	−0.3†
1434329_s_at	Mm.291826	68465	7				Adiponectin receptor 2	0.2	0.1	−0.2†
1416408_at	Mm.356689	11430	7	1		2	Acyl-coenzyme A oxidase 1, palmitoyl	0.0	−0.2	−0.1†
1416119_at	Mm.260618	22166	11	1		2	Thioredoxin 1	0.2*	0.1	0.1*
1451035_a_at	Mm.30085	58810	5	1			Aldo-keto reductase family 1, member A4 (aldehyde reductase)	0.1*	0.3*	0.1*
1416979_at	Mm.332855	66537	15	4		2	RIKEN cDNA 2510048O06 gene	0.1	0.3*	0.2*
1415882_at	Mm.182912	66092	7			3	Growth hormone-inducible transmembrane protein	−0.1	−0.3†	0.2*
1437745_at	Mm.138792	320790					Chromodomain helicase DNA binding protein 7	0.0	−0.2	0.2*
1426449_a_at	Mm.8211	18744	18		1		Praja1, RING-H2 motif-containing	0.1	0.6*	0.2*
1422130_at	Mm.5142	18164	19	4	1	1	Neuronal pentraxin 1 (**Nptx1**)	0.3*	1.8*	0.4*
1458398_at	Mm.87299	72268					RIKEN cDNA 1700027F06 gene	0.0	−0.4†	0.4*
1416543_at	Mm.1025	18024	13	3		1	Nuclear factor, erythroid-derived 2, -like 2	0.1	2.0	0.4*
1428749_at	Mm.93636	76881					Dmx-like 2	0.3*	0.5*	0.4*
1449125_at	Mm.2312	66443	12				RIKEN cDNA 2600017J23 gene	1.5*	1.0*	0.4*
1436532_at	Mm.26361	245038	2			1	Doublecortin and CaM kinase-like 3	0.6*	2.6*	0.6*
1421106_at	Mm.22398	16449	12	4	1	2	Jagged 1	0.0	−0.6	0.6*
1443915_at	Mm.41077	56456	11				Actin-like 6A	−0.3†	−0.6†	0.6*
1418260_at	Mm.125874	26559	17	3		1	Hormonally up-regulated Neu-associated kinase	−0.6†	−0.7†	0.7*
1419426_s_at	Mm.220853	18829	5	1			Chemokine (C-C motif) ligand 21b (serine)	−0.8†	−0.8†	0.7*
1421681_at	Mm.262663	83961	2			1	Neuregulin 4	−0.9†	−0.7†	0.7*
1424811_at	Mm.347969	69049	3	1			Camello-like 5	2.3*	0.9*	0.7*
1450715_at	Mm.15537	13077	1			1	P450, family 1, subfamily a, polypeptide 2 (**Cyp1a2**)	2.0*	1.4*	0.8*
1440739_at	Mm.1402	22341	4	1			Vascular endothelial growth factor C	−0.3	−0.8	0.9*
1422758_at	Mm.212446	54371	16	2	1		Carbohydrate sulfotransferase 2	−0.4	−1.1	1.0*
1450044_at	Mm.297906	14369	22	3	1		Frizzled homolog 7 (*Drosophila melanogaster*)	0.8	0.6	1.1*
1456500_at	Mm.41291	69695					Anterior pharynx defective 1c homolog (*Caenorhabditis elegans*)	−0.5	−1.5	1.1*
1426418_at	Mm.87449	71093	20	5		2	Atonal homolog 8 (*D. melanogaster*)	1.4*	0.6	1.1*

P450, cytochrome P450.
* Effects showing statistically significant up-regulation ($p < 10^{-3}$ in the nested F test).
† Effects showing statistically significant down-regulation.

levels are becoming highly reliable. Nevertheless, we believed it prudent to use real-time qRT-PCR as an independent method to evaluate mRNA levels for selected genes whose expression, by array analyses, seemed to be related to AHR status and/or TCDD treatment. We used *Cyp1a1* as a benchmark because CYP1A1 mRNA is known to be inducible via the AHR pathway. As expected, the array results and the qRT-PCR results showed that CYP1A1 mRNA is strongly up-regulated by TCDD in $Ahr^{+/+}$ mice, whereas CYP1A1 mRNA is undetectable in $Ahr^{-/-}$ mice or in untreated $Ahr^{+/+}$ mice (Fig. 4, top row). Nptx1 and Fmo3 behave very much like CYP1A1: each of these mRNAs is highly up-regulated by TCDD in $Ahr^{+/+}$ mice, whereas in $Ahr^{-/-}$ mice mRNA levels are very low in either the constitutive state or in TCDD-treated animals (Fig. 4; 2nd row). Our results show a strong AHR dependence for induction of Nptx1 and Fmo3 (Table 4 and Fig. 4).

Real-time qRT-PCR also confirms array results which show that mRNA levels for metallothionein 1 (Mt1), CYP17A1, and CYP2B20 are dramatically higher in $Ahr^{-/-}$ mice than in $Ahr^{+/+}$ mice (Fig. 4, 3rd row); none of these genes shows a significant response to TCDD in either $Ahr^{+/+}$ mice or $Ahr^{-/-}$ mice.

For Tuba8, constitutive expression is much lower in $Ahr^{+/+}$ mice than in $Ahr^{-/-}$ mice. TCDD treatment in $Ahr^{+/+}$ mice essentially "restores" Tuba8 mRNA to levels similar to those in $Ahr^{-/-}$ mice (Fig. 4, row 4). It is possible that the AHR suppresses Tuba8 when no exogenous ligand is present and that TCDD interferes with this suppression.

Functional Categorization of Responsive Genes by Ontological Analysis. High-throughput expression experiments often identify large numbers of genes whose expression is altered by biological status or by the treatment of interest. It can be difficult to discern functionally relevant patterns by simple inspection of large data sets. To characterize the gene lists, we used ontological analysis to identify functional categories that were enriched in each of the effects studied: AHR (Table 6), AHR-TCDD (Table 7), and TCDD (Supplemental Table S3).

The AHR effect is quite functionally coherent (Table 6). It includes genes for numerous proteins involved in electron transport, drug metabolism, and defense against xenobiotic toxicants, including 13 cytochrome P450s, 4 glutathione-*S*-transferases, 2 thioredoxins, and 2 metallothioneins. It also includes three separate aquaporins (water channels), which may indicate a perturbation in osmotic homeostasis within the cell, consistent with the large number of ion channels that also show altered expression. Three of the eight genes on the array that have roles in aromatic amino acid catabolism are down-regulated, and three of nine negative regulators of cell adhesion are up-regulated.

The AHR-TCDD interaction (Table 7) shows the clearest patterns. There is profound up-regulation of genes involved in electron transport, detoxification, and ribosome assembly and structure. There also is a strong down-regulation of genes involved in amino acid metabolism in general, and particularly, catabolism.

The AHR-independent TCDD effect (Supplemental Table S4) displayed little functional coherency—genes that are affected by TCDD in the absence of the AHR do not fit any particular category.

Transcription Factor Binding Sites. Classic TCDD-inducible/AHR-dependent genes such as *Cyp1a1* contain an AHRE-I motif that binds the ligand-AHR-ARNT complex and leads to enhanced transcription. A second response element, AHRE-II, has been discovered that seems to mediate the induction of rat *CYP1A2* (Sogawa et al., 2004) and several other genes (Boutros et al., 2004) by AHR agonists. We searched for AHRE-I and AHRE-II motifs in the 5′-flanking sequence of all genes whose expression was significantly influenced by the AHR or TCDD in the array experiment.

The core AHRE-I sequence (GCGTG) is found in nearly all responding genes listed in Tables 2 to 5. The majority of these core sequences probably represent random occurrence of this pentanucleotide which will occur approximately once every 256 bases (4^5 for two strands and two orientations) throughout the genome. Neither the core AHRE-I or AHRE-II binding sites nor any of the longer AHRE-I sequences that were examined in an earlier phylogenetic footprinting study (Boutros et al., 2004) showed significant enrichment in any condition. This is consistent with the lack of substantial overlap between the genes identified in previous microarray

TABLE 6
Ontological analysis of the AHR effect on transcript expression

Ontological analysis used the GOMiner software package (Zeeberg et al., 2003), which performs a two-tailed Fisher's exact test to determine over-representation of GO categories in the gene list relative to the array as a whole. The GO term and name are given, along with the total number of genes in the category and the numbers of over- and underexpressed genes that have this annotation. *p* values, shown in parentheses, are calculated for enrichment of the term as a whole and for enrichment of the term among over- and underexpressed genes alone. GO categories shown were selected from those showing statistical significance ($P < 0.001$) in the AHR effect.

GO	Total	Under	Over	Name
GO:0006725	68 ($<10^{-4}$)	7 ($<10^{-4}$)	2 (0.1505)	Aromatic compound metabolism
GO:0006118	239 ($<10^{-4}$)	13 ($<10^{-4}$)	6 (0.034)	Electron transport
GO:0016705	63 ($<10^{-4}$)	9 ($<10^{-4}$)	2 (0.1332)	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
GO:0016491	483 ($<10^{-4}$)	25 ($<10^{-4}$)	16 ($<10^{-4}$)	Oxidoreductase activity
GO:0004497	80 ($<10^{-4}$)	7 (0.0001)	4 (0.0087)	Monooxygenase activity
GO:0005792	85 ($<10^{-4}$)	7 (0.0001)	5 (0.0017)	Microsome
GO:0006082	245 (0.0001)	14 ($<10^{-4}$)	3 (0.4524)	Organic acid metabolism
GO:0006519	153 (0.0002)	11 ($<10^{-4}$)	1 (0.7912)	Amino acid and derivative metabolism
GO:0009308	174 (0.0002)	12 ($<10^{-4}$)	1 (0.832)	Amine metabolism
GO:0009310	39 (0.0003)	5 (0.0001)	1 (0.3277)	Amine catabolism
GO:0008483	27 (0.0003)	5 ($<10^{-4}$)	0 (1)	Transaminase activity
GO:0006520	116 (0.0004)	10 ($<10^{-4}$)	0 (1)	Amino acid metabolism
GO:0016769	28 (0.0004)	5 ($<10^{-4}$)	0 (1)	Transferase activity, transferring nitrogenous groups
GO:0009074	8 (0.0006)	3 (0.0001)	0 (1)	Aromatic amino acid family catabolism
GO:0009063	30 (0.0006)	5 ($<10^{-4}$)	0 (1)	Amino acid catabolism
GO:0007162	9 (0.0009)	0 (1)	3 (0.0001)	Negative regulation of cell adhesion

analyses and in our current direct test of AHR activation. It is possible that the 19-h exposure to TCDD in our array study resulted in some secondary responses that could obscure enrichment in both AHRE-I and AHRE-II binding sites. This hypothesis is supported by an enrichment of AHRE-I motifs in the 25 ProbeSets most highly induced by TCDD in an AHR-dependent fashion (AHR-TCDD up-regulated list). This suggests that the AHRE-I motif is solely involved in TCDD-dependent induction through the AHR, rather than repression or ligand-independent induction. The presence of confounding secondary effects would be particularly relevant for the AHRE-II because AHRE-II was found to be generally depleted in liver-expressed genes.

To characterize patterns of other transcription-factor binding sites in addition to AHRE-I and AHRE-II motifs, we used a library-based TFBS search. Genes were separated according to their direction of change for both the AHR effect and the AHR-TCDD effect, yielding four lists of coexpressed genes. We identified two basic groups of results. First, a series of TFBS were found to be consistently enriched or depleted in all four sets of coexpressed genes tested (Table 8). These motifs seem to represent liver-specific transcription factors, such as HNF3. When such motifs are under-represented, they may represent nonhepatic transcription factors (e.g., AHRE-II) or transcription factors antagonized by the AHR. The latter class might include E2F motif, in which the AHR can corepress E2F-dependent expression through interactions with rubidium (Marlowe et al., 2004). Second, several TFBS were found to be enriched or depleted in only one or two of the sets of coexpressed genes (Table 9). These motifs may represent particular mechanisms of AHR regulation. For instance, genes repressed by TCDD through the AHR are

TABLE 7
Ontological analysis of the AHR-TCDD interaction on transcript expression

Ontological analysis used the GOMiner software package (Zeeberg et al., 2003), which performs a two-tailed Fisher's exact test to determine over-representation of GO categories in the gene list relative to the array as a whole. The GO term and name are given, along with the total number of genes in the category and the numbers of over- and underexpressed genes that have this annotation. p values, shown in parentheses, are calculated for enrichment of the term as a whole and for enrichment of the term among over- and underexpressed genes alone. GO categories shown were selected from those showing statistical significance ($P < 0.001$) in the AHR effect. GO categories shown were selected from those showing statistical significance ($p < 0.001$) in the AHR-TCDD effect.

GO	Total	Under	Over	Name
GO:006725	68 ($<10^{-4}$)	3 (0.0095)	7 (0.0002)	Aromatic compound metabolism
GO:006412	349 ($<10^{-4}$)	0 (1)	22 ($<10^{-4}$)	Protein biosynthesis
GO:006118	239 ($<10^{-4}$)	3 (0.1994)	19 ($<10^{-4}$)	Electron transport
GO:0016616	67 ($<10^{-4}$)	2 (0.0692)	11 ($<10^{-4}$)	Oxidoreductase activity, acting on the CH-OH group of donors, NAD, or NADP as acceptor
GO:009310	39 ($<10^{-4}$)	4 (0.0001)	3 (0.0315)	Amine catabolism
GO:004497	80 ($<10^{-4}$)	2 (0.0938)	10 ($<10^{-4}$)	Monooxygenase activity
GO:0030529	226 ($<10^{-4}$)	0 (1)	21 ($<10^{-4}$)	Ribonucleoprotein complex
GO:009058	687 ($<10^{-4}$)	7 (0.1519)	31 ($<10^{-4}$)	Biosynthesis
GO:0019843	7 ($<10^{-4}$)	0 (1)	4 ($<10^{-4}$)	rRNA binding
GO:005840	137 ($<10^{-4}$)	0 (1)	19 ($<10^{-4}$)	Ribosome
GO:005792	85 ($<10^{-4}$)	2 (0.1039)	12 ($<10^{-4}$)	Microsome
GO:008152	4159 ($<10^{-4}$)	29 (0.3312)	109 ($<10^{-4}$)	Metabolism
GO:004499	4 (0.0001)	0 (1)	3 ($<10^{-4}$)	Dimethylaniline monooxygenase (*N*-oxide-forming) activity
GO:0042254	62 (0.0001)	0 (1)	8 ($<10^{-4}$)	Ribosome biogenesis and assembly
GO:007046	59 (0.0001)	0 (1)	8 ($<10^{-4}$)	Ribosome biogenesis
GO:006631	89 (0.0003)	3 (0.0196)	6 (0.0049)	Fatty acid metabolism
GO:006519	153 (0.0003)	8 ($<10^{-4}$)	4 (0.288)	Amino acid and derivative metabolism
GO:0015036	15 (0.0004)	0 (1)	4 (0.0001)	Disulfide oxidoreductase activity
GO:0016789	76 (0.0005)	0 (1)	8 (0.0001)	Carboxylic ester hydrolase activity
GO:004364	16 (0.0005)	0 (1)	4 (0.0001)	Glutathione transferase activity
GO:009074	8 (0.0007)	3 ($<10^{-4}$)	0 (1)	Aromatic amino acid family catabolism

TABLE 8
Transcription factor binding sites common to all conditions

p values for transcription factor binding sites that were found to be over-represented in all four of the effects studied. Here, AHR($\downarrow$) refers to genes with negative AHR effects, whereas AHR-TCDD($\uparrow$) refers to genes with positive interactions. The ID number refers to the JASPAR identifier of the matrix used for testing the specific TFBS. The p values given here were obtained by dinucleotide randomization studies (1000 iterations). Of the 15 TFBS here, only five are under-represented (MA0054, MA0103, MA0036, and MA0024), whereas 10 are over-represented. Many of these TFBS are likely to be liver-specific rather than AHR-specific.

ID	Transcription Factor Name	AHR ($\downarrow$)	AHR ($\uparrow$)	AHR-TCDD ($\downarrow$)	AHR-TCDD ($\uparrow$)
MA0041	HFH-2 FORKHEAD	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
MA0042	HFH-3 FORKHEAD	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
MA0055	Myf bHLH	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
MA0047	HNF-3β FORKHEAD	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
MA0015	CF2-II ZN-FINGER, C2H2	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
MA0094	Ubx HOMEO	0.001	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
MA0017	COUP-TF NUCLEAR RECEPTOR	0.014	0.001	0.003	$<10^{-3}$
MA0088	Staf ZN-FINGER, C2H2	$<10^{-3}$	0.010	$<10^{-3}$	$<10^{-3}$
MA0086	Snail ZN-FINGER, C2H2	0.035	$<10^{-3}$	0.116	0.003
MA0057	MZF_5−13 ZN-FINGER, C2H2	$<10^{-3}$	0.014	0.183	0.164
MA0054	MYB.ph3 TRP-CLUSTER	0.997	0.955	0.946	0.898
Custom	AHRE-II	0.957	0.897	0.892	0.999
MA0103	δEF1 ZN-FINGER, C2H2	0.969	0.923	0.981	0.999
MA0036	GATA-2 ZN-FINGER, GATA	1	1	1	1
MA0024	E2F unknown	1	1	1	1

enriched for p53 binding motifs, indicating a potential cross-talk between these pathways.

Chromosomal Locations of Responsive Genes. It is possible that genes which respond to a particular regulatory molecule such as the AHR might occur in clusters or "hotspots" in the genome (Cohen et al., 2000). We mapped the chromosomal location for named genes as depicted in Fig. 5. A sliding-window analysis identified some hotspots of differential expression. One notable hotspot for the AHR effect is on chromosome 8, where carboxylesterase-2 and -5 are in close proximity to a RIKEN gene (2210023G05Rik); this enrichment is statistically significant at $p < 10^{-3}$. The AHR-TCDD effect is much more spatially clustered than the AHR effect. One notable cluster for AHR-TCDD involves genes encoding four glutathione S-transferase proteins on chromosome 3 (Gstm1, Gstm2, Gstm3, and Gstm4); this group of glutathione S-transferase genes may have arisen by gene duplication and remain in proximity on chromosome 3. Another cluster, on chromosome 6, involves three unrelated genes: a solute carrier (Slc6a13), a protease (Usp18), and α-tubulin (Tuba8) with $p < 10^{-3}$.

Although there are some significant clusters, it is clear from data in Fig. 5 that genes whose expression is affected by the AHR in a ligand-dependent or -independent manner are widely dispersed across the mouse genome, with the exception of sex chromosomes. The sex chromosomes contain few genes that seem to be regulated by the AHR, at least in liver; there are no hepatically expressed AHR-regulated genes on the Y chromosome and only a handful on the X chromosome. Although the X chromosome generally is gene-rich, it shows minimal differential expression related to AHR, with nonrandom under-representation for both for the AHR effect ($p = 0.004$) and the AHR-TCDD interaction ($p = 0.002$).

Discussion

Our array experiments in $Ahr^{-/-}$ versus $Ahr^{+/+}$ mice are intended to comprehensively define the separate sets of genes whose expression is significantly affected by AHR status per se or by the interaction of TCDD with AHR in mouse liver. This is a prelude to the longer-term goal of determining which AHR-regulated genes are central to normal physiolo-

TABLE 9
AHR-specific transcription factor binding sites identified

A selection of statistically significant TFBSs identified as depleted (i.e., HLF) or enriched (all others) in at least one contrast. ↓ refers to genes with negative effects, whereas ↑ refers to genes with positive effects. p values were derived from 1000 iterations of dinucleotide randomization. The enrichment of SP1 sites in genes induced by TCDD in an AHR-dependent fashion (such as $Cyp1a1$) is supportive of the known direct interactions between the AHR and SP1. On the other hand, p53-regulated genes generally are proapoptotic/cell-defense genes; thus, the TCDD-dependent down-regulation of such genes is surprising and may contribute to some of the acute toxic effects of TCDD exposure.

Effect	ID	TF Name	p Value
AHR (↓)	MA0039	Gklf	$<10^{-3}$
AHR (↑)	MA0068	Pax-4	$<10^{-3}$
AHR (↑)	MA0065	Peroxisome proliferator-activated receptor-γ	$<10^{-3}$
AHR-TCDD(↓)	MA0106	p53	0.001
AHR-TCDD(↑)	MA0079	SP1	$<10^{-3}$
AHR-TCCD(↑)	MA0043	HLF	0.993
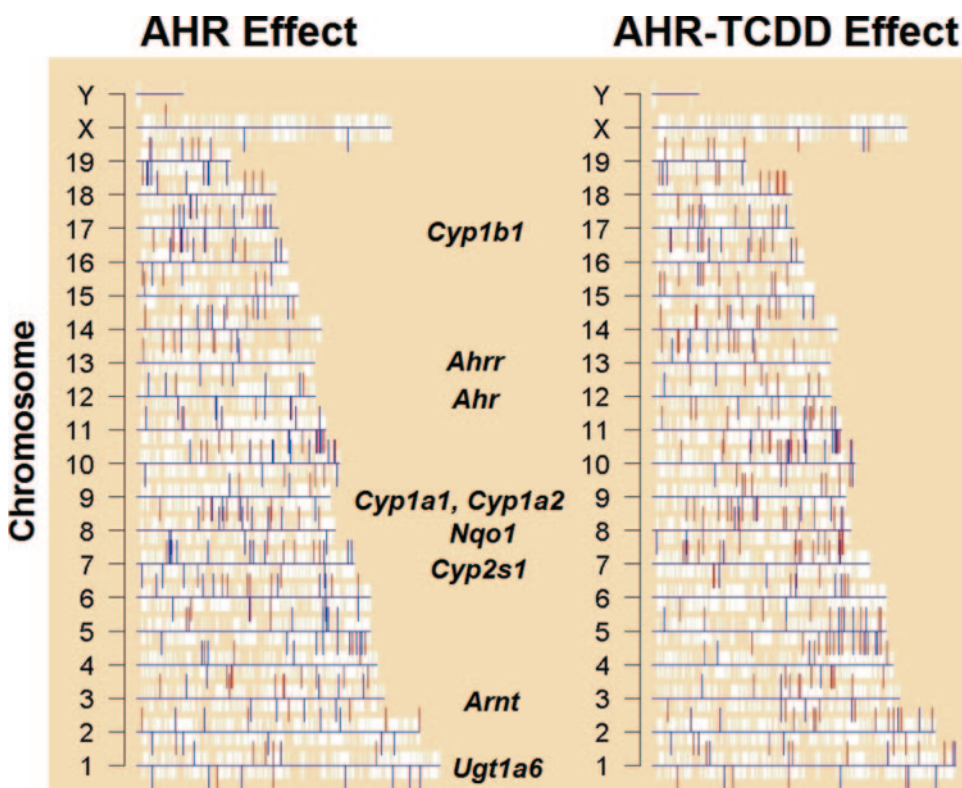
Fig. 5. Chromosomal distribution of genes that respond to the AHR or to the AHR-TCDD interaction. Genes that exhibited a statistically significant response to AHR or to the AHR-TCDD interaction were mapped to the genome using the geneplotter package of the BioConductor open-source project. The chromosomes that contain genes encoding the AHR, ARNT, and prototypical AHR-responsive genes ($Cyp1a1$, $Cyp1a2$, $Cyp1b1$, $Cyp2s1$, $Nqo1$, $Ugt1a6$, and $Ahrr$) are indicated. Red bars represent positions of genes that are up-regulated, and blue bars indicate down-regulated genes. White bars represent the locations of all other genes contained on that chromosome that are not affected by AHR status or by AHR-TCDD interaction.

gy/development and which genes are dysregulated by dioxins in a manner that evokes toxicity. Our functional analyses (Tables 6 and 7) reveal that AHR status and TCDD exposure influence the expression of genes that carry out a wide variety of functions.

Genes Related to Reproduction, Growth, and Development. $Ahr^{-/-}$ mice exhibit reduced fecundity (Lahvis et al., 2000). The reasons are not clear, but female reproduction is susceptible to disruption by alterations in estrogen function. One ongoing challenge in dioxin research is to mechanistically rationalize the extensive cross-talk between estrogen-signaling pathways and dioxin-signaling pathways (Beischlag and Perdew, 2005). Our study shows that expression of both Esr1 (ERα) itself and two of its closely related proteins, Esrra and Esrrg, can be regulated by the AHR. With the extensive intertwining and antagonism of estrogen and AHR pathways, it seems that they are reciprocally regulated. That is, the AHR modulates Esr1 levels, thereby potentially dampening estrogen effects. We postulate that basal AHR levels are, in turn, partly regulated by estrogens through Esr1 or one of the Esr-related receptors. In the absence of TCDD, hepatic Esr1 levels are 3.5-fold lower in $Ahr^{+/+}$ than in $Ahr^{-/-}$ mice. The estrogen-receptor-related α gene (Esrra) also is moderately down-regulated by the AHR in a TCDD-independent manner (Supplemental Table S2). Moreover, our data show that estrogen-receptor-related γ (Esrrg) is dramatically induced (11-fold) by TCDD in an AHR-dependent fashion (Table 4). The function of Esrrg is largely unknown. Esrrg does not bind estrogenic ligands but can homodimerize and occupy a wide range of estrogen response elements, potentially antagonizing the action of the major ligand-dependent estrogen receptors, Esr1 (ERα) and Esr2 (ERβ) (Razzaque et al., 2004). Thus, the high degree of Esrrg induction by TCDD constitutes a novel mechanism by which TCDD interferes with estrogen signaling in development and reproduction.

Our study also reveals that the constitutive level of CYP17A1 mRNA is 30-fold higher in $Ahr^{-/-}$ mice than in $Ahr^{+/+}$ mice (Table 2 and Fig. 4). CYP17 enzymes are essential for biosynthesis of both glucocorticoids and sex steroid precursors. It is possible that excessive hepatic CYP17A1 in livers of $Ahr^{-/-}$ mice disrupts homeostasis of reproductive steroids and impairs fecundity, because this enzyme has 17α-hydroxylase activity and 17,20-lyase activity, as well as activity as a squalene monooxygenase, which can alter cholesterol biosynthesis. TCDD or the dioxin-like PCB126 has been reported to decrease CYP17A expression in rat testis (Mebus et al., 1987), but our results show that hepatic CYP17A1 expression is unaltered by TCDD exposure for 19 h, regardless of Ahr genotype.

Genes Related to Cell Cycle, Cell Growth, Differentiation, and Apoptosis. Evidence continues to expand that the AHR plays fundamental roles in the cell cycle and apoptosis (Hanlon et al., 2005). We found neuronal pentraxin I (*Nptx1*) to be induced more than 60-fold in livers of $Ahr^{+/+}$ mice treated with TCDD. In the nervous system, Nptx1 is induced by hypoxia and is involved in apoptotic cell death in neurons undergoing stress. Nptx1 is expressed in liver but its function there is unclear. Induction of Nptx1 by TCDD may indicate that Nptx1 is broadly responsive to a wide variety of environmental stressors.

Proteinase Inhibitors. Proteinase inhibitors play major roles in tissue maintenance by reducing degradation of the extracellular matrix by enzymes such as matrix metalloproteinases (MMPs). Up-regulation of proteinase inhibitors in TCDD-exposed tissues may be beneficial because TCDD can induce MMPs. We found that MMP-24 was up-regulated 2-fold by TCDD in livers of $Ahr^{+/+}$ mice (Supplemental Table S2), but this was accompanied by a 30-fold induction of the proteinase inhibitor Serpine1 (Table 4). Our experiments revealed that Serpina12 mRNA is dramatically reduced in livers of $Ahr^{-/-}$ mice compared with $Ahr^{+/+}$ mice, but the effect of TCDD is modest (Table 2 and Fig. 4). It is possible that some of the liver pathology that afflicts $Ahr^{-/-}$ mice (Walisser et al., 2004b) is related to deficiency of Serpina12.

Genes Related to Toxicity or to Defense from Toxicity of Xenobiotic Chemicals. Our array experiment shows that TCDD elicits the expected very high induction of mRNAs for CYP1A1, CYP1A2, CYP1B1, and Nqo1 in $Ahr^{+/+}$ mice along with induction of the conjugating enzymes Gstm1 and Ugt1a6. We also found (Table 4) a remarkably high induction of the flavin monooxygenase enzymes Fmo2 and Fmo3. Flavin monooxygenase enzymes play important roles in detoxification of many foreign chemicals, including psychoactive drugs, pesticides, and dietary-derived compounds, but previously were believed not to be inducible in mammals (Cashman, 2002). Flavin monooxygenase enzyme induction may be a further adaptive mechanism by which the AHR fosters protection from xenobiotic chemicals.

Constitutive PEPCK (Pck1) expression was much higher in livers of $Ahr^{-/-}$ mice than in livers of $Ahr^{+/+}$ mice. PEPCK is a key enzyme in gluconeogenesis, and studies in rodents have shown that acute toxicity of TCDD may be associated with reduced PEPCK activity, together with appetite suppression and inhibition of gluconeogenesis (Viluksela et al., 1999). We found that TCDD treatment decreased Pck1 mRNA levels in $Ahr^{+/+}$ mice but not in $Ahr^{-/-}$ mice (Fig. 4), lending support to the potential AHR-dependent role of PEPCK in dioxin toxicity.

Another striking finding in our array experiments was the profoundly lower expression of mRNAs for the metallothioneins Mt1 and Mt2 in $Ahr^{+/+}$ mice than in $Ahr^{-/-}$ mice (Table 2). Metallothioneins are considered a major defense mechanism against DNA damage and apoptosis (Cherian et al., 2003). We speculate that the high constitutive levels of Mt1 and Mt2 in $Ahr^{-/-}$ mice reflect a state of oxidative stress in livers of these animals compared with their wild-type counterparts caused by an insufficiency of other protective mechanisms against oxidative stress.

Our functional analysis revealed broad-scale dysregulation of genes involved in protein synthesis. A total of 19 ribosomal transcripts had their expression moderately up-regulated by TCDD in an AHR-dependent manner, and eight are involved in ribosome biogenesis, suggesting that TCDD is stimulating the assembly of new ribosomes. Expression of 12 genes involved in amino acid catabolism is altered by TCDD in an AHR-dependent manner, and fully eight of these are down-regulated. Furthermore, we see that Eif3s6 (formerly Int-6), a critical member of the EIF3 translation initiation complex and its interacting protein, Eif3s6ip, both are up-regulated by TCDD in an AHR-dependent manner. These two interacting proteins mediate cross-talk between the translation initiation complex, the COP9 signalosome, and the 26S protea-

some. These observations suggest that TCDD initiates a broad cellular effort to increase protein translation by increasing ribosome assembly and decreasing amino acid catabolism, possibly through Eif3-mediated changes in protein degradation rates. In fact, increased plasma amino acid levels have been observed in TCDD-treated rats (Viluksela et al., 1999).

AHR-Mediated Alteration of Gene Expression: Possible Mechanisms for Up-Regulation and Down-Regulation. As shown in Tables 2, 4, and 5, the core sequence for AHRE-I is "available" in nearly all genes that exhibited up-regulation in response to AHR status or to the AHR-TCDD interaction. The "full" AHRE-I sequence is found in 12 of the 25 genes that were most highly up-regulated by the AHR-TCDD interaction but in only one of the genes that were most greatly down-regulated (Table 4).

The mere presence of an AHRE motif is not sufficient to establish that this motif is responsible for regulating the expression of a particular gene. In addition to AHRE motifs, we mapped other TFBSs and found a set of regulatory elements common to all four gene lists (Table 8) that includes 10 enriched and five depleted TFBSs. The identity of several of the enriched TFBSs strongly supports the hypothesis that these are general liver-specific promoter elements (e.g., HFH-3). An alternative mechanism has emerged by which the AHR or TCDD might down-regulate gene expression. Ray and Swanson (2004) reported that TCDD suppresses the expression of p53 and p16^{INK4a} by provoking methylation of promoters in these genes in an AHR-dependent fashion. In our study, p53 binding sites are over-represented in genes down-regulated by the TCDD in an AHR-dependent fashion (Table 9).

To sum up, our key findings are that the AHR affects the expression of large, separable gene batteries in the presence or absence of TCDD. Furthermore, virtually all effects of TCDD on gene expression require the AHR. Key dysregulated pathways include estrogen signaling, energy metabolism, and protein synthesis. Almost as many genes are responsive to AHR status per se as are responsive to the AHR-TCDD interaction, which supports the concept that the AHR plays important roles in normal development and physiology, not just in response to toxic environmental chemicals. Array studies provide menus of genes that are plausible candidates to be involved in particular biological or toxicological outcomes. The challenge now is to link AHR-mediated expression of specific genes and gene batteries to those phenotypic outcomes.

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