

# Regulation of the *Cyp2a5* Gene Involves an Aryl Hydrocarbon Receptor-Dependent Pathway

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Received October 11, 2004; accepted January 18, 2005

## ABSTRACT

We have investigated the role of the aryl hydrocarbon receptor (AHR) in the regulation of the *Cyp2a5* gene. The C57BL/6 and DBA/2 mouse strains with a genetically determined difference in AHR function were used to study the CYP2A5 induction by typical AHR ligands, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-methylcholanthrene. The CYP2A5 mRNA up-regulation in these mouse strains showed a difference in response, typical for AHR-regulated genes, both by TCDD in cultured primary hepatocytes and by 3-methylcholanthrene *in vivo*. In primary hepatocytes, TCDD caused a 3-fold elevation of the CYP2A5 protein level and a similar induction of the CYP2A5-catalyzed coumarin 7-hydroxylation activity. In reporter gene assays, the *Cyp2a5* promoter region –3033 to +10 mediated a 2- to 5-fold induction of luciferase activity by TCDD

treatment in primary hepatocytes and in Hepa-1 hepatoma cells with an intact AHR/AHR nuclear translocator (ARNT) complex. In Hepa-1 variant cell lines with deficiencies in the AHR/ARNT complex, the absence of ARNT abolished the induction. A putative AHR response element (XRE) was identified in the *Cyp2a5* promoter at the position –2514 to –2492 and found to interact with the AHR/ARNT heterodimer. Transfection experiments combined with mutation of the XRE site indicated that the site partly mediates the TCDD induction of *Cyp2a5*. An additional AHR-dependent mechanism also regulates the proximal promoter of the *Cyp2a5* gene. In conclusion, our studies showed that AHR ligands up-regulate *Cyp2a5* transcriptionally by an AHR/ARNT-dependent mechanism and established *Cyp2a5* as a novel AHR-regulated gene.

The mouse cytochrome P450 CYP2A5 and its human ortholog CYP2A6 metabolize several toxic substances, such as nitrosamines and aflatoxins (Camus et al., 1993; Pelkonen et al., 1997b). In addition to hepatocytes, CYP2A5 and CYP2A6 are expressed in some extrahepatic tissues, especially nasal mucosa (Kaipainen and Lang, 1985; Su et al., 1996; Koskela et al., 1999). The regulation of CYP2A5 is complex and significantly different from that of the other major xenobiotic-metabolizing P450 enzymes, and both transcriptional and post-transcriptional mechanisms seem to be essential (Glisovic et al., 2003b). Coumarin 7-hydroxylase (COH) activity, catalyzed predominantly by CYP2A5 and CYP2A6 (Pelkonen et al., 1997a), is inducible by a number of structurally diverse compounds, including phenobarbital, rifampicin, pyrazole,

and its derivatives, as well as porphyrinogenic substances (Donato et al., 2000). In addition, CYP2A5 is elevated in mouse liver tumors (Kobliakov et al., 1993). A hepatotoxin pyrazole induces CYP2A5 by a post-transcriptional mechanism involving binding of heterogenous nuclear ribonucleoprotein A1 (hnRNP A1) to the 3' untranslated region of CYP2A5 mRNA, with subsequent stabilization of the mRNA (Glisovic et al., 2003a). Otherwise, the mechanisms behind CYP2A5 induction are still mainly unknown.

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor involved in the regulation of several genes. The activated AHR forms a heterodimer together with the AHR nuclear translocator (ARNT). The heterodimer binds to the xenobiotic response elements (XREs) in the promoter regions of the target genes and interacts with other transcription factors of the transcription complex. The CYP1 family members *CYP1A1*, *CYP1A2*, and *CYP1B1* are well known AHR-regulated genes, and several ligands of AHR, such as polycyclic aro-

This work was financially supported by the National Technology Agency of Finland grant from the Drug 2000 program (to O.P.) and Academy of Finland grant 51610.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.  
doi:10.1124/mol.104.008078.

**ABBREVIATIONS:** P450, cytochrome P450; COH, coumarin 7-hydroxylase; hnRNP, heterogenous nuclear ribonucleoprotein; AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; XRE, xenobiotic responsive element; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Sp1, stimulating protein 1; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; ANOVA, analysis of variance; EMSA, electrophoretic mobility shift assay; AP-1, activator protein-1.

matic hydrocarbons, are substrates of these P450 enzymes (Hankinson, 1995).

It was believed previously that among the cytochrome P450s, only the members of the CYP1 family are regulated by the AHR pathway. A novel P450 form CYP2S1 was recently identified and found to be controlled by AHR (Rivera et al., 2002). Evidence also suggests that certain CYP2A enzymes can be up-regulated by ligands of AHR. For example, the rat lung CYP2A3 is up-regulated by 3-methylcholanthrene (Kimura et al., 1989), and the rat liver CYP2A1 is induced by 3-methylcholanthrene and Aroclor 1254 (Thomas et al., 1981). The CYP2A5-catalyzed COH activity was also elevated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mouse liver slices (Gokhale et al., 1997). Furthermore, the hamster CYP2A8 is up-regulated by a mechanism involving at least AHR, ARNT, and Sp1 (Kurose et al., 1999). In spite of this evidence, the possible involvement of AHR in the regulation of CYP2A genes has not been examined in detail.

In the present study, we investigated the effects of typical ligands of AHR, TCDD, and 3-methylcholanthrene on the CYP2A5 expression in liver cells. We were able to show that the mouse *Cyp2a5* gene is transcriptionally regulated by a mechanism that involves binding of a ligand-activated AHR/ARNT complex to a response element at about 2.5 kb upstream from the transcription start site. In addition, AHR mediates *Cyp2a5* induction through the proximal promoter.

## Materials and Methods

**Preparation of Primary Cultures of Hepatocytes.** Hepatocytes were isolated from male C57BL/6 (JOLA<sup>Hsd</sup>) and DBA/2 (OLA<sup>Hsd</sup>) mice (Center for Experimental Animals, University of Oulu, Oulu, Finland) aged 8 to 10 weeks. Livers were perfused with collagenase solution (Worthington Biochemicals, Lakewood, NJ) as described previously (Salonpää et al., 1994). After filtration and centrifugation, the isolated hepatocytes were dispersed in Williams' E medium (Sigma-Aldrich, St. Louis, MO) containing 20 ng/ml dexamethasone (Sigma-Aldrich), insulin/transferrin/selenate (5 mg/l insulin, 5 mg/l transferrin, 5 µg/l sodium selenate) (Sigma-Aldrich), 50 µg/ml gentamicin (Invitrogen, Paisley, Scotland), and 10% fetal bovine serum (Invitrogen) at a density of  $5 \times 10^6$  cells/100-mm dish (Falcon 3003; Falcon; BD Biosciences Discovery Labware, Bedford, MA),  $2 \times 10^6$  cells/60-mm dish (Falcon 3004),  $1 \times 10^6$  cells/one well in six-well plates (Falcon 3046), and  $3 \times 10^5$  cells/one well in 12-well plates (Falcon 3043). The cultures were maintained at 37°C in a humidified incubator for 1 to 2 h, after which nonattached cells were discarded by aspiration, and the medium was replaced by serum-free Williams' E medium. The cultures were maintained for additional 24 h before treatment with TCDD (National Cancer Institute Chemical Carcinogen Repository, Bethesda, MD) or transient transfection.

**Animals.** For in vivo studies, male DBA/2N and C57BL/6N mice as well as their F1 hybrids were obtained from IFFA-Credo (Lyon, France). Backcrosses of F1 and DBA/2N mice were generated at the International Agency for Research on Cancer. The mice were treated with 20 mg/kg 3-methylcholanthrene (Sigma-Aldrich) i.p. on 3 consecutive days. Control animals received vehicle (peanut oil) alone. The animals were killed 24 h after the last injection, and their livers were removed and immediately processed for RNA preparation. The local animal care and use committees approved the animal experiments.

**Cell Cultures.** A subclone Hepa-1c1c7 of the mouse hepatoma cell line Hepa-1 and the Hepa1c1c7 mutant strains deficient in AHR (c12) and ARNT (c4) (Hankinson, 1994) were kindly provided by Dr. Sirkku Saarikoski (Helsinki, Finland) (originally from Dr. Oliver Hankinson, UCLA, Los Angeles, CA). The cell lines were cultured in

nucleoside-free  $\alpha$ -minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). The monkey kidney cell line COS-1 (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's modified Eagle's medium with GlutaMAX (Invitrogen), containing 10% fetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were seeded to six- or 24-well plates on the day preceding the induction or transfection assays, respectively.

**RNA Preparation and Northern Blot.** Total liver RNA was prepared using the RNeasy B reagent (Tel-Test Inc., Friendswood, TX), and 20 µg was size fractionated by electrophoresis through a 1.2% agarose/formaldehyde gel and transferred on to a Gene screen Plus nylon membrane (PerkinElmer Life and Analytical Sciences, Boston, MA). RNA from cultured mouse hepatocytes (60-mm wells) and Hepa-1 cells (six-well plates) treated with TCDD or vehicle (dimethyl sulfoxide) only for 24 h was isolated by the guanidine thiocyanate-CsCl method (Chirgwin et al., 1979). Five micrograms of the total RNA was electrophoretically resolved and transferred onto Hybond-N<sup>+</sup> nylon membrane (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). The RNA was fixed by UV-crosslinking, and the membrane was hybridized with [ $\alpha$ -<sup>32</sup>P]dCTP-labeled probes. The full-length CYP2A5 cDNA was kindly provided by Dr. M. Negishi (National Institute of Environmental Health Sciences, Research Triangle Park, NC), the CYP1A2 cDNA was a gift from Dr. P. Honkakoski (University of Kuopio, Kuopio, Finland), the CYP1A1 cDNA probe was prepared as described previously (Hakkola et al., 1996), and the 18S probe was provided by Dr. H. Ruskoaho (University of Oulu).

**Cycloheximide Experiments.** DBA/2 and C57BL/6 hepatocytes were treated with 10 µg/ml cycloheximide (Merck, Darmstadt, Germany) for 1 h before the administration of the inducer to block protein synthesis. The cells were then treated with 1 µM TCDD or vehicle (dimethyl sulfoxide) only for 24 h, after which the CYP2A5 mRNA levels were measured and compared with induced cells not treated with cycloheximide.

**Coumarin 7-Hydroxylase Assay.** DBA/2 hepatocytes on 100-mm dishes were treated with 1 µM TCDD or vehicle (dimethyl sulfoxide) only for 48 h, after which the cells were scraped, washed, suspended in 200 µl of phosphate-buffered saline (PBS), and sonicated. The sonicated suspensions were centrifuged at 13,000g for 10 min. Pellets were discarded, and COH activity was measured from the supernatants as described previously (Aitio, 1978) using 100 µM coumarin (Sigma-Aldrich) as a substrate.

**Western Blotting.** Twenty micrograms of the 13,000g supernatant proteins was subjected to SDS-polyacrylamide gel (10% polyacrylamide) electrophoresis. The proteins were transferred on to a Hybond ECL nitrocellulose membrane (Amersham Biosciences UK, Ltd.). The membrane was then incubated with chicken polyclonal antibody raised against mouse CYP2A5 (kindly provided by Dr. Risto Juvonen, University of Kuopio) (1:5000 dilution) and secondary horseradish peroxidase-rabbit anti-chicken/turkey IgG (Zymed Laboratories, South San Francisco, CA) (1:20,000 dilution). After washing, the immunoreactive bands were visualized with ECL Plus Western blotting detection system (Amersham Biosciences UK, Ltd.).

**Plasmids and Transient Transfection Assays.** The *Cyp2a5* 5' -3033 to +10 (from the transcription start site) fragment of the *Cyp2a5* 5'-flanking region was amplified with PCR from DBA/2 mouse genomic DNA using Dynazyme EXT polymerase (Finnzymes, Helsinki, Finland). The fragment was then cloned into the pGL3-Basic vector (Promega, Madison, WI) in front of the luciferase reporter gene. In addition, several shorter 5' deletion constructs were prepared by PCR, using the *Cyp2a5* 5' -3033 to +10-Luc plasmid as a template and subcloning the PCR products. The MatInspector professional program using Genomatix matrixes (<http://www.genomatix.de>) was used to search for AHR/ARNT heterodimer binding sites at the -3033 to +10 promoter region of the *Cyp2a5* gene. A potential xenobiotic response element (XRE) was identified at the *Cyp2a5* 5' -2514 to -2492 region. The *Cyp2a5* 5'-XRE-rAlbTATA-

Luc plasmid was prepared by cloning this region (*Cyp2a5* -2513 GCTCACTCACGCACTCTGG -2495) in front of the rat albumin proximal promoter region -40 to +28 (Hakkola et al., 2003). The expected structures of the constructs were verified by sequencing. The expression plasmids pcDNA3-AHR (Fukunaga and Hankinson, 1996) and pcDNA/Neo-ARNT (Reisz-Porszasz et al., 1994) expressing mouse AHR and ARNT, respectively, were provided by Dr. Oliver Hankinson (UCLA).

The reporter gene constructs were transfected into mouse hepatocytes (12-well plates), Hepa-1 cells (24-well plates), or COS-1 cells (24-well plates) together with *Renilla* luciferase reporter vector (pRL3-TK) (Promega), which was used as an internal control. Then, 0.5  $\mu$ g of *Cyp2a5* 5'-Luc and 0.1  $\mu$ g of pRL3-TK were transfected per  $3 \times 10^5$  hepatocytes, and 0.65  $\mu$ g of *Cyp2a5* 5'-Luc or *Cyp1a1* 5'-Luc and 0.05  $\mu$ g of pRL3-TK were transfected per one well of Hepa-1 or COS-1 cells using Tfx-20 reagent (Promega) according to the manufacturer's protocol in Opti-MEM I medium (Invitrogen). In the co-transfection assays of COS-1 cells, 0.05  $\mu$ g of expression vector DNA was transfected per well. Twenty-four hours after the transfection, the cells were treated with 1  $\mu$ M TCDD or vehicle dimethyl sulfoxide only. The cell extracts were assayed for luciferase activity with dual-luciferase reporter assay system (Promega).

**Preparation of Nuclear Extracts.** COS-1 and Hepa-1c1c7 cells were cultured on 100-mm dishes. COS-1 cells were transfected with 12  $\mu$ g of pcDNA3-AHR, pcDNA/Neo-ARNT, or empty pcDNA3.1 expression vector (Invitrogen) per dish, using Tfx-20 reagent in Opti-MEM I medium and cultured 24 h before induction. Both COS-1 and Hepa-1 cells were treated with 1  $\mu$ M TCDD or vehicle dimethyl sulfoxide only for 24 h. Nuclear extracts were prepared according to Sreiber et al. (1989). In brief, the cells were first washed with 10 ml of PBS and then scraped to another 10 ml of PBS and pelleted by centrifugation at 110g for 5 min, suspended in 1 ml of PBS, and centrifuged at 660g for 15 s. The cell pellet was resuspended in ice-cold hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and Complete Mini protease inhibitor cocktail; Roche Diagnostics, Mannheim, Germany), and the cells were allowed to swell on ice for 15 min. The cell membranes were lysed by adding 10% solution of Tergitol (type Nonidet P-40) (Sigma-Aldrich) to a final percentage of 0.6%. The homogenate was centrifuged at 660g for 30 s, and the nuclear pellet was resuspended in ice-cold buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and Complete Mini protease inhibitor cocktail; Roche Diagnostics). The nuclear proteins were extracted by incubation at 4°C for 15 min on a shaking platform. The samples were centrifuged at 15,000g for 5 min, and the supernatant fractions containing the nuclear proteins were collected. The protein content of the nuclear extract was determined using the Bradford protein analysis method (Bradford, 1976).

**Electrophoretic Mobility Shift Assay.** Double-stranded DNA probes were prepared by annealing the desired sense and antisense oligonucleotides (*Cyp2a5* 5'-XRE; -2523 5'-CAAAGCCCCTGCTCACTCAGCACTCTGGAAGCCTGC3' -2487, consensus XRE; 5'-GAGCTCGGAGTTGCGTGAGAAGAGCC3'; Denison et al., 1988). Single-stranded oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX). Double-stranded oligonucleotides were 5' end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP and T<sub>4</sub> polynucleotide kinase and then purified using the QIAquick nucleotide removal kit (QIAGEN, Venlo, The Netherlands). Ten micrograms of COS-1 nuclear extract, binding buffer (25 mM HEPES, pH 7.9, 10% glycerol, 50 mM KCl, 0.5 mM EDTA, and Complete Mini protease inhibitor cocktail; Roche Diagnostics), 1  $\mu$ g/ $\mu$ l single-stranded DNA, and 0.04 pmol (30,000 cpm) of labeled oligonucleotide probe were incubated at room temperature (22°C) for 30 min in a final volume of 15  $\mu$ l. For competition experiments, unlabeled competitor oligonucleotides (5–100-fold excess) were added to the mixtures. The sequence of the mutated *Cyp2a5* 5'-XRE oligonucleotide was the same as the one used in the site-directed mutagenesis. For supershift experiments, 2  $\mu$ l of goat anti-AHR polyclonal antibody (ab2100; Abcam Limited, Cambridge, UK)

was added to the nuclear extracts and preincubated on ice for 20 min. The samples were separated by electrophoresis through 6% polyacrylamide gel, and the retarded complexes were detected by autoradiography.

**Site-Directed Mutagenesis.** Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The mutations were introduced into *Cyp2a5* 5' -3033 to +10-Luc plasmid using mutated oligonucleotides -2523 CAAAGCCCCTGCTCACT**ATAG**CACTCTGGAAGCCTGC -2487. The core sequence according to the Genomatix matrix is underlined, and the mutated nucleotides are shown in bold. Mutations were chosen based on the results from Shen and Whitlock (1992). Correct assembly of the mutations was confirmed by sequencing.

**Statistical Analysis.** Student's *t* test was used for comparisons between two groups. Comparisons of several groups were done with one-way ANOVA followed by the least significant difference post hoc test. Differences were considered significant when *p* < 0.05.

## Results

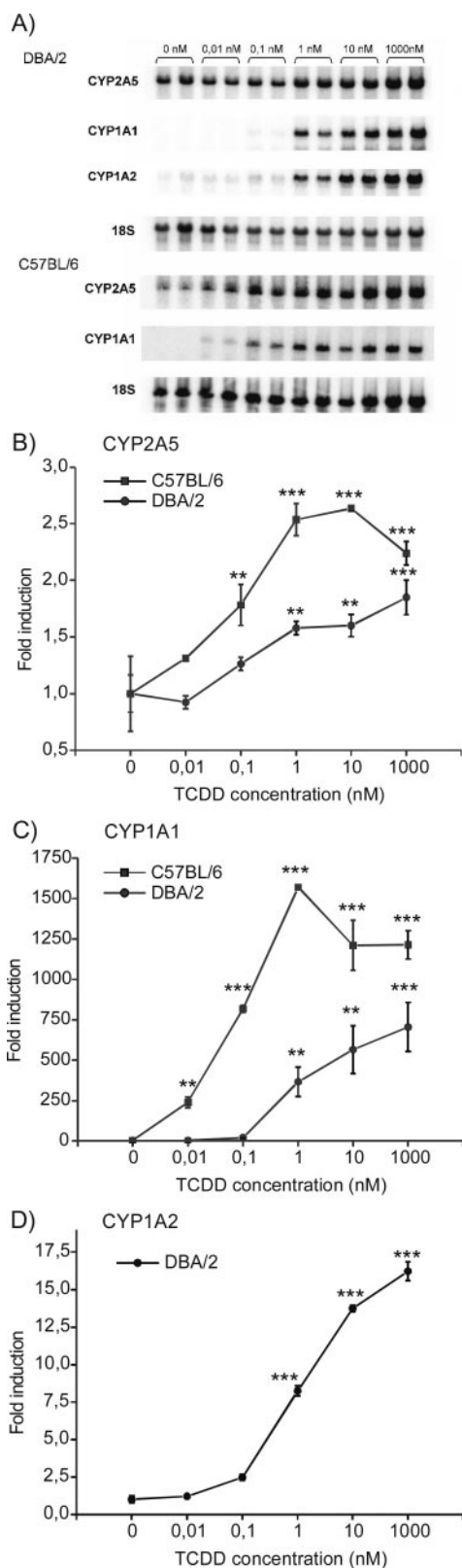
**Induction of CYP2A5 by TCDD.** Primary hepatocyte cultures derived from livers of DBA/2 and C57BL/6 mice were used to investigate the inducibility of the *Cyp2a5* gene by TCDD. These mice show a well characterized, genetically determined differential response to AHR ligands; the DBA/2 mouse AHR having about 10-fold lower affinity to TCDD than that of the C57BL/6 mouse (Chang et al., 1993; Ema et al., 1994). The *Cyp2a5* gene was found to be induced dose dependently by TCDD in both mouse strains, as determined by the mRNA levels (Fig. 1). However, maximum induction was reached with 1  $\mu$ M TCDD in DBA/2 hepatocytes compared with only 10 nM TCDD in C57BL/6 hepatocytes. This induction pattern was found to correlate with the CYP1A1 and CYP1A2 induction.

Cycloheximide chase was used to study whether new protein synthesis is needed for the induction of *Cyp2a5* by TCDD. DBA/2 and C57BL/6 hepatocytes were treated with 10  $\mu$ g/ml cycloheximide for 1 h before the 24-h TCDD treatment. TCDD induced CYP2A5 mRNA regardless of cycloheximide treatment, indicating that de novo protein synthesis is not needed for up-regulation (data not shown).

**TCDD Increases CYP2A5 Protein Levels and COH Activity.** The effect of TCDD on CYP2A5 protein expression was studied next. DBA/2 hepatocytes express a higher level of CYP2A5 than the C57BL/6 strain and were therefore used (Lush and Andrews, 1978). Primary hepatocytes were treated with 1  $\mu$ M TCDD for 24 h, and the 13,000g supernatant fraction was prepared. The CYP2A5 apoprotein levels were determined by immunoblotting. TCDD increased the CYP2A5 protein concentration 2.4-fold, as judged from the intensity of the immunorecognizable bands (Fig. 2, A and B). COH activity also increased 2.9-fold (Fig. 2C).

**In Vivo Induction of CYP2A5 mRNA.** The induction of CYP2A5 in vivo was studied using another well known AHR ligand, 3-methylcholanthrene. 3-Methylcholanthrene is a weaker ligand than TCDD and induces the expression of CYP1A1 and the CYP1A2 in C57BL/6 but not in DBA/2 mice (Ema et al., 1994). The mice were treated with 3-methylcholanthrene or vehicle alone for 72 h, and the liver mRNA levels were determined by RNA blotting. Both CYP1A2 and CYP2A5 mRNA levels were readily induced by 3-methylcholanthrene in the C57BL/6 strain. In contrast, in the DBA/2 strain, which has low ligand-binding affinity of AHR, the





**Fig. 1.** Dose-response effect of TCDD on CYP2A5, CYP1A1, and CYP1A2 mRNA expression in DBA/2 and C57BL/6 mice hepatocytes. Hepatocytes were treated with increasing amounts of TCDD or vehicle (dimethyl sulfoxide) only for 24 h. A, 5  $\mu$ g of total RNA was electrophoresed, blotted, and hybridized with mouse CYP2A5, human CYP1A1, mouse CYP1A2, and rat 18S probes. B to D, densitometric quantification of CYP2A5, CYP1A1, and CYP1A2 mRNA blots. The values are normalized against 18S control levels, and the means  $\pm$  range of two normalized samples are

CYP1A2 and the CYP2A5 mRNAs were poorly induced. In the F1 hybrid mouse, too, the CYP1A2 and the CYP2A5 mRNAs were strongly induced similarly to the C57BL/6 strain (Fig. 3A).

**Genetic Studies on Coinduction of CYP1A2 and CYP2A5 in DBA/2N and C57BL/6N Mice and Their Offspring Exposed to 3-Methylcholanthrene.** To further examine the genetics of CYP2A5 inducibility by 3-methylcholanthrene, we generated backcrosses of F1 and DBA/2 mice and treated them with 3-methylcholanthrene. Both CYP2A5 and CYP1A2 displayed gender differences in expression, and the results were therefore analyzed separately for each gender. Two male mice (of a total of four) had a clearly responsive phenotype (amounts CYP1A2 mRNA comparable with those of 3-methylcholanthrene-treated male C57BL/6 mice), whereas the other two had low levels of CYP1A2 mRNA after 3-methylcholanthrene treatment (nonresponsive phenotype, comparable with 3-methylcholanthrene-treated male DBA/2 mice) (Fig. 3B.). In female mice, two of three were found to be responsive. The induction of CYP2A5 correlated with CYP1A2 induction in both genders, indicating genetically determined coregulation of the *Cyp1a2* and *Cyp2a5* genes by 3-methylcholanthrene.

**TCDD Induces *Cyp2a5* by a Transcriptional Mechanism.** The involvement of transcriptional regulation in the induction of the *Cyp2a5* gene by TCDD was studied next. The *Cyp2a5* 5'-flanking region -3033 to +10 and a series of 5'-deleted, shorter fragments from the same region were cloned in front of a luciferase reporter gene, and the resulting constructs were transfected into mouse primary hepatocytes and COS-1 cells. In DBA/2 hepatocytes, TCDD increased luciferase activity 2.4- to 5.9-fold, depending on the construct (Fig. 4). A similar induction profile was seen when transfecting constructs into C57BL/6 hepatocytes (data not shown). The highest relative luciferase activity was obtained with *Cyp2a5* 5' -3033 to +10-Luc. In COS-1 cells not expressing AHR or ARNT, no induction by TCDD could be demonstrated. However, cotransfection of the AHR and ARNT expression vectors to these cells increased the luciferase activity of the *Cyp2a5* 5' -3033 to +10-Luc construct 4.7-fold. None of the shorter, 5'-deleted *Cyp2a5* promoter constructs were affected by AHR/ARNT cotransfection (data not shown). These results with COS-1 cells suggest transcriptional regulation of the *Cyp2a5* gene by the AHR/ARNT complex with the critical regulatory site between -3033 and -2013 base pairs at the CYP2A5 promoter. However, in liver cells also the proximal promoter was found to be responsive.

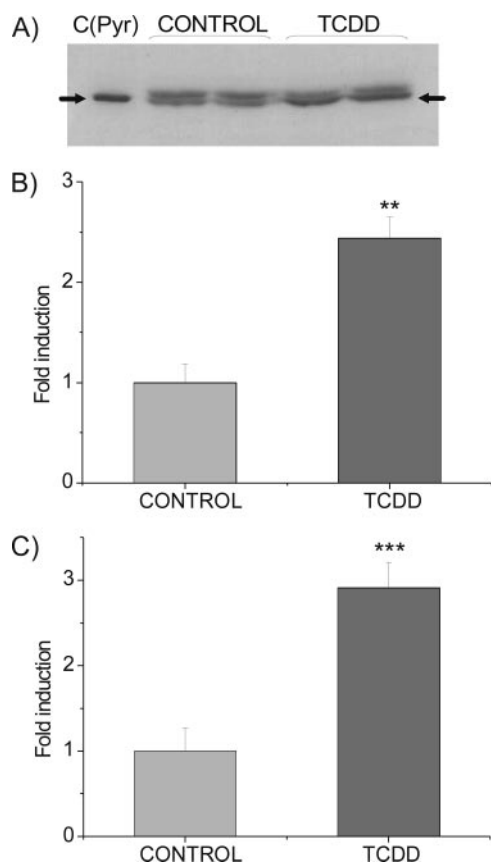
**Induction of *Cyp2a5* by TCDD Requires AHR Complex.** An AHR-mediated mechanism is known to be responsible for the induction of most TCDD-regulated genes. To further assess the contribution of AHR to the induction of the *Cyp2a5* gene, the mouse hepatoma cell line Hepa-1c1c7 (wild type) and its mutant strains deficient in AHR (c12) and ARNT (c4) were transiently transfected by *Cyp2a5* 5' -3033 to +10-Luc plasmid and treated with 1  $\mu$ M TCDD or vehicle only. TCDD induced luciferase expression 2.2-fold in wild-type Hepa-1 cells, but only 1.4-fold in AHR-deficient cells. No

compared with untreated cells (0 nM TCDD). Difference to untreated cells \*\*\*,  $p < 0.001$  and \*\*,  $p < 0.01$  (one-way ANOVA followed by least significant difference post hoc test). The experiments with both strains were repeated, and these independent experiments gave similar results.

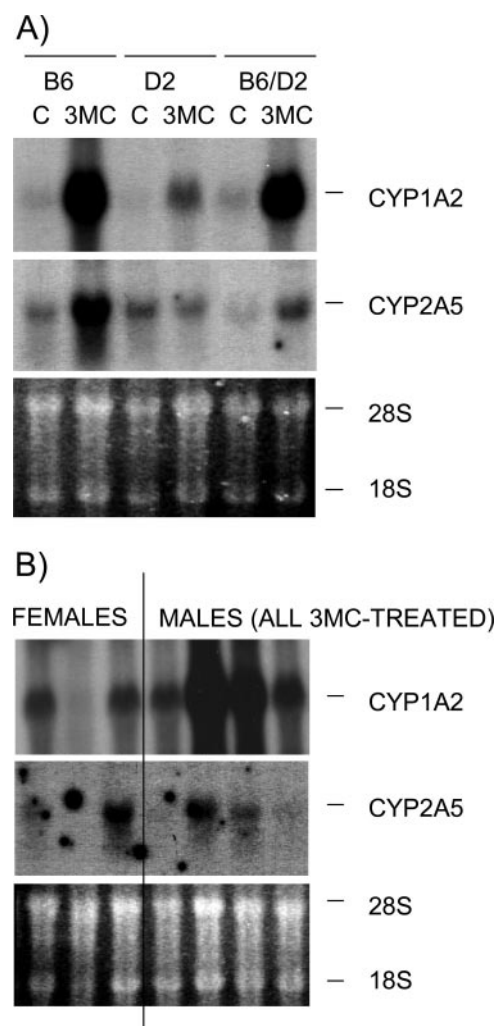
induction was seen in ARNT-deficient cells (Fig. 5). This indicates that the AHR-ARNT complex is necessary for *Cyp2a5* induction by TCDD. Similar results were obtained when transfecting shorter *Cyp2a5* 5' promoter plasmids into Hepa-1 cells (data not shown), indicating that both in primary hepatocytes and in Hepa-1 cells, the proximal promoter region also contributed to the *Cyp2a5* TCDD response. As a control, the CYP1A1 mRNA induction by 1  $\mu$ M TCDD was measured in wild-type and mutant Hepa-1c1c7 cell lines. CYP1A1 mRNA was induced 66 times in wild-type cells and 5.9 times in AHR-deficient cells; no induction was detected in ARNT-deficient cells (data not shown).

**Identification and Characterization of a Putative AHR Binding Element.** One potential XRE element was identified in the *Cyp2a5* 5'-flanking region at position -2514 to -2492 by a computer search with the MatInspector professional program (<http://www.genomatix.de>). Binding of the AHR complex to this putative XRE sequence was elucidated

by using electrophoretic mobility shift assay (EMSA). A double-stranded, end-labeled *Cyp2a5* 5'-XRE oligonucleotide was incubated with the nuclear extract from COS-1 cells transfected with the expression vectors for AHR or/and ARNT and treated with TCDD or vehicle (dimethyl sulfoxide) only. The resulting DNA-protein complexes were separated by gel electrophoresis. A retarded complex was only detected when both AHR and ARNT were present in the nuclear extract. A weak complex was seen even without TCDD treatment. This may be the result of overexpression of AHR and ARNT and/or inefficiency of the mechanisms retaining unliganded AHR in the cytosol. Complex intensity was enhanced by TCDD treatment. The AHR/ARNT-*Cyp2a5* 5'-XRE complex had the same mobility as that formed by AHR/ARNT with consensus XRE. One hundred-fold excess of unlabeled *Cyp2a5* 5'-XRE or consensus XRE could completely compete

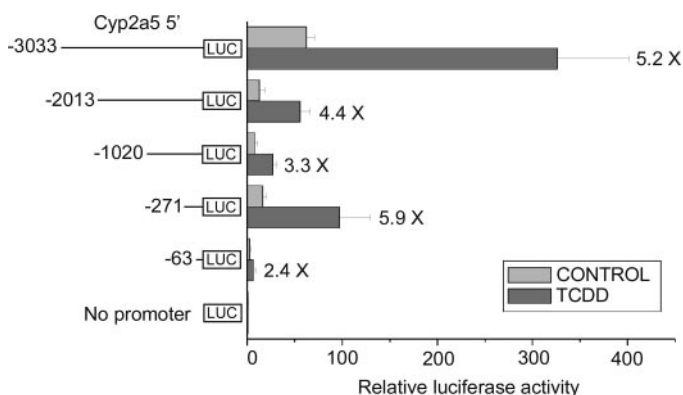


**Fig. 2.** Effect of TCDD treatment on CYP2A5 protein expression and COH activity in DBA/2 mouse hepatocytes. Mouse hepatocytes were treated with 1  $\mu$ M TCDD or vehicle only (control; dimethyl sulfoxide) for 48 h. A, Western blot analysis of 13,000g supernatants (20  $\mu$ g of total protein/lane) from control and TCDD-induced hepatocytes stained with chicken anti-CYP2A5 antibody. In vivo pyrazole-induced mouse liver microsomes were used as control (C Pyr). The lower band represents CYP2A5 protein. The anti-CYP2A5 antibody cross-reacted also with another unidentified protein. This protein is probably nonmicrosomal of origin, because it was not detected in the microsomal sample used as a control. B, densitometric quantification of Western blot. The values represent means  $\pm$  range of two samples and are normalized against the control level. The experiment was repeated, and both independent experiments gave similar results. C, COH activity of control and TCDD-induced hepatocytes. The values represent means  $\pm$  S.D. of four samples in two independent experiments. Difference to untreated cells \*\*\*,  $p < 0.001$  and \*\*,  $p < 0.01$  (Student's  $t$  test).

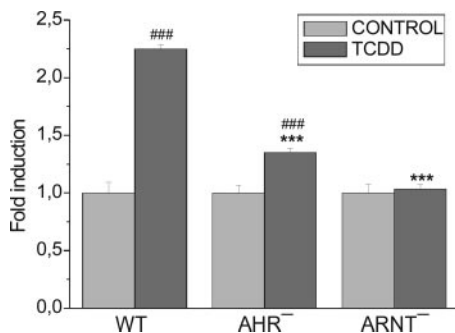


**Fig. 3.** Induction of CYP1A2 and CYP2A5 by 3-methylcholanthrene in mouse liver in vivo. A, total RNA was prepared from the livers of untreated or 3-methylcholanthrene (3MC)-treated C57BL/6, DBA/2, and F1 (B6/D2) mice and subjected to electrophoresis and RNA blotting using CYP1A2 and CYP2A5 cDNAs. The ethidium bromide-stained gel is shown for RNA loading assessment. B, induction of CYP1A2 and CYP2A5 in backcrosses. Total RNA was prepared from the livers of 3-methylcholanthrene-treated backcrosses (DBA/2  $\times$  F1) and subjected to electrophoresis and RNA blotting using CYP1A2 and CYP2A5 cDNAs. The CYP1A2 autoradiography was overexposed to detect the low levels of CYP1A2 in certain individual samples. The ethidium bromide-stained gel is shown for RNA loading assessment.

away the retarded AHR/ARNT-*Cyp2a5* 5'-XRE complex. In contrast, the AHR/ARNT-consensus XRE complex was competed slightly less efficiently by the 100-fold excess of unlabeled *Cyp2a5* 5'-XRE compared with the unlabeled consensus XRE (Fig. 6A). The relative affinity of AHR complex to the *Cyp2a5* 5'-XRE was further assessed by competition with several concentrations of unlabeled oligonucleotides. Five-fold excess of unlabeled consensus XRE oligonucleotide was able to completely compete away the retarded AHR/ARNT-*Cyp2a5* 5'-XRE complex, whereas the unlabeled *Cyp2a5* 5'-XRE oligonucleotide itself competed less efficiently. Mutated unlabeled *Cyp2a5* 5'-XRE poorly competed with nonmutated oligonucleotide (Fig. 6B). These competition experiments suggest that the AHR complex affinity to the *Cyp2a5* promoter XRE is lower than that to the consensus site. Involvement of AHR in the formation of the detected DNA/protein complex was further verified by using anti-AHR antibody,



**Fig. 4.** Effect of TCDD treatment on transcriptional activity of *Cyp2a5* 5'-luciferase constructs transfected into DBA/2 mouse hepatocytes. After transfection, the cells were treated with 1  $\mu$ M TCDD or vehicle (dimethyl sulfoxide) only for 24 h, after which luciferase activities were measured. The activities produced by the promoter constructs were normalized against cotransfected control plasmid (pRL-TK) activities. The values represent means  $\pm$  S.D. of four individual samples. Fold induction is shown for each reporter construct. The difference to TCDD-induced cells was statistically significant,  $p < 0.05$  (Student's  $t$  test), for all constructs except the control vector with no promoter. The experiment was repeated, and both independent experiments gave similar results.



**Fig. 5.** Effect of TCDD treatment on transcriptional activity of *Cyp2a5* 5'-3033 to +10-luciferase construct transfected into Hepa1c1c7 (wt), Hepa-1c1c12 (AHR<sup>-</sup>), and Hepa-1c1c4 (ARNT<sup>-</sup>) cell lines. After transfection, the cells were treated with 1  $\mu$ M TCDD or vehicle (dimethyl sulfoxide) only for 24 h, after which luciferase activities were measured. The activities were normalized against cotransfected control plasmid (pRL-TK) activities. The values represent the means  $\pm$  S.D. of four individual samples. ###, difference to untreated cells was statistically significant  $p < 0.001$  (Student's  $t$  test). \*\*\*, difference to TCDD-induced wild-type (wt) cells was statistically significant  $p < 0.001$  (one-way ANOVA followed by least significant difference post hoc test). The experiment was repeated, and both independent experiments gave similar results.

which was able to supershift the detected complexes. A similar-sized complex, supershifted by anti-AHR antibody, was also seen when using TCDD-treated Hepa-1c1c7 cell nuclear extracts (Fig. 6C). Together, these results suggest that the AHR/ARNT complex is able to bind to the identified XRE site found in the *Cyp2a5* promoter.

**AHR Complex Induces *Cyp2a5* Transcription through the XRE Site.** To investigate the functional significance of the AHR/ARNT heterodimer binding to the *Cyp2a5* promoter, the *Cyp2a5* 5' XRE site at -2514 to -2492 was cloned in front of the rat albumin TATA box in the luciferase reporter vector. The *Cyp2a5* 5' XRE rAlbTATA construct was then transfected into COS-1 cells, and the AHR and ARNT expression vectors were cotransfected. When both AHR and ARNT were cotransfected and the cells were treated with TCDD, the luciferase expression of the *Cyp2a5* 5'-XRE reporter plasmid increased 3.6-fold (Fig. 7).

The nucleotides known to be important for AHR/ARNT binding to XRE were mutated in the *Cyp2a5* 5' -3033 to +10-Luc construct with site-directed mutagenesis. The original and mutated constructs were transfected into DBA/2 mouse primary hepatocytes, and luciferase activities were measured. The mutation of the XRE site significantly reduced the induction by TCDD from 3.1- to 2.2-fold (Fig. 8), indicating that the XRE site is functional and that the AHR/ARNT complex regulates *Cyp2a5* gene expression partially by interacting with this site.

## Discussion

We demonstrate here that the AHR/ARNT complex regulates the expression of the mouse *Cyp2a5* gene. The mechanism of this regulation involves direct interaction of the activated AHR/ARNT complex with a single XRE element situated about 2.5 kilobases upstream from the transcription start site at the *Cyp2a5* promoter. An additional regulatory element, also involved in the AHR/ARNT-dependent CYP2A5 regulation, is situated at the proximal promoter region less than 300 base pairs from the transcription start site. However, this element does not seem to be an XRE, and our results do not support any direct interaction of AHR/ARNT with the DNA at this site.

TCDD and 3-methylcholanthrene, two well characterized ligands for AHR, were found to induce CYP2A5 in cultured cells and in vivo. The induction was detected at transcriptional, mRNA, protein, and catalytic activity levels. The experiments with the protein synthesis inhibitor cycloheximide indicate that protein synthesis is not necessary for the induction.

Several lines of evidence support the involvement of AHR in the *Cyp2a5* induction. First, the observed dependence of *Cyp2a5* induction of the AHR genotype in high-response C57BL/6 and low-response DBA/2 mouse strains strongly suggests that an intact AHR is necessary for CYP2A5 induction by TCDD or 3-methylcholanthrene. In cultured primary hepatocytes from the C57BL/6 and DBA/2 mouse strains, CYP2A5 mRNA induction by TCDD displayed a dose-response difference typical for AHR-regulated genes in these strains (Chang et al., 1993; Ema et al., 1994), and CYP2A5 was dose dependently coinduced with CYP1A1 and CYP1A2, although the level of CYP2A5 induction was lower. Furthermore, the CYP2A5 induction by 3-methylcholanthrene in

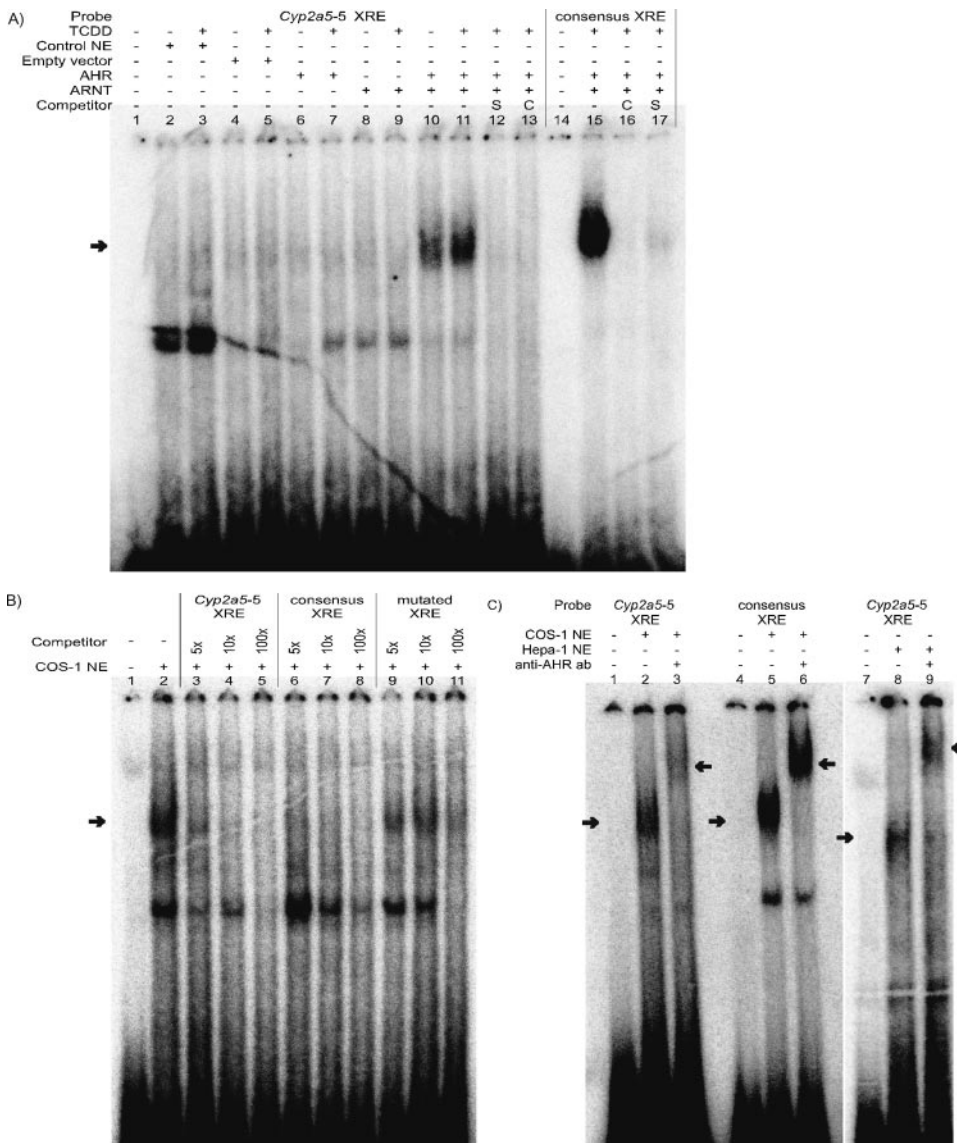


vivo followed the CYP1A2 induction AHR genotype-dependently. Second, we demonstrated in AHR and ARNT-deficient Hepa-1 cells that CYP2A5 induction requires an intact AHR complex. The induction is completely missing in the absence of ARNT and decreased in AHR-deficient cells. The probable reason for some preservation of induction in AHR-deficient cells is the low but existing levels of AHR in the cell line (Zhang et al., 1996). The low but existing level of CYP1A1 mRNA induction by TCDD in AHR-deficient cells also supports this assumption. Finally, in COS-1 cells, the *Cyp2a5* promoter was induced only after AHR and ARNT cotransfection.

The results of the present study indicated that AHR ligands regulate *Cyp2a5* predominantly through transcriptional activation. AHR regulates its target genes through a number of mechanisms. Unliganded AHR exists in a cytoplasmic complex composed of AHR, a dimer of hsp90, the immunophilin-like protein ARA9/XAP2/AIP, and the cochaperone p23 (Lees and Whitelaw, 2002). The binding of a ligand releases AHR from the complex and triggers the translocation of AHR from cytosol into the nucleus, where it forms a heterodimer with ARNT. Together, AHR and ARNT, both

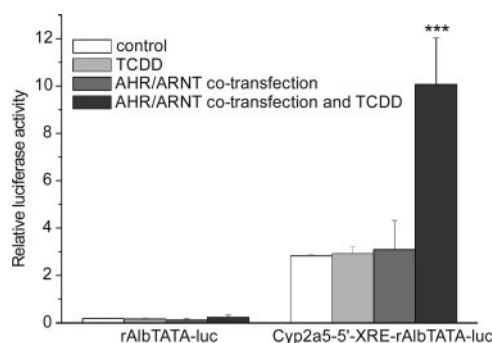
comprising the basic helix-loop-helix/Per-ARNT-Sim domains, bind to the response elements in DNA called XREs and trigger the transcription of the target genes (Hankinson, 1995). However, several alternative mechanisms involving protein-protein interactions have been described for AHR-mediated gene regulation. Ohtake et al. (2003) showed that the ligand-activated AHR-ARNT heterodimer could directly associate with estrogen receptors and activate transcription via estrogen-responsive gene promoters. AHR may also interact with retinoblastoma protein and nuclear factor- $\kappa$ B (Tian et al., 1999; Puga et al., 2000). Moreover, TCDD activates the activator protein-1 (AP-1) factor by both AHR-dependent and -independent pathways (Hoffer et al., 1996).

Sequence analysis of the 5' regulatory region of the *Cyp2a5* gene for putative AHR binding sites revealed a single XRE at the position -2514 to -2492. In EMSAs, this XRE site was able to interact with the AHR/ARNT heterodimer. The cotransfection and mutagenesis studies showed that the XRE is functional and able to mediate the TCDD induction of *Cyp2a5*. Yet, TCDD also induced the proximal promoter constructs without the XRE site. The experiments in AHR and particularly ARNT-deficient Hepa-1 cell lines suggest that

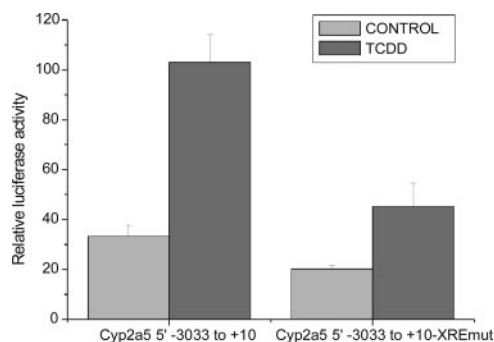


**Fig. 6.** Interaction of AHR and ARNT with *Cyp2a5* 5' XRE (-2523 to -2487) sequence or consensus XRE sequence in EMSA. A, retarded protein/DNA complex of interest is indicated with an arrow. The first lane represents the binding reaction with no protein, and the following lanes (2–11) indicate the reactions with nuclear extracts from differently treated COS-1 cells (no transfection, AHR, ARNT, or empty expression vector transfection with TCDD or dimethyl sulfoxide treatment) as indicated. Finally, the reactions with nuclear extracts from AHR- and ARNT-transfected and TCDD-treated COS-1 cells were competed with 100-fold excess of unlabeled *Cyp2a5* 5' XRE (S) and consensus XRE (C) oligonucleotides as indicated (lanes 12 and 13). The last four lanes (14–17) represent positive control reactions with labeled XRE consensus sequence without protein or incubated with nuclear extracts from AHR- and ARNT-transfected and TCDD-treated COS-1 cells and competitions as indicated. B, Competition reactions with *Cyp2a5* 5' XRE, consensus XRE, and mutated *Cyp2a5* 5' XRE. Five-, 10-, or 100-fold excess of unlabeled oligonucleotides were used to compete with labeled *Cyp2a5* 5' XRE for the binding of proteins in nuclear extracts from AHR- and ARNT-transfected and TCDD-treated COS-1 cells. C, supershift analysis of *Cyp2a5* 5' XRE and consensus XRE sequence binding proteins with anti-AHR antibody. The retarded protein/DNA complex formed in the control reaction is indicated with an arrow on the left side of the figure. The anti-AHR antibody-supershifted complex is indicated with an arrow on the right side of the figure. Lanes 7 to 9 are from different gel and represent interaction of nuclear extract proteins from TCDD-treated Hepa-1 cells with the *Cyp2a5* 5' XRE and supershift analysis with anti-AHR-antibody.

the response of the proximal promoter is also dependent on the AHR complex. There are no apparent XRE sites in the *Cyp2a5* proximal promoter, and protein-protein interactions are therefore likely to be involved. The *Cyp2a5* promoter sequence contains binding sites for numerous other transcription factors, and at least the nuclear factor I, the hepatocyte nuclear factor 4, and the D-site binding protein are known to take part in the constitutive regulation of *Cyp2a5* (Lavery et al., 1999; Ulvila et al., 2004). At the moment, none of these transcription factors are known to interact with AHR or ARNT. We recently identified hnRNPA1 as a regulator of the *Cyp2a5* gene. Our evidence suggests that this protein may act both transcriptionally and post-transcriptionally. It is worth noticing that one of the putative binding sites of hnRNPA1 at the *Cyp2a5* promoter lies in the proximal promoter region participating in AHR/ARNT-mediated regulation (Glisovic et al., 2003b). Because both toxic xenobiotics and oxidative stress activate hnRNPA1, it will be interesting



**Fig. 7.** Effect of AHR and ARNT cotransfection on the function of the *Cyp2a5* 5' XRE site in COS-1 cells. The expression vectors of AHR and ARNT were cotransfected with the *Cyp2a5* 5'-XRE-rAlbTATA-Luc reporter plasmid or the reporter plasmid without XRE into COS-1 cells. After 24 h, the cells were treated with TCDD or dimethyl sulfoxide only and incubated for 24 h, after which the cells were harvested and the luciferase activities measured. The activities produced by the studied constructs were normalized against the cotransfected control plasmid (pRL-TK) activities. The values represent means  $\pm$  S.D. of four individual samples. \*\*\*, difference to the control without cotransfection and without TCDD treatment is statistically significant  $p < 0.001$  (one-way ANOVA followed by least significant difference post hoc test). The experiment was repeated twice with similar results.



**Fig. 8.** Significance of the promoter XRE site on *Cyp2a5* induction by TCDD. The *Cyp2a5* 5' -3033 to +10-Luc with a mutated XRE site (core sequence CACGC  $\rightarrow$  ATAGC) was prepared and transfected into DBA/2 mouse primary hepatocytes. The TCDD induction of the mutated construct was compared with that of the nonmutated construct. The activities produced by the studied constructs were normalized against the cotransfected control plasmid (pRL-TK) activities. The values represent the means  $\pm$  S.D. of four individual samples. The XRE mutation reduced TCDD induction statistically significantly,  $p < 0.05$  (Student's *t* test). The experiment was repeated twice with similar results.

to find out whether interaction exists between it and the AHR in the regulation of *Cyp2a5*. Some potential AP-1 and stimulating protein 1 (Sp1) binding sites are found upstream of the *Cyp2a5* transcription start site. It has been shown that the transcription factors AP-1 and Sp1 are involved in the 3-methylcholanthrene-induced CYP2A8 expression in hamster hepatocytes (Kurose et al., 1999). However, the induction through the *Cyp2a5* proximal promoter seems to be hepatocyte-specific, because it was detected in primary hepatocytes and in the hepatoma cell line Hepa-1, but not in COS-1 cells. Therefore, hepatocyte-enriched factors in addition to ubiquitous transcription factors are likely to be necessary.

The extent of *Cyp2a5* induction by AHR is modest compared with *Cyp1* family induction and especially with that of *Cyp1a1*. *Cyp1a1* contains several copies of XREs in its 5' regulatory region, whereas only one site was identified in the *Cyp2a5* promoter, which may explain the less robust induction of *Cyp2a5*. Nevertheless, significant amounts of CYP2A5 protein and corresponding human CYP2A6 are expressed in the liver and in some extrahepatic organs, and their induction may have important consequences for the elimination of xenobiotics and the activation of carcinogens. Mouse CYP2A5 and human CYP2A6 have several similarities in their regulation and function (Donato et al., 2000). CYP2A6 is the major enzyme responsible for the elimination of nicotine, and it also activates tobacco-derived carcinogens, such as nitrosamines (Camus et al., 1993; Nakajima et al., 1996). It will be of great importance to investigate whether the human CYP2A6 enzyme similar to the mouse CYP2A5 is induced by the AHR ligands present in tobacco smoke.

In conclusion, we have shown that TCDD and 3-methylcholanthrene induce *Cyp2a5* by an AHR-dependent mechanism involving a distal XRE site and hepatocyte-specific regulation of the proximal promoter. The *Cyp2a5* thus joins, as a novel member, the group of AHR-regulated genes. The current study, in line with other investigations (Kurose et al., 1999; Rivera et al., 2002), indicates that, in addition to the CYP1 family, several members the CYP2 family are controlled by AHR.

#### Acknowledgments

The skillful technical assistance of Päivi Tyni and Ritva Tauriainen is gratefully acknowledged. We thank Dr. Sirkku Saarikoski (National Public Health Institute, Department of Mental Health and Alcohol Research, Helsinki, Finland) for valuable help with Hepa-1 cells and CYP2S1 constructs and Dr. Oliver Hankinson (Department of Pathology and Laboratory Medicine, Jonsson Comprehensive Cancer Center, Molecular Biology Institute, University of California, Los Angeles, CA) for providing the AHR and ARNT expression plasmids and the permission to use Hepa-1 mutant cell lines.

#### References

- Aitio A (1978) A Simple and sensitive assay of 7-ethoxycoumarin deethylation. *Anal Biochem* 85:488–491.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.
- Camus AM, Geneste O, Honkakoski P, Berezat JC, Henderson CJ, Wolf CR, Bartsch H, and Lang MA (1993) High variability of nitrosamine metabolism among individuals: role of cytochromes P450 2A6 and 2E1 in the dealkylation of N-nitrosodimethylamine and N-nitrosodiethylamine in mice and humans. *Mol Carcinog* 7:268–275.
- Chang C, Smith DR, Prasad VS, Sidman CL, Nebert DW, and Puga A (1993) Ten nucleotide differences, five of which cause amino acid changes, are associated with the Ah receptor locus polymorphism of C57BL/6 and DBA/2 mice. *Pharmacogenetics* 3:312–321.



- Chirgwin JM, Przybyla AE, MacDonald RJ, and Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5299.
- Denison MS, Fisher JM, and Whitlock JP Jr (1988) The DNA recognition site for the dioxin-Ah receptor complex. Nucleotide sequence and functional analysis. *J Biol Chem* **263**:17221–17224.
- Donato MT, Viitala P, Rodriguez-Antona C, Lindfors A, Castell JV, Raunio H, Gomez-Lechon MJ, and Pelkonen O (2000) CYP2A5/CYP2A6 expression in mouse and human hepatocytes treated with various in vivo inducers. *Drug Metab Dispos* **28**:1321–1326.
- Ema M, Ohe N, Suzuki M, Mimura J, Sogawa K, Ikawa S, and Fujii-Kuriyama Y (1994) Dioxin binding activities of polymorphic forms of mouse and human aryl-hydrocarbon receptors. *J Biol Chem* **269**:27337–27343.
- Fukunaga BN and Hankinson O (1996) Identification of a novel domain in the aryl hydrocarbon receptor required for DNA binding. *J Biol Chem* **271**:3743–3749.
- Glisovic T, Ben David Y, Lang MA, and Raffalli-Mathieu F (2003a) Interplay between HnRNP A1 and a cis-acting element in the 3' UTR of CYP2A5 mRNA is central for high expression of the gene. *FEBS Lett* **535**:147–152.
- Glisovic T, Soderberg M, Christian K, Lang M, and Raffalli-Mathieu F (2003b) Interplay between transcriptional and post-transcriptional regulation of Cyp2a5 expression. *Biochem Pharmacol* **65**:1653–1661.
- Gokhale MS, Bunton TE, Zurlo J, and Yager JD (1997) Cytochrome P450 isoenzyme activities in cultured rat and mouse liver slices. *Xenobiotica* **27**:341–355.
- Hakkola J, Hu Y, and Ingelman-Sundberg M (2003) Mechanisms of Down-Regulation of CYP2E1 expression by inflammatory cytokines in rat hepatoma cells. *J Pharmacol Exp Ther* **304**:1048–1054.
- Hakkola J, Pasanen M, Hukkanen J, Pelkonen O, Maenpaa J, Edwards RJ, Boobis AR, and Raunio H (1996) Expression of xenobiotic-metabolizing cytochrome P450 forms in human full-term placenta. *Biochem Pharmacol* **51**:403–411.
- Hankinson O (1994) A genetic analysis of processes regulating cytochrome P4501A1 expression. *Adv Enzyme Regul* **34**:159–171.
- Hankinson O (1995) The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* **35**:307–340.
- Hoffer A, Chang CY, and Puga A (1996) Dioxin induces transcription of Fos and Jun genes by Ah receptor-dependent and -independent pathways. *Toxicol Appl Pharmacol* **141**:238–247.
- Kaipainen P and Lang M (1985) Comparison of kidney, lung and liver coumarin 7-hydroxylases in phenobarbital pretreated DBA/2J and C57BL/6J mice. *Biochem Pharmacol* **34**:1987–1991.
- Kimura S, Kozak CA, and Gonzalez FJ (1989) Identification of a novel P450 expressed in rat lung: cDNA cloning and sequence, chromosome mapping and induction by 3-methylcholanthrene. *Biochemistry* **28**:3798–3803.
- Kobliakov V, Kulikova L, Samoilov D, and Lang MA (1993) High expression of cytochrome P450 2a-5 (coumarin 7-hydroxylase) in mouse hepatomas. *Mol Carcinog* **7**:276–280.
- Koskela S, Hakkola J, Hukkanen J, Pelkonen O, Sorri M, Saranen A, Anttila S, Fernandez-Salguero P, Gonzalez F, and Raunio H (1999) Expression of CYP2A genes in human liver and extrahepatic tissues. *Biochem Pharmacol* **57**:1407–1413.
- Kurose K, Tohkin M, and Fukuhara M (1999) A novel positive regulatory element that enhances hamster CYP2A8 gene expression mediated by xenobiotic responsive element. *Mol Pharmacol* **55**:279–287.
- Lavery DJ, Lopez-Molina L, Margueron R, Fleury-Olela F, Conquet F, Schibler U, and Bonfils C (1999) Circadian expression of the steroid 15 alpha-hydroxylase (Cyp2a4) and coumarin 7-hydroxylase (Cyp2a5) genes in mouse liver is regulated by the PAR leucine zipper transcription factor DBP. *Mol Cell Biol* **19**:6488–6499.
- Lees MJ and Whitelaw ML (2002) Effect of ARA9 on dioxin receptor mediated transcription. *Toxicology* **181–182**:143–146.
- Lush IE and Andrews KM (1978) Genetic variation between mice in their metabolism of coumarin and its derivatives. *Genet Res* **31**:177–186.
- Nakajima M, Yamamoto T, Nunoya K, Yokoi T, Nagashima K, Inoue K, Funae Y, Shimada N, Kamataki T, and Kuroiwa Y (1996) Role of human cytochrome P4502A6 in C-oxidation of nicotine. *Drug Metab Dispos* **24**:1212–1217.
- Ohtake F, Takeyama K, Matsumoto T, Kitagawa H, Yamamoto Y, Nohara K, Tohyama C, Krust A, Mimura J, Chambon P, et al. (2003) Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature (Lond)* **423**:545–550.
- Pelkonen O, Raunio H, Rautio A, Pasanen M, and Lang MA (1997a) The metabolism of coumarin, in *Coumarins: Biology, Applications and Mode of Action* (O'Kennedy R and Thornes RD eds) pp 67–92, John Wiley, Chichester.
- Pelkonen P, Lang MA, Negishi M, Wild CP, and Juvonen RO (1997b) Interaction of aflatoxin B1 with cytochrome P450 2A5 and its mutants: correlation with metabolic activation and toxicity. *Chem Res Toxicol* **10**:85–90.
- Puga A, Barnes SJ, Dalton TP, Chang C, Knudsen ES, and Maier MA (2000) Aromatic hydrocarbon receptor interaction with the retinoblastoma protein potentiates repression of E2F-dependent transcription and cell cycle arrest. *J Biol Chem* **275**:2943–2950.
- Reisz-Porszasz S, Probst MR, Fukunaga BN, and Hankinson O (1994) Identification of functional domains of the aryl hydrocarbon receptor nuclear translocator protein (ARNT). *Mol Cell Biol* **14**:6075–6086.
- Rivera SP, Saarikoski ST, and Hankinson O (2002) Identification of a novel dioxin-inducible cytochrome P450. *Mol Pharmacol* **61**:255–259.
- Salonpaa P, Pelkonen O, Kojo A, Pasanen M, Negishi M, and Raunio H (1994) Cytochrome P4502A5 expression and inducibility by phenobarbital is modulated by camp in mouse primary hepatocytes. *Biochem Biophys Res Commun* **205**:631–637.
- Schreiber E, Matthias P, Muller MM, and Schaffner W (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* **17**:6419.
- Shen ES and Whitlock JP Jr (1992) Protein-DNA Interactions at a dioxin-responsive enhancer. Mutational analysis of the DNA-binding site for the liganded Ah receptor. *J Biol Chem* **267**:6815–6819.
- Su T, Sheng JJ, Lipinskas TW, and Ding X (1996) Expression of CYP2A genes in rodent and human nasal mucosa. *Drug Metab Dispos* **24**:884–890.
- Thomas PE, Reik LM, Ryan DE, and Levin W (1981) Regulation of three forms of cytochrome P-450 and epoxide hydrolase in rat liver microsomes. Effects of age, sex and induction. *J Biol Chem* **256**:1044–1052.
- Tian Y, Ke S, Denison MS, Rabson AB, and Gallo MA (1999) Ah receptor and NF- $\kappa$ B interactions, a potential mechanism for dioxin toxicity. *J Biol Chem* **274**:510–515.
- Ulvila J, Arpiainen S, Pelkonen O, Aida K, Sueyoshi T, Negishi M, and Hakkola J (2004) Regulation of Cyp2a5 transcription in mouse primary hepatocytes: roles of hepatocyte nuclear factor 4 and nuclear factor I. *Biochem J* **381**:887–894.
- Zhang J, Watson AJ, Probst MR, Minehart E, and Hankinson O (1996) Basis for the loss of aryl hydrocarbon receptor gene expression in clones of a mouse hepatoma cell line. *Mol Pharmacol* **50**:1454–1462.

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