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Regulation of the *Cyp2a5* gene involves an aryl hydrocarbon receptor dependent pathway*

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Running title: Regulation of *Cyp2a5* involves AHR

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Abbreviations: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CYP, cytochrome P450; hnRNP, heterogenous nuclear ribonucleoprotein; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive element; COH, coumarin 7-hydroxylase

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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-methylcholantrene. The CYP2A5 mRNA upregulation in these mouse strains showed a difference in response, typical for AHR -regulated genes, both by TCDD in cultured primary hepatocytes and by 3-methylcholantrene *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/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 upregulate *Cyp2a5* transcriptionally by an AHR/ARNT-dependent mechanism and established *Cyp2a5* as a novel AHR-regulated gene.

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Introduction

The mouse cytochrome P450 (CYP) 2A5 and its human orthologue 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 CYP enzymes, and both transcriptional and post-transcriptional mechanisms appear 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 posttranscriptional mechanism involving binding of hnRNP A1 (heterogenous nuclear RiboNucleoProtein A1) to the 3' UTR 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

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polycyclic aromatic hydrocarbons (PAHs), are substrates of these CYP enzymes (Hankinson, 1995).

It was believed earlier that, among the cytochrome P450s, only the members of the CYP1 family are regulated by the AHR pathway. Recently, however, a novel CYP form CYP2S1 was identified and found to be controlled by AHR (Rivera *et al.*, 2002). Evidence also suggests that certain CYP2A enzymes can be upregulated by ligands of AHR. For example, the rat lung CYP2A3 is upregulated by 3-methylcholantrene (Kimura *et al.*, 1989), and the rat liver CYP2A1 is induced by 3-methylcholantrene 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 upregulated 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-methylcholantrene, on the CYP2A5 expression in liver cells. We were able to show that the mouse *Cyp2a5* gene is transcriptionally regulated by a mechanism which 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.

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Materials and methods

Preparation of primary cultures of hepatocytes

Hepatocytes were isolated from male C57BL/6 (JOlaHsd) and DBA/2 (OlaHsd) mice (Center for Experimental Animals, University of Oulu, Finland) aged 8 to 10 weeks. Livers were perfused with collagenase solution (Worthington Biochemical Co., Lakewood, NJ, USA) as described previously (Salonpaa *et al.*, 1994). After filtration and centrifugation, the isolated hepatocytes were dispersed in William's medium E (Sigma Chemical Co., St. Louis, MO, USA) containing dexamethasone (Sigma) 20 ng/ml, ITS (insulin 5 mg/l, transferrin 5 mg/l, sodium selenate 5 µg/l) (Sigma), gentamicin (Invitrogen, Paisley, Scotland) 50 µg/ml, and 10 % fetal bovine serum (Invitrogen) at a density of 5×10^6 cells / 100-mm dish (Falcon 3003), 2×10^6 cells / 60-mm dish (Falcon 3004), 1×10^6 cells / one well in six-well plates (Falcon 3046), and 3×10^5 cells / one well in twelve-well plates (Falcon 3043). The cultures were maintained at 37°C in a humidified incubator for 1 to 2 hours, after which non-attached cells were discarded by aspiration, and the medium was replaced by serum-free William's E medium. The cultures were maintained for additional 24 hours before treatment with TCDD (National Cancer Institute Chemical Carcinogen Repository, Bethesda, MD, USA) 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-methylcholantrene (Sigma) i.p. on three consecutive days. Control animals received

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vehicle (peanut oil) alone. The animals were killed 24 hours after the last injection, and their livers were removed and immediately processed for RNA preparation. The animal experiments had been approved by the local animal care and use committees.

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, USA). The cell lines were cultured in nucleoside-free α-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, Rockville, MD) was cultured in Dulbecco's Modified Eagle Medium with GlutaMAX^{TM} (Invitrogen), containing 10 % fetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were seeded to 6-well 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 RNAzol B reagent (TEL-TEST Inc. Friendswood, Texas, USA), 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 (Dupont Co. Boston MA, USA). 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 hours was isolated by the guanidine thiocyanate-CsCl method (Chirgwin *et al.*, 1979). 5 ug of the total RNA was electrophoretically resolved and transferred on to Hybond-N+ nylon membrane (Amersham Biosciences, Little Chalfont, UK). The RNA was fixed by UV-

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crosslinking, and the membrane was hybridized with [α^{32}P] dCTP-labeled probes. The full-length CYP2A5 cDNA was kindly provided by Dr. M. Negishi (NIEHS, Research Triangle Park, NC, USA), the CYP1A2 cDNA was a gift from Dr. P. Honkakoski (University of Kuopio, Finland), the CYP1A1 cDNA probe was prepared as described (Hakkola *et al.*, 1996), and the 18S probe was provided by Dr. H. Ruskoaho (University of Oulu, Finland).

Cycloheximide experiments

DBA/2 and C57BL/6 hepatocytes were treated with 10 µg/ml cycloheximide (Calbiochem, Merck KGaA, Darmstadt, Germany) for 1 hour prior to 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 hours, after which the CYP2A5 mRNA levels were measured and compared to 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 13000 *g* for 10 min. Pellets were discarded, and coumarin 7-hydroxylase (COH) activity was measured from the supernatants as described previously (Aitio, 1978) using 100 µM coumarin (Sigma) as a substrate.

Western blotting

20 µg of the 13 000 *g* supernatant proteins was subjected to SDS-polyacrylamide gel (10 % polyacrylamide) electrophoresis. The proteins were transferred on to a Hybond ECL nitrocellulose membrane (Amersham Biosciences). The membrane was then incubated with

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chicken polyclonal antibody raised against mouse CYP2A5 (kindly provided by Dr. Risto Juvonen, University of Kuopio, Kuopio, Finland) (1:5000 dilution) and secondary HRP-rabbit anti-chicken/turkey IgG (Zymed, San Francisco, CA) (1:20000 dilution). After washing, the immunoreactive bands were visualized with ECL+plus Western Blotting Detection System (Amersham Biosciences).

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 - -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, Los Angeles, CA, USA).

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The reporter gene constructs were transfected into mouse hepatocytes (12-well plates), Hepa-1 cells (24-well plates) or COS-1 cells 1 (24-well plates) together with Renilla luciferase reporter vector (pRL3-TK) (Promega), which was used as an internal control. 0.5 μg of *Cyp2a5* 5'-Luc and 0.1 μg of pRL3-TK were transfected per 3×10^5 hepatocytes, and 0.65 μg of *Cyp2a5* 5'-Luc or *Cyp1a1* 5'-Luc and 0.05 μg 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 μg of expression vector DNA was transfected per well. 24 h after the transfection, the cells were treated with 1 μM 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 μg 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 μM TCDD or vehicle dimethyl sulfoxide only for 24 h. Nuclear extracts were prepared according to Screiber *et al.* (1989). Briefly, the cells were first washed with 10 ml of PBS, then scraped to another 10 ml of PBS and pelleted by centrifugation at 110 *g* for 5 min, suspended in 1 ml of PBS, and centrifuged at 660 *g* for 15 s. The cell pellet was resuspended in cold, hypotonic buffer A (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; Complete Mini protease inhibitor cocktail, Roche Diagnostics GmbH, 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 P40) (Sigma) to a final percentage of 0.6 %. The homogenate was centrifuged at 660 *g* for 30 s, and the nuclear pellet was resuspended in cold buffer C (20 mM HEPES pH 7.9; 0.4 M

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NaCl; 1 mM EDTA; 1 mM EGTA; Complete Mini protease inhibitor cocktail, Roche Diagnostics GmbH, Germany). The nuclear proteins were extracted by incubation at +4°C for 15 min on a shaking platform. The samples were centrifuged at 15 000 g 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'CAAAGCCCCTGCTCACTCACGCACTCTGGAAGCCTGC3' -2487, consensus XRE; 5' GAGCTCGGAGTTGCGTGAGAAGAGCC3' (Denison *et al.*, 1988)). Single-stranded oligonucleotides were purchased from Sigma Genosys (St. Louis, MO, USA). Double-stranded oligonucleotides were 5' end-labeled with [γ^{32}P]ATP and T_4 polynucleotide kinase and then purified using the QIAquick nucleotide removal kit (Qiagen, Venlo, The Netherlands). 10 µg of COS-1 nuclear extract, binding buffer (25 mM Hepes pH 7.9; 10 % glycerol; 50 mM KCl; 0.5 mM EDTA; Complete Mini protease inhibitor cocktail, Roche Diagnostics GmbH, Germany), 1 µg/µl ssDNA 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 µl. 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 µl 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

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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, USA) 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 $p<0.05$.

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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 tenfold 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 μM 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 or not new protein synthesis is needed for the induction of *Cyp2a5* by TCDD. DBA/2 and C57BL/6 hepatocytes were treated with 10 μg/ml cycloheximide for 1 hour prior to the 24-hour TCDD treatment. TCDD induced CYP2A5 mRNA regardless of cycloheximide treatment, indicating that *de novo* protein synthesis is not needed for upregulation (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 μM TCDD for 24 hours, and the 13 000 *g* supernatant fraction was prepared. The CYP2A5 apoprotein levels were determined by immunoblotting. TCDD increased the CYP2A5 protein concentration 2.4-fold, as judged

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from the intensity of the immunorecognizable bands (Fig. 2A-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-methylcholantrene. 3-methylcholantrene 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-methylcholantrene 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-methylcholantrene in the C57BL/6 strain. In contrast, in the DBA/2 strain, which has low ligand-binding affinity of AHR, the 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-methylcholantrene

To further examine the genetics of CYP2A5 inducibility by 3-methylcholantrene, we generated backcrosses of F1 and DBA/2 mice and treated them with 3-methylcholantrene. Both CYP2A5 and CYP1A2 displayed gender differences in expression, and the results were therefore analyzed separately for each gender. Two male individuals (out of a total of four) had a clearly responsive phenotype (amounts CYP1A2 mRNA comparable to those of 3-methylcholantrene -treated male C57BL/6 mice), while the other two had low levels of CYP1A2 mRNA after 3-methylcholantrene treatment (non-responsive phenotype, comparable to 3-methylcholantrene -treated male DBA/2 mice) (Fig. 3B.). In female mice, two out three were found to be responsive. The induction of CYP2A5 correlated with CYP1A2 induction in

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both genders, indicating genetically determined co-regulation of the *Cyp1a2* and *Cyp2a5* genes by 3-methylcholantrene.

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 – 5.9-fold, depending on the construct (Fig. 4). A similar induction profile was seen when transfecting constructs into C57BL/6 hepatocytes (results 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, co-transfection 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 co-transfection (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 bp 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

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plasmid and treated with 1 μM 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 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 (results 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 μM 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 due to over-expression 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. 100-fold excess of unlabeled *Cyp2a5* 5'-XRE

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or consensus XRE could completely compete 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. 5-fold excess of unlabeled consensus XRE oligonucleotide was able to completely compete away the retarded AHR/ARNT-*Cyp2a5* 5'-XRE complex while the unlabeled *Cyp2a5* 5'-XRE oligonucleotide itself competed less efficiently. Mutated unlabeled *Cyp2a5* 5'-XRE poorly competed with non-mutated 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, 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). Collectively, 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

In order 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 co-transfected. When both AHR and ARNT were co-transfected and the cells were treated with TCDD, the luciferase expression of the *Cyp2a5* 5'-XRE reporter plasmid increased 3.6-fold (Fig.7).

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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-fold 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.

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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 kb 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-methylcholantrene, 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-methylcholantrene. 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 co-induced with CYP1A1 and CYP1A2, although the level of CYP2A5 induction was lower. Furthermore, the CYP2A5 induction by 3-methylcholantrene *in vivo* followed the CYP1A2 induction AHR genotype-

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dependently. Secondly, 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 reason for some preservation of induction in AHR-deficient cells is probably due to the low, but existing levels of AHR in the cell line (Zhang *et al.*, 1996). This assumption is also supported by the low, but existing level of CYP1A1 mRNA induction by TCDD in AHR-deficient cells. Finally, in COS-1 cells, the *Cyp2a5* promoter was induced only after AHR and ARNT co-transfection.

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 co-chaperone 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 (bHLH-PAS) domains, bind to the response elements in DNA called xenobiotic response elements (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 can directly associate with estrogen receptors and activate transcription via estrogen-responsive gene promoters. AHR may also interact with retinoblastoma protein and NF-κB (Puga *et al.*, 2000; Tian *et al.*, 1999). Moreover, TCDD activates the AP-1 factor by both AHR-dependent and -independent pathways (Hoffer *et al.*, 1996).

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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 co-transfection 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 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 (NF-I), the hepatocyte nuclear factor 4 (HNF-4), and the D-site binding protein are known to take part in the constitutive regulation of *Cyp2a5* (Ulvila *et al.*, 2004; Lavery *et al.*, 1999). Currently, none of these transcription factors are know to interact with AHR or ARNT. Recently, we 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). Since hnRNPA1 is activated by both toxic xenobiotics and oxidative stress, it will be interesting to find out whether interaction exists between it and the AHR in the regulation of *Cyp2a5*. Some potential activator protein 1 (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-methylcholantrene -induced CYP2A8 expression in hamster hepatocytes (Kurose *et al.*, 1999). However, the induction through the *Cyp2a5* proximal promoter appears to be hepatocyte-specific, as it was detected in primary hepatocytes and in the hepatoma cell line Hepa-1, but not in COS-1 cells. Therefore,

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hepatocyte-enriched factors in addition to ubiquitous transcription factors are likely to be necessary.

The extent of *Cyp2a5* induction by AHR is modest compared to *Cyp1* family induction and especially that of *Cyp1a1*. *Cyp1a1* contains several copies of XREs in its 5' regulatory region, while 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 (Nakajima *et al.*, 1996; Camus *et al.*, 1993). It will be of great importance to investigate if 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-methylcholantrene 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 (Rivera *et al.*, 2002; Kurose *et al.*, 1999), indicates that, in addition to the CYP1 family, several members the CYP2 family are controlled by AHR.

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Footnotes

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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 hours. A) 5 μg of total RNA was electrophoresed, blotted, and hybridized with mouse CYP2A5, human CYP1A1, mouse CYP1A2 and rat 18S probes. B, C, D) Densitometric quantification of CYP2A5, CYP1A1 and CYP1A2 mRNA blots. The values are normalized against 18S control levels, and the means ± range of two normalized samples are compared to untreated cells (0 nM TCDD). Difference to untreated cells *** $p<0.001$ and ** $p<0.01$ (one-way ANOVA followed by LSD Post Hoc test). The experiments with both strains were repeated, and these independent experiments gave similar results.

Fig. 2. Effect of TCDD treatment on CYP2A5 protein expression and COH activity in DBA/2 mouse hepatocytes. Mouse hepatocytes were treated with 1 μM TCDD or vehicle only (Control; dimethyl sulfoxide) for 48 h. A) Western blot analysis of 13 000 *g* supernatants (20 μg 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 non-microsomal 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 + 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

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hepatocytes. The values represent means + SD 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-methylcholantrene in mouse liver *in vivo*. A) Total RNA was prepared from the livers of untreated or 3-methylcholantrene (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-strained gel is shown for RNA loading assessment. B) Induction of CYP1A2 and CYP2A5 in backcrosses. Total RNA was prepared from the livers of 3-methylcholantrene -treated backcrosses (DBA/2 X F1) and subjected to electrophoresis and RNA blotting using CYP1A2 and CYP2A5 cDNAs. The CYP1A2 autoradiography was overexposed in order to detect the low levels of CYP1A2 in certain individuals. The ethidium bromide-strained gel is shown for RNA loading assessment.

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 µM TCDD or vehicle (dimethyl sulfoxide) only for 24 hours, after which luciferase activities were measured. The activities produced by the promoter constructs were normalized against co-transfected control plasmid (pRL-TK) activities. The values represent means +SD 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.

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Fig. 5. Effect of TCDD treatment on transcriptional activity of *Cyp2a5* 5' –3033 to +10 – luciferase construct transfected into Hepa1c1c7 (wt), Hepa-1c1c12 (AHR$^-$) and Hepa-1c1c4 (ARNT$^-$) cell lines. After transfection, the cells were treated with 1 μM TCDD or vehicle (dimethyl sulfoxide) only for 24 hours, after which luciferase activities were measured. The activities were normalized against co-transfected control plasmid (pRL-TK) activities. The values represent the means + SD of four individual samples. ### The difference to untreated cells was statistically significant $p<0.001$ (student's t test). *** The difference to TCDD-induced wt cells was statistically significant $p<0.001$ (one-way ANOVA followed by LSD Post Hoc test). The experiment was repeated, and both independent experiments gave similar results.

Fig. 6. Interaction of AHR and ARNT with *Cyp2a5* 5' XRE (-2523 to –2487) sequence or consensus XRE sequence in EMSA. A) The 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 12-13). 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) Competion reactions with *Cyp2a5* 5' XRE, consensus XRE and mutated *Cyp2a5* 5' XRE. 5-, 10- or 100-fold excess of unlabelled oligonucleotides were used to compete with labeled *Cyp2a5* 5' XRE for the binding of proteins in nuclear extracts from

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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 7-9 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.

Fig. 7. **Effect of AHR and ARNT co-transfection on the function of the *Cyp2a5* 5' XRE site in COS-1 cells.** The expression vectors of AHR and ARNT were co-transfected with the *Cyp2a5* 5' –XRE-rAlbTATA-Luc reporter plasmid or the reporter plasmid without XRE into COS-1 cells. After 24 hours, the cells were treated with TCDD or dimethyl sulfoxide only and incubated for 24 hours, after which the cells were harvested and the luciferase activities measured. The activities produced by the studied constructs were normalized against the co-transfected control plasmid (pRL-TK) activities. The values represent means +SD of four individual samples. *** The difference to the control without co-transfection and without TCDD treatment is statistically significant $p<0.001$ (one-way ANOVA followed by LSD 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 → ATAGC) was prepared and transfected into DBA/2 mouse primary hepatocytes. The TCDD induction of the mutated construct was compared with that of the non-mutated construct. The activities produced by the studied constructs were normalized against the co-transfected control plasmid (pRL-TK) activities. The values represent the means +SD of four individual samples.

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The XRE mutation reduced TCDD induction statistically significantly, $p<0.05$ (Student's t test). The experiment was repeated twice with similar results.

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Fig. 1.

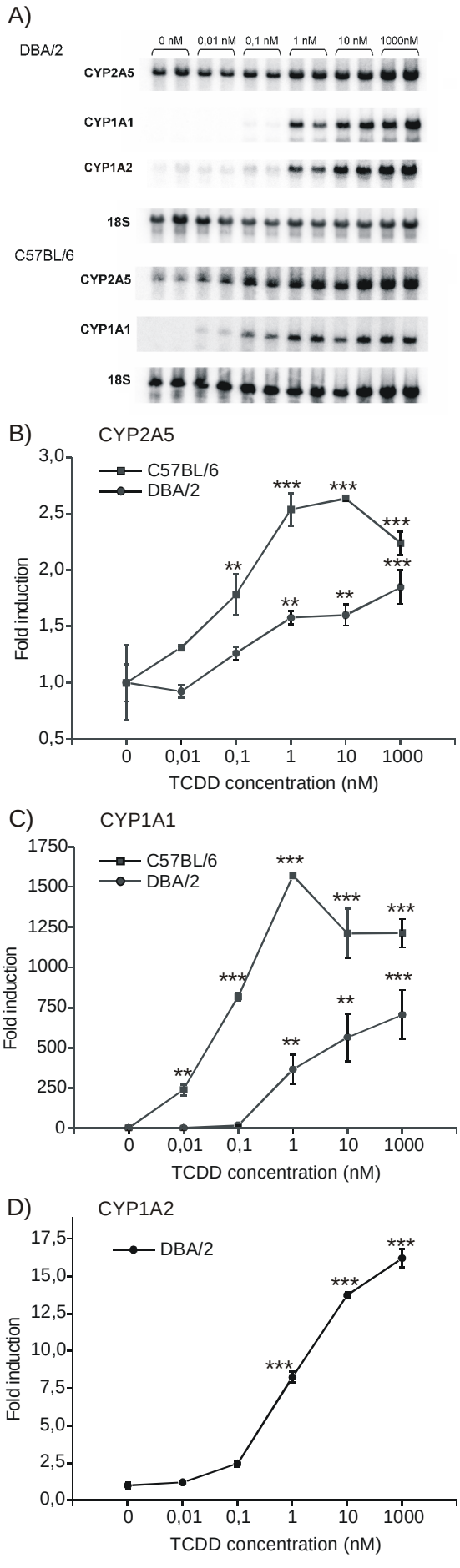

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Fig.2.

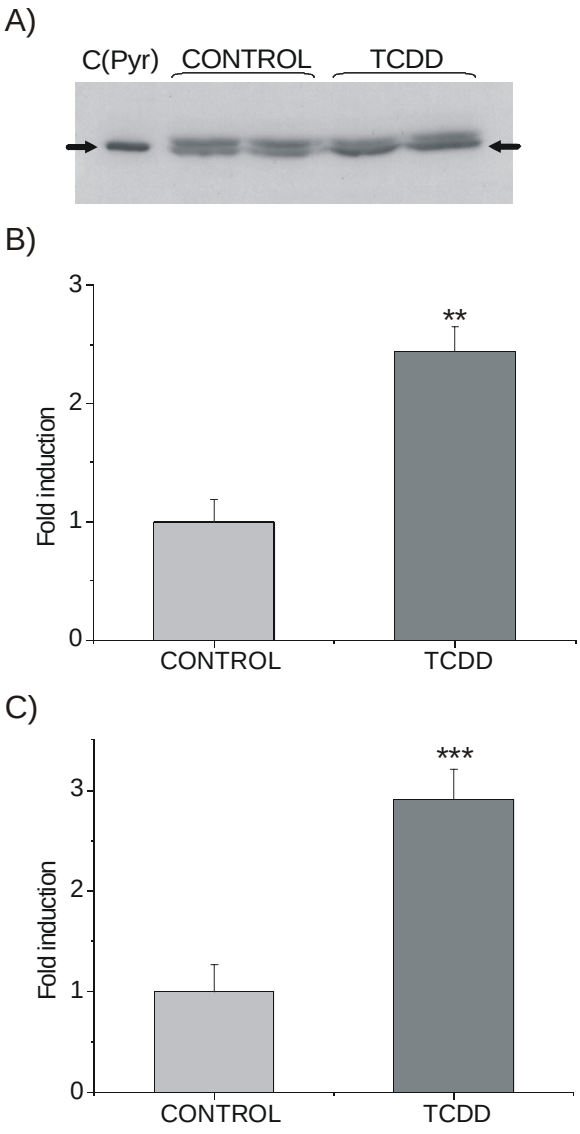

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Fig. 3

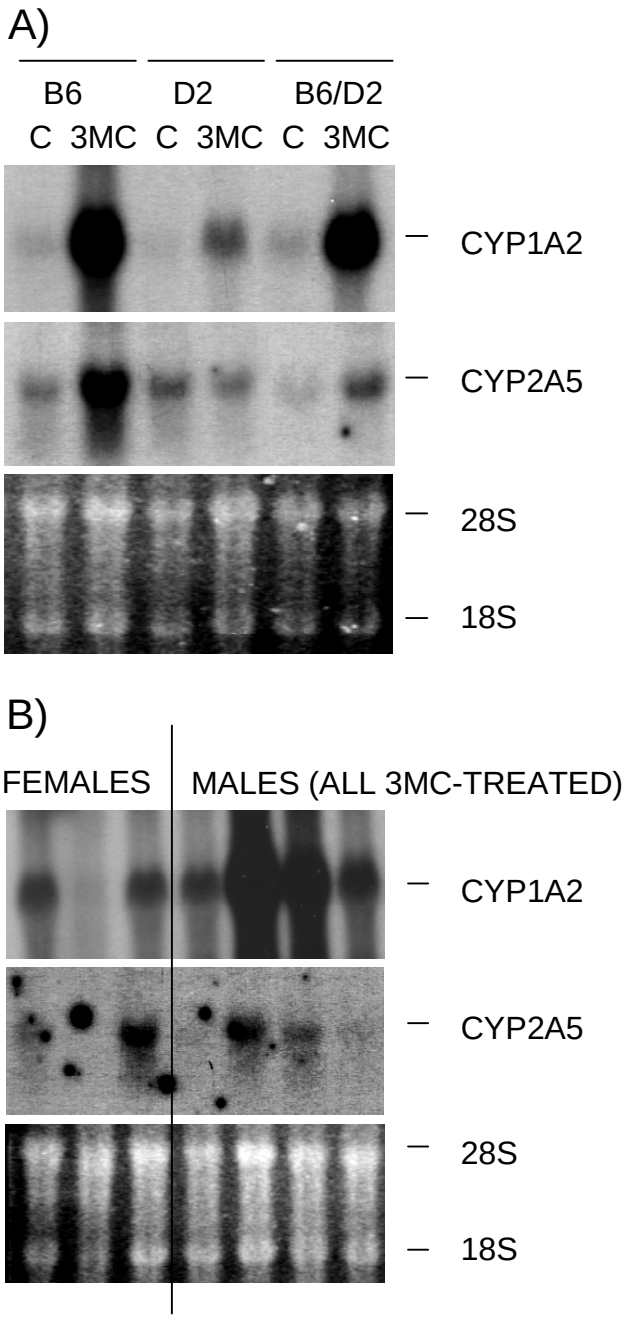

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Fig.4.

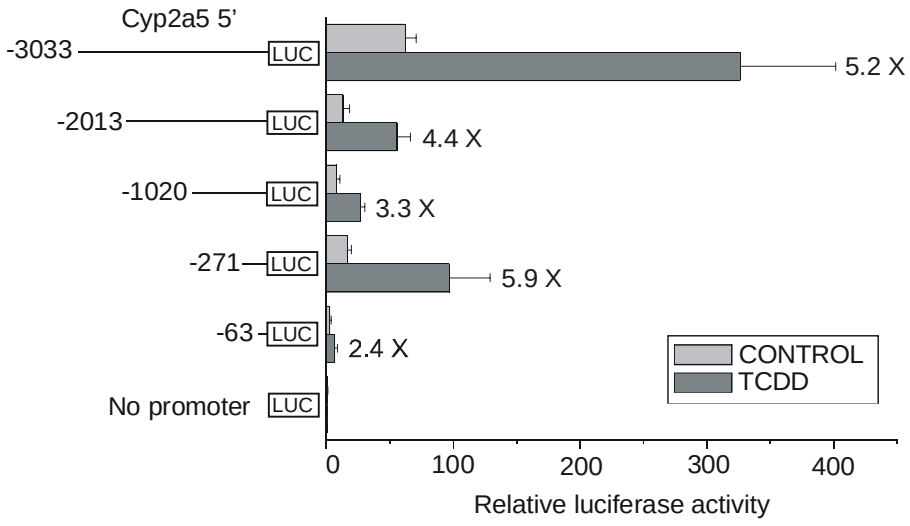

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Fig.5.

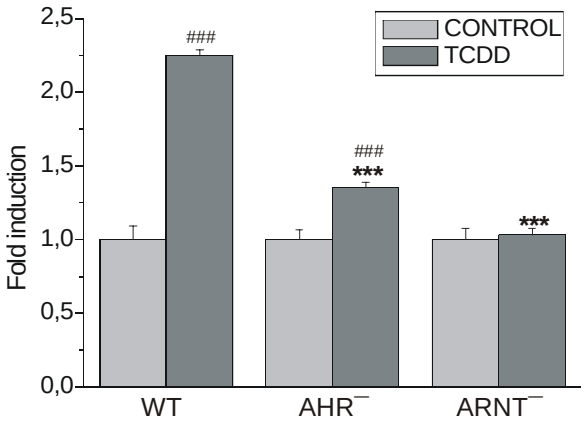

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Fig. 6.

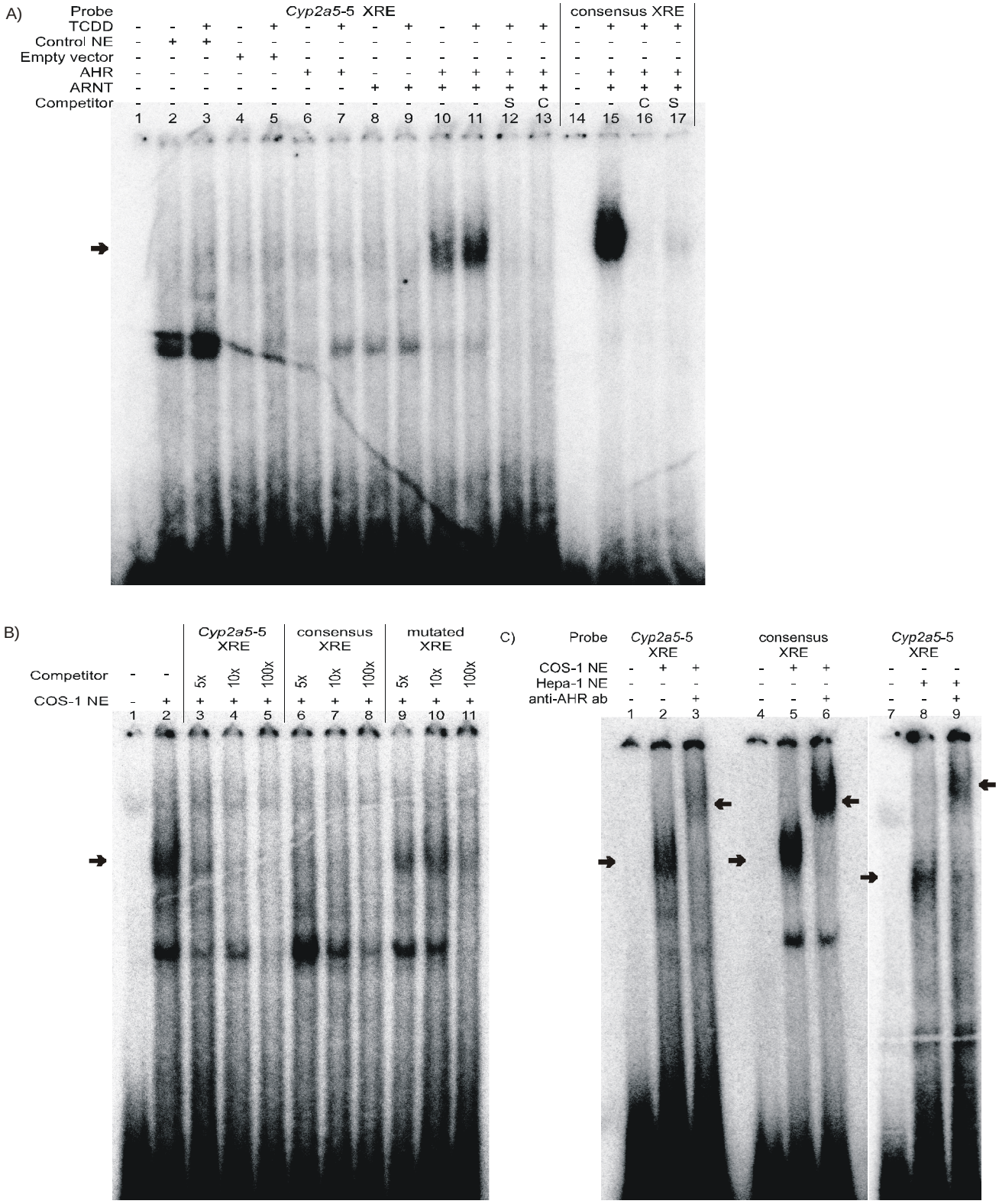

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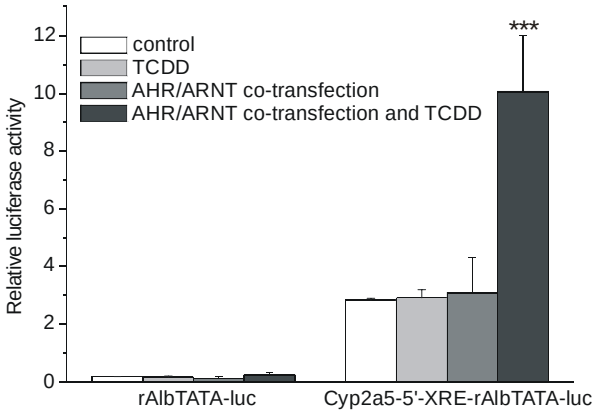
Fig.7.



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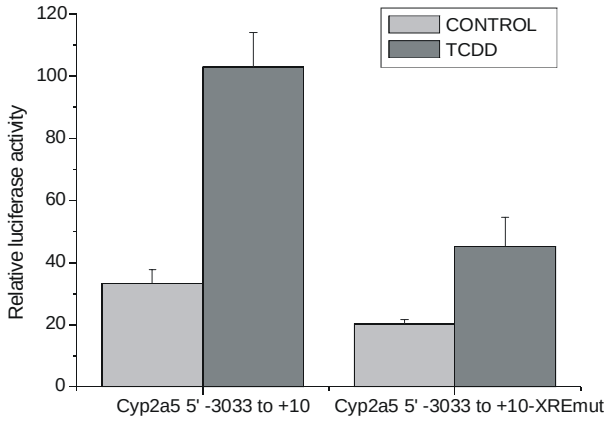

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