MOLECULAR CLONING OF THE GUINEA PIG CYP1A2 GENE 5'-FLANKING REGION: IDENTIFICATION OF FUNCTIONAL AROMATIC HYDROCARBON RESPONSE ELEMENT AND CHARACTERIZATION OF CYP1A2 EXPRESSION IN GPC16 CELLS

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ABSTRACT:

Aromatic hydrocarbon (AH) effects are mediated by binding of the AH receptor and its heterodimeric partner aromatic hydrocarbon nuclear translocator to specific response elements on DNA (AHREs). CYP1A2 expression is induced by AHs, yet AHREs have been identified in CYP1A2 genes of only two species and their functional role assessed only in the human gene. There have been few analyses of CYP1A2 gene regulation in nonhepatic cells. To gain further insight into CYP1A2 regulation, we cloned the initial 1.2 kilobases (kb) of the guinea pig CYP1A2 gene 5’-flanking region and characterized CYP1A2 expression in guinea pig colon adenocarcinoma cells (GPC16). Two putative AHRE sites were identified (−830 and −575 bp). They are considerably more proximal than the functional AHRE found in the human CYP1A2 gene (−2.5 kb). GPC16 cells expressed CYP1A2 after treatment with AH, enabling characterization of the putative AHRE sites in a homologous cell line. Double-stranded oligonucleotide probes, corresponding to each putative AHRE, bound in an AH-induced and specific manner to nuclear proteins prepared from GPC16 cells. In transfection analyses, only the distal site mediated AH-induced reporter gene activity. Mutation of this site suppressed AH-induced activity, supporting the concept that it is involved in AH-mediated induction of CYP1A2. However, the low level of AH-induction by the wild type suggests that other factors modulate AH-response by the CYP1A2 gene.

The cytochromes P450 comprise a large gene superfamily of enzymes involved in the metabolism of numerous environmental agents and endogenous compounds (Nebert and Gonzalez, 1987). Members of the mammalian CYP1A family (CYP1A1 and CYP1A2) metabolize aromatic hydrocarbons (AHs)1. In this role they have been considered agents of toxicity and carcinogenesis. CYP1A2 is a major contributor to the metabolism of heterocyclic amines and aflatoxin B1 to carcinogenic compounds (McManus et al., 1990), but it also metabolizes commonly consumed agents, such as caffeine and acetaminophen (Buters et al., 1996; Peters et al., 1999). In addition, CYP1A family members metabolize steroids, retinoids, and arachidonic acid (Rifkind et al., 1995; Raner et al., 1996; Lee et al., 2003). These endogenous substrates participate in development and differentiation, maintenance of homeostasis, hormone response, and modification of signal transduction processes. Induction of CYP1A1 and CYP1A2 by AHs or other exogenous agents can, potentially, alter levels of these physiologically active substances, resulting in environmentally or diet-induced dysfunction and disease.

Current understanding of the mechanism of CYP1A1 induction by AHs has been summarized in a recent review (Okino and Whitlock, 2000). Briefly, AH ligands bind to a cytoplasmic aromatic hydrocarbon receptor (AHR), which is associated with a chaperone complex containing heat shock protein 90, AHR-interacting protein, and other cytoplasmic factors. The liganded AHR enters the nucleus where, released from its chaperone complex, it dimerizes with its partner, ARNT (Heid et al., 2000). The AHR/ARNT heterodimer interacts with response elements on the DNA variously called dioxin response elements (DREs), xenobiotic response elements (XREs), or aromatic hydrocarbon response elements (AHREs). The binding of AHR/ARNT at one or more of the multiple AHREs identified in the CYP1A1 gene ultimately results in increased transcription.

It is often assumed that the regulation of the CYP1A2 gene is identical to that of CYP1A1 because it is responsive to AHs. However, AHR/ARNT binding sites have been reported in CYP1A2 genes of only two species and characterized only for the human gene (Quattrochi and Tukey, 1989; Strom et al., 1992; Quattrochi et al.,...
1994). The two putative AHR/ARNT binding sites previously identified in the human gene are located much further 5′ to the proximal –1 kb of the guinea pig CYP1A2 gene. We characterized these sites in a homologous cell line, guinea pig adenocarcinoma cells (GPC16), in which we demonstrated, for the first time, AH-induced expression of both CYP1A1 and CYP1A2. Both sites were capable of binding nuclear proteins prepared from GPC16 cells in an AH-induced manner, but only one (–830) was capable of mediating AH-induced reporter gene activity. Concatamers of oligonucleotides corresponding to this site were able to replicate the fold induction in reporter gene activity, and mutation of the site suppressed AH-induced activity, further supporting its involvement in AH-mediated induction of the guinea pig CYP1A2. However, the level of AH-induced reporter gene activity was considerably less than that reported for CYP1A1, indicating that other elements modulate CYP1A2 response to AH.

Materials and Methods

Animals and Drug Treatment. Male English short-haired (Hartley) guinea pigs (775–850 g; Camm Research Laboratories, Wayne, NJ), were fed standard laboratory chow ad libitum in a controlled lighting environment (lights on at 6:00 AM, lights off at 7:00 PM). Before sacrifice, they were injected with sodium pentobarbital (Diabutal, 60 ng/kg body weight; Diamond Laboratories, Des Moines, IA). Tissues for RNA and DNA preparation were removed, trimmed of fat, immediately frozen in liquid nitrogen, and stored in liquid nitrogen until use. Animal care and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Preparation of Genomic DNA and Genomic DNA Clones. Guineapig genomic DNA was prepared according to published protocols (Davis et al., 1986). Using the Universal GenomeWalker Kit (BD Biosciences Clontech, Palo Alto, CA), five pools or "libraries" of uncloned, adaptor-ligated genomic DNA fragments were constructed. Each library was amplified by PCR using the GenomeWalker adaptor primers and a gene-specific primer (5′-GGCAGGTGCAGGAGTACAGGATTG-3′) designed against the 5′ end of published cDNA sequence for guinea pig CYP1A2 available in GenBank (Accession numbers: D50457, U23501) (nt 3–29, D50457). The longest PCR products (1.2 kb) were obtained from the library created using the restriction enzyme Stul. These were subcloned into pCR2.1 using the Original TA Cloning Kit (Invitrogen, Carlsbad, CA). The distance between the putative first intron and the end of the known cDNA sequence, judged by comparison with the rat CYP1A2 gene (Sogawa et al., 1985), did not allow for creation of a true nested primer for genome walking. Therefore, the veracity of the clones was confirmed by PCR. The Stul library served as the template, and primers corresponded to the 5′ end of the cDNA, insert from that used for genome walking (nt 8–31, U23501), and sequence at the 3′ end of the 1.2-kb clone (nt –255 to –273, 5′-CTTAGCTGCTCTGACCTTGAGAG-3′). PCR products were subcloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen).

Sequence Analysis. Initial sequence of two 1.2-kb insert-bearing clones was obtained using the adaptor and gene-specific primers. Full-length sequence for each was obtained by "walking," using oligonucleotides corresponding to the sequence as it became known. Sequence for the subcloned PCR products was obtained using oligonucleotides corresponding to M13 primer sites flanking the plasmid cloning site.

Sequencing was performed by core facilities at New York University School of Medicine and University of Colorado Health Sciences Center. GCG Wisconsin programs (Accelrys, San Diego, CA) available at New York University were used for sequence analysis and in comparing guinea pig CYP1A2 sequence with other CYP1A2 gene sequences available in GenBank (Accession numbers: human, U02993, AF253322; rat, K03241; mouse, AB042202, M10022, X00479; hamster, D10914; rabbit, X05686) and with information available in published papers (human [Ikeya et al., 1989; Quattrochi and Tukey, 1989; Quattrochi et al., 1994], rabbit [Strom et al., 1992], rat [Sogawa et al., 1985; mouse [Gonzalez et al., 1985; Ikeya et al., 1989; Uchida et al., 2002]). Potential binding sites for transcription factors were identified using MatInspector v2.2 (Quandt et al., 1995) available through TRANSFAC, the transcription factor database (Heinemeyer et al., 1998).

Potential sites of transcription were examined using a Primer Extension System (Promega, Madison, WI), following instructions provided by the manufacturer. 32P-labeled antisense oligonucleotide primer, corresponding to the proximal primer used in genome walking, was hybridized with total RNA prepared from liver tissue of 3MC-treated guinea pigs and extended using reverse transcriptase.

Reporter Constructs. The enhancer region encompassing both putative AH/ARNT binding sites (–459 to –936) was sequentially subcloned through pBluescript into the reporter vector pSEAP-Promoter (BD Biosciences Clontech), taking advantage of available restriction enzyme sites. The pSEAP2 vector contains the reporter gene, secreted alkaline phosphatase (SEAP), driven by an SV40 promoter. Fragments containing only one of the putative AH/ARNT binding sites were obtained by cleaving the –500-bp fragment with PvuII. These were subcloned directly into the NruI site of the pSEAP-Promoter. Concatamers of the more distal site (–830) were prepared by blunt ligation of ds oligonucleotides corresponding to its sequence (see below) and inserted into the Nru site of pSEAP-Promoter. The set used in this study contained two copies of the site (5′-GGCCGTTGCGTGCTCTTGGCGCTGCGCTGGTTGCGCTT-3′). To facilitate site-directed mutational analysis in the context of the full-length 1.2-kb promoter region, the entire segment was subcloned into pGL3 reporter vectors (Promega), which contain the reporter gene luciferase. The 1.2-kb segment minus a 3′ fragment encompassing the TATA box (–93 to –1204) was subcloned into the pGL3 promoter vector containing the SV40 promoter, while the entire 1.2-kb segment (–1 to –1204) was subcloned into the pGL3 basic vector, which lacks a promoter. Mutagenesis, performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and oligonucleotides mutated in the core binding sequence of the AHRE (5′-CGT/C/GT-3′), was confirmed by sequencing.

Cell Culture, Drug Treatment, RT-PCR, Northern and Western Blot Analysis, and Transfection. The guinea pig colon cell line GPC-16 (ATCC CCL 242) was purchased from the American Type Culture Collection (Rockville, MD). The cells were plated at 1 × 10^5 in 35-mm wells for RNA analysis and transfection experiments and at 5 × 10^5 in 150-mm dishes for preparation of microsomes and nuclear extracts. They were grown in Eagle’s minimum essential medium (MEM) containing phenol red as an indicator of pH, sodium pyruvate (110 µg/ml) and Earle’s balanced salt solution (90%) with fetal bovine serum (10%) in the presence of antibiotics (100 U/ml penicillin G, 100 µg/ml streptomycin) in an atmosphere of 5% CO2 at 37°C. The cells were treated with TCDD or 3MC, at concentrations given below, dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO was 0.1%. Controls received 0.1% DMSO only.

To characterize mRNA expression, cells were treated 36 to 48 h after plating with TCDD (1 and 10 nM) or 3MC (0.1 and 1 µM) for 0 to 48 h. RNA was prepared using RNA-Stat-60 (Tel-Test Inc., Friendswood, TX). RNA integrity was assessed by electrophoresis and staining of samples separated on formaldehyde-containing agarose gels. RT-PCR analyses for levels of CYP1A1, CYP1A2, ARNT mRNA were performed using guinea pig-specific primers (Table 1) and were normalized by comparison with RT-PCR products for β-actin, as previously described (Black et al., 1998b). Primers for ARNT corresponded to unpublished cDNA sequence for their periodicity/ ARNT/simple-minded domains obtained in our laboratory in collaboration with Drs. Mark Hahn and colleagues, Woods Hole Oceanographic Institute (Woods Hole, MA) (Black et al., 1998a). The correspondence of the ARH
Microsomes were prepared after 18 and 36 h of treatment and analyzed for levels of CYP1A1 and CYP1A2 immunoreactive protein, as previously described (Black et al., 1998b). Two primary antibodies were used: a monoclonal antibody, raised against scup CYP1A1, that recognizes CYP1A1 of many vertebrate species and shows little cross-reactivity with CYP1A2 (Mab 1 described by Black et al., 1998b). Reactions with peroxidase-labeled secondary antibodies were visualized using diaminobenzidine or enhanced chemiluminescence (Amersham Biosciences Inc., Piscataway, NJ).

Transfections were performed 36 to 48 h after plating, at 50 to 65% confluency, using LipofectAMINE (Invitrogen). DNA of the reporter vector, containing the reporter constructs described above (1 µg per 35-mm dish) and LipofectAMINE (3 µg per 35-mm dish) were prepared, combined, and plated, according to the manufacturer’s instructions. Control transfections were carried out in a similar fashion. Reporter vectors lacking the CYP1A2 insert served as a negative control. As a positive control, some cells were transfected with control vectors, provided by the manufacturer. To assess transfection efficiency, some cultures were cotransfected; pSEAP was paired with a vector-containing gene sequence for β-galactosidase or Dual-Glo Luciferase Assay Systems and the manufacturer’s instructions. Control transfections were carried out in a similar manner. These probes were end-labeled with [32P]ATP, purified before use, according to published protocols (Ausubel et al., 1990). Oligonucleotides corresponding to the more distal site (~830, but mutated in the core binding sequence (~5'-GCGT/CG/T-3') were prepared in a similar manner. These probes were end-labeled with γ[32P]-ATP, purified on Sepharose columns (TE SELECT-D G-25; Eppendorf-5 Prime, Inc., Boulder, CO) and quantitated by fluorescence spectroscopy. EMSA was performed essentially as described by Denison et al. (1995). The final reaction mixture contained 10 µg of protein, 25 mM Hepes, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol (v/v), with 1 to 3 µg of poly(dI:dC) and 180 to 200 mM KCl, depending on the probe used. Unlabeled competitors were included in some reactions, as indicated in the figure legends, at 25- to 100-fold the concentration of the labeled probes. An oligonucleotide corresponding to the consensus sequence for the NF-κB binding site (5'-AGTT-GAGGAGACTTTCGCCAGC-3'; Promega) was selected for use as a nonspecific probe.

**Table 1**

<table>
<thead>
<tr>
<th>Source/Access. No.</th>
<th>Strand</th>
<th>Nucleotides</th>
<th>Sequence</th>
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<td>Sense</td>
<td>5'-GACTTCAGGCTGGTCTGACGAC-3'</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>5'-GCATGTCAGGGTCTGACGAC-3'</td>
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<tr>
<td>CYP1A2/D23501</td>
<td>Sense</td>
<td>5'-ATGCCATATCGGTTGCTGACGAC-3'</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>5'-GTGACTATCGGTTGCTGACGAC-3'</td>
<td></td>
</tr>
<tr>
<td>ARNT</td>
<td>Sense</td>
<td>5'-CAGGAGCTATTGGGCGATCAC-3'</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>5'-CAGGAGCTATTGGGCGATCAC-3'</td>
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<tr>
<td>β-actin/HUMA-CCYBB</td>
<td>Sense</td>
<td>5'-TACAGCGGCGCTACACTGTCGCCAC-3'</td>
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<td></td>
<td>Antisense</td>
<td>5'-TACAGCGGCGCTACACTGTCGCCAC-3'</td>
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Transfections were also performed using a cell line derived from a human hepatocarcinoma (HepG2) and isolated rat hepatocytes. HepG2 cells were grown under conditions described previously (Quattoch and Tukey, 1989; Quattoch et al., 1998). Rat hepatocytes were isolated and maintained as primary cultures, according to published protocols (Li et al., 1991). The culture medium for hepatocytes included vitamin C (50 µg/ml) and dexamethasone (10^{-7} M); both are important for maintaining optimal expression of CYP1A2 (Silver et al., 1990; Mori et al., 1997), as well as insulin (0.25 U/ml). HepG2 cells and isolated rat hepatocytes were transfected using LipofectAMINE and treated with TCDD (1 nM) and 3MC (0.1 nM), as previously described (Black et al., 1998). Sense and antisense oligonucleotides corresponding to the mouse XRE (DRE3) (5'-GAGCTCGGAATTCCTGGAAAGGC-3') (Denison et al., 1998) and guinea pig CYP1A2 putative AHR/ARNT binding sites (5'-805: 3'-GCGCGGCGGCTGCCTCTC-3'; RNT2: 5'-75: 3'-TCTACGTCGTCGCTCTCTCAGC-3') were treated with 19 bp ds oligonucleotide probes and gel-purified before use, according to published protocols (Ausubel et al., 1990). Oligonucleotides corresponding to the more distal site (~830, but mutated in the core binding sequence (~5'-GCT/CG/T-3') were prepared in a similar manner. These probes were end-labeled with γ[32P]-ATP, purified on Sepharose columns (TE SELECT-D G-25; Eppendorf-5 Prime, Inc., Boulder, CO) and quantitated by fluorescence spectroscopy. EMSA was performed essentially as described by Denison et al. (1995). The final reaction mixture contained 10 µg of protein, 25 mM Hepes, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol (v/v), with 1 to 3 µg of poly(dI:dC) and 180 to 200 mM KCl, depending on the probe used. Unlabeled competitors were included in some reactions, as indicated in the figure legends, at 25- to 100-fold the concentration of the labeled probes. An oligonucleotide corresponding to the consensus sequence for the NF-κB binding site (5'-AGTT-GAGGAGACTTTCGCCAGC-3'; Promega) was selected for use as a nonspecific probe.

**Results**

**Molecular Cloning of the Guinea Pig CYP1A2 5'-Flanking Region and Comparison of Proximal Promoter with Other CYP1A2 Genes**. Two 1.2-kb genomic clones were obtained from guinea pig DNA by genome walking using a primer corresponding to a sequence of guinea pig CYP1A2 cDNA flanking the 5' end of the coding region (GenBank Accession numbers D50457 and U23501). Their sequences were identical and showed the expected overlap with...
the 5'-flanking region of the cDNA (GenBank, Accession number AY007245). Primer extension allowed estimation of potential start sites for transcription (Fig. 1). The relatively short leader sequence (∼58 bp) is consistent with that reported for CYP1A2 in other species [human, 62 or 64 bp (Ikeya et al., 1989; Quattrochi et al., 1994, respectively), rabbit, 59 bp (Strom et al., 1992; X05686); rat and mouse, 60 bp (Gonzalez et al., 1985; Sogawa et al., 1985)]. Further comparison of the guinea pig 5'-flanking sequence with sequence reported for other CYP1A2 genes revealed two stretches of highly conserved sequence (80–90%) within the proximal 150 bp. One (∼15 to −40) encompasses the TATA box. The other (∼50 to −150) includes highly conserved core sequences for several transcription factor binding sites: NF-1 and GATA, as well as CAAT and E boxes. Further 5', the sequences diverged from one another considerably.

Consistent with previous reports for CYP1A2, no sequence was found that corresponded to the basic transcription element found in CYP1A1 and other genes involved in xenobiotic metabolism (Ikeya et al., 1989; Fujii-Kuriyama et al., 1992).

Identification of AHRE Sites and Comparison with Those of Other CYP1A2 Genes. Computer analysis of more distal sequence identified two putative AHR/ARNT binding sites centered around −830 and −575 in the guinea pig gene. For the sake of simplicity, we called these putative binding sites AHR/ARNT 1 (R/NT1) and AHR/ARNT 2 (R/NT2), respectively. They possessed the canonical five-base pair sequence (GCGTG) characteristic of the AHR/ARNT binding sequence (Denison et al., 1988) and had high overall identity (R/NT1, 89.5%; R/NT2, 78.9%) with the 19-bp consensus sequences defined for the mouse DRE (Yao and Denison, 1992) (Fig. 2). The four-base pair core (CGTG) is essential for binding (Denison et al., 1988; Quandt et al., 1995), but sequence flanking this core can affect binding. R/NT1 had substitutions of two nucleotides in the binding consensus sequence (15, A/T to C and 19, C/G to T) and one in the functional binding consensus sequence (15, A/T to C). R/NT2 had substitutions of one base pair in the binding consensus sequence (19, C/G to A) and two in the functional binding consensus sequence (5, C/G to A and 6, T to C).

Characterization of CYP1A2 mRNA and Protein Expression in GPC16 Cells. GPC16 cells have been used in studies of AHR activation (Aarts et al., 1995; Heath-Pagliuso et al., 1998), making them a reasonable choice for further characterization of the putative AHRE sites in guinea pig CYP1A2. However, to our knowledge, GPC16 cells had not been characterized for expression of the target genes, CYP1A1 or CYP1A2. RT-PCR and Northern blot analyses
Fig. 3. Characterization of AHR, ARNT, and CYP1A expression in GPC16 cells.

A. RT-PCR analysis of mRNA levels following 18-h treatment with TCDD or 3MC at two different doses. Controls (0) were treated with the vehicle alone. PCR products of the expected full length for both CYP1A1 and CYP1A2 (~1.55 kb) increased following treatment with either TCDD or 3MC, whereas PCR products for AHR and ARNT showed little change. The arrow indicates the full-length PCR product for CYP1A2. The 700-bp PCR product represents an alternative splice of CYP1A2 previously described (Black et al., 1998). β-Actin levels are shown for comparison. Primers used are given in Table 1. RNA was assessed by ethidium bromide staining of agarose/formaldehyde gels. B. Northern blot analysis of CYP1A1 and CYP1A2 mRNA levels following 18-h treatment with TCDD or 3MC at two different doses. Controls (0) were treated with the vehicle alone. Probes corresponded to 3' regions of the full-length cDNA. Hybridization of the CYP1A1 cDNA probe is compared in an overnight exposure. Hybridization of CYP1A2 cDNA probe is compared in a 2-day exposure. C. RT-PCR analysis of mRNA levels in cultures treated with 1 nM TCDD (T), 0.1 μM 3MC (M), or the vehicle alone (−) for different lengths of time (0–48 h). Primers used are identical to the ones in A. Only the full-length PCR products are shown for CYP1A2. D. Western blot analysis of microsomal proteins prepared from cultures treated for 18 h with 1 nM TCDD (T), 0.1 μM 3MC (M), or the vehicle alone (−). Liver microsomes from guinea pigs treated with 3MC (M) are shown for comparison. The antibody used for CYP1A1 (53 kDa) was a monoclonal antibody made against scup CYP1A1 that has little cross-reactivity with CYP1A2. The antibody for CYP1A2 was a polyclonal antibody made against hamster CYP1A2 and has some cross-reactivity with CYP1A1. The arrow indicates CYP1A2 (56 kDa). The amounts of microsomal protein loaded were 10 μg for liver and 20 μg for GPC16 cells. Reactive proteins for these blots were visualized using peroxidase-labeled secondary antibodies and diaminobenzidine.
consensus sequence for NF-κB (Fig. 4, A and B). Competition by unlabeled XRE was more effective than that by the unlabeled R/NT probes, probably reflecting differences in the consensus sequence flanking the core binding sequences, noted above. Yao and Denison (1992) showed that substitutions in positions 5 and 6 of mouse DRE3, seen in R/NT2, have no effect on TCDD-inducible complex formation. However, substitutions in nucleotides 15 and 19 of the DRE oligomers, seen in both R/NT sites, significantly decreased AH-induced nuclear protein binding affinity of DRE oligomers.

Binding of labeled ds oligonucleotides corresponding to R/NT1 and R/NT2 to nuclear proteins prepared from GPC16 cells was also AH-induced and specific (Fig. 4, B and C). The binding of R/NT1 and R/NT2 was greater to nuclear proteins from cultures treated with TCDD than to nuclear proteins from control cultures treated with the vehicle alone. The induced binding of both labeled R/NT probes was completed by unlabeled homologous oligonucleotide, as well as by unlabeled oligonucleotide corresponding to the other R/NT site (Fig. 4B) and to the mouse XRE (DRE3) (Fig. 4C), but not by the unlabeled nonspecific probe, NF-κB (Fig. 4, B and C). However, the DNA-protein complex formed by R/NT2 was consistently less intense than that formed by R/NT1, and unlabeled R/NT2 was a less effective competitor of R/NT1 than of R/NT2. Oligonucleotides corresponding to R/NT1 in which the core binding site had been mutated did not bind to nuclear proteins from control or treated cultures, nor did they compete for binding (Fig. 4D). In some cases, two induced complexes could be distinguished (Fig. 4, C and D), consistent with the previous report of two distinct protein complexes in assays using guinea pig hepatic cytosol and mouse XRE (DRE3) (Bank et al., 1995). Both of the complexes reported by Bank et al. contained AHR and bound to the DRE with the same specificity; differences in subunit composition were suggested as an explanation for the different sizes of the protein-DNA complexes. These remain to be elucidated.

The functionality of the two AHR/ARNT sites was tested by transient transfection of host cells with reporter constructs containing these sites and analysis of reporter gene activity after treatment with TCDD or 3MC (Fig. 5). GPC16 cells, HepG2 cells, and primary cultures of isolated rat hepatocytes were transfected with pSEAP2-Promoter vector containing a segment encompassing both AHR/ARNT binding sites (−936 to −459). Treatment with TCDD increased SEAP activity and resulted in a 2- to 3-fold induction of the SEAP reporter molecule in all three cell culture systems (Fig. 5, A and B). Treatment of GPC16 cells transfected with a reporter construct containing R/NT1, but not R/NT2 (−936 to −633), also resulted in an approximately 2-fold induction of SEAP activity. However, cells transfected with a reporter construct encompassing R/NT2, but not R/NT1 (−633 to −459), showed no detectable, inducible SEAP activity (data not shown). These results suggest that the inducible reporter gene activity of the 477-bp fragment (−936 to −459) is mediated by R/NT1, but not R/NT2.

To test if the R/NT1 site alone could mediate inducible reporter gene activity, host cells were transiently transfected with reporter constructs containing a concatamer of ds oligonucleotides corresponding to its sequence. TCDD treatment resulted in fold induction of SEAP activity that was not significantly different from that mediated by the reporter construct encompassing R/NT1 (−936 to −633) or the larger construct encompassing both AHR/ARNT binding sites (−936 to −459) (Fig. 5C). This supports the concept that the 19-bp site at −830 is functional, i.e., capable of mediating response to aromatic hydrocarbons, but it does not exclude the possibility that adjacent elements modulate this activity.

To test the functionality of R/NT1 in the context of the full-length 1.2-kb 5′ flanking region, GPC16 cells were transfected with reporter constructs in the pGL3 vector containing the wild-type promoter or one in which R/NT1 had been mutated in the core binding sequence. TCDD treatment resulted in a fold induction of luciferase reporter gene activity, whether driven by a heterologous SV40 promoter or by the homologous promoter, that was not significantly different from that seen with the R/NT1 containing fragment (−936 to −633) or concatamers of R/NT1 in the pSEAP-2 promoter vector (Fig. 5D). Mutation of the core binding sequence at −830 suppressed this AH-induced reporter gene activity. These results indicate that the homologous promoter is functional and further support the concept that the 19-bp site at −830 is a functional AHRE. We have chosen to call the apparently functional site AHRE1.

**Discussion**

CYP1A2 has been regarded as a “liver-specific” gene whose AH inducibility resides solely in hepatic tissue, in contrast to CYP1A1, which is inducible in many tissues. However, CYP1A2 has recently been reported to be expressed in a number of tissues and cell lines...
Values represent the mean fold induction of TCDD-treated over control cultures. Values of 1.2P and 1.2B were not significantly different (P < 0.05). B, comparison of fold induction of SEAP activity mediated by reporter constructs containing both R/N1 and R/N2. Cells were treated with TCDD (T), 3MC (M), or the vehicle alone (C) for 24 h. Activity is expressed as pmoles per microgram of protein. Values represent the mean ± S.D. of triplicate cultures from a representative experiment for each cell type. Asterisks indicate values significantly different from controls (P < 0.05). B. Comparison of fold induction of SEAP activity mediated by reporter constructs containing both R/N1 and R/N2 (936 to 459 bp) in HepG2 cells, GPC16 cells, and isolated rat hepatocytes. Cells were treated with TCDD (T), 3MC (M), or the vehicle alone (control cultures). Values represent mean fold induction of treated over control cultures ± S.D. for at least three experiments, each consisting of duplicate or triplicate cultures. C, comparison of fold induction of SEAP activity in GPC16 cells mediated by a reporter construct containing a four-repeat concatamer of AHR1 with the fold induction of reporter constructs encompassing R/N1 (936 to 633) or both AHR/ARNT binding sites (936 to 459). Reporter constructs encompassing R/N2 (633 to 459) (not shown) mediated no detectable induction of SEAP activity. Values represent the mean fold induction of TCDD-treated over control cultures ± S.D. for at least three experiments, each consisting of duplicate or triplicate cultures. D, comparison of fold induction of luciferase activity in GPC16 cells mediated by three different reporter constructs in the pGL3 vector. In the first, pGL3, driven by an SV40 promoter, contains the entire 1.2-kb 5' flanking segment minus its TATA region (1.2P). In the second, pGL3, lacking the SV40 promoter, contains the entire 1.2-kb 5' flanking region and is driven by the CYP1A2 promoter (1.2B). In the third, the pGL3 vector contains the 1.2-kb 5' flanking region in which the core-binding site of R/N1 has been mutated (1.2Mut). Values represent the mean fold induction of treated over control cultures ± S.D. for at least five experiments, each consisting of triplicate cultures. Values of 1.2P and 1.2B were not significantly different (P < 0.05). Asterisks indicates that 1.2Mut levels were significantly different from those of 1.2P and 1.2B (P < 0.05).

(Ahn et al., 2002), including the human colon carcinoma cell line LS-180 (Li et al., 1998). Because its extrahepatic expression has only recently been recognized, there have been few analyses of CYP1A2 gene regulation in nonhepatic cells. In this article, we report initial cloning of the 5'-flanking region of the guinea pig CYP1A2 and demonstrate CYP1A2 expression in a guinea pig colon adenocarcinoma cell line, GPC16, for the first time. We identified two putative AHR/ARNT binding sites in the first 1.2 kb of the guinea pig CYP1A2 and used the homologous GPC16 cells to characterize one of these as a functional AHRE. This is the only AHRE to be characterized in CYP1A2, other than that previously characterized in the human gene (Quattrochi et al., 1994), and is one of few analyses of CYP1A2 gene regulation in extrahepatic cells.

Because CYP1A2 metabolizes and is induced by AHs, it has been assumed that CYP1A2 genes are regulated in a manner similar to CYP1A1. However, CYP1A2 genes do not contain the well-characterized basic transcription element just proximal to the TATA box that is found in the promoter of many cytochromes P450, including CYP1A1, and several other genes involved in xenobiotic metabolism (Ikeya et al., 1989). Furthermore, although CYP1A1 genes contain multiple AHREs in the initial −1 kb, CYP1A2 genes appear to have fewer functional AHREs, diversely placed. In the human CYP1A2 gene, most putative binding sites identified are considerably more distal (−2 to −3.7 kb and beyond) (Quattrochi and Tukey, 1989; Quattrochi et al., 1994; Corchero et al., 2001) and, thus far, only one site (−2500) has been shown to be functional (Quattrochi et al., 1994). No AHREs have been identified in the initial −4 kb of the mouse Cyp1a2, suggesting more distal location in this species as well (Uchida et al., 2002; GenBank accession number AB042202). However, in the guinea pig CYP1A2 gene, as well as in the rabbit CYP1A2 gene (Strom et al., 1992), putative AHR/ARNT binding sites are located within the first −1 kb of the 5'-flanking region and a computer search performed as a part of our analysis revealed putative AHR/ARNT binding sites in the rat at −925 bp and in the hamster at −445 and −1645 bp. Although the possibility of more distal sites in the guinea pig CYP1A2 gene, as well as in those of other species for which distal sequence is not yet available, cannot be excluded, our data indicate that one proximal AHRE site in the guinea pig CYP1A2 gene is functional.

It is important to note, however, that although the CYP1A2 promoter is functional, the level of AH-induced CYP1A2 reporter gene activity is lower than that reported for CYP1A1. In HepG2 cells, instead of 50-fold levels of CYP1A1 reporter gene activity induced by TCDD (1 nM) in transient transfection assays (Postlind et al., 1993), the human CYP1A2 shows responses of 1.8- to 6.5-fold (Aitchison et al., 2000; Postlind et al., 1993, respectively). The 2- to 3-fold levels of TCDD (1 nM)-induced reporter gene activity shown here for guinea pig CYP1A2 in three cell culture systems are within the range of levels reported for human CYP1A2 in HepG2 cells. The reasons for the consistently lower level of AH-responsiveness of CYP1A2 are not clear at this time, but low response suggests that other factors are involved in achieving the levels of CYP1A2 expression seen in vivo after AH exposure. These could include: 1) elements that mediate and/or modify AH-response located within, proximal or distal to regions containing the functional AHREs demonstrated thus far, such as more distal AHREs that act synergistically and/or sites for other transcription factors that suppress or enhance AH response; 2) interaction of AHR and/or ARNT with receptors or cofactors involved in other signaling pathways; or 3) post-transcriptional mechanisms altering mRNA stability.

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