A Novel Promoter Element Containing Multiple Overlapping Xenobiotic and Hypoxia Response Elements Mediates Induction of Cytochrome P4502S1 by Both Dioxin and Hypoxia

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Cytochrome P4502S1 (CYP2S1) is expressed at high levels in epithelial tissues and is inducible by 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) via the aryl hydrocarbon receptor (AHR). Transcriptional initiation of mouse Cyp2s1 was found to occur at three regions, ~198, 102, and 22 nucleotides from the translational initiation codon. Approximately 400 nucleotides upstream of its translational initiation codon, mouse Cyp2s1 contains three overlapping xenobiotic-responsive element (XRE) sequences, which make a major contribution toward dioxin inducibility. Each XRE sequence in this trimeric XRE can bind the AHR/aryl hydrocarbon receptor nuclear translocator (ARNT) dimer in a dioxin-dependent fashion in vitro and can mediate dioxin-dependent transcription. Cyp2s1 is also markedly inducible by hypoxia. Induction is dependent on hypoxia-inducible factor-1 (HIF-1) and is mediated in large part by three overlapping hypoxia response elements (HREs) embedded within the trimeric XRE segment. Although each HRE within this segment can bind HIF-1α/ARNT in vitro, the most 3′ HRE contributes the most toward hypoxia inducibility. AHR/ARNT and HIF-1α/ARNT dimers bind to the region containing the trimeric XRE segment of the endogenous Cyp2s1 gene in vivo in a dioxin-dependent fashion and hypoxia-dependent fashion, respectively. These observations identify a novel regulatory casette that mediates changes in Cyp2s1 expression.

The cytochrome P450 superfamily consists of at least 57 genes in humans and 102 genes in mice (1). In general, mammalian cytochrome P450s in families 1–4 metabolize foreign compounds (xenobiotics), although they also frequently metabolize endogenous molecules, such as steroids and fatty acids. Families 1–4 are often also inducible by xenobiotics. Members of family 1 are inducible by the potent tumor promoter, 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin), and carcinogenic polycyclic aromatic hydrocarbons via the aryl hydrocarbon receptor (AHR) (2). Rylander et al. (3) identified human CYP2S1 by performing a homology search in a sequence data base, whereas we cloned mouse Cyp2s1 as a dioxin-inducible transcript in the mouse hepatoma cell line, Hepa-1, in which cells it is maximally induced about 10-fold (4). CYP2S1 is unusual for a non-CYP1 family member in being inducible by dioxin (although another Cyp2 family member, Cyp2a5, has recently been shown also to be dioxin-inducible (5)). Human CYP2S1 has also been shown to be inducible in skin by coal tar, which contains high concentrations of polycyclic aromatic hydrocarbon ligands for AHR. Human CYP2S1 can convert all-trans-retinoic acid to the catabolic products, 4-hydroxyretinoic acid and 5,6-epoxyretinoic acid (6), and it has been reported that the enzyme can metabolize naphthalene to two products (7). Both mouse and human CYP2S1 are expressed robustly in most epithelial surfaces and tissues, including the lung and intestinal tract and at all stages of embryogenesis (3, 8–10).

The facts that all other previously well characterized dioxin-inducible cytochrome P450s (CYP1A1, CYP1A2, and CYP1B1) contribute significantly to the activation of a variety of procarcinogens in mammals, that CYP2S1 is highly expressed in epithelial tissues exposed to the environment, that its expression pattern is similar to that of the procarcinogen-metabolizing enzyme CYP1B1 (8, 11), and that the Cyp2s1 gene is located in a cluster with other CYP2 family members that metabolize xenobiotics, including procarcinogens, suggest that Cyp2s1 may play a role in metabolic activation or deactivation of procarcinogens present in the environment. The enzyme may also play a role in the metabolism of endogenous compounds (such as retinoic acid) involved in the differentiation and/or mainte-

6 The abbreviations used are: RACE, rapid amplification of cDNA ends; AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; XRE, xenobiotic-responsive element; HIF-1, hypoxia-inducible factor-1; HRE, hypoxia response element; EMSA, electrophoretic mobility shift assay; CREB, cAMP-responsive element-binding protein.
nance of epithelial issues and conceivably in the metabolism of pharmaceutical drugs. Characterization of the regulation of Cyp2s1, which is the objective of this paper, is therefore an important goal.

Dioxin and polycyclic aromatic hydrocarbon induction of gene transcription is mediated by AHR. After binding ligand, AHR translocates into the nucleus and dimerizes with the related aryl hydrocarbon nuclear translocator (ARNT) protein. This dimer then binds to regulatory elements flanking these genes, thereby up-regulating their rates of transcription. The principal regulatory element so involved is the xenobiotic-responsive element (XRE). The core consensus sequence for a functional XRE is 5'-CACGNC(A/C)-3'. Nucleotides flanking the core XRE sequence can affect the functional efficiency of the XRE, and some of these appear to make contact with the AHR/ARNT dimer (12).

Cells and the whole organism exhibit an adaptive response to low oxygen tension (hypoxia), depending in part on increases in mRNAs for a number of genes involved in glucose uptake and metabolism, angiogenesis, and cell survival. Transcriptional up-regulation is mediated principally by hypoxia-inducible factor 1 (HIF-1), which is a dimer of HIF-1α and ARNT (HIF-1β). Under hypoxia, the HIF-1α/ARNT dimer binds to hypoxia response elements (HREs) located in the regulatory region of responsive genes, thereby stimulating their transcription. The core sequence of the HRE, 5'-CAGGG(T/C)-3', resembles that of the XRE (13). ARNT, which binds the 5'-CAC-3' half-site of the XRE (14, 15), also presumably binds this sequence in the HRE. During normoxia, the hypoxic response is negated by a number of mechanisms. These include, but are not limited to, oxygen-dependent association of the Von Hippel-Lindau E3 ubiquitin-protein ligase with HIF-1α and the oxygen-dependent dissociation of the AHR/ARNT dimer (12).

We provide evidence here that dioxin induction of mouse Cyp2s1 is mediated in large part by a novel complex regulatory element, consisting of three overlapping XREs, located at ~400 nucleotides upstream of the Cyp2s1 translational start site. Each of the component XREs of the trimeric XRE has the capacity to bind the AHR/ARNT dimer and mediate dioxin-dependent transcription. In addition, we show that Cyp2s1 is inducible by hypoxia, and induction is mediated in part by three overlapping HREs contained within the trimeric XRE segment.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—The HepG2 (human hepatocellular carcinoma) cell line was obtained from the American Type Culture Collection (Manassas, VA). The c4 cell line is an ARNT-deficient mutant strain of the mouse hepatoma cell line Hepa1c1c7 (Hepa-1) previously isolated in this laboratory (18, 19). Cell lines were cultured in nucleoside-free α-minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum (Omega, Tarzana, CA), 100 units/ml penicillin, 100 μg/ml streptomycin (Gemini Bio-Products, Woodland, CA), and 2.5 μg/ml amphotericin (Omega). Dioxin (Wellington Laboratories, Guelph, Canada) dissolved in Me2SO (final concentration 0.01% or 0.1%) was used at 10 nM (Hepa-1 cells) or 100 nM (HepG2 cells). The hypoxic treatment was performed in a hypoxia tissue culture incubator (Forma) flushed with a gas mixture containing 1% oxygen, 5% carbon dioxide, and 94% nitrogen. The cells were exposed to hypoxia at ~60% confluence.

**Antibodies**—The affinity-purified rabbit antibodies to AHR and ARNT have been described previously (20). The HIF-1α polyclonal rabbit antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An affinity-purified polyclonal antibody to the Cyp2s1 peptide sequence Cys-REGELIQAE, corresponding to amino acids 144–152 in mouse Cyp2s1 and amino acids 143–152 in human CYP2S1 was generated. The peptide was synthesized and high pressure liquid chromatography-purified by the UCLA Peptide Synthesis Core Facility. The peptide was conjugated to keyhole limpet hemocyanin protein by the cysteine in the peptide, which is not native to Cyp2s1. The conjugated protein was mixed with Freund’s adjuvant and injected into rabbits. Affinity-purified primary antibody was purified from the final bleed’s sera by using a Sulfolinium column (made using the above peptide) using the manufacturer’s directions (Pierce).

5'-Rapid Amplification of cDNA Ends (5'-RACE) Analysis—Antisense primers were designed to the 5’ end of the coding region just within the initiating codon (5’-GCAGGCCAGC-AGCAG-3’). The RNA was isolated by using the Fast 2.0 mRNA isolation kit (Invitrogen) as described earlier (4). 5'-RACE was then carried out using the 5’-RACE kit (Promega, Madison, WI) according to the manufacturer’s instructions. The products of the 5’ cDNA reaction were cloned into pTarget (Promega) and transfected into JM109 bacterial cells. Transforms were grown on selective media. Thirty individual colonies were grown in liquid culture, purified using the Mini Plasmid Isolation kit (Qiagen, Valencia, CA), and sequenced.

**Quantitation of mRNAs**—For Northern blot analysis, mRNA was isolated using the Fast Track 2.0 mRNA isolation kit according to the manufacturer’s instructions (Invitrogen). Blotting was performed according to standard protocols. Labeling of the probes was done by random primed [32P]dATP incorporation (Prime-A-Gene Labeling Kit; Promega). A full-length cDNA for mouse Cyp2s1 was used as probe. Quantitation of the expression was done by using the 455SI PhosphorImager (Amersham Biosciences). In some experiments, as indicated, levels of specific mRNAs were measured by Taqman quantitative real time PCR. Cells were grown in 15-cm dishes (for isolating RNA for Northern blot analysis) or 60-mm diameter dishes (for Taqman analysis) and treated with hypoxia and or dioxin, as indicated. Total RNA was isolated using Triazol (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was done using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. PCR was performed on the iCycler thermal cycler (Bio-Rad). The primer and probe sequences were as follows: mouse Cyp2s1 probe sequence, 5’-FAM-GGCACAGGAGAAGAACAGACC-CAGG-3’ (where FAM is 6-carboxyfluorescein); 5’ primer sequence, 5’-TTGACGCTTCTCTGCTAAAG-3’; 3’ primer...
sequence, 5′-GCAAGTTTCTTCGGTGAATTCT-3′; mouse 36B4 probe sequence, 5′-FAM-CCTCCAGGGAGGGAGG-3′; 5′ primer sequence, 5′-AGATGCAGCCAGATCCGGCAT-3′; 3′ primer sequence, 5′-GTTCCTGCCCACTCGACC-3′.

Western Blot Analysis—HeLa-1 or c4 (ARNT-deficient) cells were treated for 1 or 2 days (as indicated) with Me2SO (vehicle), assayed with the Bio-Rad 5000-0006 protein assay) was reduced by boiling in the presence of 0.5% β-mercaptoethanol, followed by separation using SDS-PAGE through a 10% polyacrylamide gel. Proteins were immobilized onto Immobilon-P Western membrane (Millipore, Bedford, MA) and probed with the ECL luminescence kit (Amersham Biosciences) and BioMax MR film (Eastman Kodak Co.).

Generation of Cyp2s1 Promoter Luciferase Reporter Constructs—The coding regions for mouse Cyp2s1 were used to search the mouse genomic DNA data bases at the NCBI. A bacterial artificial chromosome (BAC Clone 216016, Research Genetics (RESGEN, Huntsville, AL), catalog number RPCI-23C) was obtained that contained several kilobases of 5′-flanking genomic sequence from mouse Cyp2s1. Using this bacterial artificial chromosome clone as a template, a 5.35-kb fragment was amplified with primers 5′-AGCACGGGCTCGAGGCGG-GAATTCTAGGATGGAGGAGG-3′ and 5′-AATGATACGTTTGGGGGCTCT-3′, and pelletted by centrifugation at 200 × g for 2 min at 4 °C. The pellets were then suspended in SDS-PAGE sample buffer (2% SDS, 0.1 M Tris-HCl, pH 6.8, 5 mM EDTA, 9% glycerol, 5% bromphenol blue, nonreducing), vortexed, and sonicated for 1-3 s intervals. Thirty-six μg of each sample (assayed with the Bio-Rad 5000-0006 protein assay) was used at 1:2000 dilution. Proteins were detected with the ECL luminescence kit (Amersham Biosciences) and BioMax MR film (Eastman Kodak Co.).

Generation of Mutants of the Trimeric XRE Sequence on Mouse Cyp2s1 by Site-directed Mutagenesis—We used PCR to generate three guanine to thymine substitutions within the core XRE sequence (i.e. 5′-CACCAG(C/A)-3′ to 5′-CCTCAG(C/A)-3′), individually and in combination, in order to destroy one or more of the putative xenobiotic-responsive elements within the mouse trimeric XRE segment (see Fig. 2A). Sense primers (3′) containing the base pair substitution(s) and the HindIII restriction site at their 5′ end were paired with the antisense primer used in all constructs, which was located just 3′ to the initiating ATG (5′-CTTATGTTTTTGGGCTCTTCCA-3′). The template in these reactions was the 422-bp construct mentioned earlier. PCR primer sequences were as follows: mutant 1, 5′-(AAGTAAAGCTTGGGAGACACCTCATACTGCACGC-3′); mutant 2, 5′-(AAGTAAAGCTTGGGAGACACCTCATACTGCACGC-3′); mutant 3, 5′-(AAGTAAAGCTTGGGAGACACCTCATACTGCACGC-3′); mutant 1 + 2, 5′-(AAGTAAAGCTTGGGAGACACCTCATACTGCACGC-3′); mutant 1 + 2 + 3, 5′-(AAGTAAAGCTTGGGAGACACCTCATACTGCACGC-3′); mutant 1 + 3, 5′-(AAGTAAAGCTTGGGAGACACCTCATACTGCACGC-3′); mutant 1 + 2 + 3, 5′-(AAGTAAAGCTTGGGAGACACCTCATACTGCACGC-3′). The PCR products so generated were cut with HindIII restriction site at their 5′ end were paired with the antisense primer used in all constructs, which was located just 3′ to the initiating ATG (5′-CTTATGTTTTTGGGCTCTTCCA-3′). The template in these reactions was the 422-bp construct mentioned earlier. PCR primer sequences were as follows: mutant 1, 5′-(AAGTAAAGCTTGGGAGACACCTCATACTGCACGC-3′); mutant 2, 5′-(AAGTAAAGCTTGGGAGACACCTCATACTGCACGC-3′); mutant 3, 5′-(AAGTAAAGCTTGGGAGACACCTCATACTGCACGC-3′); mutant 1 + 2, 5′-(AAGTAAAGCTTGGGAGACACCTCATACTGCACGC-3′); mutant 1 + 2 + 3, 5′-(AAGTAAAGCTTGGGAGACACCTCATACTGCACGC-3′). The PCR products so generated were cut with HindIII restriction site at their 5′ end were paired with the reverse primer used in all constructs, which was located just 3′ to the initiating ATG (5′-CTTATGTTTTTGGGCTCTTCCA-3′). The template in these reactions was the 422-bp construct mentioned earlier.
ACGACGACACCC-3'; mutant 6, 5'-AAGTAAGCTT-5'-GGGACAGGACCCACCC-3'; mutant 4 + 6, 5'-AAGTAAGCTTGGGAGACAACCTCTTGTGGACGCAACC-3'; mutant 5, 5'-AAGTAAGCTTGGGAGAACACCACCTGGCGACGACACC-3'. The PCR products generated were ligated into the pGL3-promoter-vector (Promega) digested with Cyp2s1 XRE sequences were synthesized, annealed, and sequenced. Constructs were sequenced.

Generation of pGL3-SV40 Promoter-4xm2S1XRE Reporter Constructs—The sequences corresponding the sense and antisense strands of four copies of wild type and mutated trimeric Cyp2s1 XRE sequences were synthesized, annealed, and inserted into pGL3-promoter-vector (Promega) digested with Nhel and BglII.

Luciferase Activity Assay—The constructs were transfected into either Hepa-1 or HepG2 cells growing at ~50% confluence in 12-well plates. Transfection of 0.5–1.0 µg of pGL3Basic reporter plasmid, amplified in E. coli, was assayed in a luminometer for firefly and Renilla luciferase activity. Nuclear extracts of dioxin-stimulated, reactions were incubated at 0 °C for 10 min in the presence of serum-free medium. Stoichiometric equivalency of the luciferase and used as a probe. Nuclear extracts of dioxin-stimulated, reactions were incubated at 0 °C for 10 min in the presence of serum-free medium. Stoichiometric equivalency of the luciferase was determined by the manufacturer’s instructions in the presence of serum-free medium. Stoichiometric equivalency of the various Cyp2s1/Luc constructs was maintained while holding the total DNA quantity steady, through the addition of the vector pBluescript-SK (lacking eukaryotic promoters) to the transfection complexes. Three h later, medium volumes were doubled with 2× serum-containing medium. Twenty-four h later, cells were exposed to 10 or 100 nM dioxin (Hepa-1 and HepG2, respectively) and/or 1% O₂ for 18 h. Cells were lysed with passive lysis buffer (Promega), scraped, and transferred to 1.5-ml tubes. Lysates were cleared by centrifugation at 10,000 × g for 1 min and transferred to fresh 1.5-ml tubes. The lysates were assayed in a luminometer for firefly and Renilla luciferase activity sequentially using the dual luciferase reporter assay system (Promega) and the BG-1 Luminometer (GEM Biomedical, Inc., Cambridge, UK).

Electromobility Shift Assay—Double-stranded oligonucleotides containing the wild type trimeric XRE sequence from the mouse Cyp2s1 gene, 5'-GATCCACATGCACGCACGCACGCACACACACGACACAG-3', and containing an XRE sequence from the mouse Cyp1a1 gene, 5'-GATCCGCTTCTTCTCAGGCAACTCCACACC-3', were labeled with [γ-32P]ATP by T4 polynucleotide kinase and used as a probe. Nuclear extracts of dioxin-stimulated (60 min) Hepa-1 or HepG2 cells were prepared as described (21). 10 µg each of the nuclear extracts were preincubated in a buffer containing 20 mM Hepes, pH 7.9, 1 mM dithiothreitol, 1 mM MgCl₂, 40 mM KCl, 5% glycerol, and 1.5 µg of poly(dI-dC) in a total reaction volume of 15 µl. Where indicated, reactions were incubated at 0 °C for 10 min in the presence or absence of 1 µl of antibody for ARNT. 1.6 × 10⁶ cpm of probe was added with or without a 200-fold excess of unlabeled double-stranded oligonucleotides harboring the wild type 3× XRE sequence or one of the various mutated sequence derivatives. The reaction mixtures were incubated for 20 min at room temperature and then subjected to electrophoresis in a nondeaturing 4.5% polyacrylamide gel at 4 °C. To detect HIF-1 binding to this sequence, an electromobility shift assay was performed as described above except for using a buffer containing 10 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 1 mM MgCl₂, 50 mM KCl, 50 mM NaCl, 5% glycerol, and 0.1 µg of sonicated salmon sperm DNA in a total reaction volume of 20 µl. Nuclear extracts were prepared from Hepa-1 or HepG2 cells treated by 1% O₂ for 4 h. For co-treatment with dioxin and hypoxia, the cells were cultured under 1% O₂ for 4 h, and the dioxin (10 nM for Hepa-1 and 100 nM for HepG2, respectively) was added 1 h before the end of hypoxia treatment. An antibody for HIF-1α purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) was used for the supershift assay. A double-stranded oligonucleotide containing a HRE sequence from the human EPO gene, 5'-GATCGGCTCTTCTCACGCAAC-3', was used as a positive control probe.

Inhibition of mRNA Synthesis Experiments—Hepa-1 cells were seeded at 1.8 × 10⁶ cells in a 60-mm dish. The following day, the cells were treated with 5 µg/ml actinomycin D (Calbiochem) for 30 min to inhibit endogenous gene transcription or left untreated. Cells were then treated with 10 nM dioxin, 1% O₂, or Me₂SO vehicle for 10 h in the presence or absence of 5 µg/ml actinomycin D. Total RNA was isolated from the cells by the Qiagen RNEasy mini kit according to the manufacturer’s protocol. Reverse transcription was performed on 2 µg of RNA using SuperScript III RNase H Reverse Transcriptase (Invitrogen). SYBR-green real time PCRs were performed using the Bio-Rad iQ supermix reagent according to the manufacturer’s protocol. Primers to amplify the Cyp2s1 and the 36B4 genes were described earlier. Assays were run on the Fast 7500 thermalcycler (Applied Biosystems).

Chromatin Immunoprecipitation—Hepa-1 and c4 cells were grown in α-minimal essential medium supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, and 1% Fungizone (Invitrogen). Cells were seeded in 100-cm tissue culture dishes so that they would be 85% confluent the next day. The following day, cells were treated with 10 nM dioxin, 1% O₂, or Me₂SO vehicle at 21% O₂, or 10 nM dioxin plus 1% O₂ for 1 or 3 h. Cross-linking was achieved by the addition of 1% formaldehyde at 37 °C for 10 min. Cells were rinsed with ice-cold phosphate-buffered saline and collected in 1 ml of ice-cold phosphate-buffered saline plus protease inhibitor solution (Roche Applied Science). Cells were pelleted at 600 × g for 5.5 min at 4 °C, resuspended in 0.25 ml of SDS cell lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS, and Roche Complete protease inhibitor mixture), and incubated on ice for 10 min. Cell lysates were sonicated twice at high power for 8 min, alternating between 30 s on and 30 s off using a Bioruptor cell sonicator (Diagenode Inc., New York, NY) to shear DNA fragments to sizes between 200 and 900 base pairs. Cellular debris was eliminated by centrifugation for 10 min at 13,000 rpm at 4 °C. The supernatant was then diluted 1:10 in 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.1), 1× protease inhibitor solution. Immunoprecipitation was achieved by the addition of 40 µl of a 50% slurry of protein A-agarose beads in Tris-EDTA, 2.5 µg sonicated salmon sperm DNA, bovine serum albumin solution (Upstate Biotechnology) and incubated on a rotator at 4 °C for 1 h. Supernatants were collected and placed in a new tube. The solutions were then treated with 2 µg of antibodies directed against the AHR, ARNT, or HIF-1α proteins overnight on a rotator at 4 °C. The solutions were then...
Regulation of Cytochrome P4502S1 by Dioxin and Hypoxia

RESULTS

Identification of the Transcriptional Start Sites of Mouse Cyp2s1—We used 5′-RACE to amplify the 5′ ends of Cyp2s1 mRNA. As a source of mRNA, we used Hepa-1 cells stimulated with 10 nM dioxin for 12 h. Three clusters of transcriptional start sites, positioned at about 198, 102, and 22 nucleotides upstream of the translational initiation codon, were observed (Table 1). The existence of multiple start sites is consistent with the absence of an identifiable TATA box in the Cyp2s1 promoter.

Localization of Functional Xenobiotic-responsive Elements in the Cyp2s1 5′-Upstream Region—Six elements corresponding to the core XRE sequence (5′-CACGCN(A/C)-3′) occur within 5344 bases 5′ to the translation start site of Cyp2s1. Three overlapping XREs occur between −393 and −408, and there are individual core XREs at −726, −768, and −5163. We inserted various lengths of the Cyp2s1 5′-upstream region into the luciferase reporter plasmid, pGL3Basic (which contains an enhancerless and promoterless firefly luciferase gene) in order to identify elements that respond to dioxin. All fragments started 1 nucleotide 5′ to the translational initiation codon. Equimolar concentrations of each construct were co-transfected along with the pRL-TK Renilla luciferase vector into the mouse hepatoma cell line, Hepa-1, or the human hepatoma cell line, HepG2. One day later, cells were treated with 10 nM dioxin or Me2SO vehicle and then assayed for firefly and Renilla luciferase activities. The ratio of the firefly to Renilla luciferase activity was calculated for each construct. Data for representative experiments are shown and are presented as the degree to which the above ratio was increased by dioxin treatment.

| Length of 5′-RACE product | Number of independent clones |
|---------------------------|------------------------------|
| 198                       | 3                            |
| 197                       | 2                            |
| 102                       | 9                            |
| 100                       | 1                            |
| 25                        | 1                            |
| 22                        | 1                            |
| 20                        | 1                            |

*a Number of nucleotides upstream of the translational initiation codon.

A.

![Figure 1](image1.png)

B.

![Figure 2](image2.png)
Regulation of Cytochrome P4502S1 by Dioxin and Hypoxia

A. 5'-CACATGCCACGCACGCACGCACACC-3'
Core XREs
Core HREs

B. 

C. m2s1XRE: 5'-CACATGCACGCACGCACGCACACCAG-3'
SV40 Promoter
Lac

D. 

FIGURE 2. Dioxin inducibility of derivatives of the mouse 0.422-kb pGL3Basic construct in which the trimeric XRE segment contains one or multiple inactivating mutations in its individual XREs. A, mutations generated in the trimeric XRE segment of mouse Cyp2s1. The core sequences of the three overlapping core XRE sequences and three overlapping HRE are underlined. Only one DNA strand is shown. The numbering of the mutations introduced into this sequence is also shown. B, the indicated derivatives of pGL3Basic vector containing 422 bp from the 5' region of Cyp2s1 were transfected into HepG2 cells. For each construct, the activity was calculated as the ratio of firefly luciferase to Renilla luciferase activity. The results are presented as the averages and S.D. values from three independent experiments. C, structure of the pGL3-SV40 promoter-4xm2S1XRE reporter plasmid. D, reporter gene analysis of pGL3-SV40 promoter-4xm2s1XRE and its mutant derivatives. The indicated constructs were transfected into HepG2 cells, treated with 100 nM dioxin or vehicle (Me2SO), and then analyzed for luciferase activity. For each construct, the activity was calculated as the rates of firefly luciferase to Renilla luciferase activity. The results are presented as the means and S.D. values from three independent experiments.

Dioxin inducibility of the responsive constructs was much greater in HepG2 than in Hepa-1 cells. A representative experiment using HepG2 cells is shown in Fig. 1B. The 2.0-, 1.5-, and 0.422-kb constructs were inducible by dioxin. The 0.35-kb construct exhibited similar inducibility as the 0.422-, 1.5-, and 2.0-kb constructs (data not shown). However, no induction was exhibited by the 387-bp construct, consistent with the notion that inducibility is conferred by the segment between 422 and 387 bp, encompassing the three overlapping XREs. Once again, induction of the longer constructs was less than that of the pGL3 construct containing 5.2 kb of the upstream region of the rat Cyp1a1 gene.

Analysis of the Contribution of Each of the Three Overlapping XREs to Dioxin Inducibility—We mutated each of the overlapping XREs in the pGL3 derivative containing the 422-bp fragment of Cyp2s1, individually, in all possible pairwise combinations, and altogether (Fig. 2A). This was achieved by changing the indicated guanine (') to thymine in each XRE core sequence, 5'-CACG'CN(A/C)-3'. This substitution is known to totally inactivate the XRE (23). Each substitution changed one or two flanking nucleotides of the neighboring XRE(s) in the triple XRE segment. However, these changes, from guanine to thymine at the indicated positions, 5'-G'ACACGCACG'-3', have little if any effect on the functionality of XREs in the mouse Cyp1a1 gene (12). We chose to test the dioxin inducibility of these constructs in HepG2 cells, because inducibility of the parental 422-bp construct was much greater in these cells than in the mouse Hepa-1 cells. The average results and their S.D. values from three independent experiments are presented in Fig. 2B. These results are presented as the ratio of the firefly luciferase activity (ascribed to the Cyp2s1 constructs) to the Renilla luciferase activity (ascribed to the pRL-CMV-Renilla luciferase control vector) for each transfection. Some dioxin induction occurred in the parental vector, pGL3-Basic, perhaps due to the presence of the two core XRE sequences identifiable in the vector. Compared with this background activity in pGL3-Basic, inactivation of each XRE individually diminished but did not eliminate dioxin induction. Inactivation of two XREs simultaneously either greatly reduced induction or appeared to eliminate it. Simultaneous inactivation of all three XREs eliminated induction.

In order to further investigate the role of the individual XRE components of the triple XRE segment, we inserted four copies of this segment upstream of the firefly luciferase gene driven by the SV40 promoter (Fig. 2C). Equivalent constructs containing each of the individual XRE-inactivating mutations in each of the four copies of the segment, and all combinations of these mutations were also constructed. These constructs were then transfected (along with the pRL-CMV Renilla luciferase control vector) into HepG2 cells, and luciferase activities were determined. The means and S.D. values from three independ-
ent experiments are shown in Fig. 2D. The results were similar to these obtained with the mutants generated in the 0.422-kb fragment (i.e. induction was reduced when individual XREs were mutated, even further reduced when two XREs were mutated, and eliminated when all three XREs were mutated) (Fig. 2D).

We next investigated the role of each of the three XREs in AHR/ARNT binding by electromobility shift analysis (EMSA). A short double-stranded oligonucleotide corresponding to the segment containing the three overlapping XREs was used as radioactive probe in these experiments. A shifted band was observed using nuclear extracts from both Hepa-1 and HepG2 cells treated with dioxin but not with extracts from untreated cells (Figs. 3, A and B, lanes 2 and 1, respectively). This band was eliminated by competition with a 200-fold excess of a double-stranded oligonucleotide probe corresponding to an XRE from the mouse Cyp1a1 gene (lane 11). The band migrated in the same position as the band generated with the 32P-labeled Cyp1a1 oligonucleotide (lane 13) and was supershifted with antibodies to ARNT (lane 3, and see also lane 14). These observations identify the band as corresponding to an AHR-ARNT-XRE complex. Double-stranded oligonucleotides containing the single, double, or triple XRE mutations used for the reporter gene assays above were then used as competitors in the electromobility shift assay at 200-fold higher concentrations than the radiolabeled wild-type probe. The triple mutant oligonucleotide failed to compete with the wild-type probe for binding to the AHR/ARNT dimer, whereas all of the other mutants exhibited competition.

The results obtained in the reporter assays and EMSAs with the mutant in which all three XREs are inactivated were thus consistent with one another in that simultaneous mutation of all XREs eliminated the response to dioxin and also eliminated binding to the AHR/ARNT dimer. The results of both types of assay with the individual XRE mutants are also consistent with each other, since these mutants only slightly reduced dioxin induction and were able to compete with the wild-type sequence for binding to the AHR/ARNT dimer. The double mutants (1 + 2, 2 + 3, and 1 + 3), although exhibiting similar minimal induction in the reporter assays as the pGL3 basic vector, did not exhibit as dramatically curtailed induction as the triple mutant, 1 + 2 + 3, indicating that the former mutants may in fact retain some responsiveness to dioxin. This interpretation is compatible with the observation that at a 200-fold excess relative to the labeled wild-type sequence, these sequences competed effectively with regard to AHR/ARNT binding to the triple XRE sequence in EMSA, indicating that they can bind the triple XRE sequence, although possibly with reduced affinity. In summary, the reporter gene and electrophoretic gel mobility shift assays clearly demonstrate that each XRE in the trimeric XRE segment is capable of binding the AHR/ARNT dimer and mediating dioxin-dependent transcription.

Modification of the Flanking Sequences of the XREs in the Trimeric XRE in an Attempt to Optimize Response to Dioxin—The identity of the nucleotides flanking the core sequence can affect the functionality of the XRE. The flanking sequence of the three XREs in the trimeric XRE segment conforms to the optimal sequence for function, except at the nucleotide at the 3′-position upstream of the core sequence (11). In each XRE in the trimeric segment, this is an adenine (5′-A(C/T)GCACG-CAC-3′). Previous reporter gene studies have concluded that this nucleotide needs to be a thymine in order for the XRE to respond to dioxin in Hepa-1 cells (24, 25). However, in similar studies, it was concluded that XREs with an adenine in this position are functional in another mouse hepatoma cell line and in HepG2 cells (26). We wished to investigate this issue and, in particular, to address whether the relatively low dioxin inducibility of Cyp2s1 in Hepa-1 cells is caused by the presence of adenines at this position. We therefore generated several derivatives of the 422-bp pGL3-Basic construct in which the above adenine was converted to thymine in individual XREs or two XREs at a time, as indicated (Fig. 2A). In Mutant 4, the 3′-position of the most 5′ XRE was converted from adenine to thymine. This change does not affect the extended sequence of either of the other two XREs or the core sequence of any of the XREs, and the response of this construct to dioxin should therefore be compared with the wild-type sequence. In Mutant 5, the sequence of the middle XRE is “optimized,” whereas the 5′
Regulation of Cytochrome P4502S1 by Dioxin and Hypoxia

The effect of changing adenines located 3 nucleotides upstream of the core XRE sequences in the trimeric XRE segment to thymines on the transcriptional response to dioxin. The mutant derivatives of the mouse 422-bp pGL3Basic construct used in this experiment are illustrated in Fig. 2A. Each construct was co-transfected with the pRL-CMV Renilla control vector into Hepa-1 cells, which were treated with 10 nM dioxin or Me2SO vehicle and subsequently analyzed for luciferase activities. The results are presented as the ratio of the firefly luciferase activity to Renilla luciferase activity for each transfection. The means and S.D. values from three separate experiments are shown.

A.

B.

FIGURE 5. Hypoxic induction of Cyp2s1. A, Hepa-1 cells were treated for various times with 1% O2, and then analyzed for Cyp2s1 mRNA by Northern blot analysis. B, hypoxic induction of the Cyp2s1 protein. Hepa-1 or its ARNT-deficient mutant derivative, c4, was treated for 48 h with dioxin and/or 1% O2 and then analyzed for the Cyp2s1 protein by Western blot analysis.

XRE is inactivated. The response of this mutant should therefore be compared with Mutant 1, in which the 5′ XRE is inactivated. In Mutant 6, the sequence of the most 3′ XRE is “optimized,” whereas the middle XRE is inactivated. In Mutant 4 + 6, both the 5′ and 3′ XREs are “optimized,” whereas the middle XRE is inactivated. The behaviors of Mutants 6 and 4 + 6 should therefore be compared with that of mutant 2 (in which the middle XRE is inactivated). The above derivatives were tested for dioxin inducibility. The means and S.D. values from three independent experiments are presented in Fig. 4. Conversion of adenine to thymine in the XREs was found to have no discernable effect on dioxin inducibility. This supports the notion that XREs with adenine at this position respond to dioxin as efficiently as XREs containing thymine at this position in Hepa-1 cells and are at variance with the conclusions made in Refs. 24 and 25. Our results, moreover, failed to provide an explanation for the relatively poor dioxin inducibility of Cyp2s1 in Hepa-1 cells.

Hypoxic Induction of Cyp2s1—We found that treatment of Hepa-1 cells with 1% O2 lead to a marked increase in Cyp2s1 mRNA (Fig. 5A). Twelve-fold induction occurred after 18 h of hypoxia treatment. Hypoxia also induced the Cyp2s1 protein, and induction was approximately additive with that of dioxin (Fig. 5B). Hypoxic (and dioxin) induction of the Cyp2s1 protein did not occur in the ARNT-deficient c4 mutant of Hepa-1 cells, demonstrating that induction depends upon HIF-1. (We previously demonstrated that dioxin induction of Cyp2s1 depends on AHR and ARNT (4)).

Dioxin and Hypoxic Induction of Cyp2s1 Is Dependent on de Novo Cyp2s1 mRNA Synthesis—Hepa-1 cells were pretreated for 30 min with the RNA synthesis inhibitor, actinomycin D, exposed for a further 10 h to 10 nM dioxin, Me2SO vehicle, or 1% O2 in the presence of actinomycin D, and then analyzed for Cyp2s1 mRNA expression. Control cells received dioxin, vehicle, or hypoxia in the absence of actinomycin D. Actinomycin D treatment completely eliminated induction of Cyp2s1 mRNA by both dioxin and hypoxia (Fig. 6). These results indicate that induction of Cyp2s1 by these two agents occurs via de novo synthesis of Cyp2s1 mRNA rather than by Cyp2s1 mRNA stabilization, at least during early times after treatment. More extensive studies would be required to exclude the possibility that mRNA stabilization contributes to induction after longer times of treatment.

Mechanism of Hypoxic Induction of Cyp2s1—A number of HRE core sequences, 5′-CACG(T/C)-3′, were identified within 5344 bases 5′ to the translational start site of Cyp2s1. We used a subset of the same deletion constructs that we had used to identify the regions of the gene responsive to dioxin, to search for elements responsive to hypoxia. The averages and S.D. values from three separate experiments are presented in Fig. 7A. These results demonstrate that the 5344-bp 5′ segment confers a high degree of hypoxia inducibility. Inducibility was nearly as great in the 422-bp construct but was nearly eliminated in the 249-bp construct, indicating that the hypoxia responsiveness in the 5344-bp upstream region is ascribable to a considerable degree to a segment between 422 and 249 bp. Interestingly, hypoxia induction in the 1.5 and 2 kb constructs was less than that in either the 5 kb or 422 bp constructs, suggesting that an element(s) negatively affecting hypoxia induction may reside in the region between 2 kb and 422 bp.
The region between 422 and 249 bp contains the trimeric XRE sequences. We investigated whether this segment confers hypoxia responsiveness, using the 4xm2s1XRE construct (see Fig. 2C). This construct was inducible by hypoxia in both Hepa-1 and HepG2 cells. The means and S.D. values from three independent experiments are presented in Fig. 7, A and B. As with the dioxin response, hypoxia inducibility was considerably greater in the latter cells than in the former cells. Three overlapping HRE core sequences, 5′-CACG(T/C)-3′, are contained within the trimeric XRE sequence. Furthermore, these core elements are all embedded in sequences that are identical to the extended consensus sequence for the HRE, 5′-(C/G/A)(G/C/- A)CACG(T/C)(A/G/C)(C/G/T)-3′ (33), except for the most 5′ HRE, which differs from this consensus at the most 5′ nucleotide. We then examined the hypoxia responses of mutant derivatives, 1, 2, 3, 1 + 2, 2 + 3, 1 + 3, and 1 + 2 + 3 of the 4xm2s1XRE reporter plasmid, since mutations 1, 2, and 3 each inactivate a different core HRE sequence (see Fig. 2A). The results obtained with these constructs in Hepa-1 and HepG2 cells were consistent with each other. The mutation inactivating the 5′ core HRE had little if any effect on the degree of hypoxic induction. The mutation inactivating the central core HRE reduced hypoxic induction only modestly. The inactivating mutation in the 3′ core HRE completely eliminated hypoxic induction. These results indicate that the hypoxic response mediated by this segment is ascribable mostly to the 3′ HRE. In fact, since mutation 2 changes the extended sequence of the most 3′ HRE from that of the consensus, it is possible that the effect of this mutation is due to its deleterious effect on this HRE rather than the middle HRE and that hypoxic induction is totally ascribable to the 3′ HRE. We then performed EMSA with the same triple XRE oligonucleotide that we used for analysis of AHR/ARNT binding. Nuclear extracts from both Hepa-1 and HepG2 cells were used in these experiments and generated similar results (Fig. 8, A and B). A specific retarded band was observed with extracts from hypoxia-treated but not normoxia-treated cells (lanes 1 and 2). This band was either supershifted or eliminated with antibodies to ARNT and HIF-1α (lanes 3 and 4) and was eliminated by inclusion of a 200-fold excess of an unlabeled oligonucleotide corresponding to the bona fide HRE from the human erythropoietin gene (lane 12). Furthermore, a band at this position was generated when extracts from hypoxia-treated cells but not from normoxia-treated cells were incubated with the 32P-labeled erythropoietin HRE oligonucleotide, and this band was supershifted or eliminated with antibodies to HIF-1α (lanes 16–18). These observations all identify this band as corresponding to the HIF-1α/ARNT-HRE complex. The intensity of this band was greater with HepG2 extracts than with Hepa-1 extracts, which correlates with the greater hypoxic induction of the 4xm2s1XRE-luciferase construct in the former cell line. The HIF-1α/ARNT band was eliminated when a 200-fold excess (relative to the 32P-labeled m2s1XRE probe) of unlabeled oligonucleotides corresponding to the same probe but containing mutations 1, 2, 3, 1 + 2, 1 + 3, or 2 + 3 were included in the incubation mixture (lanes 6–10). However, the band was not eliminated when a 200-fold excess of unlabeled mutant 1 + 2 + 3 probe was used (lane 11). This indicates that each HRE in the trimeric XRE segment is capable of binding HIF-1α/ARNT in vitro and that all have to be inactivated to eliminate binding. This contrasts with the results from the reporter gene assays, which indicated that the 5′ and middle HRE confer little if any hypoxia inducibility. One possible explanation for this discrepancy is that the 5′ and middle HREs bind the HIF-1α/ARNT dimer but with less affinity than the 3′ HRE and with insufficient affinity to drive transcription of the reporter gene.

Despite the above discrepancy, the results of the reporter gene and EMSA experiments support the notion that hypoxic induction of Cyp2s1 is mediated to a considerable degree by the HREs embedded within the trimeric XRE sequence and that the 3′ HRE is the most important in this regard. Finally, it is of interest that the binding of the HIF-1α/ARNT dimer to the trimeric XRE apparently occurs in preference to binding of the AHR/ARNT dimer with nuclear extracts prepared from cells treated with both dioxin and hypoxia (lanes 13–15), suggesting that the HIF-1α/ARNT dimer has greater affinity for the trimeric XRE (HRE) sequence than the AHR/ARNT dimer.

Dioxin-dependent Binding of AHR and Hypoxia-dependent Binding of ARNT to the Trimeric XRE Region in Vivo—We performed chromatin immunoprecipitation analysis as a means of demonstrating binding of the AHR/ARNT and HIF-1α/ARNT dimers to the Cyp2s1 gene in living Hepa-1 cells. Fifty minutes of dioxin treatment led to recruitment of AHR and ARNT to the Cyp2s1 gene, whereas no such recruitment occurred in c4, an ARNT-deficient mutant derivative of the Hepa-1 cell line. Fifty minutes of hypoxia treatment led to ARNT recruitment to the Cyp2s1 gene in Hepa-1 but not c4 cells. Simultaneous treatment with dioxin and hypoxia led to recruitment of both AHR and ARNT. (Etidium bromide-stained gels are shown in Fig. 9A, and results of real time PCR analysis of the same experiment are shown in Fig. 9B.) Since the hypoxia-dependent recruitment of ARNT to Cyp2s1 appeared not to be particularly robust after 50 min of hypoxia treatment (perhaps due to a significant period of time being required for the cells to experience hypoxia after the culture dishes were transferred to the atmosphere containing 1% O2), we also tested longer times of exposure to hypoxia and dioxin (Fig. 9, C and D). In this experiment, we analyzed recruitment of HIF-1α rather than ARNT.
FIGURE 7. Mechanisms of hypoxic induction of Cyp2s1. A, constructs containing the indicated segments from the 5’-flanking region of mouse Cyp2s1 cloned into pGL3Basic were co-transfected with the pRL-CMV Renilla control vector into Hepa-1 cells. One day later, the cells were treated for 4 h with 1% O2 or normoxia and then assayed for firefly and Renilla luciferase activities. The ratio of the firefly to Renilla luciferase activity was calculated for each construct. Data are presented as the ratio of firefly luciferase activity to Renilla luciferase activity for each construct and represent the mean values and S.D. values from three independent experiments. Shown is reporter gene analysis of pGL3-Promoter-4xm2s1XRE and its mutant derivatives exposed to hypoxia. The indicated constructs were transfected into Hepa-1 (B) or HepG2 (C) cells, treated with 1% hypoxia or normoxia for 24 h, and then analyzed for luciferase activities. For each construct, the activity was calculated as the ratios of firefly luciferase to Renilla luciferase activity. The results are presented as the averages and S.D. values from three independent experiments.
Dioxin induced recruitment of AHR, and maximal levels were achieved after about 1 h of treatment. In contrast, hypoxia-induced recruitment of HIF-1α was much greater after 3 h than after 1 h. Treatment with both dioxin and hypoxia induced recruitment of both AHR and HIF-1α. Since we used PCR primers flanking the trimeric XRE for amplification in the above experiments, these results indicate that dioxin induces AHR/ARNT recruitment over the trimeric XRE region of the Cyp2s1 gene and that hypoxia induces HIF-1α/ARNT recruitment over this same region. These experiments performed on the endogenous Cyp2s1 gene in its normal chromosomal setting support the results of the reporter gene and EMSA assays in demonstrating a role for the trimeric XRE sequence in both dioxin and hypoxia induction.

**DISCUSSION**

We have identified a compact regulatory segment in the Cyp2s1 gene containing three overlapping XREs and three...
Regulation of Cytochrome P450 2S1 by Dioxin and Hypoxia

overlapping HREs that appears to make a major contribution to both dioxin and hypoxia induction of the gene. As far as we are aware, no segments containing multiple XREs or multiple HREs, let alone segments containing overlapping sets of both elements, have been reported previously for any gene. It is of interest that two overlapping core XREs and two overlapping core HREs are located at an equivalent position upstream of the human CYP2S1 gene. Furthermore, dioxin and hypoxia can induce CYP2S1 in human cells (4).7

Several experimental approaches that have previously been used to investigate the potential roles that nucleotides flanking the XRE and HRE core sequences may play in transcription factor binding or gene transcription, including analysis of mutated XRE and HRE sequences in reporter gene assays and EMSAs (the latter in the presence or absence of methylation interference or protection), selection and amplification of AHR/ARNT binding sites, and ligation-mediated PCR in vivo footprinting, suggest that the AHR/ARNT and HIF-1α (or HIF-2α)/ARNT dimers contact nucleotides flanking the XRE and HRE core sequences, respectively. These investigations indicated that the XRE sequence contacted by the AHR/ARNT dimer may be up to 10 nucleotides in length, whereas that contacted by the HIF-2α/ARNT dimer may comprise eight nucleotides (12, 15, 23, 27). This suggests that although all three XRE elements in a particular trimeric XRE segment are unlikely to be occupied simultaneously by AHR/ARNT dimers, it is possible that the most 5’ and 3’ core XRE elements can be occupied simultaneously. Similarly, it is possible that the most 5’ and 3’ HRE cores can be occupied simultaneously by HIF-1α/ARNT dimers. It is conceivable that there is cooperative recruitment of two separate AHR/ARNT dimers or two separate HIF-1α/ARNT dimers or separate AHR/ARNT and HIF-1α/ARNT dimers to a particular trimeric XRE sequence, thereby augmenting the function of the closely apposed individual XRE and HRE elements in the trimeric segment, and that this may have provided selective pressure for its evolutionary development. Our EMSA analysis indicates that the HIF-1α/ARNT dimer has a greater affinity for the trimeric XRE (HRE) segment in the Cyp2s1 promoter than the AHR/ARNT dimer.

An adenine at the −3-position has been reported to eliminate the dioxin-dependent transcriptional activation potential of an XRE in Hepa-1 cells while having little effect on its binding affinity for the AHR/ARNT dimer (24). However, we found that converting some of these adenines to thymines, thereby generating a putatively “optimal” XRE sequence, had little if any effect on dioxin inducibility. It is possible that the recruitment of closely juxtaposed dioxin-ligated AHR/ARNT dimers on the trimeric XRE sequence allows for transcriptional activation even in the context of adenines located at their −3-positions, perhaps via cooperative recruitment of co-activator proteins.

We discovered that Cyp2s1 is inducible by hypoxia in mouse Hepa-1 cells. Induction depends upon ARNT, indicating that it is mediated by HIF-1. The mechanism(s) of hypoxia induction can differ between different genes. For example, transcriptional induction of the erythropoietin gene (EPO) occurs via media-

7 S. T. Saarikoski, S. P. Rivera, and O. Hankinson, unpublished data.
this enhanced expression of Cyp2s1 could affect the response of tumors to chemotherapeutic agents. The induction of Cyp2s1 by dioxin (and presumably also polycyclic aromatic hydrocarbons) in epithelial tissues exposed to the environment could affect the response of these tissues to any procarcinogens that are substrates for the enzyme. These issues all represent interesting topics for further investigation.

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