Multiple Enhancer Units Mediate Drug Induction of CYP2H1 by Xenobiotic-Sensing Orphan Nuclear Receptor Chicken Xenobiotic Receptor

CHRISTOPH HANDSCHIN, MICHAEL PODVINEC, RENATE LOOSER, REMO AMHERD,[1] and URS A. MEYER

Division of Pharmacology/Neurobiology, Biozentrum of the University of Basel, Basel, Switzerland

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Binding of nuclear receptors to drug-responsive enhancer units mediates transcriptional activation of cytochromes P-450 (P-450) by drugs and xenobiotics. In previous studies, a 264-base-pair (bp) phenobarbital-responsive enhancer unit (PBRU) located at −1671 to −1408 upstream of the chicken CYP2H1 transcriptional start-site increased gene expression when activated by the chicken xenobiotic-sensing orphan nuclear receptor CXR. In extension of these studies, we now have functionally analyzed a second distal drug-responsive element and delimited a 643- and a 240-bp PBRU located between 5 and 6 kilobases upstream of the transcriptional start site of CYP2H1. Both PBRUs were activated by CXR after treatment with different drugs. A nuclear receptor binding site, a direct repeat-4 (DR-4) hexamer repeat, was identified on the 240-bp PBRU. Site-directed mutagenesis of this DR-4 abolished activity in reporter gene assays in the chicken hepatoma cells leghorn male hepatoma as well as transactivation of the 240-bp PBRU by CXR in CV-1 cells. CXR bound to this PBRU in electromobility shift assays and the complex remained unaffected by unlabeled 240-bp PBRU with a mutated DR-4. In cross-species experiments, both the human xenobiotic-sensing nuclear receptors pregnane X receptor and constitutive androstane receptor bound to this element, suggesting sequence conservation between chicken and mammalian PBRUs and between the DNA binding domains of these receptors. Of two orphan nuclear receptors involved in cholesterol and bile acid homeostasis, only chicken liver X receptor (LXR) but not chicken farnesoid X receptor bound to the 240-bp PBRU. These results suggest that CYP2H1 induction is explained by the combined effect of multiple distal enhancer elements interacting with multiple transcription factors, including CXR and LXR.

Numerous xenobiotic and endobiotic substances are metabolized by members of the cytochrome P-450 (P-450) gene superfamily in the liver and to a lesser extent in extrahepatic tissues such as intestine, skin, or brain (Waxman and Azaroff, 1992; Nelson et al., 1996). Drug-metabolizing P-450s mainly belong to the CYP1, CYP2, CYP3, and CYP4 families. A subset of these P-450s can be transcriptionally induced by their own substrates and other compounds (Waxman and Azaroff, 1992). Phenobarbital (PB) represents one of the five prototypical inducer classes; the others are represented by aromatic hydrocarbons (dioxin), glucocorticoids (dexamethasone), peroxisome proliferators (clofibrate), and ethanol. PB and PB-type inducers affect predominantly the transcription of the CYP2B, CYP2C, and CYP3A subfamily genes in addi-

tion to at least 50 other genes in the liver (Waxman and Azaroff, 1992; Frueh et al., 1997; Kemper, 1998). Transcriptional induction of P-450s by PB has been observed in different species such as mammals, birds, and bacteria (Waxman and Azaroff, 1992; Kemper, 1998).

Recently, enhancer regions in the 5′-flanking region of PB-inducible P-450s have been isolated in chicken, rat, mouse, and human, and DNA binding proteins associated with these elements have been identified (for reviews, see Kemper, 1998; Savas et al., 1999; Waxman, 1999; Honkakoski and Negishi, 2000; Zelko and Negishi, 2000). Important mediators of the induction process are transcription factors of the nuclear receptor superfamily, in particular the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) in mammals and the xenobiotic-sensing receptor in chicken (CXR) (Honkakoski et al., 1998; Kliewer et al., 1998; Handschin et al., 2000). Although PXR, CAR, and CXR pre-

ABBREVIATIONS: P450, cytochrome P450; PB, phenobarbital; PXR, pregnane X receptor; CAR, constitutive androstane receptor; CXR, chicken xenobiotic receptor; kb, kilobase(s); bp, base pair(s); PBRU, phenobarbital-responsive enhancer unit; PBREM, phenobarbital-responsive enhancer module; LMH, leghorn male hepatoma; RXR, 9-*cis*-retinoic acid receptor; LXR, liver X receptor; FXR, farnesoid X receptor; PCR, polymerase chain reaction; DR, direct repeat; CAT, chloramphenicol acetyltransferase; ELISA, enzyme-linked immunosorbent assay; DMEM/F-12, Dulbecco's modified Eagle's medium/Ham's F-12; NF-1, nuclear factor-1; USF, upstream stimulatory factor; NR-1/2, nuclear receptor-1/2.

dominantly regulate CYP3As, CYP2Bs, and CYP2Hs, respectively, these receptors have considerable overlap in their inducer profiles and can interchangeably bind to their respective response elements even across species boundaries (Jones et al., 2000; Xie et al., 2000b).

In chicken liver, CYP2Hs are major PB-inducible enzymes that are closely related to mammalian CYP2Cs (Mattschoss et al., 1986). In an initial analysis, a 4.8-kb PB-responsive enhancer fragment was identified (Hahn et al., 1991), which later could be dissected into two enhancer elements with a size of about 1.3 kb and 264 bp, respectively (Dogra et al., 1999; Handschin and Meyer, 2000). The 264-bp PB-responsive enhancer unit (PBRU) is activated by the same transcription factors that activate the rat CYP2B2 163-bp PBRU, the mouse Cyp2b10 51-bp PB-responsive enhancer module (PBREM), and the human CYP2B6 51-bp PBREM (Handschin and Meyer, 2000; Handschin et al., 2000). Nevertheless, the contribution of additional, more distal enhancer elements of CYP2H1 in the induction response and their relatedness to mammalian enhancers remain an open question.

In this study, we functionally analyzed a 1.3-kb fragment located between −5896 and −4528 in the 5′-flanking region of CYP2H1 by reporter gene assays in the chicken hepatoma cell line LMH. We could derive two independent PBRUs within these 1.3 kb. Proteins binding to and activating these PBRUs were identified and our results indicate that the novel PBRUs are closely related to the previously identified CYP2H1 264-bp PBRU (Handschin and Meyer, 2000). Thus, all chicken PB-responsive enhancers discovered so far strongly resemble mammalian PBRUs, allowing a comparison of the sequences of nuclear receptor binding sites and of putative auxiliary transcription factors modulating PB induction to derive a common pattern of all known PBRUs.

Materials and Methods

Reagents. Dexamethasone, 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), 5-pregnen-3β-ol-20-one-16α-carbonitrile, rifampicin, and clotrimazole (1-[o-chlorotrityl]-imidazole) were obtained from Sigma (Buchs, Switzerland). Peter Sinclair (VA Hospital, White River Junction, VT) generously provided propylisopropylacetamide. Glutethimide and β-naphtoflavone were purchased from Aldrich (Buchs, Switzerland). 5-Ethyl-5-phenyl-barbituric acid sodium salt (phenobarbital sodium salt) was obtained from Fluka (Buchs, Switzerland). Poly(dI-dC)∗poly(dI-dC) was from Amersham Pharmacia Biotech (Dübendorf, Switzerland). All other reagents and supplies were obtained from standard sources. Cell culture media, sera, and tissue culture reagents were purchased from Life Technologies (Basel, Switzerland), unless noted otherwise.

Plasmids. The subcloning of the coding regions of chicken CXR, chicken RXRγ, and human CAR into the expression vector pSG5 (Stratagene, Basel, Switzerland) has been described previously (Handschin et al., 2000). The expression vector for human PXR, pSG5-hPXR, was kindly provided by Dr. S. A. Kliewer (Department of Molecular Endocrinology, Glaxo Wellcome Research and Development, Research Triangle Park, NC). The cloning and characterization of the chicken liver X receptor (LXR) and farnesoid X receptor (FXR) will be described elsewhere.

Cloning of CYP2H1 Subfragments. The 1369-bp fragment was amplified by PCR from the 4.8-kb enhancer in pBLCAT5 (Handschin and Meyer, 2000) by using the primers 5′-TCA TGT CTG GAT CTC GAA GC-3′ and 5′-CGG GAT CCC GTC CTC TAG ATA GTG GGC A-3′ and was subcloned into the reporter gene vector pBLCAT5 after

digestion with *Bam*HI and *Hin*dIII. The 726- and 643-bp elements (Fig. 1) were obtained by digesting the 1369-bp pBLCAT5 vector with *Hin*dIII/*Sph*I or *Sph*I/*Bam*HI, respectively, followed by Klenow reaction and religation of the truncated, blunt-end constructs. The 726-bp pBLCAT5 construct was digested with *Hin*dIII/*Bsp*MI or *Bsp*MI/*Bam*HI to exclude the 299- or the 427-bp elements, respectively, followed by Klenow reaction and religation. We used the 643-bp pBLCAT5 as template, and the 324-bp element was amplified by PCR with the primers 5′-TCA TGT CTG GAT CTC GAA GC-3′ and 5′-CGG GAT CCC GTT GCA ACA AAA TAT TGT CA-3′, the 319-bp element with the primers 5′-CCC AAG CTT GGG TCA TAT TTT TCA CAC C-3′ and 5′-CGG GAT CCC GTC CTC TAG ATA GTG GGC A-3′, and the 260-bp element with the primers 5′-CCC AAG CTT GGA GAC TCA TCT GAA ATT TAC-3′ and 5′-CGG GAT CCC GTA GCC ATC GTC ATT AAC CG-3′. The primers 5′-CCC AAG CTT GCA TGA AAC ACG GAG ATA CTT-3′ and 5′-CGG GAT CCC GGA CTG TTA TGA AAT GCT CTG-3′ were used to amplify the 240-bp element from the 726-bp pBLCAT5. These four PCR products were digested with *Hin*dIII and *Bam*HI and subcloned into pBLCAT5. All constructs were verified by sequencing.

Site-Specific Mutagenesis. Mutations in the putative nuclear receptor binding half-sites (Fig. 2) were introduced by PCR with standard overlap techniques as described previously (Handschin and Meyer, 2000). Briefly, subfragments were amplified with overlapping, mutated primers and vector primers. These subfragments were chosen as template in a second round of PCR by using the vector primers alone for amplification of the full-length, mutated fragment. The vector primers in the first and the second round of PCR were 5′-TCA TGT CTG GAT CTC GAA GC-3′ and 5′-TTC GCC AAT GAC AAG ACG C-3′ and the 240-bp pBLCAT5 construct was used as template for the single mutations. The complete, mutated 240-bp fragments were then digested with *Bam*HI and *Hin*dIII and cloned into the pBLCAT5 vector. The 5′ half-site of the DR-4 element (AGTTCA) was mutated into a *Sac*II recognition site (CCGCGG) by using the primers 5′-AGT GCC GCG GTG CAA GTT CTT GTT CCT CGT G-3′ and 5′-TGC ACC GCG GCA CTG GAT TAG GAG GTG GAC A-3′ and denominated HS1. The 3′ half-site (AGTTCT) was replaced by a *Eco*RV recognition site (GATATC) with the primers 5′-TGC AGA TAT CTG TTC CTC GTG GAA TGC AGG TC-3′ and 5′-AAC AGA TAT CTG CAT GAA CTC ACT GGA TTA G-3′ and the mutant was called HS2. To obtain the double mutant (double), HS1 pBLCAT5 construct was used as template for PCR amplification with the primers 5′-TGC AGA TAT CTG TTC CTC GTG GAA TGC AGG TC-3′ and 5′-AAC AGA TAT CTG CAC CGC GGC ACT GGA TTA G-3′. All PCR products were cut with *Hin*dIII and *Bam*HI followed by subcloning into pBLCAT5. The constructs were verified by digestion with *Sac*II and *Eco*RV in addition to sequencing.

Culture and Transfection of LMH Cells. LMH cells were cultured and transfected as described previously (Handschin and Meyer, 2000). Briefly, LMH cells were cultivated in Williams' E medium supplemented with 10% fetal calf serum, 1% glutamine (2 mM), and 1% penicillin/streptomycin (50 IU/ml) on gelatin-coated dishes. Transfections were performed using the FuGENE 6 Transfection reagent (Roche Molecular Biochemicals, Rotkreuz, Switzerland) in serum-free suspension according to the supplier's manual. This procedure ensured equal transfection efficiencies for control and treated cells. The cells were then plated on six-well dishes and medium was replaced 4 h after transfection by induction or control medium, respectively.

Analysis of Reporter Gene Expression. Sixteen hours after drug treatment, the cells were harvested and nonradioactive chloramphenicol acetyltransferase (CAT) assays were performed using the CAT-ELISA kit according to the supplier's manual (Roche Molecular Biochemicals). Cell extracts were also used for the determination of protein concentration with the use of the ESL (Exact, Sensitive, Low Interference) protein assay for normalization of specific CAT expression to total protein content (Roche Molecular Biochemicals).

Transcriptional Activation Assays. CV-1 cell transactivation assays were carried out as described previously (Handschin et al., 2000). Briefly, CV-1 cells were maintained in DMEM/F-12 medium supplemented with 10% fetal bovine serum. Before experiments, cells were split 1:10 in DMEM/F-12 without phenol red, supplemented with 10% charcoal-stripped fetal bovine serum. After a 3-day incubation, cells were plated onto six-well dishes at a density of 625,000 cells/well. Transfection mixes contained 150 ng of receptor expression vector, 400 ng of reporter plasmid, 800 ng of β-galactosidase expression vector (pSV-βGalactosidase; Promega, Catalys AG, Wallisellen, Switzerland) and carrier plasmid, in total 2.5 μg of DNA per well. Cells were transiently transfected using LipofectAMINE reagent (Life Technologies) according to the manufacturer's instructions. Twenty-four hours after transfection, the medium was replaced by DMEM/F-12 without phenol red, supplemented with 10% delipidated, charcoal-stripped fetal calf serum (Sigma) containing the inducer compounds of interest. Cells were then incubated for an additional 24 h, after which cell extracts were prepared and assayed for chloramphenicol acetyl transferase as described above. At the same time, β-galactosidase activities were determined by adding 180 μl of chlorophenol red-1β-D-galactopyra; Roche Molecular Biochemicals) substrate solution (0.5 mM chlorophenol red-1β-D-galactopyra, 20 mM 2-mercaptoethanol, 60 mM Na_2HPO_4, 45 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) to 20 μl of the cell lysates. After 10 min of incubation at 37°C, absorption at 550-nm wavelength was measured using a Labsystems Multiskan RC microplate reader (Labsystems GmbH, Frankfurt am Main, Germany). CAT concentrations were then normalized against measured absorption at 550 nm to compensate for intersample variations in transfection efficiencies.

Gel Mobility Shift Assays. Electromobility-shift assays were performed as described previously (Handschin et al., 2000). Chicken CXR, chicken RXRγ, human CAR, and human PXR were synthesized in vitro using the TNT T7 Quick Coupled Transcription/Translation system (Promega, Catalys AG) according to the manufacturer's instructions. Probes were labeled with Klenow enzyme in the presence of radiolabeled [α-^{32}P]ATP and the probe was purified over a Biospin 30 chromatography column (Bio-Rad, Glattbrugg, Switzerland). A volume of labeled oligonucleotide corresponding to 100,000 cpm was used for each reaction in 10 mM Tris, pH 8.0, 40 mM KCl, 0.05% Nonidet P-40, 6% glycerol, 1 mM dithiothreitol, 0.4 μg/μl bovine serum albumin, 0.2 μg poly(dI-dC)∗poly(dI-dC), and 2.5 μl of the in vitro synthesized proteins. To test for supershifts, 0.5 μl of monoclonal anti-mouse-RXR rabbit antibody (kindly provided by Dr. P. Chambon, Institut de Génétique et Biologie Moléculaire et Cellulaire, Université Louis Pasteur, Illkirch, France) were added to the reaction mix. This antibody has been positively tested for cross-reaction with the chicken RXRγ in Western blots (data not shown). The mix was incubated for 20 min at room temperature and subsequently electrophoresed on a 6% polyacrylamide gel in 0.25× Tris/borate/EDTA buffer followed by autoradiography at −70°C.

Computer Analysis of Putative Transcription Factor Binding Sites. The different enhancer elements were analyzed using the MatInspector program (Quandt et al., 1995) to detect putative transcription factor binding sites stored in the TRANSFAC version 5.0 transcription factor binding sites database (Wingender et al., 2000). All the matrices of the vertebrate group were compared with the PBRUs with a threshold of 0.75 core similarity and 0.85 matrix similarity. The number of hits of the single matrices was compared with their relative expectancy on the respective number of bp and only matrices with higher occurrence than would be expected on a random sequence of the same length as the PBRUs were considered for further analysis. Of those, nonhepatic transcription factors and
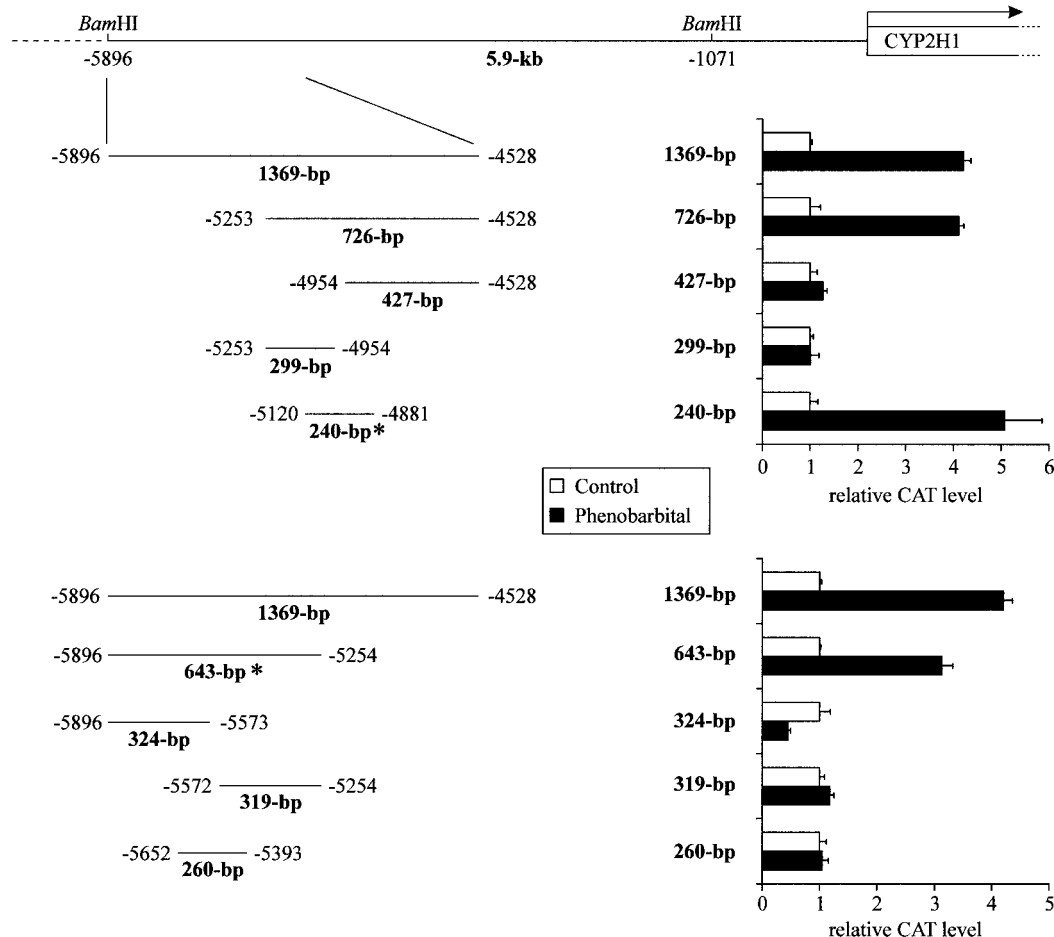
Fig. 1. Functional analysis of a 1.3-kb PB-responsive fragment from the chicken CYP2H1 5'-flanking region. Subfragments of the 1.3-kb element were generated by restriction digests and PCR as described under *Materials and Methods*. These fragments were subcloned into the pBLCAT5 reporter gene vector and subsequently transfected into LMH cells. The cells were treated with 400 μM PB for 16 h and CAT-ELISAs were performed. Relative CAT expression was standardized against untreated control cells and expressed in fold induction. Values are the average of three independent experiments, and error bars represent standard deviation. Asterisks (∗) indicate the 240- and 643-bp fragments subjected to further analyses.

transcription factors that did not appear on all PBRUs or at least on the two chicken PBRUs were discarded.

Results

Isolation of PB-Responsive Enhancer Elements in CYP2H1 5′-Flanking Region. In two independent studies, the 5′-1.3-kb section of the original 4.8-kb enhancer element of chicken CYP2H1 was found to be inducible by PB (Dogra et al., 1999; Handschin and Meyer, 2000). We analyzed this region ($-5896/-4528$) to locate PBRUs and the corresponding transcription factors. Subfragments were generated either by restriction digests or by PCR as described under *Materials and Methods*, subcloned into the pBLCAT5 reporter gene vector containing a heterologous thymidine kinase promoter, and transfected into LMH cells. A heterologous promoter was used because the CYP2H1 1.1-kb promoter region exhibits high constitutive activity in the LMH cells as described in previous experiments in chicken primary hepatocytes (data not shown). After induction with 400 μM PB for 16 h, cells were harvested and the CAT reporter gene level determined. As shown in Fig. 1, enzymatic digestion of the 1369-bp fragment into a 643-bp ($-5896/-5254$) and a 726-bp fragment ($-5253/-4528$) revealed that both elements are still inducible between 3- and 4-fold. Serial restriction digestion of the 726-bp fragment lead to a 240-bp element ($-5120/-4881$) that retained about 5-fold PB inducibility (Fig. 1A), whereas the same approach on the 643-bp fragment did not produce an obvious PBRU because none of the three overlapping subfragments showed PB induction in reporter gene assays (Fig. 1B). Further analysis of this fragment was not pursued at this time. In comparison, the previously described CYP2H1 264-bp PBRU was about 8-fold induced in this set of experiments (data not shown).
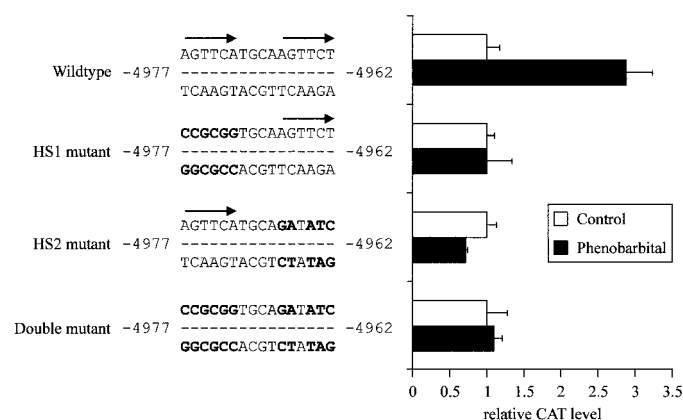
Site-Directed Mutagenesis of Putative Nuclear Receptor Binding Site. PB induction of the 726-bp fragment was abolished when cutting with *Bsp*MI at position -4954 (Fig. 1A). We searched for canonical nuclear receptor half-

sites in this region on the 240-bp PBRU and detected two hexamers that fit the consensus $AG^T/_GTCA$ sequence (at $-4977/-4972$ and $-4944/-4939$). Both half-sites were mutated but only site-directed mutagenesis of the 5′ half-site had an effect on PB inducibility of the 240-bp PBRU (data not shown). In the vicinity, an imperfect half-site (AGTTCT) was subsequently identified at $-4967/-4962$, which, together with the first half-site, formed a direct repeat with a spacing of four nucleotides (DR-4). These hexamers were mutated by PCR, and the respective 240-bp PBRUs subcloned into the reporter gene vector pBLCAT5 and transfected into LMH cells. CAT levels were measured after a 16-h induction with 400 μM PB. Site-directed mutagenesis of either one of these half-sites (HS1 mutant and HS2 mutant) or of both together (double mutant) drastically reduced PB induction of the 240-bp PBRU (Fig. 2), establishing an important role of this nuclear receptor recognition sequence for PB induction on the 240-bp PBRU.

Activation of PBRUs by Orphan Nuclear Receptor CXR. Previously, we identified a 264-bp PBRU in the CYP2H1 5′-flanking region that contains a DR-4 element and that is activated by the chicken orphan nuclear receptor CXR (Handschin and Meyer, 2000; Handschin et al., 2000). Having also detected a DR-4 element in the 240-bp PBRU, we tested whether CXR could activate the PBRUs found in the 1.3-kb element. The PBRUs cloned in pBLCAT5 were transfected into CV-1 cells together with CXR in the expression vector pSG5 and the cells were induced for 24 h before CAT levels were determined. Both the 240- and the 643-bp PBRU were activated by CXR by using different drugs in the same manner as the 264-bp PBRU reported previously (Handschin et al., 2000). Metyrapone (400 μM), propylisopropylacetamide (250 μM), glutethimide (500 μM), and clotrimazole (10 μM) were the most potent compounds, inducing the 240-bp PBRU between 4- and 8-fold and the 643-bp PBRU between 3- and 4-fold (Fig. 3A). PB (400 μM), dexamethasone (50 μM), β-naphthoflavone (10 μM), 5-pregnen-3β-ol-20-one-16α-carbonitrile (50 μM), and rifampicin (100 μM) had only low effects on both elements (Fig. 3A).

Mutations in the DR-4 element abolished PB induction of the 240-bp PBRU in reporter gene assays in the LMH cells. The double mutant 240-bp PBRU was therefore transfected into CV-1 cells together with CXR and stimulated with either 400 μM PB or 10 μM clotrimazole for 24 h, and CAT levels compared with those of the wild-type 240-bp PBRU. Inductions by clotrimazole (7-fold) and PB (2-fold) of the wild-type 240-bp PBRU could not be reproduced using the double mutant 240-bp PBRU (Fig. 3B). CXR apparently is responsible for drug induction of both PBRUs via the DR-4 element on the 240-bp PBRU and by an as-yet-unknown element on the 643-bp PBRU.

CXR Binds to DR-4 on 240-bp PBRU. Electromobility shift assays were performed to check whether CXR directly binds to the 240-bp PBRU or whether drug induction of the 240-bp PBRU by CXR is due to an indirect effect. As shown in Fig. 4, neither CXR nor chicken RXRγ alone bound to radiolabeled 240-bp PBRU (Fig. 4, lanes 2 and 3). CXR/RXRγ heterodimers formed a complex on the 240-bp PBRU (Fig. 4, arrow b) that was supershifted (Fig. 4, arrow c) when adding anti-RXR antibodies (Fig. 4, lanes 4 and 5). This protein-DNA complex signal could be decreased by adding a 50-fold excess of unlabeled, wild-type 240-bp PBRU but not by add-

Fig. 2. Site-directed mutagenesis of the DR-4 element in the 240-bp PBRU. The hexamer half-sites of the DR-4 element within the 240-bp PBRU were mutated in a PCR-based approach as described under *Materials and Methods*. Mutations are depicted in boldface. The mutated 240-bp constructs were cloned into the pBLCAT5 reporter gene vector and subsequently transfected into LMH cells. After 16-h treatment with 400 μM PB, CAT levels were determined using a CAT-ELISA. The relative CAT expression was standardized against untreated control cells and expressed as fold induction. Values are the average of three independent experiments, and error bars represent standard deviation.

ing a 50-fold excess of unlabeled DR-4 double mutant 240-bp PBRU (Fig. 4, lanes 6 and 7). The DR-4 therefore is the direct binding site for CXR on the 240-bp element and this receptor-DNA interaction is responsible for drug induction by CXR.

The 240-bp PBRU Is a Target for Different Orphan Nuclear Receptors. A high degree of similarity was found between the chicken CYP2H1 264-bp PBRU and mammalian PBRUs from rat, mouse, and human (Handschin and Meyer, 2000; Zelko and Negishi, 2000). We could show interchangeability of avian and mammalian PBRUs and avian and mammalian orphan nuclear receptors, suggesting that the same mechanisms are responsible for drug induction from chicken to human (Handschin and Meyer, 2000; Handschin et al., 2000). To expand findings from the 264-bp PBRU, binding of human PXR and human CAR to the 240-bp PBRU was tested

in electromobility shift assays. None of the receptors bound alone to the 240-bp PBRU (Fig. 5A, lanes 2–5) but as heterodimers with chicken RXRγ, both human PXR and human CAR were able to bind to the 240-bp PBRU as did the chicken orphan nuclear receptor CXR (Fig. 5A, lanes 6, 8, and 10, arrow b). The complexes formed by these receptors were supershifted when adding anti-RXR antibody (Fig. 5A, lanes 7, 9, and 11, arrow c), establishing specific binding of these receptors to the 240-bp PBRU.

The response of P-450s to drugs can be influenced by oxysterols and bile acids both at the mRNA and reporter gene level (J. C. Ourlin and U. A. Meyer, unpublished observations). These compounds are the endogenous ligands of the orphan nuclear receptors LXR and FXR (for review, see Repa and Mangelsdorf, 2000). We cloned the chicken orthologs of LXR and FXR and tested whether they bind to the 240-bp PBRU in electromobility shift assays. With chicken RXRγ as heterodimerization partner, LXR and CXR bound to the 240-bp PBRU, whereas FXR bound neither alone nor as a heterodimer with RXRγ (Fig. 5B, lanes 2–8). Adding anti-RXR antibody supershifted both the CXR/RXRγ and the LXR/RXRγ complexes bound to the radiolabeled probe (Fig. 5B, lanes 9 and 11). The effect of oxysterols on drug-inducible P-450s and the binding affinity of LXR to DR-4 elements may result in cross talk between LXR and xenobiotic-sensing orphan nuclear receptors, a question that is under current investigation in our laboratory.

Thus, the 240-bp PBRU was found to be a target of multiple chicken receptors that modulate induction of CYP2H1.

A

B
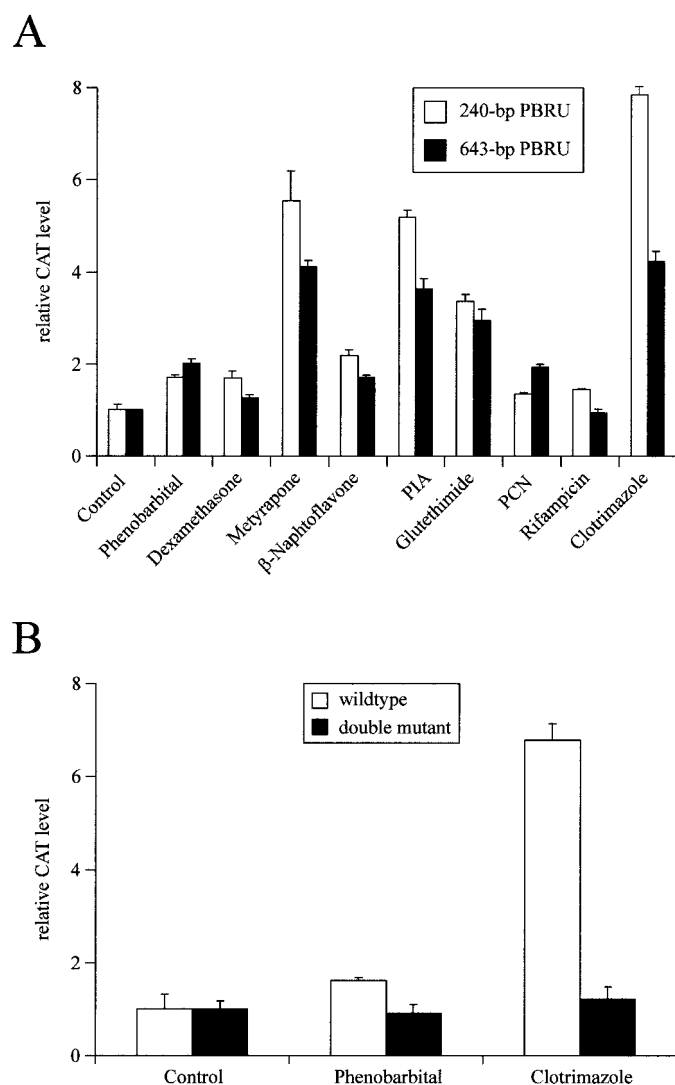
Fig. 3. CXR activates the 240- and 643-bp PBRUs in CV-1 cell transactivation assays. A, CV-1 cells were cotransfected with an expression plasmid for CXR and a CAT reporter plasmid containing either the 240- or 643-bp PBRU depicted in Fig. 1. Cells were then treated with drugs for 24 h and cell extracts were analyzed for CAT expression normalized against β-galactosidase levels. PIA, propylisopropylacetamide; PCN, 5-pregnen-3β-01-20-one-16α-carbonitrile. B, same experimental procedure as in A but using either the wild-type 240-bp or the DR-4 double mutant 240-bp PBRU reporter gene constructs. Values are the average of three independent experiments, and error bars represent standard deviation.
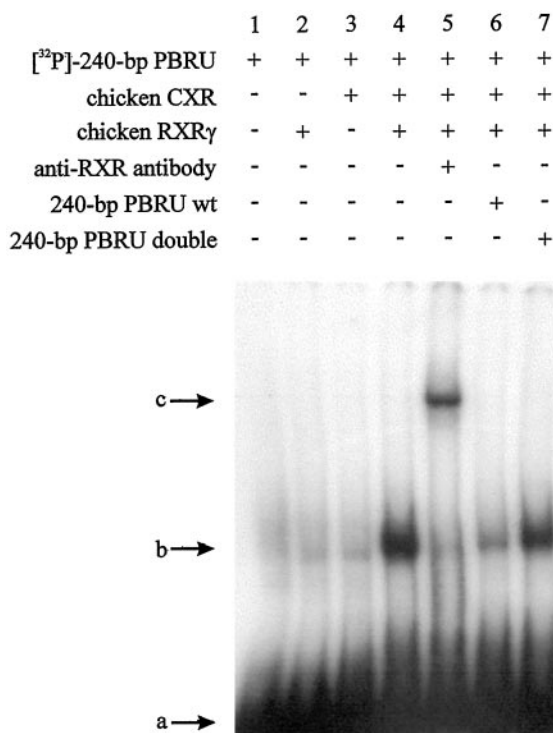
Fig. 4. CXR binds to the DR-4 of the CYP2H1 240-bp PBRU. Radiolabeled 240-bp PBRU was incubated with in vitro transcribed/translated chicken RXRγ (lanes 2 and 4–7), CXR (lanes 3–7), anti-RXR antibody (lane 5), 50-fold molar excess of unlabeled wild-type 240-bp PBRU (lane 6), and 50-fold molar excess of unlabeled DR-4 double mutant 240-bp PBRU (lane 7) as competitors in electromobility shift assays. Arrows depict unbound probe (a); the complex of CXR, RXRγ, and probe leading to a shift (b); and, together with anti-RXR antibody, to a supershift (c).

The cross-species interchangeability of PB-responsive elements now includes the 240-bp PBRU, and present data also suggest that similar transcription factors are involved in mediating drug induction as those interacting with the chicken 264-bp and the mammalian PBRUs.

Discussion

The 4.8-kb element from the chicken CYP2H1 5′-flanking region was the first known drug-inducible enhancer fragment of the CYP2 family (Hahn et al., 1991). Two major PB-responsive regions within these 4.8 kb were derived by functional analysis of the flanking region by using reporter gene assays in transiently transfected primary chicken hepatocyte cultures and the chicken hepatoma cell line LMH (Dogra et al., 1999; Handschin et al., 2000). One report expressed doubts that the transcription factors activating the chicken and rodent PBRUs would be similar and postulated that either the fundamental mechanism of drug induction is different or that there is a as yet undiscovered common site of PB action in aves and mammals (Dogra et al., 1999). We demonstrated that this element strikingly resembles rodent and human drug-responsive enhancers and that a similar nuclear receptor binding site is responsible for drug induction (Handschin and Meyer, 2000). Furthermore, cloning of

the chicken orphan nuclear receptor CXR identified a candidate receptor that mediates drug induction via this element (Handschin et al., 2000). CXR is closely related to both mammalian xenobiotic-sensing receptors PXR and CAR; therefore, the basic mechanism of drug induction is more conserved from chicken to humans than previously assumed (Handschin et al., 2000).

From the analysis of large flanking regions, it was suspected that, apart from the 264-bp PBRU, additional elements may contribute to overall CYP2H1 response to drugs (Dogra et al., 1999; Handschin and Meyer, 2000). Here, we report the identification and characterization of two additional distal enhancer elements from the chicken CYP2H1 5′-flanking region at −5896/−5254 and −5120/−4881. Both the 643- and the 240-bp PBRU were activated by CXR after drug treatment and a DR-4 nuclear receptor binding site was identified on the 240-bp PBRU. So far, no difference between the 264- and 240-bp PBRUs was found concerning activation pattern or nuclear receptors binding to these elements. Interestingly, the DR-4 element that confers drug induction on the 240-bp PBRU is also located at the very 3′ end of a 299-bp fragment that was not inducible in our analysis (Fig. 1). These findings indicate that sequence elements flanking the DR-4 or additional transcription factor binding sites are required for drug induction. Al-

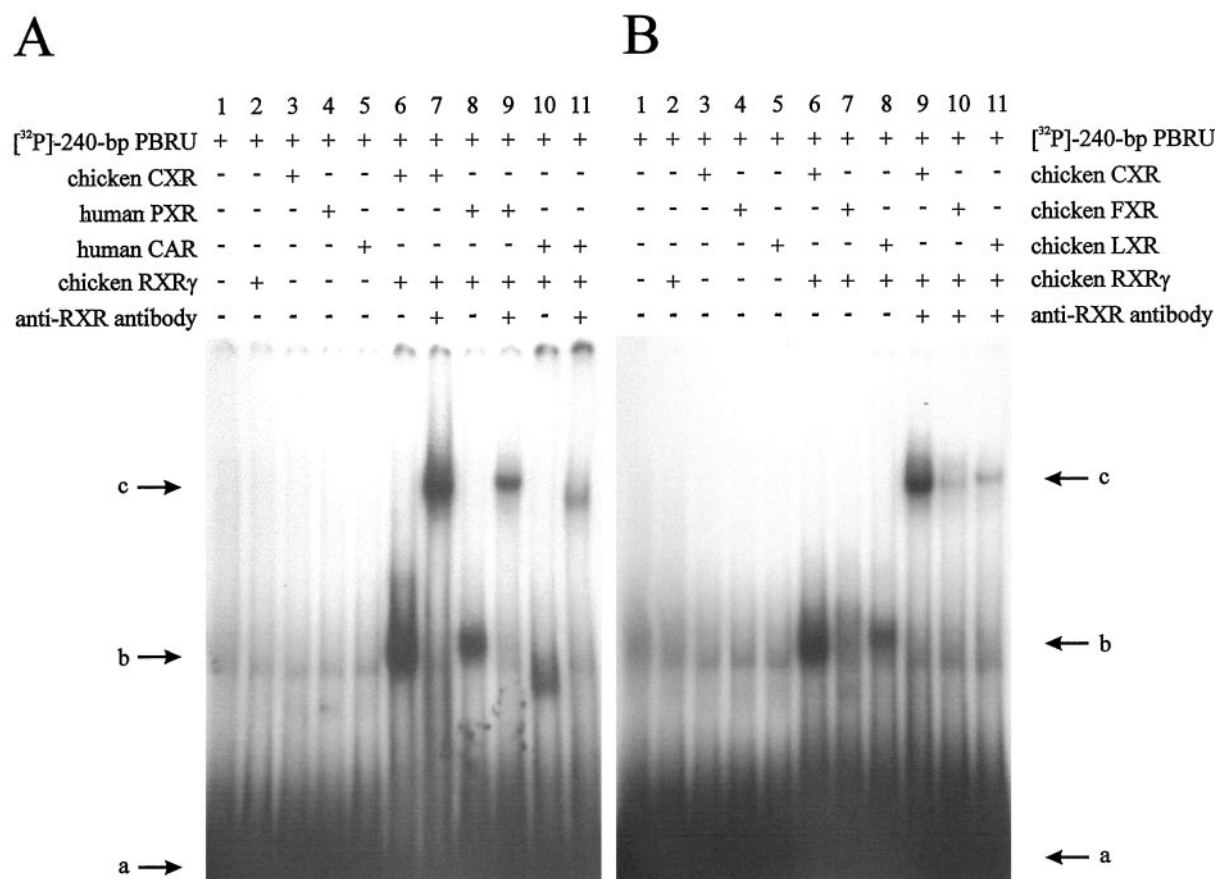**Fig. 5.** Human xenobiotic-sensing receptors PXR and CAR and the chicken oxysterol receptor LXR bind to the 240-bp PBRU. A, radiolabeled 240-bp PBRU was incubated with in vitro transcribed/translated chicken RXRγ (lanes 2 and 6–11), CXR (lanes 3, 6, and 7), human PXR (lanes 4, 8, and 9), human CAR (lanes 5, 10, and 11), and anti-RXR antibody (lane 7, 9, and 11) in electromobility shift assays. Arrows depict unbound probe (a); the complex of CXR, hPXR, or hCAR with RXRγ and probe leading to a shift (b); and, together with anti-RXR antibody, to a supershift (c). B, radiolabeled 240-bp PBRU was incubated with in vitro transcribed/translated chicken RXRγ (lanes 2 and 6–11), CXR (lanes 3, 6, and 9), chicken FXR (lanes 4, 7, and 10), chicken LXR (lanes 5, 8, and 11), and anti-RXR antibody (lane 9–11) in electromobility shift assays. Arrows depict unbound probe (a); the complex of CXR, hPXR, or hCAR with RXRγ and probe leading to a shift (b); and, together with anti-RXR antibody, to a supershift (c).

though CXR activates the 643-bp PBRU in CV-1 cell transactivation assays, several sequence elements within the 643-bp PBRU might be necessary for activity. In comparison with the CYP2H1 264- and 240-bp PBRUs that contain DR-4 elements mediating drug induction, the 643-bp PBRU is apparently organized in a different way that is currently under investigation in our laboratory.

To get a broad overview over the structure of chicken PBRUs and mammalian PBRUs, we compared the chicken CYP2H1 264- and 240-bp PBRUs with the rat CYP2B2 163-bp PBRU (Trottier et al., 1995; Stoltz et al., 1998), the mouse Cyp2b10 (−2426/−2250), and the human CYP2B6 177-bp (−1772/1596) PBRUs (Honkakoski and Negishi, 1997; Sueyoshi et al., 1999) regarding transcription factor binding sites by using the MatInspector algorithm as described under *Materials and Methods*. Recently, the rat CYP2B2 163-bp PBRU, which is not equivalent to the 51-bp PBREM located within the 163-bp PBRU, was found to have several transcription factor binding sites responsible for maximal induction (Trottier et al., 1995; Stoltz et al., 1998; Paquet et al., 2000). Therefore, instead of analyzing the 51-bp PBREMs from mouse and human, we used the initially described mouse Cyp2b10 177-bp PBRU (Honkakoski and Negishi, 1997) and the corresponding 177-bp from the human CYP2B6 5′-flanking region. Several sites were found that have been described previously to be involved in the induction process (Fig. 6A). All PBRUs contained activator protein-1, CAAT/enhancer-binding protein and glucocorticoid receptor binding sites. A nuclear factor-1 (NF-1) recognition site was detected in all elements except the human CYP2B6 177-bp PBRU under the chosen conditions of the MatInspector program, although this site has also been described in the human PBRU (Sueyoshi et al., 1999). A CAAT/enhancer-binding protein-homologous protein 10 binding site was only found in the chicken and the rat PBRUs, recognition sites for hepatic nuclear factor-3 were present in all PBRUs except the rat, GATA sites were located in the chicken and the human PBRUs, whereas exclusively the chicken PBRUs contained upstream stimulatory factor (USF) recognition sites. Finally, all but the chicken CYP2H1 264-bp PBRU contained estrogen receptor binding sites (Fig. 6A).

The glucocorticoid response element in the rat CYP2B2 163-bp PBRU is essential for conferring maximal phenobarbital responsiveness (Stoltz et al., 1998). Moreover, mice lacking a functional glucocorticoid receptor have a lower basal Cyp2b level, steroids are no longer able to induce these enzymes, and PB induction of Cyp2bs is diminished by 37% compared with wild-type mice (Schuetz et al., 2000). Recent observations showed that estrogens activate the orphan nuclear receptor CAR in induction of the mouse Cyp2b10 and that estrogen receptor-related receptor binds to the 51-bp PBREM (for review, see Zelko and Negishi, 2000). Estrogen receptors' binding sites are potentially important in modulating sex steroid effects, affect gender-specific CYP induction, or be binding sites for the repressing estrogen receptor-related receptor.

NF-1 sites were reported to be positive accessory sites in

A

	CYP2H1 240-bp	CYP2H1 264-bp	CYP2B2 163-bp	Cyp2b10 177-bp	CYP2B6 177-bp
AP-1	2	5	2	2	4
C/EBP	3	3	1	2	3
CHOP	1	1	1	-	-
ER	1	-	1	1	1
GATA	7	5	-	-	2
HNF-3	2	1	-	1	1
GR	1	1	2	2	2
NF-1	3	2	2	2	-
USF	1	4	-	-	-

B

```
                        NR1              NF-1              NR2
                 ←──────  ←──                    ←──       ←──
240-bp 2H1  -4960 CAAGAACTTGCATGAACTCACTGGATTAGGAGGTGGACAAACAAAGATCTTC -5011
                  | ||||| || || ||    ||     ||||   |       ||
264-bp 2H1  -1653 CCTGAACTTCCTTGCCCTTT.CAGAGACCGAGCCAATACATAGCAATCTGTC -1603
                  ||| ||||| | ||| ||    |||  ||| ||| |||| |||||| ||
163-bp 2B2  -2235 TCTGTACTTCCTGACCTTGGCACAGTGCCACCATCAACTTGACTGACACC -2184
                  ||||||||||||||||||||||||||||| |||||||||||||||||||
51-bp 2b10  -2339 TCTGTACTTCCTGACCTTGGCACAGTGCCACCATCAACTTGCCTGACACC -2289
                  ||||||||||||||||||     ||| ||| ||| |||| |||||| |
51-bp 2B6   -1683 ACTGTACTTCCTGACCCTGAAGAGGTGGCAGCATGGACTTTCCTGAACCA -1733
```

Consensus   HctGWACTTBCHTGacCttDVHRVagtVSSA.ScaDNVaMLHDMMDDHHNYc

the PB-responsive enhancers in rat and mouse (Honkakoski and Negishi, 1998; Stoltz and Anderson, 1999). NF-1 and CAR/RXR heterodimers can both bind simultaneously and independently to the overlapping NF-1 and NR-1 sites and NF-1 binding increases drug-responsiveness of the 51-bp PBREM (Kim et al., 2001). GATA proteins are ubiquitously expressed regulators of tissue-specific gene expression containing a DNA binding domain composed of two adjacent homologous zinc fingers (Molkentin, 2000). USF have also been implicated in early stress response or growth signals by activating mitogen-activated protein kinase phosphatase-1 (Sommer et al., 2000). Moreover, USF competes with the aryl-hydrocarbon receptor-aryl hydrocarbon nuclear translocator complex for binding to xenobiotic-response elements of CYP1A1, thereby inhibiting CYP1A1 in rabbits (Takahashi et al., 1997), and both USF and activator protein-1 are involved in regulating the PB-inducible chicken heme oxygenase-1 (Lu et al., 2000). PBRUs obviously are complex regulatory units involved in the response of the cell to various stimuli.

The exact role of most of these putative transcription factor binding sites on PBRUs is not clear; at least some of them seem to be important auxiliary factors involved in drug induction. Strikingly, most of them are involved in stress response and might aid the cell in purging potentially toxic compounds such as drugs. Moreover, these transcription factors are predominantly expressed in liver or are responsible for tissue-specific expression of their target genes. Apart from these sites, all PBRUs include DR-4 nuclear receptor binding sites. The mammalian CYP2B PBREMs are characterized by two distinct DR-4 sites (NR-1 and NR-2), whereas the chicken CYP2H1 has a single DR-4 (Handschin and Meyer, 2000; Zelko and Negishi, 2000). However, sequence comparisons reveal very high conservation of this NR-1 element between CYP2H1 and CYP2B PBRUs (Fig. 6B). Studies in the rat 163-bp PBRU have shown that NR-1 and NR-2 are not equivalent and that several transcription factors are needed at the NR-1 site for conferring maximal PB induction (Paquet et al., 2000). Because both NR-1 and NR-2 alone are sufficient for PB induction in the context of reporter gene assays by using a thymidine kinase promoter (Honkakoski and Negishi, 1998; Sueyoshi et al., 1999) and no NR-2 is present in the chicken PBRUs, NR-1 clearly is a crucial element in drug induction.

We conclude that conservation of the mechanism of drug induction from birds to mammals is observed at different levels. Nuclear receptors activated by xenobiotic and endobiotic compounds are closely related between these species, PBRUs share similar recognition elements for these receptors with high sequence conservation, and contain comparable binding sites for additional transcription factors. Moreover, the concept of multiple distal enhancer elements conferring drug induction is evident in human CYP3A4, where PXR response elements are located near the promoter and at about 7 kb upstream of the transcriptional start site and three additional distal *cis*-acting elements are critical for maximal xenobiotic responsiveness (Goodwin et al., 1999). DNaseI hypersensitivity assays of 5′-flanking regions of rodent CYP2B genes revealed two hypersensitive sites at −2.3/ −2.2 and in the proximal promoter (Luc et al., 1996). Thus, chicken and mammalian drug induction mechanisms are essentially identical. The species differences in inducer speci-

ficity are due to differences in the ligand binding domains of the nuclear receptors (Jones et al., 2000; Xie et al., 2000a). However, many details of the response need to be investigated, in particular the mechanisms of receptor activation, dimer formation, DNA binding, and coactivator or corepressor recruitment.

References

Dogra SC, Davidson BP, and May BK (1999) Analysis of a phenobarbital-responsive enhancer sequence located in the 5′ flanking region of the chicken CYP2H1 gene: identification and characterization of functional protein-binding sites. *Mol Pharmacol* **55:**14–22.

Frueh FW, Zanger UM, and Meyer UA (1997) Extent and character of phenobarbital-mediated changes in gene expression in liver. *Mol Pharmacol* **51:**363–369.

Goodwin B, Hodgson E, and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* **56:**1329–1339.

Hahn CN, Hansen AJ, and May BK (1991) Transcriptional regulation of the chicken CYP2H1 gene. *J Biol Chem* **266:**17031–17039.

Handschin C and Meyer UA (2000) A conserved nuclear receptor consensus sequence (DR-4) mediates transcriptional activation of the chicken CYP2H1 gene by phenobarbital in a hepatoma cell line. *J Biol Chem* **275:**13362–13369.

Handschin C, Podvinec M, and Meyer UA (2000) CXR, a chicken xenobiotic-sensing orphan nuclear receptor, is related to both mammalian pregnane X receptor (PXR) and constitutive androstane receptor (CAR). *Proc Natl Acad Sci USA* **97:**10769–10774.

Honkakoski P and Negishi M (1997) Characterization of a phenobarbital-responsive enhancer module in mouse P450 Cyp2b10 gene. *J Biol Chem* **272:**14943–14949.

Honkakoski P and Negishi M (1998) Regulatory DNA elements of phenobarbital-responsive cytochrome P450 CYP2B genes. *J Biochem Mol Toxicol* **12:**3–9.

Honkakoski P and Negishi M (2000) Regulation of cytochrome P450 (CYP) genes by nuclear receptors. *Biochem J* **347:**321–337.

Honkakoski P, Zelko I, Sueyoshi T, and Negishi M (1998) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* **18:**5652–5658.

Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, et al. (2000) The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinol* **14:**27–39.

Kemper B (1998) Regulation of cytochrome P450 gene transcription by phenobarbital. *Prog Nucleic Acid Res Mol Biol* **61:**23–64.

Kim J, Min G, and Kemper B (2001) Chromatin assembly enhances binding to the CYP2B1 PBRU of NF-1, which binds simultaneously with CAR/RXR and enhances CAR/RXR-mediated activation of the PBRU. *J Biol Chem* **276:**7559–7567.

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, et al. (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92:**73–82.

Lu TH, Shan Y, Pepe J, Lambrecht RW, and Bonkovsky HL (2000) Upstream regulatory elements in chick heme oxygenase-1 promoter: a study in primary cultures of chick embryo liver cells. *Mol Cell Biochem* **209:**17–27.

Luc PV, Adesnik M, Ganguly S, and Shaw PM (1996) Transcriptional regulation of the CYP2B1 and CYP2B2 genes by C/EBP-related proteins. *Biochem Pharmacol* **51:**345–56.

Mattschoss LA, Hobbs AA, Steggles AW, May BK, and Elliott WH (1986) Isolation and characterization of genomic clones for two chicken phenobarbital-inducible cytochrome P-450 genes. *J Biol Chem* **261:**9438–9443.

Molkentin JD (2000) The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. *J Biol Chem* **275:**38949–38952.

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, et al. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6:**1–42.

Paquet Y, Trottier E, Beaudet MJ, and Anderson A (2000) Mutational analysis of the CYP2B2 phenobarbital response unit and inhibitory effect of the constitutive androstane receptor on phenobarbital responsiveness. *J Biol Chem* **275:**38427–38436.

Quandt K, Frech K, Karas H, Wingender E, and Werner T (1995) MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* **23:**4878–84.

Repa JJ and Mangelsdorf DJ (2000) The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. *Annu Rev Cell Dev Biol* **16:**459–481.

Savas U, Griffin KJ, and Johnson EF (1999) Molecular mechanisms of cytochrome P-450 induction by xenobiotics: an expanded role for nuclear hormone receptors. *Mol Pharmacol* **56:**851–857.

Schuetz EG, Schmid W, Schütz G, Brimer C, Yasuda K, Kamataki T, Bornheim L, Myles K, and Cole TJ (2000) The glucocorticoid receptor is essential for induction of cytochrome P-4502B by steroids but not for drug or steroid induction of CYP3A or P-450 reductase in mouse liver. *Drug Metab Dispos* **28:**268–278.

Sommer A, Burkhardt H, Keyse SM, and Luscher B (2000) Synergistic activation of the mkp-1 gene by protein kinase A signaling and USF, but not c-Myc. *FEBS Lett* **474:**146–150.

Stoltz C and Anderson A (1999) Positive regulation of the rat CYP2B2 phenobarbital response unit by the nuclear receptor hexamer half-site-nuclear factor 1 complex. *Biochem Pharmacol* **57:**1073–1076.

Stoltz C, Vachon MH, Trottier E, Dubois S, Paquet Y, and Anderson A (1998) The CYP2B2 phenobarbital response unit contains an accessory factor element and a

putative glucocorticoid response element essential for conferring maximal phenobarbital responsiveness. *J Biol Chem* **273:**8528–8536.

Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P, and Negishi M (1999) The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J Biol Chem* **274:**6043–6046.

Takahashi Y, Nakayama K, Itoh S, Fujii-Kuriyama Y, and Kamataki T (1997) Inhibition of the transcription of CYP1A1 gene by the upstream stimulatory factor 1 in rabbits. Competitive binding of USF1 with AhR Arnt complex. *J Biol Chem* **272:**30025–30031.

Trottier E, Belzil A, Stoltz C, and Anderson A (1995) Localization of a phenobarbital-responsive element (PBRE) in the 5′-flanking region of the rat CYP2B2 gene. *Gene* **158:**263–268.

Waxman DJ (1999) P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. *Arch Biochem Biophys* **369:**11–23.

Waxman DJ and Azaroff L (1992) Phenobarbital induction of cytochrome P-450 gene expression. *Biochem J* **281:**577–592.

Wingender E, Chen X, Hehl R, Karas H, Liebich I, Matys V, Meinhardt T, Pruss M, Reuter I, and Schacherer F (2000) TRANSFAC an integrated system for gene expression regulation. *Nucleic Acids Res* **28:**316–319.

Xie W, Barwick JL, Downes M, Blumberg B, Simon CM, Nelson MC, Neuschwander-Tetri BA, Brunt EM, Guzelian PS, and Evans RM (2000a) Humanized xenobiotic response in mice expressing nuclear receptor SXR. *Nature (Lond)* **406:**435–439.

Xie W, Barwick JL, Simon CM, Pierce AM, Safe S, Blumberg B, Guzelian PS, and Evans RM (2000b) Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. *Genes Dev* **14:**3014–3023.

Zelko I and Negishi M (2000) Phenobarbital-elicited activation of nuclear receptor CAR in induction of cytochrome P450 genes. *Biochem Biophys Res Commun* **277:**1–6.

Address correspondence to: Dr. Urs A. Meyer, Division of Pharmacology/Neurobiology, Biozentrum of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland. E-mail: urs-a.meyer@unibas.ch