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Regulation of Human *CYP2C9* by the Constitutive Androstane Receptor: Discovery of a New Distal Binding Site

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ABSTRACT

The CYP2C subfamily metabolizes many clinically important drugs. These genes respond to prototypical inducers such as phenobarbital and rifampicin, yet little has been reported on the mechanisms of induction. This report examines the regulation of *CYP2C9* with respect to two specific receptors thought to be involved in phenobarbital (PB) induction, the constitutive androstane receptor (CAR) and pregnane X receptor (PXR). Transfection of either mouse CAR (mCAR) or human CAR (hCAR) into HepG2 cells results in increased CYP2C9 mRNA content. Inducers further increased this response in CAR transfected cells. mCAR but not hCAR conferred drug inducibility to the proximal −2145 bp of the *CYP2C9* promoter in luciferase assays. Further examination of a −2925-bp promoter construct revealed that hCAR cotransfection increased activity 20-fold. Gel shift assays confirmed the presence of a distal PB-responsive enhancer module-like enhancer module, CAR-responsive enhancer (CAR-RE), between −2900 and −2841 bp consisting of two DR-5 nuclear receptor binding motifs capable of binding hCAR, mCAR, and, to a lesser extent, human PXR. The majority of binding and hCAR activation is derived from the NR1 portion of the CAR-RE. PB treatment did not further increase the hCAR activation in any of the constructs. In summary, a new CAR/PXR binding site was identified in the *CYP2C9* promoter, and this site seems to constitutively regulate transcription via a CAR-dependent mechanism; however, it could not be shown to account for PB inducibility of the gene.

Cytochrome P450 enzymes make up a superfamily of metabolic enzymes that serve a variety of functions in living organisms (Nelson et al., 1996). Often, P450s can metabolize xenobiotics to more hydrophilic derivatives to facilitate excretion, and can serve as a protective barrier against xenobiotics by directed expression in barrier tissues such as liver, intestine, and kidney (Gonzalez, 1988). The P450 superfamily in humans is composed of more than 50 distinct genes; however, the majority of human metabolism of clinical drugs is carried out by the *CYP1*, *CYP2*, *CYP3*, and *CYP4* gene families (Handschin et al., 2001). The CYP2C subfamily is an important class of enzymes composed of four isoforms (CYP2C9, CYP2C19, CYP2C8, and CYP2C18) that are thought to metabolize approximately 20% of known drugs (Goldstein 2001). Specifically, CYP2C19 stereoselectively hydroxylates the *S*-enantiomer of mephenytoin (anticonvulsant), and can also metabolize the antiulcer drug omeprazole, the β-adrenergic receptor blocker propranolol, and the anxiolytic diazepam (Andersson et al., 1992). CYP2C8 has been shown to metabolize the anticancer drug paclitaxel (Taxol) and is thought to catalyze the oxidation of endogenous arachidonic acid into vasodilating metabolites (epoxyeicosatrienoic acids) and to play a role in retinoic acid metabolism (Rifkind et al., 1995). The CYP2C9 isoform is the most clinically important member of this subfamily, metabolizing many prescribed drugs, such as the hypoglycemic drug tolbutamide, the anticoagulant warfarin, the anticonvulsant phenytoin, the diuretic torsemide, and several nonsteroidal anti-inflammatory drugs such as diclofenac, ibuprofen, piroxicam, and acetylsalicylic acid, along with endogenous compounds such as arachidonic acid (Goldstein and de Morais 1994). From this abbreviated list of CYP2C substrates, it is clear that this subfamily is an important component of the human defense system against xenobiotic exposure.

Along with many P450 isoforms, the CYP2C subfamily is thought to be inducible in humans by pretreatment with phenobarbital and other prototypical inducers such as rifampicin and dexamethasone (Gerbal-Chaloin et al., 2001). This

ABBREVIATIONS: P450, cytochrome P450; PB, phenobarbital; PXR, pregnane X receptor; CAR, constitutive androstane receptor; RT-PCR, reverse transcription-polymerase chain reaction; CAR-RE, constitutive androstane receptor responsive element; DMSO, dimethyl sulfoxide; TCPOBOP, (1,4-bis[2-(3,5-dichloropyridyloxy)]benzene); XS, *Xho*I-*Stu*I; XH, *Xho*I-*Hin*dIII; XE, *Xho*I-*Eco*RI; SV40, simian virus 40 promoter; MEM, minimal essential medium; hCAR, human constitutive androstane receptor; mCAR, mouse constitutive androstane receptor; 2898, distal constitutive androstane receptor binding module of CYP2C9 from −2900 to −2840 bp; ND, no-drug; hPXR, human pregnane X receptor; kb, kilobase(s); bp, base pair(s).

inducibility can lead to significant drug-drug interactions that may affect the pharmacokinetic profiles of endogenous and xenobiotic substrates (Fuhr, 2000; Moore and Kliewer; 2000). Recently, significant progress has been made in unraveling the mystery of the drug induction mechanism in mammals (Kliewer et al., 1999; Waxman, 1999; Zelko and Negishi, 2000). Specific enhancer regions in the 5′ flanking region of PB-inducible genes have been identified in mouse, human, rat, and chicken, and specific DNA binding receptors have been identified and shown to bind to these promoter elements and facilitate transcriptional activation constitutively and in response to drug treatment. Among these receptors, the pregnane X receptor (PXR), also called steroid and xenobiotic receptor (Blumberg et al., 1998), and the constitutive androstane receptor (CAR) are thought to play prominent roles in mouse, rat, and human, whereas the chicken xenobiotic receptor has been identified to play an analogous role in chickens (Handschin et al., 2000). Although significant progress has been made in unraveling the mechanisms of induction in CYP2B and CYP3A subfamilies, little research has been reported on the CYP2C subfamily.

For the CYP2C subfamily, it has been reported that rifampicin and barbiturates can induce metabolism in vivo (Zhou et al., 1990; Chan et al., 1994). Recently, Gerbal-Chaloin et al. (2001) demonstrated that PB, rifampicin, and dexamethasone can up-regulate *CYP2C8*, *CYP2C9*, and *CYP2C19* transcript levels in primary human hepatocytes. Although an earlier study reported that *CYP2C9* and *CYP2C19* are not inducible by PB or rifampicin (Runge et al., 2000), the majority of reports indicate that *CYP2C8*, *CYP2C9*, and perhaps *CYP2C19* are transcriptionally up-regulated by these prototypical inducers.

To examine the potential roles of CAR and PXR in drug induced transcriptional activation of CYP2Cs, we performed RT-PCR to test the effects of overexpression of CAR on CYP2C mRNA content in HepG2 cells; we cloned the *CYP2C9* promoter sequences into luciferase reporter constructs and cotransfected the reporter with CAR and PXR to elucidate their roles in regulating transcription and drug induction; we identified a new CAR/PXR binding site at −2898 bp (CAR-RE) from the translational start; and we examined the ability of this new regulatory element to respond to transfection of CAR in the presence and absence of prototypical inducers in luciferase reporters.

Materials and Methods

Materials. Phenobarbital (sodium salt) was purchased from Sigma-Aldrich (St. Louis, MO). Androstenol was purchased from Steraloids, Inc., (Newport, RI). Rifampicin, clotrimazole, DMSO, and other common reagents not listed were purchased from Sigma-Aldrich or standard sources. 1,4-bis[2-(3,5-Dichloropyridyloxy)]benzene (TCPOBOP) was a kind gift from Dr. James Sideway (AstraZeneca, Hässle, Mölndal, Sweden). Cell culture media was purchased from Sigma-Aldrich. Fetal bovine serum and trypsin/EDTA were purchased from Invitrogen (Carlsbad, CA). Oligonucleotides were purchased from Genosys, Inc. (The Woodlands, TX) at 50 μM scale and desalted.

Isolation of Total RNA and RT-PCR Analysis. Total RNA was extracted using TRIzol Reagent (Invitrogen). RT-PCR analysis was performed in two steps by initial reaction with Superscript II (Invitrogen) reverse transcriptase, followed by PCR with *Taq* DNA Polymerase (Invitrogen). For the RT reaction, 200 ng of total RNA was combined with 2 μl (40 units) of RNase inhibitor (PerkinElmer, Boston, MA), 1× first strand buffer (final), 10 mM dithiothreitol (final concentration), 0.5 mM dATP, dTTP, dGTP, and dCTP (each, final), and 1 μl (200 units) of Superscript II to total volume of 20 μl, and incubated at 42°C for 50 min, then inactivated at 70°C for 15 min, and stored at −20°C or immediately used in PCR analysis. PCR was performed with 2 μl of RT template (1 μl for β-actin controls), 1× *Taq* buffer, 0.4 mM dNTP, 2 mM $MgCl_2$, 5 pmol of each primer, and 1 unit of *Taq* polymerase. Specific primers for *CYP2C9* (65.815 forward and 65.903 reverse) are shown in Table 1 with annealing temperature implemented. β-Actin primers used are also shown in Table 1. PCR reactions were initiated with a 95°C denaturation step for 30 s, then cycled 35 times at 94°C for 30 s, at appropriate melting temperature for 30 s, and 72°C for 30 s. PCR products were analyzed on 3% agarose gels and stained with ethidium bromide.

Plasmids and Cloning of *CYP2C9* Promoter Region. pGL3-Basic, pGL2-Enhancer, and pGL3-Promoter were purchased from Promega (Madison, WI). pGL3-tk and the nuclear receptor expression plasmids pSG5-hPXR, pCR3.1-hCAR, pCR3-mCAR, and pGEMT-hRXR were provided by author M.N. *CYP2C9* 5′ flanking region from −2145 bp to +61 bp was cloned previously in our laboratory into the pGL2-Enhancer luciferase reporter system and named 2.2Luc/E along with deletions *Xho*I-*Stu*I (XS), *Xho*I-*Hin*dIII (XH), *Xho*I-*Pst*I, and *Xho*I-*Eco*RI (XE) (Ibeanu and Goldstein 1995). #4/P/For was prepared by cloning the *Sac*I fragment from 2.2Luc/E

TABLE 1
Primers

Primer	Primer Sequence (5′ → 3′)
CYP2C9-65.815F	AGGAAAAGCACAACCAACCA
CYP2C9-65.903R	TCTCAGGGTTGTGCTTGTC
β-Actin-F	GAGCTATGAGCTGCCTGACG
β-Actin-R	CACTTGCGGTGCACGATG
2C9-212NheIF	GCTAGCGAGTGGACAATGGAAC
2C9-1NcoIR	CATGCCATGGTTGAAGCCTTCTC
2C9-2923F/NheIF	GCTAGCCCATAGAATGTACAAC
2C9-2898fullF	CTAGAATGAACCCTACATAAACTATGAGCTTTGGTTGAT
2C9-2898fullR	CTAGAATGAACCAACATTGACGCATCATCATCAACCAAA
2C9-NR1F	CTAGAATGAACCCTACATAAACTAT
2C9-NR1R	CTAGATAGTTTATGTAGGGTTCATT
2C9-NR2F	CTAGATGCGTCAATGTTGGTTCA
2C9-NR2R	CTAGAATGAACCAACATTGACGC
2B6-NR1F	CTAGACTGTACTTTCCTGACCCTG
2B6-NR1R	CTAGCAGGGTCAGGAAAGTACAGT
QX-2C9-m1818F	GTTATAAAACAAACTCTTCTCTGGTCTCAATCTAGTCAA
QX-2C9-m1818R	TTGACTAGATTGAGACCAGAGAAGAGTTTGTTTTATAAC
QX-2C9-m2898F	GTACAACACAAAGAAGGGACCCTACATAAACTATG
QX-2C9-m2898R	CATAGTTTATGTAGGGTCCCTTCTTTGTGTTGTAC

into *Sac*I digested and dephosphorylated pGL3-SV40. Deletions of this construct were made by cleavage with *Kpn*I and *Stu*I, *Hin*dIII, *Pst*I, *Bsa*I, or *Eco*RI. These cleavage products were flushed with Klenow (New England Biolabs, Beverly MA) and dNTPs, then blunt-end ligated overnight. 2C9Basal promoter construct was prepared by insertion of a PCR amplicon from −212 bp to −1 bp generated from the 2.2Luc/E template with primers 2C9-212NheIF and 2C9-1NcoIR (Table 1) using *Nhe*I and *Nco*I recognition sites to the 5′ and 3′ termini, respectively. 2C9-3kb#5 was constructed by first subcloning a 3-kb amplicon of *CYP2C9* from a human PAC clone (Incyte Genomics, Palo Alto, CA) DNA with primers 2C9-2923F/*Nhe*I and 2C9-1NcoIR (Table 1) into the pCRTopoTA vector (Invitrogen, Carlsbad, CA). After sequence verification with genomic sequence data (National Center for Biotechnology Information), *Nhe*I and *Nco*I sites were used to remove the *CYP2C9* promoter region and insert into pGL3-B. 2898f-tk-Luc was constructed by ligating a double-stranded oligonucleotide sequence identical to the *CYP2C9* sequence from −2898 bp to −2839 bp into pGL3-TK at *Nhe*I sites with oligonucleotides 2C9-2898fullF and 2C9-2898fullR. 2898r-2C9Basal-Luc was prepared by ligating double-stranded oligonucleotides 2C9-NR1F and 2C9-NR1R 2C9Basal at the *Nhe*I site in reversed orientation. $(NR1)_{2XF}$-2C9Basal-Luc was also created by oligonucleotide ligation of double stranded 2C9-NR1 oligonucleotides with two copies in the forward orientation into 2C9Basal via *Nhe*I sites. 2C9-3kb#5 mutants m1818, m2898, and m1818&m2898 were generated with the QuikChange site-directed mutagenesis (Stratagene, La Jolla, Ca) strategy to sequences with primers QX-2C9-m1818F, QX-2C9-m1818R, QX-2C9-m2898F, and QX-2C9-m2898R (Table 1) following the manufacturers' recommended procedure. All sequences were verified by Dye terminator DNA sequencing (Applied Biosystems, Foster City, CA).

Culture and Transfection of HepG2 Cells. HepG2, g2car-3, and g2hCAR-8 cells were cultured in Eagle's minimal essential medium (MEM) with 31 mg/l penicillin and 50 mg/l streptomycin and supplemented with 10% fetal bovine serum. Primary human hepatocytes were kindly provided by Dr. Edward LeCluyse (School of Pharmacy, University of North Carolina at Chapel Hill, NC). Primary hepatocytes were maintained in Williams E medium with 31 mg/l penicillin and 50 mg/l without phenol red, and supplemented with insulin-transferrin-selenous acid (Collaborative Biomedical Products, Bedford, MA) on collagen-coated plates (Biocoat, Fort Washington, PA). Transfections were performed with CellPhect (Amersham Biosciences, Piscataway, NJ) calcium phosphate-mediated transfection reagent or Effectene transfection reagent (QIAGEN, Valencia, CA) using manufacturers' recommended procedures. All cells were plated in 24-well plates, transfected for 12 to 18 h, and washed with media before drug treatment. g2car-3 cells were kindly provided by author M.N., whereas the stable hCAR cell line g2hCAR-8, analogous to the mCAR containing g2car-3, was generated by selecting for G418 resistant colonies after transfection of endotoxin free pCR3.1-hCAR and analyzing for activity with *CYP2B6* $(NR1)_5$-tk-Luc and Western blotting for CAR expression.

Trancriptional Activation Assays. HepG2 and g2car-3 cells (Sueyoshi et al., 1999) were maintained in Eagle's MEM with 10% fetal bovine serum. Cells were passaged at a density of approximately 100,000 cells/well in 24-well plates. Transfections typically included 100 ng of receptor, 100 ng of reporter, and 25 ng of pRL-SV40 transfection control with the exception of the 2C9-3kb#5 reporter, which has lower expression levels, and requires 400 ng of reporter with 5 ng of pRL-SV40 to provide detectable signal. This requirement is consistent with a negative regulating region of the *CYP2C9* promoter described previously (Ibeanu and Goldstein 1995). Twelve to 24 h after transfection, cells were washed per manufacturer's recommendations, treated with drugs or controls, and incubated for 24 or 48 h. Cells were subsequently lysed with 100 μl of passive lysis buffer (Promega) for 2 h at room temperature with gentle rocking. Dual luciferase assays (Promega) were then performed on cell lysates per manufacture's procedure.

Gel Mobility Shift Assays. Electrophoretic gel mobility assays were performed by methods analogous to the procedure described by Honkakoski et al. (1998). Briefly, human RXR, human CAR, mouse CAR, and human PXR were synthesized in vitro using the TNT Quick-Coupled In Vitro Transcription/Translation system (Promega) following the manufacturer's protocol. Probes were labeled with [γ-^{32}P]dCTP and the probe was purified by Microspin G-25 columns (Amersham Biosciences). A volume of 150,000 cpm of labeled probe was applied to each binding reaction in 10 mM HEPES, pH 7.6, 0.5 mM dithiothreitol, 15% glycerol, 0.05% Nonidet P-40, 50 mM NaCl, 2 μg of poly(dI-dC), and 1 to 2 μl of in vitro transcribed/translated proteins to a final reaction volume of 10 μl. The reactions were incubated at room temperature for 20 min, then loaded on 5% acrylamide gels in Tris/acetate/EDTA buffer, dried, and exposed to film for 6 to 18 h at −70°C.

Results

RT-PCR of *CYP2C9* in HepG2, g2car-3, and g2hCAR-8 Cells. To examine the possible mechanisms mediating drug induction, we compared *CYP2C9* mRNA content of human HepG2 cells with that of mRNA found in cells transiently transfected with human CAR as well as cells stably transfected with mouse or human CAR (hCAR), g2car-3, and g2hCAR-8, respectively. Figure 1A shows that stable transfection of mCAR (g2car-3 cells) results in a significant increase in constitutive *CYP2C9* transcript in untreated cells, whereas no detectable product is observed in either untreated HepG2 cells or cells treated with xenobiotics. In cells stably transfected with mCAR, *CYP2C9* mRNA was significantly elevated by 0.2% DMSO vehicle, whereas *CYP2C9*

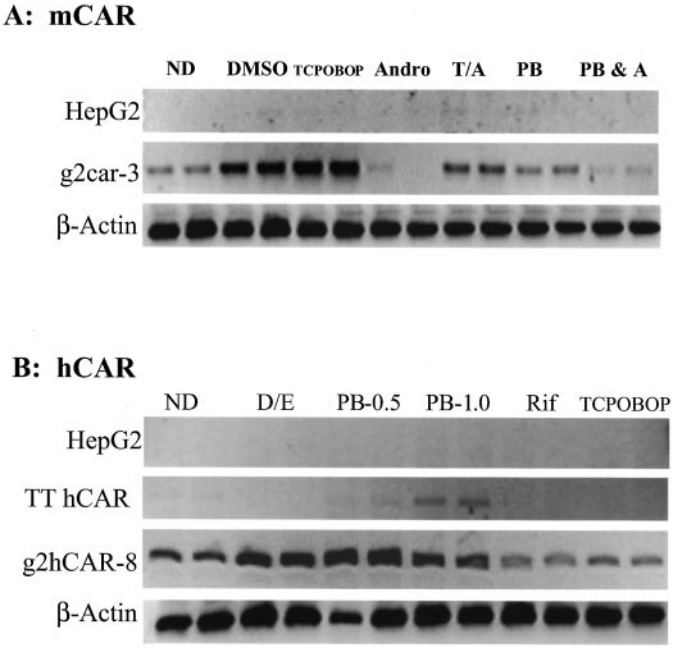


Fig. 1. Total RNA was isolated from HepG2 cells and derivatives transfected with CAR. RT-PCR was performed as described under *Materials and Methods*. A, specific primers for *CYP2C9* were applied in RT-PCR analyses of transcript levels of RNA from HepG2 and a stably transfected mCAR derivative (g2car-3) treated for 48 h with no drug (ND-PB control), DMSO (0.2%), TCPOBOP (250 nM), androstenol (Andro; 10 μM), TCPOBOP + androstenol (T&A), PB (500 μM-Na^+ salt), and PB + androstenol. B, analogous experiments to A, investigating the hCAR response in both stably (g2hCAR-8) and transiently transfected cells treated with no drug, DMSO/ethanol vehicle (D/E), 0.5 mM PB, 1.0 mM PB, 10 μM rifampicin (Rif), and 250 nM TCPOBOP.

transcripts were further increased upon addition of the potent mCAR inducer TCPOBOP (250 nM) relative to DMSO vehicle controls. The constitutive activation of *CYP2C9* by mCAR is extensively repressible with 10 μM androstenol to quantities below those of the DMSO control and untreated cells. This androstenol repression of *CYP2C9* mRNA can be derepressed by coaddition of TCPOBOP. PB treatment alone at 500 μM had little effect on *CYP2C9* mRNA in g2car-3 cells relative to the no drug vehicle; however, PB treatment did produce a marginal derepression of the androstenol repression when both effectors were present. In contrast, rifampicin produced no significant effects on *CYP2C9* mRNA in g2car-3 cells (data not shown). These data demonstrate that mCAR can regulate transcription and drug induction of the *CYP2C9* gene in a manner analogous to that reported for human *CYP2B6*.

Stable human CAR transfection in HepG2 cells (g2hCAR-8 cells) results in highly elevated constitutive levels of *CYP2C9* transcript relative to untransfected HepG2 cells (Fig. 1B). A similar but less pronounced constitutive activation was found when hCAR was transiently transfected into HepG2 cells, a result consistent with lower hCAR content in cell transfected transiently versus stably. PB treatment at 0.5 and 1.0 mM produced further elevation of *CYP2C9* mRNA in both transiently and stably transfected HepG2 cells relative to the no-drug vehicle (ND). Treatment with rifampicin (10 μM) did not induce *CYP2C9* transcription, but significantly repressed the constitutive activity of hCAR relative to its D/E vehicle. Finally, in contrast to our observations in g2car-3 cells (Fig. 1A), TCPOBOP (250 nM) treatment does not induce *CYP2C9* with stably transfected hCAR, an observation consistent with other reports of differences in regulation by mouse versus human CAR (Moore et al., 2000). In summary, these data indicate that CAR plays a role in the expression of *CYP2C9* and could potentially play a role in PB inducibility in humans.

Drug Response of *CYP2C9* Proximal Promoter Region. To further evaluate the role of CAR in regulation of *CYP2C9*, we used luciferase promoter assays of the 5′ flanking region from −2145 bp to +61 bp. In previous studies, this region was cloned into the pGL2-Enhancer vector in a construct termed 2.2Luc/E (Ibeanu and Goldstein 1995). Transfection studies with this construct demonstrated dose-dependent TCPOBOP and PB induction in g2car-3 cells consistent with RT-PCR analyses (data not shown). Similar induction responses were also observed for this region in luciferase promoter constructs #4/P/For and #3/P/Rev driven by the SV40 promoter (data not shown). These data indicate that there is a mCAR responsive DNA element within the proximal −2145 to + 61 bp of the *CYP2C9* promoter.

To localize the responsive region that seems to confer mCAR dependent drug induction, we examined deletions of 2.2 Luc/E. In g2car-3 cells a 2-fold activation was observed with 1 mM PB (Fig. 2). This response is maintained upon deletion of the *Xho*I/*Stu*I fragment (XS) of 2.2 Luc/E to a length of −1877 bp to +61 bp. Further deletion to *Xho*I/*Hin*dIII (XH) to −1454 bp results in the loss of mCAR dependent PB inducibility that is also absent in subsequent deletions of −990 and −362 bp. Simultaneous experiments in HepG2 and g2hCAR-8 cells indicate no PB induction is observable for 2.2Luc/E or its deletions. Taken together, these studies suggest that although mCAR seems to mediate PB induction of −2 kb, hCAR is not effective. One possible explanation is that hCAR-mediated PB inducibility may be controlled by more distal elements of the *CYP2C9* promoter.

Receptor Activation and Drug Response of the −3kb *CYP2C9* Promoter Region. Due to the availability of high throughput genomic sequence (HTGS), we were able to analyze −5 kb of the *CYP2C9* promoter sequence for potential CAR/PXR binding sites based on homology with established sites from the literature. Using the GCG Wisconsin Package (Accelrys, Princeton, NJ) findpatterns tool, we discovered a putative CAR/PXR binding cassette within the proximal −3 kb of the promoter sequence with similar topology to PBREM (CYP2B) and XREM (CYP3A) (Fig. 3). Analogous to phenobarbital responsive promoter elements in *CYP2B1*, *CYP2B2*, *CYP2B6*, and *Cyp2b10*, this module consists of two direct repeat motifs spaced by a short sequence (22 bp in this case). Both of these putative nuclear receptor binding sites have a DR-5 topology, each consisting of two aggtca-like direct repeats spaced by 5 bp. By comparison, CYP2B elements in mouse and human have been found as DR-4 motifs, whereas CYP3A genes are predominantly DR-3 or ER-6 (Fig. 3). By comparison with known xenobiotic responsive elements in P450s, the topology of this putative CAR/PXR binding site warranted detailed examination.

To address the potential role of this module in *CYP2C9* transcriptional regulation, we cloned the proximal −3 kb of 5′-flanking region into pGL3-Basic. This construct, termed 2C9-3kb#5, was transfected in HepG2 cells under various conditions with nuclear receptors hCAR, mCAR, and hPXR (Fig. 4A). Relative to no-receptor controls, hCAR cotransfection produces an extensive activation (22-fold) of 2C9-3kb#5. Transient transfection with mCAR activates this construct to a lesser extent (4-fold), whereas hPXR addition had no effect on basal levels of reporter activity. PB treatment of HepG2 cells without exogenous receptor produces a 2-fold activation. PB treatment did not further increase luciferase activity in cells cotransfected with hCAR, although it marginally increased reporter activity with mCAR (1.6-fold). PB treatment also marginally induced reporter levels in cells transfected

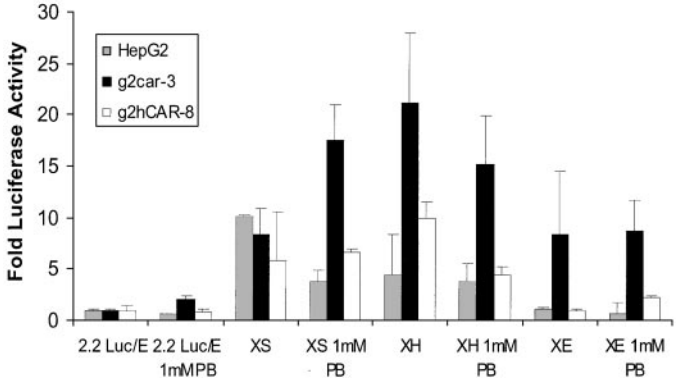


Fig. 2. 2.2Luc/E and the pRL-SV40 control plasmid were transfected into HepG2 and stably transfected derivative cell lines g2car-3 (mCAR) and g2hCAR-8 (hCAR) for 12 to 18 h. Cells were washed with PBS, fed, and treated with 1 mM PB for 48 h. Cells were lysed with passive lysis buffer and luciferase assays were performed using the manufacturer's recommended procedure. Fold Luciferase Activity was normalized to the 2.2Luc/E-ND control. XS, XH, and XE are sequential deletions of 2.2 Luc/E at −1877, −1454, and −362 bp, respectively. These data represent results obtained from two independent experiments with S.D. indicated between replicate samples.

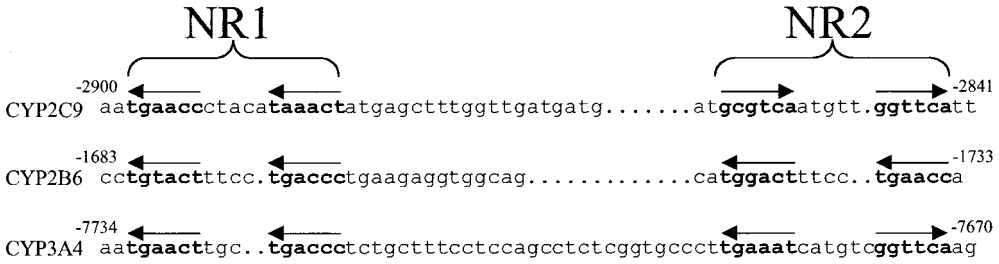


Fig. 3. Distal promoter elements identified in *CYP2C9*, *CYP2B6*, *CYP1H1*, and *CYP3A4* thought to bind nuclear receptors and activate transcription via enhancer mechanisms. NR1 sequences from all four genes are shown in bold type. Spacers are inserted to align receptor binding half-sites from the different genes.

with hPXR relative to hPXR alone, whereas, in contrast, the hPXR ligand rifampicin did not induce reporter activity with hPXR. In total, these data suggest that hCAR can strongly activate reporter activity within the proximal −3 kb of the *CYP2C9* gene, whereas hPXR is essentially ineffective under the conditions implemented, and fails to account for rifampicin inducibility of the *CYP2C9* gene.

Transfections of 2C9-3kb#5 in primary human hepatocytes were also performed (Fig. 4). For these experiments, 0.1 μM dexamethasone was applied to the media to maximize receptor response (LeCluyse et al., 2000). These data provide similar results to those observed in HepG2 cells transfected with hCAR (data not shown). The magnitude of this response (3-fold) is much lower, but this may reflect endogenous hCAR levels or lower transfection efficiencies. Thus, in both primary human hepatocytes and HepG2 cells, hCAR cotransfection activates transcription of the proximal −3kb of the *CYP2C9* promoter. These data for 2C9-3kb#5 demonstrate that hCAR can regulate constitutive transcriptional activity of the proximal −3 kb of the *CYP2C9* promoter.

Element Identification and Gel Shift Analysis of Binding. To specifically examine the potential of the 60-bp PBREM-like region at −2898 bp (termed "2898 module") of the *CYP2C9* promoter in nuclear receptor-mediated regulation, we first tested its ability to bind transcription factors CAR and PXR. Initially, we examined the full 2898 module shown in Fig. 3. Both putative sites, NR1 and NR2, resemble imperfect AGGTCA motifs known for many nuclear receptor binding sites, including CAR and PXR. To test the binding capabilities of this putative regulatory module, we designed oligonucleotide probes to the 2898 module, labeled the double stranded segment, and performed binding assays with in vitro transcribed/translated nuclear receptors hCAR, mCAR, and hPXR. These binding studies produced an intense band with hCAR/hRXR proteins that was not observed with hCAR alone and was competed out by addition of 5-fold excess cold competitor double-stranded oligonucleotide (data not shown). Similar but somewhat lower intensity binding was observed for mCAR and hPXR with the 2898 module. To further probe the specificity of binding to the 2898 module, we tested the individual NR1 and NR2 components with each nuclear receptor. Figure 5 contains a representative example of the results of these analyses. Here, the *CYP2B6* NR1 sequence was used as a positive control and demonstrates strong binding with hCAR/hRXR and mCAR/hRXR, whereas hPXR/hRXR binds with significantly lower affinity, consistent with published reports that CAR and PXR can bind to the identical enhancer elements (Xie et al., 2000). This binding was completely lost by competition with 5-fold excess cold competitor. By comparison, the NR1 sequence from the *CYP2C9* 2898 module also binds strongly to hCAR/hRXR and mCAR/

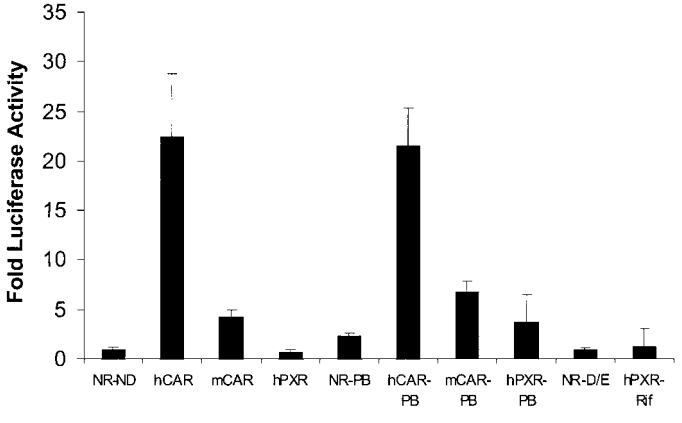


Fig. 4. Luciferase assays were performed on HepG2 cells transiently transfected with 2C9-3kb#5, a luciferase construct containing −2928 to −1 bp of the *CYP2C9* promoter region. 100 ng of nuclear receptor expression plasmids for hCAR, mCAR, and hPXR were cotransfected for 12 to 18 h, followed by 48-h incubation with PB (1 mM), TCPOBOP (250 nM), 50:50 DMSO/ethanol (D/E) vehicle, clotrimazole (10 μM), or rifampicin (Rif; 10 μM). Cells were lysed with PLB and assays via manufacturer's recommended procedure. These data represent independent analyses and S.D. values between replicate samples are indicated.

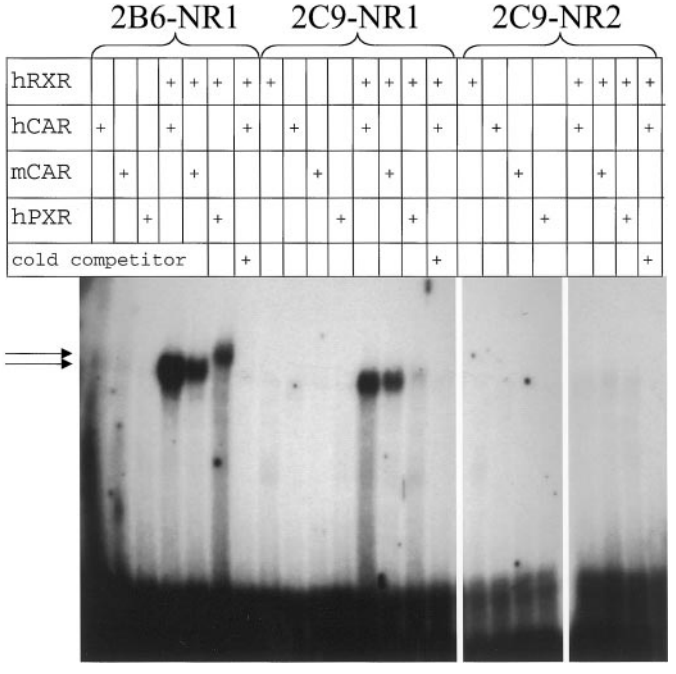


Fig. 5. Electrophoretic mobility shift assays were performed as described under *Materials and Methods*. Oligonucleotides for human *CYP2B6* NR1, *CYP2C9* NR1, and *CYP2C9* NR2 were labeled with [^{32}P]dCTP (Amersham Bioscieces), and nuclear receptors were synthesized in vitro (Promega Quick Coupled TNT) and combined at room temperature for 20 min in binding reactions. Nonradiolabeled competitor was added at 5-fold excess for the hCAR/hRXR binding interaction.

hRXR; however, a much weaker (clearly observable upon longer exposure) affinity to hPXR/hRXR was observed. Although the overall intensity of the *CYP2C9* NR1 binding seems slightly lower than the *CYP2B6* NR1 control, when one considers the somewhat lower efficiency of the *CYP2C9* NR1 labeling reaction in this experiment, the affinity is comparable. However, the putative NR2 binding site of the 2898 module produces much weaker binding with all three receptors under identical conditions. Upon longer exposure, this NR2 binding is observable; however, it is considerably weaker than the NR1 binding to CAR. In total, the putative 2898 module of *CYP2C9* from −2900 kb to −2840 kb can bind the nuclear receptors CAR>PXR only in the presence of RXR, and the binding seems to arise predominantly through the NR1 segment.

2898 Module Reporter Assays. Once binding to nuclear receptors CAR and PXR was established, we began to explore the potential role of the 2898 module in the regulation of promoter activity. From the 2C9-3kb#5 transfection experiments, we observed a prominent activation upon hCAR cotransfection, but neither hCAR nor hPXR seemed to regulate drug induction by phenobarbital, rifampicin, or TCPOBOP in this transfection-based promoter system in either HepG2 cells or primary hepatocytes. To further test whether induction was controlled by our newly discovered nuclear receptor binding site, we generated additional luciferase reporter constructs for transfection experiments. Within a constructed 2C9Basal luciferase reporter (−212 to −1 bp), we inserted either the complete 2898 module (2898-2C9Basal) or two copies of the CAR-RE (NR1) segment (NR1-2XF-2C9Basal) upstream of the basal promoter sequence. Transfection experiments in HepG2 cells resulted in reporter activation with cotransfected hCAR analogous to that observed in experiments with the proximal −3 kb of the *CYP2C9* promoter (Fig. 6A). This increase was also observed with the 2898 module inserted into the heterologous tk promoter (data not shown) and indicates that within HepG2 cells, this enhancer element can confer elevated transcriptional activity via a hCAR mediated mechanism. A similar, but less prominent response is observed for mCAR cotransfected cells from both reporters shown in Fig. 6A. hPXR cotransfection failed to increase reporter activity over untreated control. These luciferase reporter experiments in HepG2 cells seem consistent with our DNA binding studies, which demonstrate that hCAR, mCAR, and, to a much lower extent, hPXR can bind to this newly discovered enhancer site within the *CYP2C9* gene and, taken together, offer a similar story to that observed for the full-length 2C9-3kb#5 reporter.

Further examination of these reporters in primary human hepatocytes was performed, and the results are shown in Fig. 6B. Here, hCAR cotransfection results in a 3-fold increase in reporter activity for the 2898-2C9Basal plasmid, whereas a seemingly additive 6-fold activation is observed for the NR1-2XF-2C9Basal construct with regard to the high affinity CAR-RE segment. Conversely, neither mCAR nor hPXR cotransfection resulted in activation of either construct in primary human hepatocytes. Consistent with observations with the longer 2C9-3kb#5 reporter, it seems that hCAR, but not mCAR cotransfection can activate transcription of the 2898 module in primary human hepatocytes. To further evaluate the enhancer properties of the CAR-RE segment, we tested its ability to confer PB inducibility in the context of the basal *CYP2C9* promoter in HepG2 cells and primary human hepatocytes (data not shown). We found that PB treatment (1 mM) produced no additional increase in reporter activity in hCAR-cotransfected cells, and rifampicin (10 μM) did not induce reporter activity in hPXR-cotransfected cells. In total, these in vitro data indicate that the CAR binding site at −2898 bp of the *CYP2C9* promoter responds to hCAR in a manner analogous to that observed for the longer −3kb reporter. However, we could not account for PB or rifampicin inducibility with or without CAR or PXR transfection under the conditions of our experiments.

To further probe the nature of the hCAR interaction with CAR-RE and potential effects on PB inducibility, we titrated the cotransfected quantity of hCAR expression plasmid, and assayed for luciferase activity of the NR1-2XF-2C9Basal reporter. In HepG2 cells (Fig. 7A), cotransfection with 10 pg (20 pg/ml) or 100 pg of pCR3-hCAR had only marginal effects on reporter activity. Further 10-fold increase to 1 ng of pCR3-hCAR resulted in a significant 5-fold activation that was indistinguishable from that observed for 10 ng. A 15-fold activation was observed at the highest quantity of hCAR (100

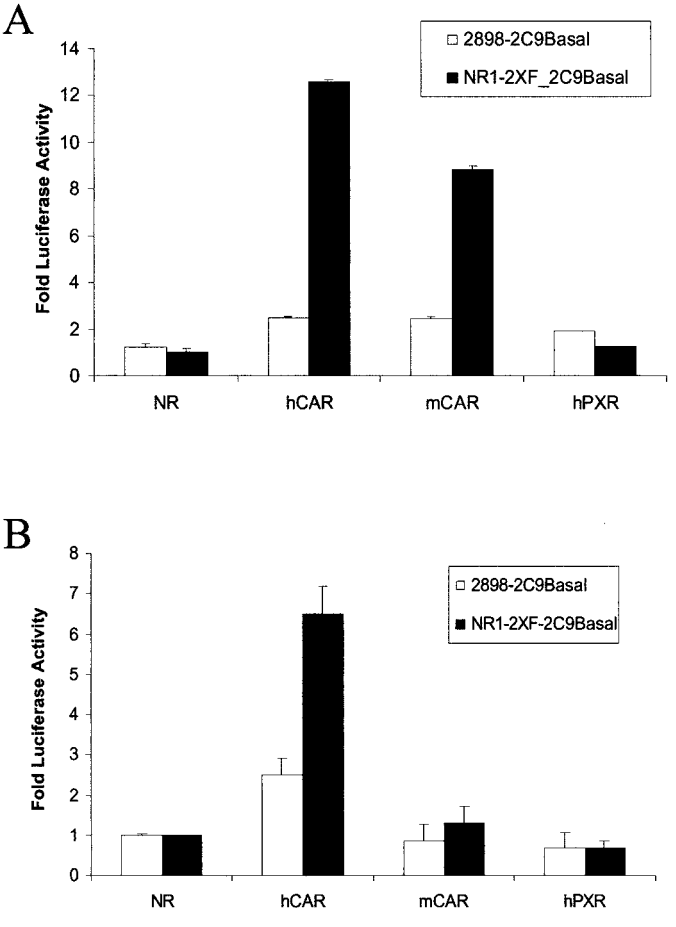


Fig. 6. Luciferase assays were performed on HepG2 cells (A) and primary human hepatocytes (B) transiently transfected with 2898-2C9Basal and NR1-2XF-2C9Basal reporters of the full PBREM-like module, and the high affinity NR1 segment, respectively, driven by the −212 bp to −1 bp region of the *CYP2C9* promoter. One hundred nanograms of each nuclear receptor expression vector was cotransfected with 100 ng of reporter and 10 ng of pRL-SV40 internal control. Transfections were incubated for 12 to 18 h, then washed with media and further incubated for 48 h. These data represent independent analyses, with S.D. values indicated between replicate samples.

ng), the amount used in all other cotransfection experiments. Upon reducing the strong basal activity of hCAR by limiting its quantity, we examined the effects of PB treatment at each of the quantities tested above. From Fig. 7A, PB did not significantly induce reporter activity at any of the hCAR quantities, consistent with our previous observations that hCAR-dependent PB inducibility is not observable in any of our promoter assays. An analogous experiment was conducted in primary human hepatocytes (Fig. 7B). Here, a marked 3-fold increase was observed at 100 pg of pCR3-hCAR (3-fold) and was further increased to 5-fold at 10 ng, both of which are identical to responses observed in HepG2 cells. However, at 100 ng of hCAR expression plasmid, only a 5-fold activation is observed, which is significantly lower than the 15- to 17-fold response observed in HepG2 cells. Decreased transfection efficiencies observed for primary human hepatocytes may provide a clue to this significant difference in reporter activity. PB treatment had no effect at any of the points, consistent with previous observations. In summary, these data demonstrate that the quantity of hCAR expression plasmid transfected, and thus hCAR receptor, is directly related to the level of reporter activation of our CAR-RE, yet reducing the quantity of hCAR protein does not afford observable PB inducibility.

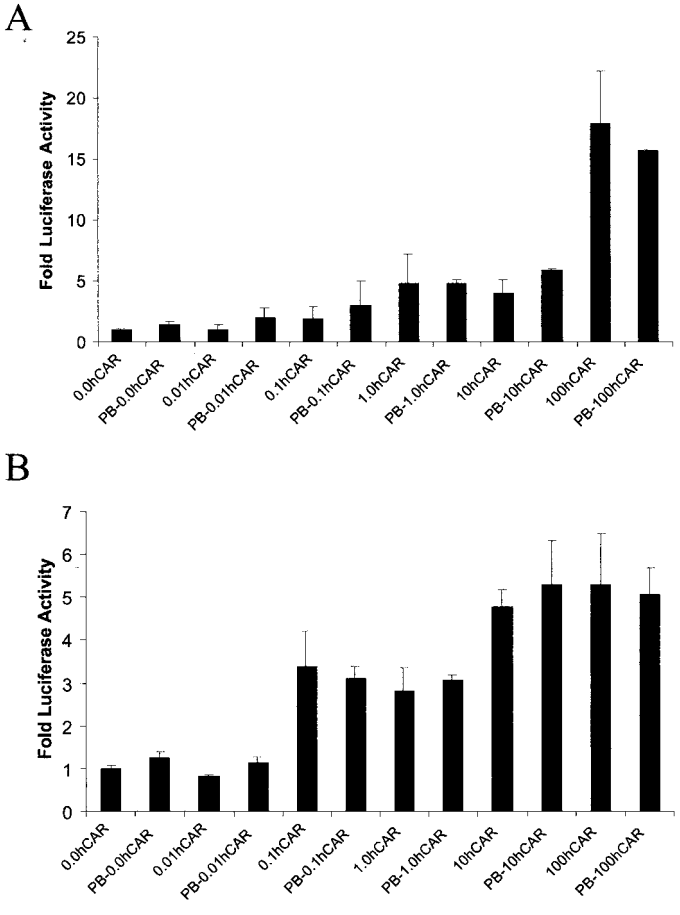


Fig. 7. Luciferase assays were performed on HepG2 cells (A) and primary human hepatocytes (B) transiently transfected with NR1-2XF-2C9Basal. pCR3.1-hCAR (10 pg, 100 pg, 1 ng, 10 ng, and 100 ng) were cotransfected with 100 ng of reporter and 10 ng of pRL-SV40 internal control. Transfections were incubated 12 to 18 h, then washed with media and treated with no drug or PB (1 mM) for 48 h. These data represent independent analyses, and S.D. values between replicate samples are indicated.

Mutational Analysis of Known CAR Binding Sites. To examine the specific contribution of the newly discovered CAR binding site at −2898 bp relative to a CAR-binding site at −1818 bp recently identified by Gerbal-Chaloin et al. (2002), we mutated each site individually and in combination (Fig. 8A). Transfection of these constructs into HepG2 cells with and without transfection of hCAR was performed, and the results are shown in Fig. 8B. Cotransfection of 50 ng of hCAR activated the 2C9-3kb#5 wild-type reporter approximately 10-fold. Mutation of the CAR-RE at −2898 alone decreased CAR activation ~60%. Mutation of the CAR binding site at −1818 bp alone resulted decreased CAR activation by ~50%. These data demonstrate that mutation of either element alone does not completely abolish CAR activation in the context of the proximal −3 kb of the promoter. However, double mutation of both elements resulted in complete loss of hCAR activation in HepG2 cells. These data demonstrate that both elements are required for full activation by CAR, and that no other CAR activated enhancer sites seem to be localized within the proximal −3 kb of the promoter.

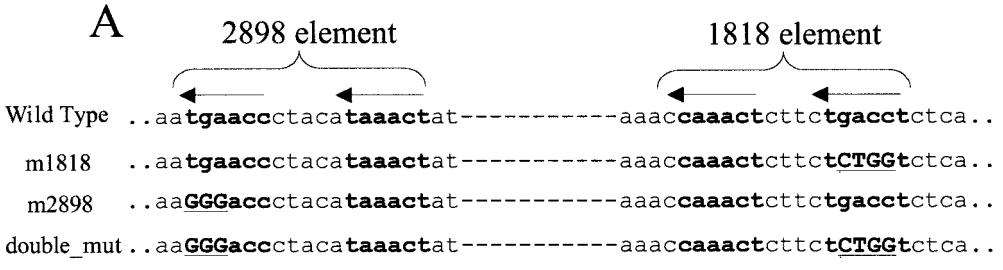
Analogous experiments performed in primary human hepatocytes were completely consistent with data in HepG2 cells. Here mutation of the element at 2898 decreased CAR activation 60%, mutation of the element at −1818 bp decreased CAR activation 42%, and mutation of both elements completely eliminated activation from transiently transfected hCAR (Fig. 8C). In the absence of exogenously transfected CAR, basal promoter activity was decreased by ~30%, probably due to constitutive levels of CAR in primary human hepatocytes. From these data, it is clear that hCAR activates *CYP2C9* transcription via two CAR binding sites at −2898 and −1818 bp of the *CYP2C9* promoter.

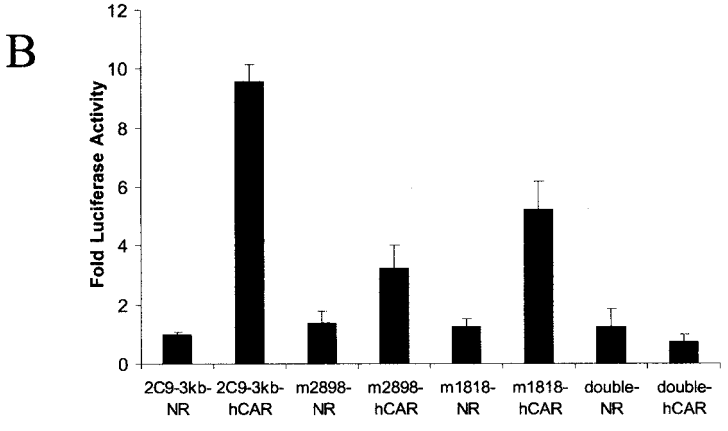
Discussion

One of the most significant findings of the present study was that stable transfection of human or mouse CAR in HepG2 cells increases both constitutive content of CYP2C9 mRNA and confers transcriptional drug inducibility (PB and TCPOBOP, respectively). This is the first report demonstrating that CAR transfection can up-regulate CYP2C9 mRNA content. Our finding that mCAR can regulate basal and drug responsive (androstenol repression, TCPOBOP activation and derepression) transcription of human *CYP2C9* is consistent with previous reports for *CYP2B6* (Sueyoshi et al., 1999); however, an mCAR mediated mechanism fails to explain the process in humans. hCAR, unlike mCAR is poorly repressed by androstenol, as has been reported previously (Moore et al., 2000; Gerbal-Chaloin et al., 2002). The observation that hCAR can up-regulate basal transcription of *CYP2C9* is consistent with reports that neither the human nor mouse isoforms require apparent ligand binding to activate transcription (Choi et al., 1997). Thus, CAR can up-regulate transcription of *CYP2C9* in HepG2 cells, and understanding the mechanism of this response may help unravel the mechanisms of induction of the CYP2Cs by PB and other drugs in humans.

Our investigations of the proximal −2 kb *CYP2C9* promoter revealed a 2- to 3-fold mCAR-dependent PB and TCPOBOP induction that could not be demonstrated in hCAR-transfected cells. Thus, the mRNA response to PB treatment observed with transfected hCAR could not be ac-

counted for in the proximal −2kb, and we examined more distal regions of the promoter. We discovered that at −2898 bp from the translation start site, a pair of putative CAR/PXR binding sites were present in a configuration similar to that of PBREM (CYP2B) and XREM (CYP3A) (Sueyoshi and Negishi 2001). We hypothesized that this unique module, composed of two DR-5 motifs, might account for PB and/or rifampicin inducibility in human *CYP2C9* via CAR and/or PXR dependent mechanisms. Our demonstration that CAR and to a lesser extent PXR could bind the module further supported this idea. This is the first example of a CAR/PXR binding site with a DR-5 structure; however, nuclear receptors are known to have spacers as large as 8 bp (Kast et al., 2002). Upon cloning and analyzing reporter assays with a longer 3-kb segment of the *CYP2C9* promoter, our most prominent response was a marked increase in reporter activity with the cotransfection of hCAR. Further investigation revealed that the presence of the CAR-RE (NR1) portion of the 2898 module was sufficient to confer hCAR mediated activation to the basal *CYP2C9* promoter in both HepG2 cells and primary human hepatocytes. We also found that hCAR expression increased reporter activity of the CAR-RE response element in a concentration-dependent manner. This increase is consistent with a specific interaction between the receptor and the CAR-RE. These findings indicate that the CAR-RE is involved in hCAR mediated activation of *CYP2C9* transcription. A recent study by Gerbal-Chaloin et al. (2002) suggests that the presence of a regulatory element at −1818 containing a DR-4 motif may also be involved in CAR activation. However, mCAR activates their motif with similar intensity to hCAR. This is consistent with our observations with mCAR and hCAR in the proximal −2kb of the promoter, yet our studies with the larger −3 kb region indicate that hCAR is much more effective in activating the longer −3-kb





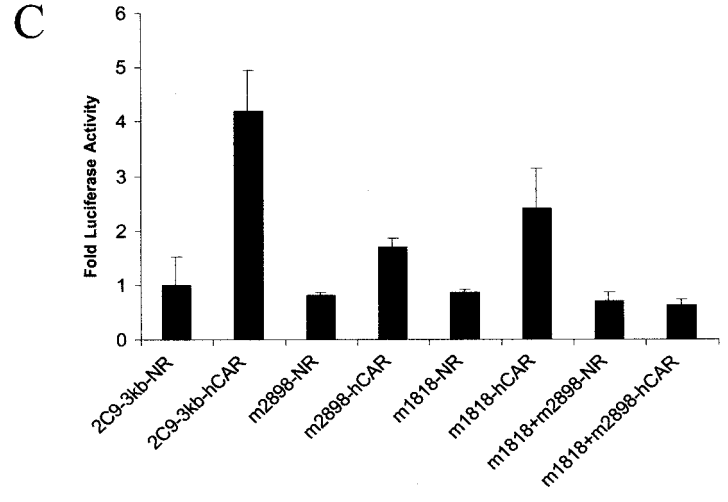


Fig. 8. Luciferase assays were performed on HepG2 (B) and primary human hepatocytes (C) transiently transfected with the wild-type 2C9-3kb#5 and mutants indicated using 50 ng of pCR3.1-hCAR. Transfections were incubated 12 to 18 h, then washed with media and incubated for 48 h. These data represent independent analyses, and S.D. values are indicated between replicate samples.

reporter than mCAR. This difference is consistent with our finding that the newly discovered CAR-RE at −2898 bp binds and is activated more effectively with hCAR than mCAR. To assess the relative contribution of each binding site to full hCAR response, we mutated each site individually and in concert. From these experiments, it seems that both elements are involved in CAR activation because only simultaneous mutation of both elements eliminates hCAR activation. This also indicates that no additional cryptic elements are responsive to hCAR within this proximal −3 kb region. Whether human CAR is localized in the cytosol or nucleus in human hepatocytes remains unknown; thus, the interpretation of the basal activity differences of the 2C9-3kb#5 mutants in primary human hepatocytes remains difficult to reconcile. If hCAR regulates basal activity, then mutation of the CAR binding sites should significantly reduce untreated activity compared with wild-type, but only if CAR is localized in the nucleus. If CAR is localized in the cytosol, as is the case for mCAR in primary mouse hepatocytes, it could explain why our transient transfection response is greatly reduced compared with HepG2 cells, with only a percentage of transfected hCAR overwhelming the sequestration mechanism and entering the nucleus to activate transcription. In addition, cytosolic localization of CAR would produce small-to-no change in basal reporter levels (as was observed in our experiments) between wild type and mutants. In conclusion, the mechanism of CAR activity in humans remains unclear; however, our observations with CYP2C9 mRNA and reporter constructs should provide additional knowledge to help elucidate these complex processes.

One goal of our original studies was to elucidate the mechanisms of rifampicin and PB induction for *CYP2C9* by nuclear receptors CAR and PXR. Although hCAR transfection confers detectable transcriptional activation to the endogenous *CYP2C9* gene in HepG2 cells, our reporter data suggests that PB or rifampicin induction may not be localized in the proximal −3 kb of the gene. The lack of an observable PXR mediated rifampicin induction with CAR-RE was not surprising based on the low binding affinity of PXR to the element; however, Gerbal-Chaloin et al. (2002) reported significant binding of PXR to the enhancer at −1818 bp. It is unclear why hPXR-mediated rifampicin induction was observed in *CYP3A4* controls but not in the *CYP2C9* reporters. Gerbal-Chaloin et al. (2002) demonstrated that mCAR-dependent activation of the *CYP2C9* proximal promoter is repressible by androstenol and derepressible with TCPOBOP, which is consistent with our observations. However, mouse CAR is not present in human cells, and no such drug activation of CYP2C9 by hCAR, via either of these elements, has been demonstrated. Although hCAR transfection confers detectable transcriptional activation to the endogenous *CYP2C9* gene in HepG2 cells, our reporter data does not localize PB or rifampicin induction within the proximal −3 kb of the gene. Rifampicin and PB induction of *CYP2C9* may also conceivably be mediated by alternate receptors or more distal elements that have yet to be examined. For *Cyp2b10*, it has been reported that knockouts of mCAR in mice abolishes PB inducibility; however, other genes remain inducible in the CAR knockout mice (Ueda et al., 2002). Thus, CAR-independent mechanisms are apparently responsible for PB induction of some genes, which could conceivably include human *CYP2C9*. Alternatively, essential cofactors may be expressed at low levels due to dedifferentiation (Dixon and Ginsberg, 1993) in HepG2 cells or primary hepatocytes and limit our ability to model the in vivo process. Moreover, mCAR differs from human CAR in several aspects (Moore et al., 2000). Mouse CAR is repressed by androstenol, and such drugs as PB and TCPOBOP can derepress this repression. Mouse CAR normally resides in the cytosol but is in the nucleus of HepG2 cells. Its localization in heterogeneous populations of human primary hepatocytes is not well documented. PB inducibility was observed with mCAR but not hCAR in our reporter assays of the proximal −2 kb. Therefore, elements that regulate drug induction in response to mCAR could possibly be important in human drug response, and the lack of response to hCAR in a heterologous system could be misleading. In total, the role of human CAR in regulating basal and PB induced transcription of all cytochrome P450 genes remains an interesting topic for future research.

In conclusion, we have identified the nuclear receptor CAR (from both mouse and human) as an effective regulator of *CYP2C9* transcription at both the mRNA level and in promoter assays. We have demonstrated that mouse and human CAR differentially regulate transcription, with only mCAR conferring drug response in reporter assays. Finally, we identified a CAR/PXR binding site at −2898 bp of the *CYP2C9* 5′ flanking region (CAR-RE) that can confer strong hCAR dependent activation to luciferase reporters. Because PB and rifampicin induction could not be associated with a specific enhancer element within the first 3 kb of the *CYP2C9* promoter, further investigation will be required to unravel the mechanism of drug-drug interactions in humans. However, our experiments clearly show a role for hCAR in the constitutive activation of *CYP2C9* in humans and show that two CAR binding elements are important in constitutive *CYP2C9* regulation: a new distal element (CAR-RE) (−2898) and one at −1818 bp. Both the proximal and distal elements seem to act in concert to regulate *CYP2C9* expression.

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