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# Transcriptional Regulation of the Human *CYP3A4* Gene by the Constitutive Androstane Receptor

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## ABSTRACT

Cytochrome P450 3A4 (CYP3A4), the predominant P450 expressed in adult human liver, is both constitutively expressed and transcriptionally activated by a variety of structurally diverse xenochemicals. In this study, we examined the role of the constitutive androstane receptor (CAR), a member of the steroid/retinoid/thyroid hormone receptor superfamily, in the transcriptional regulation of *CYP3A4*. Herein, we demonstrate that CAR is capable of *trans*-activating expression of the *CYP3A4* gene, both in vitro and in vivo. Induction of *CYP3A4* is dependent on cooperativity between elements within the promoter proximal region of the gene and the distal xenobiotic-responsive enhancer module. CAR responsiveness was shown to be primarily mediated by two high-affinity binding motifs located within the *CYP3A4* gene 5′-flanking region, approximately 7720 and 150 bases upstream of the transcription initiation site. Importantly, the human CAR response elements also mediate *trans*-activation of *CYP3A4* by the human pregnane X receptor, suggesting that interplay between these receptors is likely to be an important determinant of *CYP3A4* expression.





The cytochromes P450 (P450) are a superfamily of heme-thiolate-containing proteins involved in the oxidative metabolism of a plethora of endogenous and exogenous compounds (Nelson et al., 1996). Cytochrome P450 3A4 (CYP3A4), the predominant P450 isoform constitutively expressed in adult human liver, is transcriptionally activated by a variety of structurally diverse compounds, including rifampicin, phenobarbital (PB), mifepristone, and clotrimazole (Maurel, 1996). *trans*-Activation of P450 genes by xenochemicals increases the organism's capacity to metabolize and ultimately excrete toxins and carcinogens (Denison and Whitlock, 1995; Waxman, 1999).

Until recently, the molecular mechanisms underlying the transcriptional activation of the *CYP3A4* gene were poorly understood. A number of independent studies have demonstrated that the human pregnane X receptor (hPXR) is activated by compounds that are known *CYP3A4* inducers, including drugs, steroids, and environmental chemicals (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998; Schuetz et al., 1998). Ligand-activated hPXR binds hormone response elements (HREs) in the 5′-flanking region of the *CYP3A4* gene as a heterodimer with the 9-*cis* retinoic acid receptor-$\alpha$ (RXR$\alpha$) (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998; Goodwin et al., 1999). Targeted disruption of PXR in mice results in selective loss of xenobiotic inducibility of the murine *Cyp3a11* gene, but does not affect constitutive expression in liver or intestine (Xie et al., 2000a; Staudinger et al., 2001). Although PXR-RXR$\alpha$ heterodimers bind an HRE in the promoter proximal region of *CYP3A4* (prPXRE, bases −172 to −149) (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998), activation of the native *CYP3A4* promoter is dependent upon the presence of a distal xenobiotic-responsive enhancer module (XREM, bases −7836 to −7208) (Goodwin et al., 1999). Cooperativity between promoter proximal and distal PXR-response elements is central to the PXR-mediated *trans*-activation of *CYP3A4*.

Recently, Negishi and coworkers demonstrated that induction of *CYP2B* genes by PB and a variety of structurally unrelated compounds collectively known as "PB-like" inducers [e.g., chlorpromazine, methoxychlor, and 1,1,1-trichloro-1,2-bis(*o*,*p*′-

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**ABBREVIATIONS:** P450, cytochrome P450; PB, phenobarbital; hPXR, human pregnane X receptor; PXR, pregnane X receptor; HRE, hormone response element; RXR$\alpha$, 9-*cis* retinoic acid receptor-$\alpha$; prPXRE, proximal pregnane X receptor response element; XREM, xenobiotic-responsive enhancer module; CAR, constitutive androstane receptor; mCAR, murine constitutive androstane receptor; hCAR, human constitutive androstane receptor; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; kb, kilobase; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair(s).

chlorophenyl)ethane] was mediated by the constitutive androstane receptor (CAR) (Honkakoski et al., 1998; Kawamoto et al., 1999; Sueyoshi et al., 1999). Overexpression of murine CAR (mCAR) in HepG2 cells conferred PB responsiveness on the endogenous *CYP2B6* gene and heterologous reporter gene constructs containing the *CYP2B6* PB-responsive enhancer module (Sueyoshi et al., 1999). In mammalian cell lines and yeast, mCAR is transcriptionally active in the absence of exogenous ligand (Forman et al., 1998; Kawamoto et al., 1999; Sueyoshi et al., 1999). The constitutive *trans*-activational capacity of mCAR is repressed by the steroids androstanol (5$\alpha$-androstan-3$\alpha$-ol) and androstenol (5$\alpha$-androst-16-en-3$\alpha$-ol) (Forman et al., 1998). In HepG2 cells, the ligand-dependent repression of mCAR is reversed by PB and PB-like inducers (Kawamoto et al., 1999; Sueyoshi et al., 1999). More recently, naturally occurring and xenobiotic mCAR and human CAR (hCAR) agonists were identified (Moore et al., 2000; Tzameli et al., 2000). In similarity to human and mouse PXR, hCAR and mCAR exhibited divergent activation profiles (Lehmann et al., 1998; Moore et al., 2000).

In the liver and primary cultures of hepatocytes, mCAR is sequestered in the cytoplasm and only translocates to the nucleus after exposure of the cell to PB or PB-like inducers. The PB-induced nuclear translocation is uncoupled by concomitant exposure to okadaic acid an inhibitor of phosphatases 1 and 2A, suggesting that dephosphorylation of mCAR is required for its nuclear compartmentalization (Kawamoto et al., 1999). Thus, the PB-induced dephosphorylation of CAR seems to be critical step in *CYP2B* induction (Kawamoto et al., 1999). In support of this observation, previous reports have documented the importance of phosphorylation status in *CYP2B* induction (Sidhu and Omiecinski, 1995; Nirodi et al., 1996; Honkakoski and Negishi, 1998). Importantly, nuclear translocation of mCAR in HepG2 cells is spontaneous and does not seem to be dependent on ligand binding and/or modification of phosphorylation status (Kawamoto et al., 1999; Sueyoshi et al., 1999). Thus, cDNA-directed expression of mCAR resulted in transcriptional activation of the endogenous *CYP2B6* gene in the absence of inducer. Moreover, although mCAR ligands, namely, androstanol and androstenol, inhibited the *trans*-activational capacity of mCAR in HepG2 cells they did not block nuclear translocation of the protein (Sueyoshi et al., 1999). Targeted in vivo disruption of mCAR completely abrogates PB and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP)-mediated induction of the murine *Cyp2b10* gene (Wei at al., 2000), demonstrating the pivotal role of this receptor in the xenobiotic induction of some P450s. The mechanisms by which PB and PB-like inducers modify the cellular localization of CAR seem to involve a leucine-rich region near the C terminus of the CAR protein that has been designated a xenochemical response signal (Zelko et al., 2001).

Interestingly, mCAR-RXR$\alpha$ heterodimers are capable of binding the prPXRE (bases $-172$ to $-149$) of the human *CYP3A4* gene. Additionally, a reporter gene construct containing multimerized copies of this motif was transcriptionally activated by mCAR (Sueyoshi et al., 1999; Tzameli et al., 2000; Xie et al., 2000b). Although *trans*-activation of the native *CYP3A4* promoter by mCAR was not reported, the ability of both CAR-RXR$\alpha$ and PXR-RXR$\alpha$ heterodimers to interact with the same nuclear receptor-binding motif in the *CYP3A4* promoter suggests that interplay between nuclear receptors is likely to be a significant factor in *CYP3A4* regulation. This contention is supported by the recent findings that hPXR can *trans*-activate the human *CYP2B6* gene (Goodwin et al., 2001) and the mouse *Cyp2b10* gene (Xie et al., 2000b) through response elements that also interact with hCAR.

Accordingly, the present study was designed to investigate the role of CAR in the transcriptional regulation of *CYP3A4*. CAR was demonstrated to be capable of *trans*-activating *CYP3A4* expression. Moreover, hCAR-binding motifs in the proximal promoter and distal enhancer regions of *CYP3A4* were mapped and shown to be identical to those described previously for hPXR (Goodwin et al., 1999).

## Experimental Procedures

**Materials.** The mammalian expression vector pSG5 and SuperScript II reverse transcriptase were from Stratagene (La Jolla, CA). PCR primers, TaqMan probes, and PCR master mix were sourced from Applied Biosystems (Foster City, CA). FuGENE 6 transfection reagent was provided by Roche Applied Science (Castle Hill, NSW, Australia).

**Reporter Gene Constructs and Expression Vectors.** Preparation of the chimeric *CYP3A4*-luciferase reporter gene constructs, including the constructs containing mutated HREs, was described in detail previously (Goodwin et al., 1999). The structure of the p3A4-362 and p3A4-362(7836/7208ins) is shown in Fig. 1A. Site-directed mutagenesis of the prPXRE in the CYP3A4 promoter (as shown in Fig. 3) was designed to disrupt both the ER-6 motif as well as an overlapping imperfect DR-4 motif (AACTCAaaggAGGTCA). The hCAR and hRXR$\alpha$ expression vectors pSG5-hCAR and pSG5-hRXR$\alpha$, respectively, were generously provided by Dr. Steven A. Kliewer (GlaxoSmithKline Research, Research Triangle Park, NC).

**Transient Transfection of Mammalian Cells.** The human hepatoblastoma cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA) and maintained in antibiotic-free Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. Cells ($1 \times 10^5$) were inoculated into 24-well plates 24 h before transfection with FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Typically, cells were transfected with 80 ng of luciferase reporter gene construct, 30 ng of $\beta$-galactosidase control vector (pCMV$\beta$), and 0 to 100 ng of receptor expression vector (adjusted to 100 ng with pSG5). Subsequently, cells were cultured for 48 h in fresh medium supplemented with 10% charcoal-stripped serum. Luciferase activities were determined on cell lysates using a commercially available system (Promega, Madison, WI). $\beta$-Galactosidase assays were performed as described previously (Foster et al., 1988).

**Electrophoretic Mobility Shift Assay.** EMSA of putative hCAR-hRXR$\alpha$-binding motifs was performed using in vitro transcribed/translated hCAR and hRXR$\alpha$ exactly as described previously (Goodwin et al., 1999).

**Quantitation of CYP3A4 and PXR mRNA Levels.** CYP3A4 and hPXR mRNA levels were examined by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). HepG2 cells were transfected in 60-mm-diameter culture dishes, as described above, with the pSG5-hCAR expression vector (0–1600 ng adjusted to 1600 ng with pSG5). The cells were cultured for a further 48 h before extraction of RNA using a commercially available reagent (TRIzol; Invitrogen, Carlsbad, CA). cDNA was synthesized from 5 $\mu$g of total RNA using random hexamers and Superscript II reverse transcriptase according to the manufacturer's instructions. An aliquot of each cDNA synthesis reaction (1 $\mu$l) was subjected to PCR amplification using a Prism 7700 real-time PCR platform (Applied Biosystems). Primers and TaqMan probes were as follows: CYP3A4 151 to 323 bp, forward primer TTGTCCTACCATAAGGGCTTTTGT, reverse primer AAAGGCCTCCGGTTTGTGA, probe 210 to 238 bp FAM-AGTGTGGGGCTTTTATGATGGTCAACAGC-TAMRA; and hPXR

1448 to 1528 bp, forward primer CCCAGCCTGCTCATAGGTTC, reverse primer GGGTGTGCTGAGCATTGATG, probe 1469 to 1497 bp FAM-TGTTCCTGAAGATCATGGCTATGCTCACC-TAMRA. Results were normalized against β-actin determined using a commercially available TaqMan kit (catalog no. 401846; Applied Biosystems). Both the CYP3A4 and hPXR probes were designed to cross intron-exon junctions to avoid interference from genomic DNA. Moreover, the CYP3A4 primers and probe were designed so as to exclude other CYP3As, particularly the highly homologous CYP3A7 cDNA. Cycle parameters for all PCR were 50°C for 2 min then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

**Trangenic Mice.** A transgene was constructed by linking the *CYP3A4* gene 5′-flanking region, extending from the *Kpn*I site at −13 kilobase pairs to +53 bp downstream of the transcription start site, to an *Escherichia coli lacZ* reporter gene. The latter comprises the coding region for the bacterial enzyme β-galactosidase flanked by DNA sequences for eukaryotic translational start and stop signals, simian virus 40 transcriptional termination and polyadenylation signals, and an intron. Mice carrying the CYP3A4/*lacZ* transgene were created by microinjection of the DNA constructs into the pronuclei of zygotes harvested from FVB/N strain mice. Microinjection and manipulation of embryos were carried out by standard techniques (Hogan et al., 1994). Stable transgenic mouse lines were established by breeding from transgenic founders identified by Southern analysis and the line used for the present studies was termed CYP3A4-13kb-9/4. This transgenic model will be described in detail elsewhere (G. R. Robertson, B. Goodwin, J. Field, C. Liddle, in preparation). Mice ($n$ = 3/group) were treated with 3 mg/kg TCPOBOP or vehicle alone (corn oil) daily for 3 days by intraperitoneal injection as described previously (Wei at al., 2000) before sacrifice on day 4. β-Galactosidase activity was visualized in cut sections of liver by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

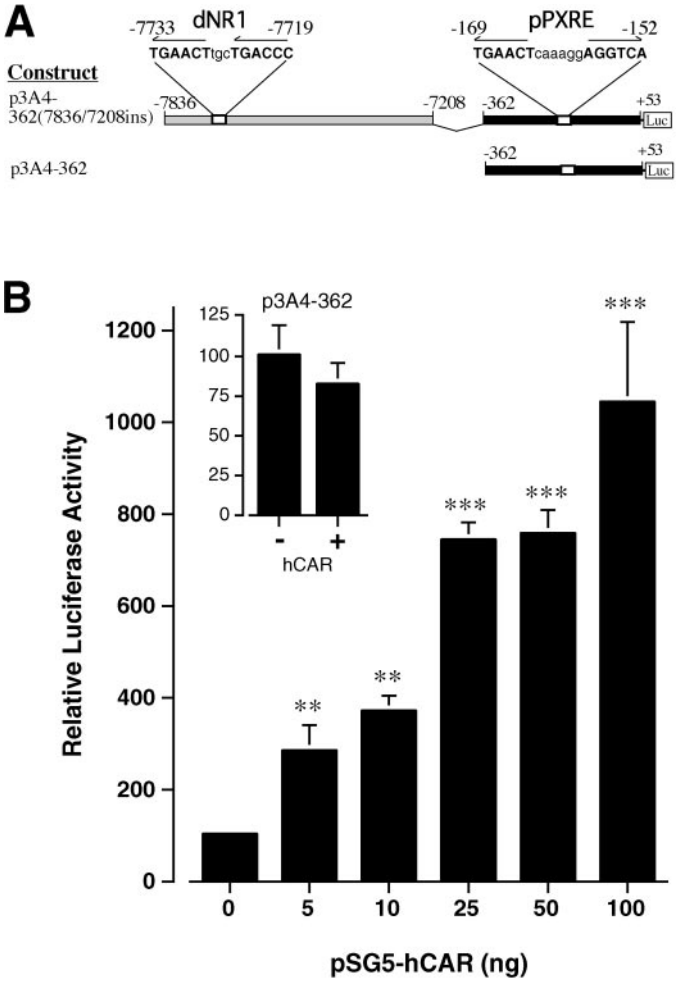



**Fig. 1.** *trans*-Activation of the chimeric *CYP3A4*-luciferase reporter gene constructs by hCAR. A, structure of the p3A4-362(7836/7208ins) and p3A4-362 constructs. The high-affinity hPXR binding motifs in the promoter proximal and XREM region (bases −7836 to −7208) of *CYP3A4*, prPXRE, and dNR1, respectively, and their positions relative to the transcription initiation site are shown. The nuclear receptor half-sites are delineated by horizontal arrows. B, p3A4-362(7836/7208ins) construct (80 ng) was transiently transfected into HepG2 cells in the presence of increasing amounts (0–100 ng) of an hCAR expression vector, pSG5-hCAR, as described under *Experimental Procedures*. The total amount of expression vector was adjusted to 100 ng by the addition of pSG5. The effect of exogenous hCAR expression (25 ng of pSG5-hCAR) on the activity of p3A4-362 is also shown (inset). Luciferase values were normalized to β-galactosidase. Data are mean ± S.D. of four individual transfections from a single representative experiment performed on two separate occasions. **, $p < 0.01$; ***, $p < 0.001$, relative to zero hCAR control.

**Statistics.** Multiple comparisons were performed by factorial analysis of variance. Post hoc comparisons between categories were accomplished using the Bonferroni/Dunn test.

## Results

The ability of hCAR to *trans*-activate the *CYP3A4* 5′-flanking region was examined by transient transfection of HepG2 cells. As shown in Fig. 1B, inset, the native *CYP3A4* proximal promoter (bases −362 to +53) did not confer hCAR responsiveness on luciferase reporter gene expression. In contrast, the p3A4-362(7836/7208ins) construct, which contains the XREM region (bases −7836 to −7208) in addition to the proximal promoter, was enhanced in a dose-dependent manner by cDNA-directed expression of hCAR (Fig. 1B). Maximal induction of reporter gene expression (approximately 8- to 10-fold) was observed with 25 to 100 ng of pSG5-hCAR (Fig. 1B). Subsequent cotransfection experiments were performed with 25 ng of pSG5-hCAR.

The interaction between hCAR-hRXRα heterodimers and putative nuclear receptor-binding motifs located in the XREM and proximal promoter regions of *CYP3A4* was examined by EMSA using in vitro-translated hCAR and hRXRα (Fig. 2). In keeping with previous reports (Sueyoshi et al., 1999), an everted repeat with a six-base spacer (ER-6) within the proximal promoter known to bind hPXR-hRXRα heterodimers (prPXRE) complexed hCAR-hRXRα. Additionally, a direct repeat with a three-base spacer (DR-3) within the XREM (dNR1) bound hCAR-hRXRα with high affinity (Fig. 2B). Extended autoradiography revealed that additional motifs within the XREM, referred to as dNR2 and dNR3 (Fig. 2A), were capable of weakly complexing hCAR-hRXRα heterodimers (data not shown). Importantly, the affinity of dNR2 and dNR3 for hCAR-hRXRα was substantially lower than either dNR1 or prPXRE (Fig. 2, B and C; data not shown).

The relative affinity of prPXRE, dNR1, and dNR2 for hCAR-hRXRα was examined by competition binding studies. A 5-fold molar excess of unlabeled dNR1 effectively competed with $^{32}$P-labeled prPXRE for hCAR-hRXRα (Fig. 2C). Indeed, these competition-binding studies indicated that the affinity of dNR1 for hCAR-hRXRα was approximately 4- to 5-fold higher than that of prPXRE. Thus, CAR-RXRα heterodimers exhibit higher affinity for DR-3 than ER-6 elements. In support of this observation, the intensity of the band-shift resulting from complexation of hCAR-hRXRα with dNR1 was significantly stronger than that seen when prPXRE was used

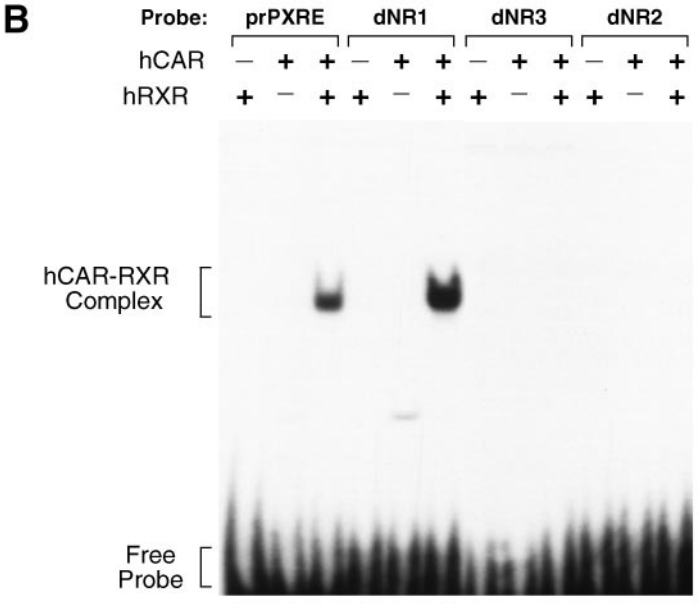
as a probe (Fig. 2B). In comparison, a 200-fold molar excess of unlabeled dNR2 failed to effectively compete with prPXRE for hCAR-hRXR$\alpha$ binding (Fig. 2C).

The relative contribution of the two high-affinity hCAR-hRXR$\alpha$-binding motifs, dNR1 and prPXRE, to the hCAR-mediated *trans*-activation of p3A4-362(7836/7208ins) was examined by site-directed mutagenesis. Cotransfection of pSG5-hCAR and p3A4-362(7836/7208ins) resulted in an 8- to 9-fold induction in reporter gene activity (Fig. 3). Mutation of dNR1 in the context of this construct resulted in a 56% reduction in hCAR responsiveness. Thus, cotransfection of pSG5-hCAR and the p3A4-362(7836/7208ins) construct harboring a mutated dNR1 site resulted in a 4-fold increase in luciferase expression. Similarly, mutation of prPXRE decreased hCAR-mediated *trans*-activation by approximately 45% (5-fold induction). Although the prPXRE in the context of the p3A4-362 construct (Fig. 1B, inset) has no inherent ability to confer hCAR inducibility on the luciferase reporter gene, this element seems to cooperatively interact with elements within the XREM region. This functional cooperativity was further investigated by linking the XREM region to a minimal thymidine kinase promoter (−105 to +52 bp). The presence of the heterologous promoter completely abrogated hCAR-mediated expression (data not shown), in contrast to the partial loss of expression seen when the prPXRE alone was mutated. Mutation of both dNR1 and prPXRE removed approximately 85% of the wild-type hCAR responsiveness. The residual hCAR inducibility (1.5-fold) of this construct is most probably mediated by the low-affinity hCAR-hRXR$\alpha$-binding motifs described above, namely, dNR2 and dNR3. These data are summarized in Fig. 3.

In HepG2 cells, exogenously expressed mCAR is known to spontaneously translocate to the nucleus. In this system, nuclear compartmentalization of the receptor and *trans*-ac-

**A**

| Probe | Sequence | Position |
|---|---|---|
| prPXRE | ata **TGAACT** caaagg **AGGTCA** gtg | -172 to -149 |
| dNR1 | gaa **TGAACT** tgc **TGACCC** tct | -7736 to -7716 |
| dNR2 | cct **TGAAAT** catgtc **GGTTCA** agc | -7696 to -7669 |
| dNR3 | ata **TATTGT** tat **TGAACT** atc | -7290 to -7270 |



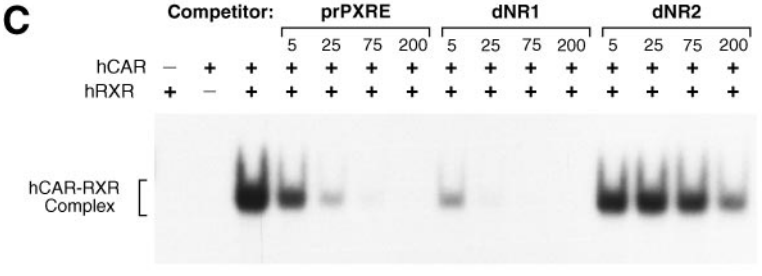




**Fig. 2.** EMSA of putative hCAR-hRXR$\alpha$-binding elements. The ability of potential hCAR-response elements to bind hCAR-hRXR$\alpha$ heterodimers was investigated using EMSA as described under *Experimental Procedures*. A, oligonucleotides used as probes. Putative nuclear receptor half-sites (uppercase) and the position relative to the *CYP3A4* transcription initiation site are shown. B, EMSA using radiolabeled prPXRE, dNR1, dNR3, and dNR2. Incubations received 1 $\mu$l of in vitro-translated hCAR, hRXR$\alpha$, or both as indicated. C, hCAR competition binding study. The ability of dNR1 and dNR2 to compete with prPXRE for binding of hCAR-hRXR$\alpha$ heterodimers was investigated by EMSA. Each lane contains 50 fmol of $^{32}$P-labeled prPXRE and in vitro-translated hCAR and hRXR$\alpha$ (0.5 $\mu$l each), as indicated. Unlabeled competitor oligonucleotides were added to the binding reaction 5-, 25-, 75-, and 200-fold molar excess.

tivation of target genes are ligand-independent. Therefore, we examined the ability of hCAR to regulate expression of the endogenous *CYP3A4* gene in HepG2 cells, a cell line that has been reported not to express significant amounts of CYP3A4 mRNA or protein. Using the sensitive technique of real-time RT-PCR *CYP3A4*-specific transcripts were routinely detected in HepG2 cells. Transient transfection of HepG2 cells with the hCAR expression vector pSG5-hCAR resulted in a dose-dependent increase in CYP3A4 mRNA levels (Fig. 4A). To exclude the possibility that the hCAR-induced increase in CYP3A4 mRNA was secondary to induction of hPXR expression, the abundance of hPXR mRNA was also determined by real-time RT-PCR. No increase in hPXR expression was observed (Fig. 4B).

To provide additional evidence for a functional role for CAR in *CYP3A4* regulation, mice bearing a transgene consisting of the *CYP3A4* 5′-flanking region (−13 kb to +53 bp) linked to a β-galactosidase reporter gene were treated with the mCAR-specific ligand TCPOBOP or vehicle ($n$ = 3/group), as described under *Experimental Procedures*. In mice receiving vehicle alone reporter gene expression was restricted to a small number of hepatocytes immediately adjacent to central veins or larger hepatic veins. In contrast, mice treated with TCPOBOP exhibited a striking induction of hepatic reporter gene expression, extending outward from central veins, such that approximately one-third of all hepatocytes exhibited positive staining for β-galactosidase (Fig. 5). Identical results were obtained for all three animals within each group.

## Discussion

The recognition that nuclear receptors capable of recognizing a range of lipophilic xenobiotic and endobiotic ligands can in turn regulate metabolizing and transporting genes has provided a new paradigm to explain how an organism is able to mount an adaptive response to potentially toxic compounds. The most extensively studied receptor in this respect is the PXR, although it is clear that there is substantial overlap between the ligand specificities of PXR and CAR (Moore et al., 2000). CYP3A4 represents a major pathway for clearance of both xenobiotics and endobiotics, and it is clear that PXR is a major mediator of transcriptional induction of this enzyme. To date, however, it is has been uncertain whether CAR is also able to *trans*-activate this enzyme in response to xenobiotic challenge.

In the present study we have shown that hCAR is capable of interacting with the regulatory 5′-flanking region of the *CYP3A4* gene. Recent reports suggested that the prPXRE of *CYP3A4*, an everted repeat of the AG(G/T)TCA hexamer separated by six nucleotides (ER-6), was capable of conferring CAR responsiveness on a heterologous thymidine kinase promoter (Sueyoshi et al., 1999; Tzameli et al., 2000). However, in the present study this element, in the context of the native *CYP3A4* promoter (bases −362 to +53), did not confer hCAR responsiveness in transient transfection studies performed in HepG2 cells. Similar observations have been made for the hPXR-mediated *trans*-activation of *CYP3A4*-reporter gene constructs (Goodwin et al., 1999). The ability of hCAR-RXRα and hPXR-RXRα heterodimers to interact with common nuclear receptor-binding motifs (Sueyoshi et al., 1999; Tzameli et al., 2000) suggested that the PXR-responsive XREM region of *CYP3A4* (bases −7836 to −7208) may also be capable of mediating *trans*-activation of *CYP3A4* by hCAR. Indeed, when the XREM region was linked to the proximal promoter, a CAR-dependent increase in reporter gene expression was observed.

To further understand the nature of the interaction between hCAR and *CYP3A4*, EMSA was performed on putative response elements within the proximal promoter and XREM. As described previously (Sueyoshi et al., 1999), the prPXRE complexed hCAR-hRXRα heterodimers. Additionally, there was a high-affinity site within the XREM, denoted as dNR1, that also bound hCAR-hRXRα. Importantly, the DNA binding profile of hCAR-hRXRα delineated in this study was highly homologous to that of hPXR-hRXRα (Goodwin et al., 1999). The importance of these DNA motifs in CAR-directed gene expression was confirmed by site-directed mutagenesis. Importantly, mutagenesis experiments revealed cooperation between dNR1 and the prPXRE, mirroring what we have observed previously for the PXR (Goodwin et al., 1999). This

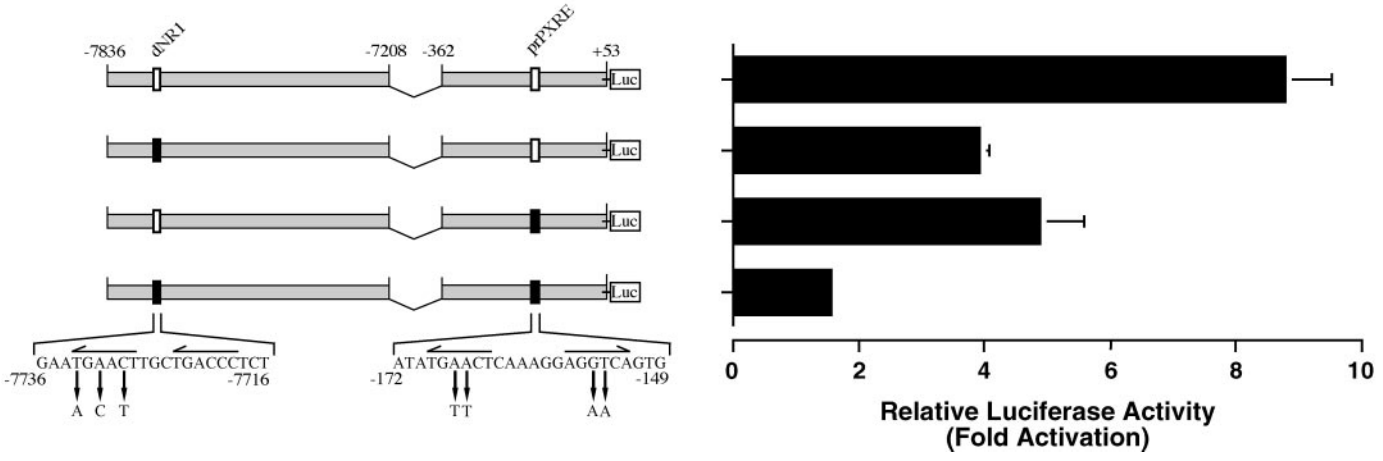



**Fig. 3.** Mutational analysis of putative hCAR response elements in the XREM and proximal promoter regions of *CYP3A4*. Putative hCAR response elements, namely, dNR1 and prPXRE, in the p3A4-362(7836/7208ins) construct were mutated as described under *Experimental Procedures*, and their hCAR responsiveness was analyzed in transient transfection studies. Reporter gene constructs (80 ng) were transfected into HepG2 cells in the presence of pSG5-hCAR (25 ng) and pCMVβ (30 ng). Cells were cultured for 48 h before harvest and determination of luciferase activity and β-galactosidase activity. Luciferase values are normalized to β-galactosidase and expressed as fold activation over parallel transfections in which pSG5-hCAR was substituted with the empty pSG5 vector (25 ng). Data are derived as described in Fig. 1.

is despite our finding that the prPXRE alone lacks the ability to mediate CAR-directed gene expression. To further examine this, the *CYP3A4* proximal promoter was replaced with a heterologous minimal thymidine kinase promoter. Surprisingly, all CAR-mediated transcription was lost, despite the presence of the XREM. This demonstrates that there is a functional dependence of the XREM on the native promoter that is independent of the prPXRE.

To determine the functional relevance of the interaction between CAR and CYP3A4, we used two entirely different models. First, we sought to determine whether hCAR was capable of regulating the endogenous *CYP3A4* gene in HepG2 cells. In this cell line exogenously expressed mCAR is known to spontaneously translocate to the nucleus and activate expression of the PB-inducible *CYP2B6* gene (Sueyoshi et al., 1999). In this system, nuclear compartmentalization of the receptor and *trans*-activation of the target gene are inducer-independent (Kawamoto et al., 1999; Sueyoshi et al., 1999). Transient transfection of hCAR resulted in a significant increase in endogenous CYP3A4 mRNA expression. Moreover, this was not mediated by an indirect effect of hCAR on endogenous PXR expression. These data demonstrate that endogenous *CYP3A4* gene in HepG2 cells is sensitive to hCAR-mediated regulation. Second, we performed a functional in vivo experiment. We were able to take advantage of the observation that the PB-like inducer TCPOBOP is a selective ligand for mCAR and does not bind to or activate mPXR (Moore et al., 2000). There is a lack of a similarly selective ligand for hCAR, making experiments performed in human models, such as primary human hepatocytes, difficult to interpret. Thus, mice carrying a *CYP3A4* regulatory transgene provide a useful system to determine the ability of CAR to *trans*-activate *CYP3A4*. The finding that TCPOBOP was able to markedly induce hepatic expression of the transgene provides strong evidence in favor of a functional role for CAR in *CYP3A4* regulation.

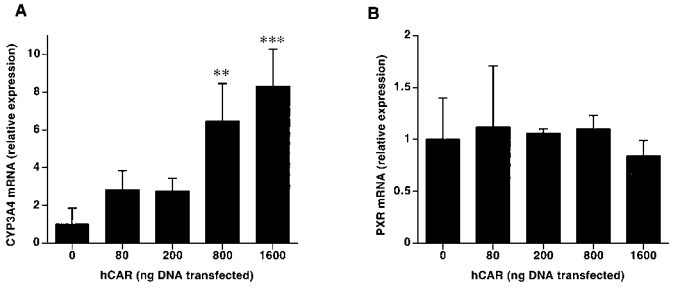



**Fig. 4.** hCAR-mediated induction of *CYP3A4* mRNA in HepG2 cells. HepG2 cells were transfected with pSG5-hCAR (0–1600 ng) as described under *Experimental Procedures* and maintained in culture for a further 48 h. Total RNA was extracted followed by real-time RT-PCR using TaqMan probes specific for CYP3A4, hPXR, and β-actin. The relative abundance of both CYP3A4 (A) and hPXR (B) is shown normalized for β-actin expression. Error bars show S.D., $n = 4$/experimental point. **, $p < 0.01$; ***, $p < 0.001$ relative to zero hCAR control.

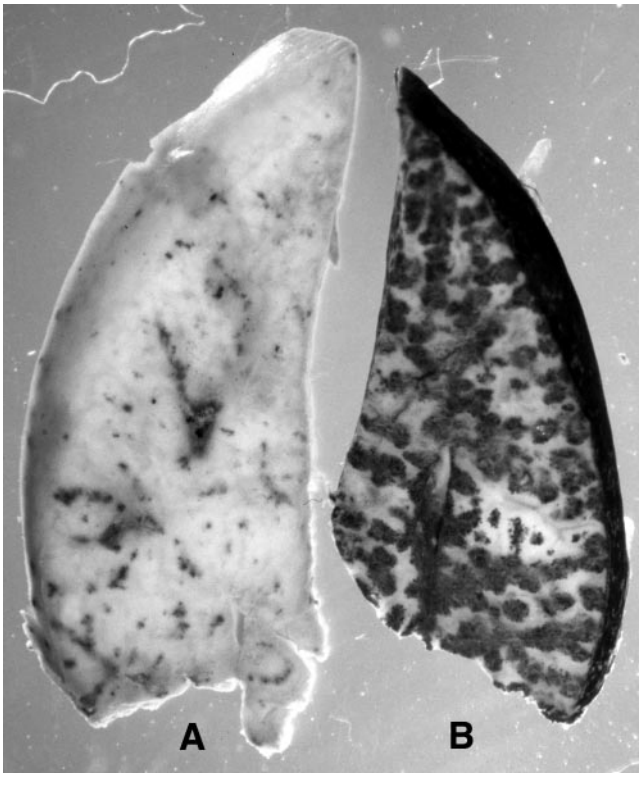



**Fig. 5.** Expression of a β-galactosidase reporter gene linked to the regulatory 5′-flanking region of *CYP3A4*, −13 kb to +53 bp, inserted into mice as a transgene. Mice were treated with vehicle alone (corn oil) (A) or the mCAR-specific ligand TCPOBOP (B), as described under *Experimental Procedures*. Hepatocytes exhibiting transgene expression are visualized as the darkly stained areas on the cut surface of the liver.

In the mouse, induction of *Cyp2b* gene expression by a range of xenochemicals, including PB, is mediated by CAR. Targeted disruption of the CAR gene completely abrogates *Cyp2b10* induction by PB and TCPOBOP (Wei et al., 2000). In contrast, disruption of the murine PXR gene does not affect PB induction of *Cyp3a11*, indicating that in the mouse CAR is capable of mediating the inductive response of *CYP3A* genes to PB as well (Staudinger et al., 2001). Given that hCAR is capable of *trans*-activating the human *CYP2B6* gene in response to PB and PB-like inducers (Sueyoshi et al., 1999), it would be reasonable to assume that *CYP3A4* and *CYP2B6* would be coordinately regulated on exposure of the hepatocyte to compounds that activate hCAR. Induction of both *CYP3A4* and *CYP2B6* expression by PB is well documented (Pichard et al., 1990; Schuetz et al., 1993; Kocarek et al., 1995; Chang et al., 1997; Gervot et al., 1999; Sueyoshi et al., 1999). Additionally, the few bone fide *CYP2B6* inducers identified to date, including TCPOBOP (Smith et al., 1993) and cyclophosphamide (Chang et al., 1997; Gervot et al., 1999), also up-regulate *CYP3A4* expression. Evidently, induction of multiple P450 genes upon exposure to potentially toxic or carcinogenic compounds increases the probability that the organism can successfully metabolize and ultimately excrete xenobiotics.

Goodwin et al. (2001) have shown that both hPXR and hCAR can mediate the PB induction of the *CYP2B6* gene in primary human hepatocytes, although hCAR predominates in this respect. It seems likely that the conformation of the HREs in the target genes is the main determinant as to which nuclear receptor predominates. In *CYP2B6* there are two adjacent DR-4 motifs, separated by only 16 base pairs (Sueyoshi et al., 1999). In contrast, in the *CYP3A4* gene the two predominant PXR/CAR binding motifs are separated by in excess of 7.5 kilobase pairs, an arrangement that seems to favor PXR-mediated induction over CAR (Goodwin et al., 1999, 2001).

In summary, CAR was shown to directly regulate the transcriptional activity of the *CYP3A4* gene, both in vitro and in

vivo. *trans*-Activation of *CYP3A4* by CAR was mediated by nuclear receptor-binding motifs located in the distal XREM and promoter proximal regions of the gene. These elements are capable of binding both hCAR-hRXR$\alpha$ and hPXR-hRXR$\alpha$ heterodimers with high affinity. The convergence of hCAR- and hPXR-mediated signaling pathways at common response elements in the *CYP3A4* gene clearly demonstrates that cross talk between these two nuclear receptors is probably an important factor in the regulation of this gene. Furthermore, the ability of CAR and PXR to regulate the same gene suggests that these proteins are integral parts of common homeostatic pathways.

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