

Identification of Constitutive Androstane Receptor and Glucocorticoid Receptor Binding Sites in the *CYP2C19* Promoter

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ABSTRACT

CYP2C19 is an important human drug-metabolizing enzyme that metabolizes a number of clinically used drugs including the antiulcer drug omeprazole, the anxiolytic drug diazepam, the β -blocker propranolol, the antimalarial drug proguanil, certain antidepressants and barbiturates, and the prototype substrate *S*-mephenytoin. Previous studies show that compounds such as rifampicin and dexamethasone induce *CYP2C19* both in vivo in humans and in vitro in human hepatocytes. This study examines the transcriptional regulation of *CYP2C19*. Analysis of the *CYP2C19* promoter revealed a single constitutive androstane receptor (CAR) binding site (CAR-RE; -1891/-1876 bp) and a glucocorticoid-responsive element (GRE; -1750/-1736 bp). Gel-shift assays showed that CAR-RE binds CAR and pregnane X receptor (PXR). Cotransfection with hCAR, mCAR, or hPXR in HepG2 cells up-regulated transcription of *CYP2C19* promoter constructs, whereas mutation of the -1891-bp

CAR-RE abolished up-regulation. Expression with hCAR also up-regulated endogenous *CYP2C19* mRNA content in HepG2 cells. Androstenediol repressed the mCAR-mediated constitutive activation of the *CYP2C19* promoter in HepG2 cells, whereas the potent mCAR ligand 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene derepressed this response. Rifampicin produced a modest increase in promoter activity in cells cotransfected with hPXR. Dexamethasone activated the -2.7-kb *CYP2C19* promoter constructs in HepG2 cells only in the presence of cotransfected glucocorticoid receptor (GR), whereas the GR antagonist mifepristone inhibits this response. Mutation of the GRE abolishes dexamethasone activation. This is the first study to identify nuclear receptor binding sites (CAR/PXR and GR) in the *CYP2C19* promoter and to suggest that these receptors may up-regulate *CYP2C19* constitutively and possibly its response to drugs.

Cytochromes P450 are a superfamily of enzymes that catalyze the oxidative metabolism of xenobiotic drugs and environmental chemicals as well as many endogenous compounds. The CYP2Cs are a major subfamily of P450s that represent approximately 20% of the P450 enzymes in human liver and metabolize a similar proportion of clinically used drugs (Goldstein, 1996). The CYP2C subfamily consists of only four genes in humans: *CYP2C9*, *CYP2C19*, *CYP2C8*, and *CYP2C18*.

CYP2C19 is a clinically important enzyme, which is polymorphic in humans (Goldstein, 1996). *CYP2C19* is less abundant in liver than the structurally related *CYP2C9*, but it has distinctly different substrate specificities. *CYP2C19* metabolizes the *S*-enantiomer of the anticonvulsant mephenytoin, the antiulcer drug omeprazole (one of the ten most prescribed drugs worldwide), certain antidepressants and barbiturates, and certain antimalarial

drugs such as proguanil, and it is partially responsible for the metabolism of the β -blocker propranolol and the anxiolytic Valium. In population studies, individuals can be divided into two distinct groups: poor metabolizers and extensive metabolizers of drugs metabolized by *CYP2C19*. It has recently been reported that the cure rate for gastric and duodenal ulcers by omeprazole is greatly affected by polymorphisms in *CYP2C19* (Furuta et al., 1998). A Japanese study showed that the most commonly used dose of omeprazole produced a cure rate of only 25 to 33% for duodenal and gastric ulcers in homozygous *CYP2C19* extensive metabolizers, whereas heterozygous individuals carrying one defective copy of the gene had a 57 to 64% cure rate, and the cure rate in poor metabolizers was 100%. These differences are attributed to differences in the rate of metabolism of omeprazole in the three groups. Because most white persons are extensive metabolizers, the rate of

ABBREVIATIONS: PXR, pregnane X receptor; CAR, constitutive androstane receptor; CAR-RE, constitutive androstane receptor-responsive element; GRE, glucocorticoid-responsive element; RU486, mifepristone; GR, glucocorticoid receptor; RXR, retinoid X receptor; DMSO, dimethyl sulfoxide; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; kb, kilobase(s); bp, base pair(s); wt, wild-type; Dex, dexamethasone; tk, thymidine kinase; h, human; m, mouse.

CYP2C19 metabolism has very important implications in the cure of gastric ulcers and subsequent prevention of gastric cancer.

Recently CYP2C8, CYP2C9, and CYP2C19 have been reported to be inducible in humans by prior exposure to drugs, leading to another possible source of variability in the metabolism of CYP2C19 substrates (Gerbai-Chaloin et al., 2001; Raucy et al., 2002). In recent years, considerable advances have been made in understanding the inducible regulation of cytochrome P450 enzymes such as CYP2B6 and CYP3A4 in both rodents and humans. CYP3A4 is regulated by various nuclear receptors, such as the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), and the orphan nuclear receptor HNF4 α (Lehmann et al., 1998; Goodwin et al., 1999, 2002). Induction of CYP3A4 by PXR is also influenced by the orphan receptor HNF4 α (Tirona et al., 2003). PXR binds various ligands such as the antibiotic rifampicin, forms a heterodimer with the retinoid X receptor (RXR), and activates gene transcription after binding both a proximal (160 bp) and a distal xenobiotic-responsive element (7.8 kb) in the 5' flanking region of CYP3A4 (Goodwin et al., 1999). PXR has recently been reported to reside in the cytoplasm of hepatocytes of untreated mice and move into the nucleus after administration of ligand (Kawana et al., 2003).

In contrast, a number of drug-metabolizing enzymes including CYP2B6 have been shown to be induced by drugs such as phenobarbital via a nuclear receptor known as the CAR (Honkakoski and Negishi, 1997, 1998; Sueyoshi et al., 1999). Induction of mouse CYP2B10 by phenobarbital is also influenced by AF-1 sites adjacent to the CAR binding site (Rivera-Rivera et al., 2003). In primary mouse hepatocytes, CAR is sequestered in the cytoplasm. Phenobarbital and other drugs initiate a complex process that is believed to involve phosphorylation, and CAR translocates to the nucleus. In the nucleus, it forms a heterodimer with RXR, binds to regulatory elements in genomic DNA, and recruits coactivators or corepressors to modulate transcription. Although CAR is sequestered in the cytoplasm in murine hepatocytes, progress in understanding the response to drugs in humans has been impeded by the lack of a human cell line or a human primary hepatocyte system that clearly retains CAR in the cytoplasm and fully mimics the in vivo situation.

Various studies indicate that the human CYP2Cs are inducible by previous exposure of humans to drugs (Zhou et al., 1990; Gerbai-Chaloin et al., 2001; Raucy et al., 2002). The degree of induction varies for different CYP2Cs and for different inducers. CYP2C8 seems to be preferentially inducible by rifampicin, whereas CYP2C9 is inducible by rifampicin, Phenobarbital, and other drugs (Raucy et al., 2002). Somewhat variable results have been reported with respect to the inducibility of CYP2C19. Raucy et al. (2002) reported that primary hepatocytes from different patients displayed different responses to rifampicin treatment. There was more pronounced induction of both CYP2C19 protein and mRNA content in hepatocytes from some patients, but there was no evidence for induction in samples from other patients. However, as early as 1990, Zhou and coworkers (1990) reported that prior treatment with rifampicin increased metabolism of mephenytoin in vivo in humans, suggesting that CYP2C19 is inducible by drugs.

Recently, the first studies of a CYP2C promoter have demonstrated that CYP2C9 is up-regulated by both proximal and

distal CAR-responsive elements and the glucocorticoid responsive element (Ferguson et al., 2002; Gerbai-Chaloin et al., 2002). The present study represents the first study of the promoter region of CYP2C19 and demonstrates its transactivation by hCAR, hPXR, and hGR.

Materials and Methods

Chemicals. 1,4-bis[2-(3,5-Dichloropyridyloxy)] benzene (TCPOBOP) was kindly provided by Dr. James Sideway (AstraZeneca, Södertälje, Sweden). Androstenediol was purchased from Steraloids (Newport, RI). Rifampicin, DMSO, dexamethasone, RU486, and other common reagents were purchased from Sigma-Aldrich (St. Louis, MO) or standard sources. Cell culture media were purchased from Invitrogen (Carlsbad, CA). Desalted oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX). Restriction enzymes were purchased from New England Biolabs (Beverly, MA).

Transient Transfection Constructs. A -2.7-kb sequence of the 5'-flanking promoter region of CYP2C19 was amplified with the use of a human CYP2C19 PAC clone (Incyte Systems, Palo Alto, CA) as the template using primers identical with those used earlier to amplify the CYP2C9 3-kb construct (Ferguson et al., 2002), then inserted into pGL3-basic (Promega, Madison, WI), which had been linearized by double digestion with *NheI* and *NcoI*. The CYP2C19 2.7-kb construct was progressively deleted from the 5' end by digestion with *MluI* and *StuI* for the -1.9-kb fragment, with *EcoRV* for the -1.4-kb fragment, and with *PmlI* for the -645-bp fragment. After gel purification, the large fragments with the pGL3-basic sequence were made flush by incubation with *Escherichia coli* Klenow fragment DNA polymerase I (New England Biolabs), and then they were self-ligated at 12°C overnight to produce the deleted CYP2C19 promoter constructs. pSG5-hPXR was provided by Steve Kliewer (GlaxoSmithKline, Welwyn Garden City, Hertfordshire, UK). The cDNA of hGR was excised from pEGFP-hGR by *BamHI* and *XhoI* digestion, subcloned into the same restriction enzyme sites of pGL3-basic, then re-excised from pGL3-basic, and finally inserted into pCR3.1 between the *NheI* and *BamHI* sites. *NheI*-linearized pGL3-tk (Sueyoshi et al., 1999) was used for the insertion of the oligonucleotides that correspond to CYP2C19-GRE (5'-ctagactcaattgaactgaattgtttgtttaa-3') and mutant CYP2C19-GRE (5'-ctagactcaattgaactgaattgaattgtttaa-3') (hexamer half-sites are indicated by bold letters and mutated nucleotides are underlined).

Cell Culture Transfection Assay and Luciferase Assay. Caco-2, HepG2, and hCAR stably transfected HepG2 cells (hereafter termed g2hCAR-8) were cultured in minimal essential medium supplemented with 10% fetal bovine serum and antibiotics at 37°C under 5.0% CO₂. One day before transfection, 1 to 1.5 \times 10⁵ cultured cells/well were seeded into 24-well plates. The transfections were performed according to the instructions for the Effectene transfection reagent (QIAGEN, Valencia, CA). CYP2C19 promoter luciferase reporter plasmid (100–200 ng) with or without specific nuclear receptor expression constructs was combined with 10 ng of pRL-SV40 (or pRL-tk), mixed with Effectene transfection reagents (QIAGEN) before transfection, and then added into cell culture medium. Twenty-four hours after transfection, the media were removed from the wells, and cell extracts were prepared for the dual luciferase analysis (Promega). Alternatively, various drugs were added to the transfected cells 18 to 24 h after transfection and left in the medium for 24 to 48 h, followed by luciferase assays. Promoter activities were calculated from firefly luciferase activities normalized against *Renilla reniformis* luciferase activities of the internal control plasmid.

Site-Directed Mutagenesis. The pGL3-basic construct, including -2.7 and -1.9 kb of the CYP2C19 promoter region, was used as the template for the site-directed mutagenesis (QuikChange site-directed mutagenesis, Stratagene, La Jolla, CA) to prepare CAR-RE- and GRE-mutated CYP2C19 promoter constructs, respectively. The primers used for mutagenesis were as follows (hexamer half-sites are

indicated by bold letters and mutated nucleotides are underlined): CAR-RE-mutant, 5'-**ccaaactcttctctg**gccaatctagtcac-3'; GRE-mutant 1, 5'-gtgactcaatt**gcctt**gaat**gttttgc**-3'; and GRE-mutant 2, 5'-ctcaatt**gaactgaatgcctt**gcttgaaatgaacc-3'. All constructs with mutations were verified by DNA sequencing.

Gel-Shift Assays. Human RXR, hCAR, and hPXR were synthesized in vitro using the TNT Quick-Coupled In Vitro Transcription Translation system (Promega) following the manufacturer's protocol. Klenow Fragment (New England Biolabs) was used to incorporate [³²P]dCTP at the 5' ends of the double-stranded oligonucleotides. Approximately 30,000 cpm of labeled probe was incubated with or without synthesized nuclear receptors in a 10-μl binding reaction containing 10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% (v/v) glycerol, 50 mM NaCl, and 1 μg of nonspecific competitor poly(dI-dC) (Sigma, St. Louis, MO). In parallel reactions, specific cold competitors or specific antibody was added to the mixture before the addition of proteins. After 20 min of incubation at room temperature, 9 μl of the reaction mixture was loaded onto a 5% nondenaturing polyacrylamide gel for electrophoresis in 0.5× Tris borate-EDTA buffer for 2 h at 150 V. The gels were dried and exposed to film. Following are the sequences of the oligonucleotides (CAR-REs) used as probes, wild-type, or mutated specific cold competitors: *CYP2C19* proximal wt, 5'-ctagaccaactcttctgacccc-3'; *CYP2C19* proximal mutated, 5'-aaacaaactcttctgccc-3'; *CYP2C19* distal wt, 5'-ctagaatgaacctacagaaact-3'; *CYP2C9* proximal wt, 5'-ctagaccaactcttctgacct-3'; and *CYP2C9* distal wt, 5'-ctagaatgaacctacataaact-3' (mutated sequence is underlined).

Reverse Transcriptase-Polymerase Chain Reaction. Total RNA was isolated from HepG2 and g2hCAR-8 cells using TRIzol lysis solution (Invitrogen). Superscript II (Invitrogen) reverse transcriptase with 50 to 100 ng of total RNA was used to synthesize cDNA, and 0.5 to 1 μl of reverse-transcribed product served as the template in polymerase chain reactions. The following primers were used (forward, reverse): *CYP2C19*, 5'-cgaggtccagagatacatc-3', 5'-tgtcatgtacacagaagt-3'; *CYP2C9*, 5'-aaaacatcaagattttgagcag-3', 5'-tctcagggtgtgcttgc-3'; actin, 5'-gagctatgagctgctgacg-3', 5'-cacttgcggtgacagatg-3'. After 35 cycles of amplification, the products were separated onto 2% agarose gels and visualized by ethidium bromide staining.

Statistics. Fisher's least significant difference test was used to make pairwise comparisons between groups. A variance-stabilizing logarithmic transformation was used in some cases in these analyses.

Results

Activation of *CYP2C19* by Nuclear Receptors

Various reports indicate that *CYP2C19*, an important *CYP2C* member in humans, is induced by drug treatment both in vivo (Zhou et al., 1990) and in vitro at the mRNA and protein levels in primary human hepatocytes (Gerbal-Chaloin et al., 2001; Raucy et al., 2002). However, the transcriptional regulation of *CYP2C19* has not been studied. To investigate whether nuclear receptors are involved in the regulation of *CYP2C19*, we cloned 2.7 kb of 5' flanking sequence of the *CYP2C19* promoter into pGL3-basic and transiently transfected this reporter into HepG2 cells with several nuclear receptor-expressing plasmids. As indicated in Fig. 1A, three orphan nuclear receptors (hCAR, mCAR, and hPXR) produced a moderate increase in *CYP2C19* promoter activity ($P < 0.01$). hCAR cotransfection produced a 2-fold elevation of promoter activity of the *CYP2C19* 2.7-kb sequence. Murine CAR produced a higher activation than hCAR, in contrast with results for the structurally related *CYP2C9*, for which hCAR was a stronger activator than mCAR (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002).

Figure 1B shows that mCAR activation can be inhibited by the mCAR ligand androstrenol and derepressed by the murine *CYP2B6* inducer TCPOBOP ($P < 0.01$ in both cases), which further indicates that CAR can play a role in the transactivation of *CYP2C19* promoter. Human PXR produced a smaller increase in activity of the *CYP2C19* 2.7-kb promoter construct than hCAR. Rifampicin, the prototypical activator for PXR, produced a slight increase (40–94%) in *CYP2C19* promoter activity when transfected with hPXR (Fig. 1C; $P < 0.01$). The activation of *CYP2C19* by hCAR was dose-responsive in both HepG2 cells and Caco-2 cells (data not shown). Both *CYP2C19* and *CYP2C9* mRNA content of HepG2 cells was increased by stable transfection with hCAR, but the increase in *CYP2C9* mRNA content was larger than the increase in *CYP2C19* mRNA content (Fig. 2A). Similarly,

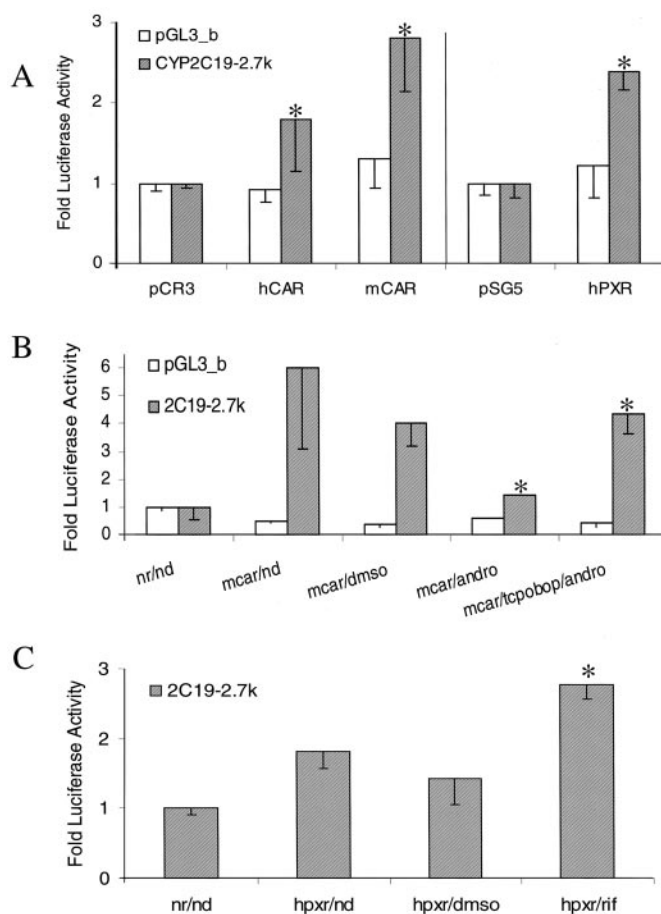


Fig. 1. The transactivation of *CYP2C19* promoter by CAR and PXR. A, to investigate the effects of transient transfection with nuclear receptor expression constructs on *CYP2C19* promoter activity, HepG2 cells ($1-1.5 \times 10^5$ /well) were transfected with 100 ng of pGL3-basic vector or *CYP2C19* 2.7-kb promoter reporter constructs either without cotransfection with nuclear receptor (nr) or with 100 ng of pCR3-hCAR, pCR3-mCAR, and pSG5-hPXR expression plasmids, respectively. Twenty-four hours later, cells were lysed and assayed for luciferase activity. *, statistical significance of $P < 0.01$ compared with HepG2 cells transfected with pCR3 control vector for hCAR and mCAR, or pSG5 control for hPXR. B, alternatively, media were refreshed after 24 h of transfection, and either no drug (nd) or various effectors were added in 0.1% DMSO/250 nM TCPOBOP or 10 μM (androstrenol), and the cells were cultured for an additional 24 h. Significant repression by androstrenol and derepression by TCPOBOP were observed ($P < 0.01$). *, rifampicin-treated cells were significantly different from DMSO control ($P < 0.01$). C, these data represent results from three independent transfections, with error bars indicating standard deviations.

hCAR produced a much stronger increase in *CYP2C9* promoter activity than in *CYP2C19* (Fig. 2B; $P < 0.01$). hPXR produced similar activation of the two genes ($P < 0.05$). These results clearly show that hCAR is involved in the transcriptional activation of *CYP2C19* and *CYP2C9*, but the extent of activation differs.

Identification of a CAR-RE of *CYP2C19*

Analysis of 2.7 kb of the *CYP2C19* promoter region using the GCG Wisconsin Package Findpatterns tool (Accelrys, Burlington, MA) with a nuclear receptor binding site search set revealed two putative CAR-binding elements: a proximal site between -1891 and -1876 bp from the translation start site and a possible second distal element between -2730 and -2714 bp (Fig. 3A). These two elements show high similarity to two CAR-REs reported in the upstream region of *CYP2C9* (Ferguson et al., 2002). However, each element differed by a single nucleotide from the CAR binding elements found in the *CYP2C9* promoter. Gel-shift assays demonstrated completely distinct effects of the two 1-bp differences on the binding abilities of the elements to hCAR/hRXR (Fig. 3B).

The proximal elements of both genes bind strongly with hCAR/hRXR to form a specific complex, which can be effectively competed out by unlabeled competitors, although the CAR-RE found in *CYP2C19* shows slightly less binding than the proximal element of *CYP2C9*. In contrast, whereas the distal element of *CYP2C9* (Ferguson et al., 2002) binds with hCAR/hRXR, no complex is observed with the distal putative element of *CYP2C19*. These results indicate that the 1-bp difference between the distal element of *CYP2C19* and *CYP2C9* resulted in undetectable CAR binding of the putative distal CAR-RE of *CYP2C19*.

To further characterize the functional role of the putative CAR-RE of *CYP2C19* on the *CYP2C19* transcription, three promoter constructs were made and examined through hCAR cotransfection experiments. The first construct is the *CYP2C19* 1.9-kb sequence, in which the putative distal CAR-RE was removed, but the proximal element was retained. The second construct, the *CYP2C19* 645-bp sequence, contains no putative CAR-RE. Finally, using site-directed mutagenesis, the core region of the proximal CAR-RE was mutated in the *CYP2C19* 2.7-kb promoter construct to gen-

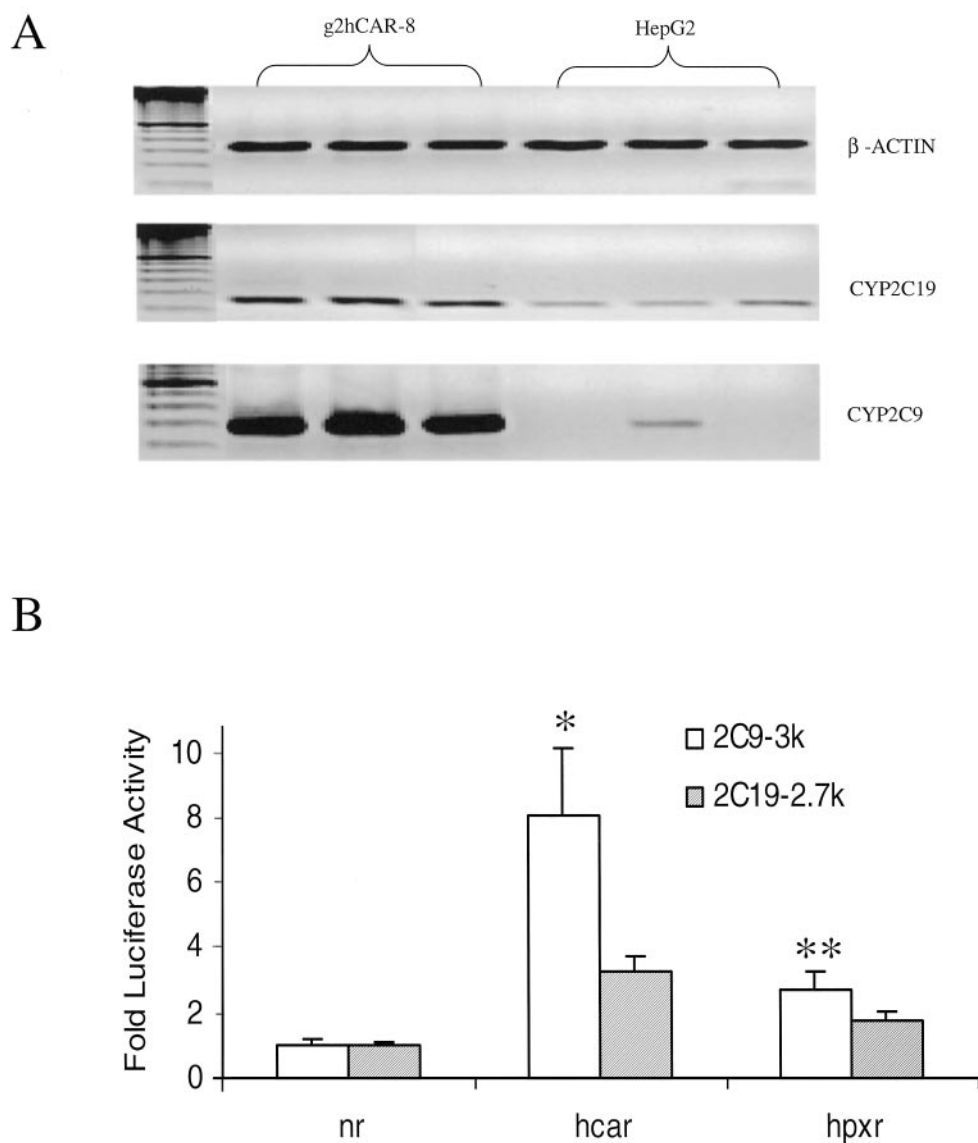


Fig. 2. Comparison of nuclear receptor transactivation of *CYP2C9* and *CYP2C19*. A, the activation of endogenous *CYP2C19* and *CYP2C9* gene transcription by hCAR in HepG2 cells. Total RNA (100 ng) isolated from triplicate cultures of HepG2 cells and hCAR stably transfected HepG2 cells (g2hCAR-8) were amplified by conventional reverse transcriptase-polymerase chain reaction, and polymerase chain reaction products were separated by 2% agarose gel electrophoresis. B, 100 ng of *CYP2C9* 3-kb and *CYP2C19* 2.7-kb promoter constructs were transfected side by side into HepG2 cells with or without nuclear receptor (nr) expression construct cotransfection. After 48 h, cell lysates were prepared for luciferase activity. Values represent the means \pm S.D. of triplicate transfection experiments. *, $P < 0.01$ and **, $P < 0.05$ indicate significantly higher activation of *CYP2C9* by hCAR and hPXR, respectively, compared with *CYP2C19*.

erate the *CYP2C19* 2.7-kb mutant, a full-length construct containing only a mutation in the CAR binding site at -1891 bp (Fig. 4A). As shown in Fig. 4B, transient transfection with hCAR activated the *CYP2C19* 1.9-kb construct but not the *CYP2C19* 645-bp construct. These data indicate the existence of a CAR-RE between -1.9 and -645 bp of the *CYP2C19* promoter, consistent with our gel-shift data. Mutation of the -1891-bp CAR-RE resulted in complete abolishment of hCAR activation. This mutation result clearly demonstrates that the proximal CAR-RE of *CYP2C19* mediates the transcriptional activation of the *CYP2C19* 2.7-kb promoter by hCAR, whereas the putative distal site is not involved in hCAR regulation.

We further tested the ability of this proximal CAR binding site to bind hPXR. Cotransfection of hPXR with *CYP2C19* promoter constructs containing a functional CAR-RE further increased reporter activity. In addition, a small but significant increase was seen with the hPXR ligand rifampicin (Fig. 1C; $P < 0.01$). To test whether hPXR could bind to the CAR-RE, electrophoretic mobility shift assays were performed to compare the binding ability of the *CYP2C19* CAR-RE with that of hCAR and hPXR. Figure 5 shows that hPXR also binds the CAR-RE. Wild-type cold competitor efficiently competed out the binding of hPXR, whereas the

mutated cold competitor did not compete with binding of hPXR to the CAR-RE. This supports the hypothesis that hPXR also recognizes the CAR-RE element and can transactivate the *CYP2C19* promoter.

Induction of *CYP2C19* Promoter by Dexamethasone

Previous studies in human primary hepatocytes have shown that dexamethasone induces *CYP2C19* (Raucy et al., 2002), but the molecular mechanism of this response has not been elucidated. Dexamethasone has been reported to directly mediate the induction of many P450 genes, including *CYP3A23* (Quattrochi et al., 1998) and *CYP2C9* (Gerbal-Chaloin et al., 2002), through interaction of the glucocorticoid receptor (GR) with GRE elements in the promoters of these genes. Other studies show that dexamethasone can also induce P450 genes indirectly by inducing the expression of nuclear receptors such as hCAR and hPXR, which then subsequently activate the *CYP3A4* promoter (Pascucci et al., 2000, 2001).

To examine the role of hGR in the regulation of transcription of *CYP2C19*, we first tested the effects of dexamethasone on the *CYP2C19* 2.7-kb promoter construct in HepG2 cells. Twenty-four hours after cotransfection with the *CYP2C19* promoter construct and pCR3.1-hGR, dexamethasone was

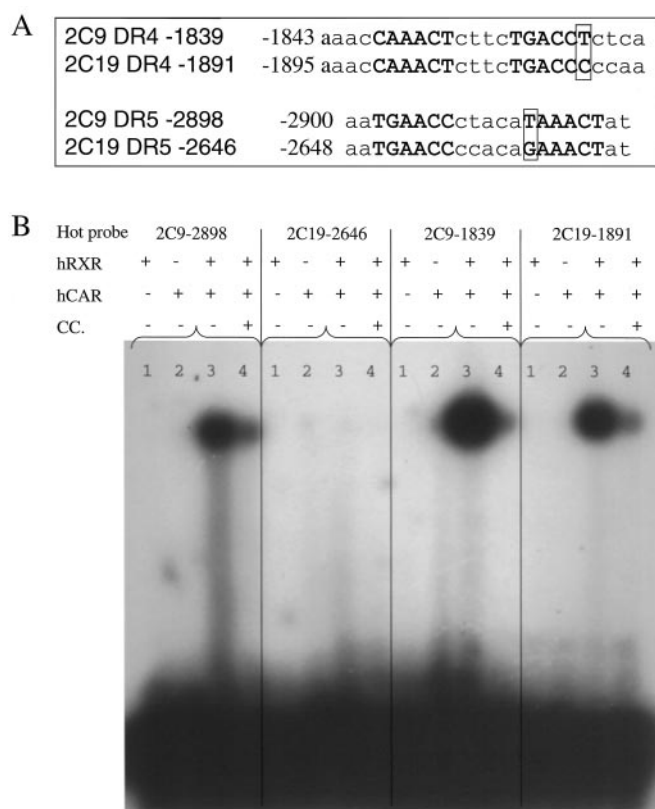


Fig. 3. Electrophoretic mobility shift assay of CAR-RE from *CYP2C19* and *CYP2C9*. A, DNA sequence comparison of putative CAR-REs found in *CYP2C9* and *CYP2C19* revealed two homologous DR4 half-sites (in capital letters) differing by only one nucleotide. Boxed letters indicate nucleotide differences within the CAR-REs of two genes. B, to compare the binding capacities of the two putative CAR-REs of *CYP2C19* and *CYP2C9* to hCAR in electrophoretic mobility shift assays, the two radiolabeled putative CAR-REs of *CYP2C9* and *CYP2C19* were incubated for 20 min at room temperature with hCAR in the presence and absence of hRXR to form possible DNA protein complexes. Fifty-fold excess of cold competitors (CC) was added to test the specificity of the complexes.

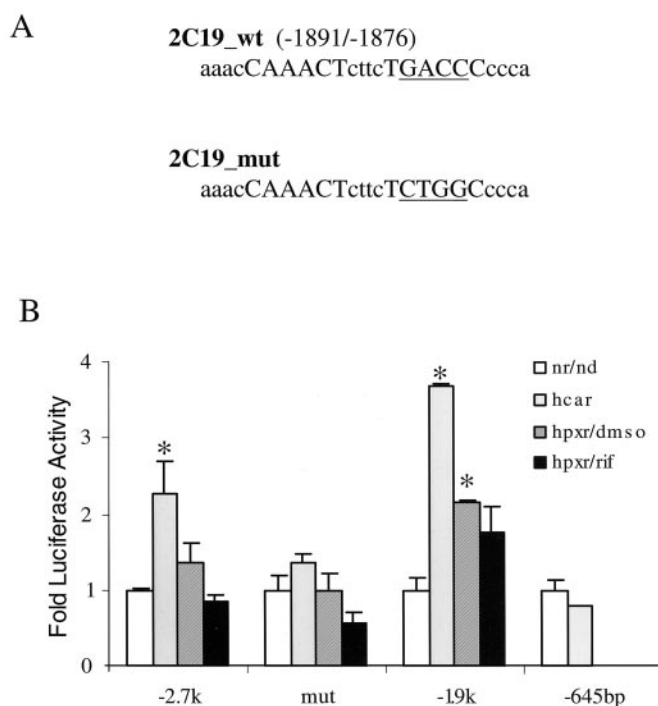


Fig. 4. Functional analysis of *CYP2C19* CAR-RE. A, schematic representation of *CYP2C19* and *CYP2C9* promoter constructs with wild-type (wt) or mutated (mut) CAR-REs (in capital letters; area to be mutated is underlined in both wt and mut). B, transient transfection in HepG2 cells with wild-type -2.7-kb, -1.9-kb, and -645-bp regions of the *CYP2C19* promoter, progressive deletions of the *CYP2C19* promoter, and the -2.7-kb promoter with a mutated -1891-bp CAR-RE (mut) was performed with 100 ng of hCAR. Samples (200 ng) of each deleted or mutated *CYP2C19* promoter reporter construct was transfected into HepG2 cells along with a pRL-SV40 internal control in the absence (nr) or presence of cotransfected pCR3-hCAR-expressing plasmids. DMSO vehicle (0.1%) or 10 μ M rifampicin (rif) was added 24 h after transfection. Luciferase analyses were performed after 24 h of transfection to test the activation of the *CYP2C19* promoter. All data reflect mean \pm S.D. of three independent transfections. *, $P < 0.01$ compared with pGL3-basic empty promoter control.

added to the medium. As shown in Fig. 6A, luciferase activity of the *CYP2C19* 2.7-kb construct was increased by dexamethasone treatment (significant increase of $P < 0.01$ versus vehicle, pGL3-basic, or empty pCR3.1 expression vector controls). Similar induction of *CYP2C19* promoter activity by dexamethasone was observed in the intestinal Caco-2 cell line (Fig. 6B). In addition, the effect seems to occur only in the presence of exogenous hGR in HepG2 cells, because dexamethasone treatment without cotransfected hGR does not result in an activation of *CYP2C19* transcription (Fig. 6A). These data demonstrate that dexamethasone induction of *CYP2C19* is localized within the proximal -2.7 kb of the promoter.

Identification of a Putative Glucocorticoid-Responsive Element in the Proximal -2.7 kb of *CYP2C19* Promoter Region

Functional characterization of the GRE of *CYP2C19*. Through the use of the same GCG Findpatterns tool, a putative GRE was localized within -2.7 kb of the *CYP2C19* promoter region (1750/1736 bp). Figure 7 shows the sequence comparison of the putative GRE element in the flanking region of *CYP2C19* relative to the GRE recently identified in *CYP2C9* (Gerbai-Chaloin et al., 2002). The two GRE half-

sites of *CYP2C19* and *CYP2C9* were identical except for nucleotide differences in the flanking regions. To verify the location of the newly identified *CYP2C19*-GRE, the *CYP2C19* 1.9-kb construct, and another deletion construct, the *CYP2C19* 1.4-kb construct, were transfected into HepG2 or Caco-2 cells (Fig. 7, B and C). As expected, only the 1.9-kb construct of the *CYP2C19* promoter, which contained the putative GRE, showed strong activation of the luciferase reporter activity by dexamethasone ($P < 0.01$). This result is consistent with the fact that the putative GRE of *CYP2C19* is between 1.9 and 1.4 kb of the promoter region of this gene.

We then mutated the GRE in the *CYP2C19* promoter construct to evaluate the specific role of this element. The 1.9-kb construct was selected as the template to generate two mutant *CYP2C19* 1.9-kb constructs by site-directed mutagenesis, each containing mutations in one of the two half-sites (Fig. 7A). Mutations within each half-site resulted in the suppression of dexamethasone induction in HepG2 and Caco-2 cells (Fig. 7, B and C). In total, these results indicate the GRE located between -1750 and -1736 bp of the *CYP2C19* promoter acts as a mediator for dexamethasone induction of *CYP2C19* transcription.

Inhibition of Dexamethasone Induction by RU486. Three copies of the *CYP2C19* GRE were cloned upstream of

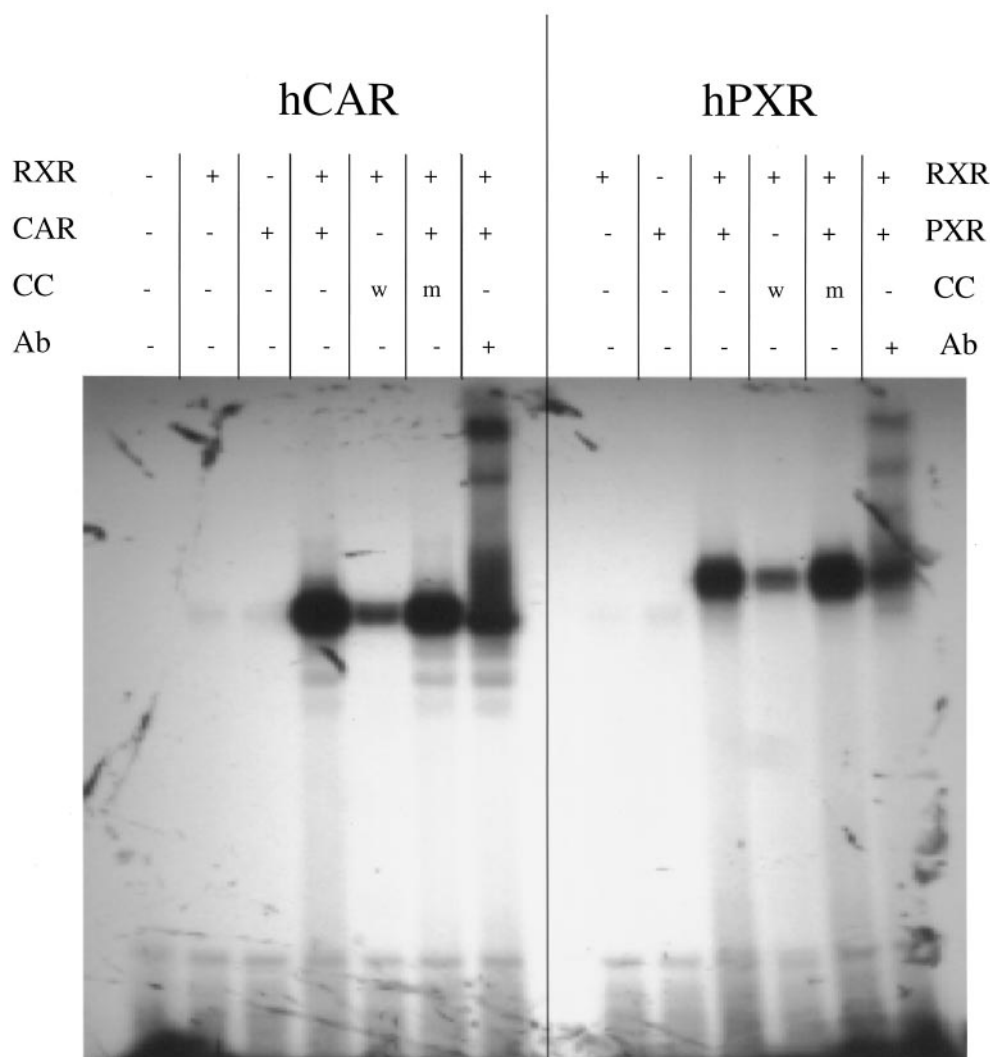


Fig. 5. Electrophoretic mobility shift assay of the *CYP2C19* CAR-RE with hCAR and hPXR. Radiolabeled *CYP2C19* CAR-RE double-stranded oligonucleotide was individually mixed with in vitro synthesized hRXR, hCAR, or hPXR alone and with combinations of hCAR/hRXR and hPXR/hRXR at room temperature for 20 min, followed by the 5% polyacrylamide gel electrophoresis separation. Molar excess (50-fold) of unlabeled wild-type (w) and mutant (m) cold competitors (CC) were added in the parallel binding reactions to show the specificity of complex. The polyclonal antibodies (Ab) to hRXR α were used to produce supershift bands in parallel experiments.

the pGL3-tk [(GRE)3-tk-pGL3-B], whereas GRE mut3 (Fig. 7A) was cloned as a negative control [(GRE-m3)-tk-pGL3-b]. Figure 8 demonstrates that 100 nM Dex strongly activates (GRE)3-tk-pGL3-b approximately 20-fold in HepG2 cells cotransfected with hGR. This activation can be almost completely abolished by the coaddition of 1 μ M of the prototype GR antagonist RU486. A construct containing mutations in both GRE half-sites did not respond to Dex treatment, demonstrating that both cotransfected GR and the putative GRE from the *CYP2C19* promoter are essential for Dex induction.

Discussion

CYP2C19 is a clinically important human drug-metabolizing enzyme that metabolizes a variety of drugs such as the anxiolytic valium, the antiulcer drug omeprazole, the β -blocker propranolol, and certain antidepressants, and it activates antimalarial prodrugs such as proguanil (Goldstein and de Moraes, 1994). Both in vivo and in vitro studies suggest that drugs such as barbiturates and rifampicin (Zhou et al., 1990; Gerbal-Chaloin et al., 2001) may induce *CYP2C19*. The present study identifies two important regulatory elements within the first 2.7 kb of the *CYP2C19* promoter. To our knowledge, this is the first study of transcriptional regulation by the promoter of *CYP2C19*. One regulatory element (CAR-RE) located between -1891 and -1876 bp from the translation start site binds the orphan

receptors CAR and PXR as a heterodimer with RXR. The second regulatory element (GRE) between -1750 and -1736 bp is activated by the glucocorticoid receptor. We demonstrate that three different types of nuclear receptors, hCAR, hPXR, and hGR, are able to up-regulate activity of *CYP2C19* reporter constructs containing these elements in cotransfection experiments in both cultured hepatocyte lines (HepG2 cells) and an intestinal cell line (Caco-2 cells). These data indicate that both CAR-RE and GRE are involved in up-regulation of *CYP2C19* transcription by hCAR and by glucocorticoids. CAR also up-regulates *CYP2C19* mRNA in human HepG2 cell lines, as has been shown for both *CYP2C9* (Ferguson et al., 2002) and *CYP3A4* (Goodwin et al., 2002), providing strong evidence for the involvement of CAR in the constitutive regulation of these three genes.

Two earlier studies reported the presence of active CAR-RE found within the proximal -3 kb of the promoter of the closely related *CYP2C9* (>90% homology), a proximal DR4 nuclear receptor binding motif at -1838 to -1823 bp (Gerbal-Chaloin et al., 2002) and a distal DR5 nuclear binding motif at -2898 to -2882 bp (Ferguson et al., 2002), each capable of binding hCAR and mCAR. In the present study,

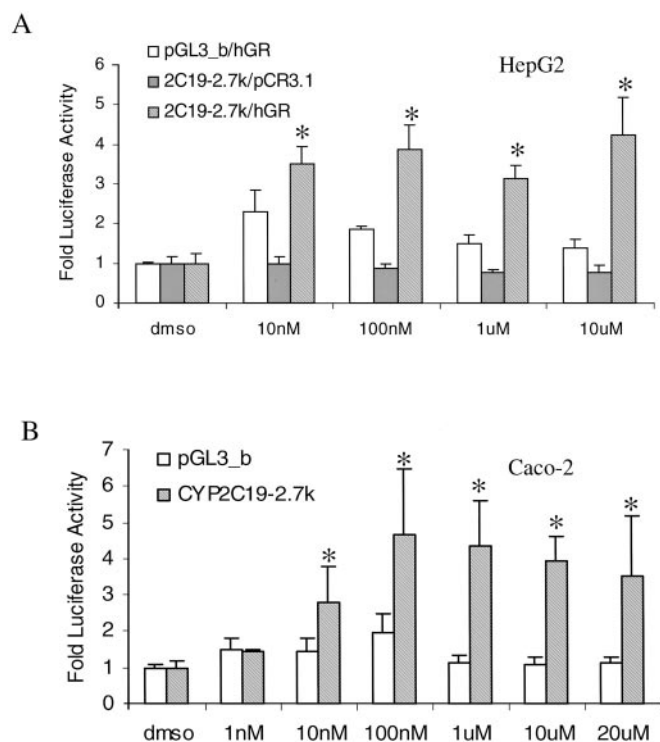


Fig. 6. Dexamethasone induction of the *CYP2C19* promoter. Induction of *CYP2C19* promoter activity by Dex was observed only with 100 ng of the *CYP2C19* 2.7-kb construct cotransfected with 100 ng of pCR3.1-hGR into 10^5 HepG2 cells (A) or Caco-2 cells (B) in the presence of pRL-tk. Twenty-four hours after transfection, the transfected cells were treated with increasing concentrations of Dex for another 24 h. After cell lysis, luciferase assays were performed to test the effects of drugs on reporter gene expression. Error bars represent means \pm S.D. of three independent transfections. *, statistical significance of $P < 0.01$ compared with the vehicle 0.1% DMSO-treated control, pGL3-basic empty promoter control, or pCR3.1-empty hGR expression constructs.

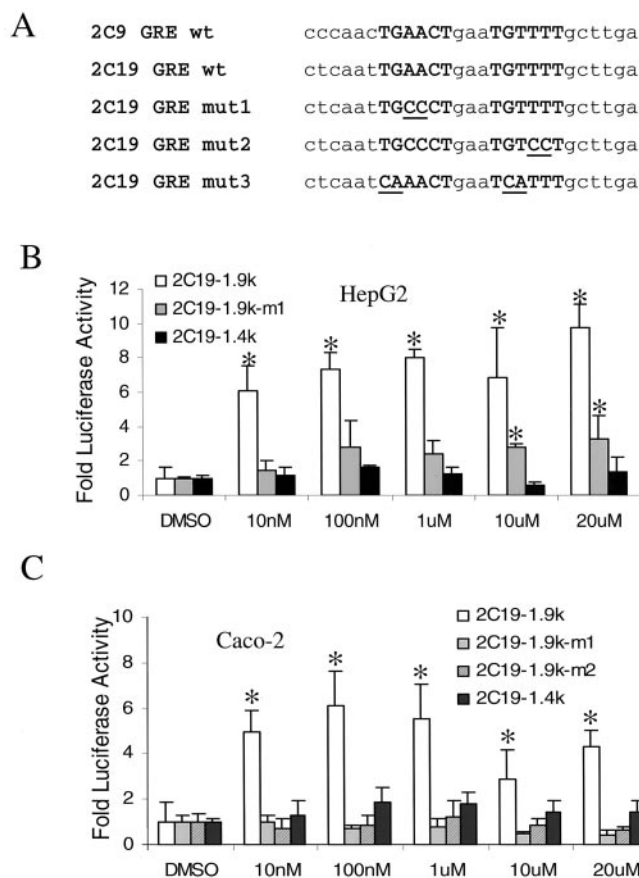


Fig. 7. Functional evaluation of the putative *CYP2C19* GRE. A, luciferase reporter constructs containing -1.4 and -1.9 kb of the 5' flanking region of the *CYP2C19* promoter with or without mutation of the GRE. Half-sites are shown in capital letters; mutations are underlined. B, GRE mediation of Dex induction of the *CYP2C19* constructs. Samples (100 ng) of wild-type or GRE-mutated *CYP2C19* promoter constructs were cotransfected with 100 ng of hGR/pCR3.1 into HepG2 cells (B) or Caco-2 cells (C), followed by 24-h Dex treatment with increasing concentrations after 1 day of transfection, and cells were assayed for luciferase activity. Error bars represent the mean \pm S.D. of three independent transfections. *, $P < 0.01$ versus the vehicle 0.1% DMSO-treated control.

we found that the promoter of *CYP2C19* also contains two putative CAR-binding elements in similar locations (a proximal DR4 located between -1891 and -1876 bp and a distal DR5 located between -2646 and -2630). However, there are 1-bp differences between the elements found in *CYP2C9* and those found in *CYP2C19*. As a result of these single base-pair differences, only one of the two putative CAR-REs in *CYP2C19* actively bound hCAR in gel-shift assays (Fig. 3B). Moreover, *CYP2C19* promoter constructs containing only the proximal CAR-RE were transcriptionally up-regulated when cotransfected with hCAR more than hPXR. The difference in the number of active CAR-REs in the first -3 kb of the *CYP2C9* and *CYP2C19* promoters is one possible explanation for data showing that stable transfection of hCAR into HepG2 cells results in a much larger increase in *CYP2C9* mRNA content than *CYP2C19* mRNA. In addition, cotransfection of hCAR produces greater activation of *CYP2C9* promoter constructs (8–18-fold) than that of *CYP2C19* promoter constructs. The proximal CAR-RE of *CYP2C9* also bound hCAR to a greater extent than the CAR-RE of *CYP2C19* in gel-shift assay. All of these data are consistent with the fact that constitutive hepatic levels of *CYP2C19* in human liver are much lower than those of *CYP2C9* (Goldstein et al., 1994) and with the hypothesis that CAR may contribute to the constitutive levels of *CYP2C9* and *CYP2C19* in human liver. This hypothesis is consistent with results of studies of the role of CAR in gene regulation in wild-type and CAR knockout mice (Wei et al., 2000; Ueda et al., 2002). These studies found that constitutive levels of mRNAs for many proteins were under the control of CAR, whereas for some genes, drug inducibility and/or constitutive expression was regulated by CAR. It is possible that CAR is somewhat active, even in the presence of added ligand, possibly because of the presence of endogenous activators in the cell as suggested recently by Pascussi and coworkers (2003), which could explain recent data suggesting the constitutive regulation of some proteins by CAR.

CAR and/or PXR may also be involved in the inducibility of

CYP2C9 and *CYP2C19* by drugs such as barbiturates and rifampicin. The findings that mCAR effectively activates both *CYP2C9* (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002) and *CYP2C19* transcription (this study) via the proximal CAR-RE support this hypothesis. The CAR repressive ligand androstenol represses this activation, and TCPOBOP, an mCAR-specific inducer, derepresses this transactivation. These data support the possibility that CAR may be a candidate mediator of drug induction for *CYP2C19* via the CAR-RE between -1891 and -1876 bp. The presence of only one active CAR binding site in the *CYP2C19* promoter may also account for reports of lesser induction of *CYP2C19* by CAR activators, including rifampicin and phenobarbital, compared with that observed for *CYP2C9* in primary human hepatocytes (Gerbal-Chaloin et al., 2002; Raucy et al., 2002). In interpreting results of studies of induction of human P450 genes in vitro, one must remember that mCAR resides in the cytoplasm and moves into the nucleus after drug treatment in a manner which is still under investigation. However, in most human cell lines, hCAR is already located in the nucleus (Kawamoto et al., 1999). Even in primary human hepatocytes, the location of CAR could vary with the state of differentiation of the cells. The absence of a suitable cell model for induction studies complicates efforts to determine whether CAR is involved in the induction of human P450 genes by drugs.

Rifampicin, the prototypical PXR ligand, has been reported to be a good inducer of *CYP2C19* both in vivo (Zhou et al., 1990) and in vitro (Gerbal-Chaloin et al., 2001). hPXR, which is the mediator of induction of many P450s such as *CYP3A4* (Goodwin et al., 1999), displayed a relatively small but significant activation of the *CYP2C19* 2.7-kb construct (~1.5-fold) in our cotransfection experiments in HepG2 cells. Rifampicin produced some further increase in this response. It is not unusual for reporter assays to produce smaller increases than those seen in vivo, but we are also pursuing the possibility that additional rifampicin-responsive PXR elements may exist outside of the promoter region we have analyzed to account for the effect reported in vivo and in hepatocytes (Zhou et al., 1990; Raucy et al., 2002). For example, a distal rifampicin-responsive XREM was identified more than 7 kb upstream of the transcription-initiation site of *CYP3A4* (Goodwin et al., 1999), in addition to a proximal PXR binding site.

We found that the *CYP2C19* promoter was activated by dexamethasone (10–100 nM) both in HepG2 cells and Caco-2 cells. We have unequivocally identified an active GRE between -1750 and -1736 bp of the *CYP2C19* promoter. The sequence of the half-sites of this GRE element are completely identical with those of *CYP2C9*-GRE (Gerbal-Chaloin et al., 2002), although differences are seen in the flanking areas. Mutation of this GRE in *CYP2C19* results in the suppression of dexamethasone responsiveness. When this element is cloned upstream of the tk promoter, it can activate the promoter activity in the presence of hGR. Moreover, the prototypical glucocorticoid receptor antagonist RU486 completely inhibits this effect, clearly showing the mediation of the direct interaction between this GRE and hGR in Dex induction for *CYP2C19*. Taken together, these data clearly explain the mechanism of induction of *CYP2C19* previously reported in human hepatocytes (Raucy et al., 2002).

In conclusion, the present study identified two critical nu-

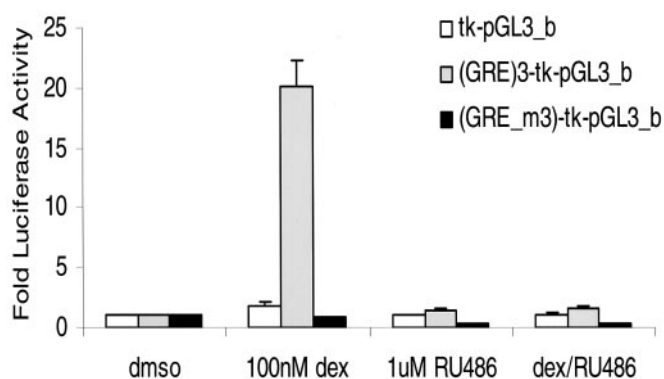


Fig. 8. RU486 inhibition of dexamethasone induction in HepG2 cells. Oligonucleotides possessing wild-type or mutated *CYP2C19* GRE were inserted upstream of the tk promoter of pGL3-basic. The luciferase constructs with the tk promoter (tk-pGL3_b) \pm the *CYP2C19* GRE (three copies of wild-type or one copy of mutated, respectively) were transfected into 1.5×10^5 HepG2 cells in the presence of cotransfected hGR expression construct and internal control pRL-tk. Culture medium was refreshed 24 h after transfection, and the corresponding drugs (indicated on the figure) were added to the medium and maintained for 36 h. Cells were finally lysed and assayed for luciferase activity. Fold luciferase activity was calculated by determining the average of the triplicate transfections with error bars indicated.

clear receptor binding sites in the promoter of CYP2C19, an important drug-metabolizing enzyme in humans, a CAR/PXR-binding element, and a GRE. *CYP2C19* contained two potential CAR/PXR binding sites that were highly homologous to two CAR binding sites previously identified in the closely related *CYP2C9* (Gerbal-Chaloin et al., 2001; Ferguson et al., 2002). However, only the proximal CAR binding site was active in *CYP2C19*, whereas both the distal and proximal CAR binding sites were functional in *CYP2C9*. The relative number of active CAR binding sites in the two enzymes may possibly contribute to the low constitutive expression of CYP2C19 in human liver compared with the higher hepatic expression of CYP2C9. The number of CAR/PXR binding sites may also confer differences in drug inducibility to the two enzymes. We also identified a functional GRE in the promoter of *CYP2C19* that clearly confers dexamethasone inducibility to CYP2C19. In total, our observations establish that the nuclear receptors hCAR, hPXR, and hGR play important roles in controlling the expression of *CYP2C19*, and unraveling these regulatory pathways is essential to understand the regulation of this clinically important human drug-metabolizing enzyme.

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