0090-9556/10/3804-591–599
DRUG METABOLISM AND DISPOSITION
U.S. Government work not protected by U.S. copyright
DMD 38:591–599, 2010

Vol. 38, No. 4
30387/3572254
Printed in U.S.A.

Hepatocyte Nuclear Factor 4α Regulates Rifampicin-Mediated Induction of *CYP2C* Genes in Primary Cultures of Human Hepatocytes

Ritu Rana, Yuping Chen, Stephen S. Ferguson, Grace E. Kissling, Sailesh Surapureddi, and Joyce A. Goldstein

Human Metabolism Section, Laboratory of Pharmacology (R.R., Y.C., S.S., J.A.G.) and Biostatistics Branch (G.E.K.), National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina; and Invitrogen Corporation/CellzDirect, Durham, North Carolina (S.S.F.)

Received September 23, 2009; accepted January 15, 2010

ABSTRACT:

CYP2C enzymes are expressed constitutively and comprise ~20% of the total cytochrome P450 in human liver. However, the factors influencing the transcriptional regulation of the *CYP2C* subfamily have only been addressed recently. In the present study, we used primary cultures of human hepatocytes to investigate the role of HNF4α in the pregnane X receptor (PXR)/rifampicin-mediated up-regulation of *CYP2C8*, *CYP2C9*, and *CYP2C19* gene expression. We first identified new proximal *cis*-acting HNF4α sites in the proximal *CYP2C8* promoter [at −181 base pairs (bp) from the translation start site] and the *CYP2C9* promoter (at −211 bp). Both sites bound HNF4α in gel shift assays. Thus, these and recent studies identified a total of three HNF4α sites in the *CYP2C9* promoter and two in the *CYP2C8* promoter. Mutational studies showed that the HNF4α sites are needed for up-regulation of the *CYP2C8* and *CYP2C9* promoters by rifampicin. Furthermore, silencing of HNF4α abolished transactivation of the *CYP2C8* and *CYP2C9* promoters by rifampicin. Constitutive promoter activity was also decreased. Quantitative polymerase chain reaction analysis demonstrated that silencing HNF4α reduced the constitutive expression of CYP2C8 (53%), CYP2C9 (55%), and CYP2C19 (43%) mRNAs and significantly decreased the magnitude of the rifampicin-mediated induction of CYP2C8 (6.6- versus 2.7-fold), CYP2C9 (3- versus 1.5-fold), and CYP2C19 (1.8- versus 1.1-fold). These results provide clear evidence that HNF4α contributes to the constitutive expression of the human *CYP2C* genes and is also important for up-regulation by the PXR agonist rifampicin.

The human CYP2C subfamily of cytochrome P450 (P450) enzymes consists of four liver enzymes, CYP2C8, CYP2C9, CYP2C18, and CYP2C19, which metabolize ~20% of all clinically prescribed therapeutic agents and a number of physiologically important endogenous molecules (Goldstein, 2001). Among the four CYP2C enzymes, CYP2C8, CYP2C9, and CYP2C19 proteins are mainly expressed in human liver (Goldstein and de Morais, 1994). CYP2C18 protein has not been identified in any tissue. Low levels of CYP2Cs are also found in extrahepatic tissues such as intestine, lung, kidney, and brain (Krishna and Klotz, 1994). Constitutive expression of *CYP2C* genes in the liver is believed to be under the control of endogenous regulatory signals such as HNF4α (Jover et al., 2001; Kamiyama et al., 2007), HNF3γ (Bort et al., 2004), and the CCAAT/enhancer-binding protein α (Jover et al., 1998). However, exposure to numerous structurally unrelated xenobiotics, including rifampicin, hyperforin, phenobarbital, and dexamethasone (Raucy et al., 2002; Madan et al., 2003; Komoroski et al., 2004), up-regulates CYP2C enzyme expression. Induction is mediated via upstream responsive elements in the *CYP2C* promoters for the xenobiotic sensing receptors CAR and PXR (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Chen et al., 2003, 2004; Ferguson et al., 2005). This activity contributes to interindividual variability of CYP2C expression in humans and affects the metabolism of certain xenobiotics in vivo (Zhou et al., 1990; Williamson et al., 1998; Niemi et al., 2001).

HNF4α acts as a central mediator of hepatocyte-specific gene expression and liver function, including control of xenobiotic detoxification, energy metabolism, bile acid synthesis, and serum protein production (Duncan et al., 1994; Stoffel and Duncan, 1997; Li et al., 2000; Hayhurst et al., 2001; Inoue et al., 2002; Kamiya et al., 2003; Tirona et al., 2003). Studies in HNF4α-deficient mice (Wiwi and Waxman, 2004) and cultured human hepatocytes (Jover et al., 2001)

This study was supported by the Intramural Research Program of the National Institutes of Health National Institute of Environmental Health Sciences [Intramural Project ZO1-ES02124].

R.R. and Y.C. contributed equally to this work.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.109.030387.

ABBREVIATIONS: P450, cytochrome P450; HNFα, hepatocyte nuclear factor α; CAR, constitutive androstane receptor; PXR, pregnane X receptor; ChIP, chromatin immunoprecipitation; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; SRC-1, steroid receptor coactivator-1; kb, kilobase(s); EMSA, electrophoretic mobility shift assay; siRNA, small interfering RNA; siHNF4, siRNA for HNF4α; bp, base pairs; RE, response element; DMSO, dimethyl sulfoxide; RT, reverse transcription; PCR, polymerase chain reaction; DR, direct repeat.

have shown the importance of HNF4α in the constitutive expression of hepatic drug-metabolizing *CYP* genes. A recent study reported that the level of HNF4α expression in liver accounted for the highest degree of collinearity among the expression of human genes involved in xenobiotic metabolism such as *CYP2A6*, *CYP2B6*, *CYP2C8*, *CYP2C9*, and *CYP2C19*. (Wortham et al., 2007). Analysis of various promoter and enhancer sequences has shown that HNF4α has a positive role in the regulation of numerous rodent and human P450s (Akiyama and Gonzalez, 2003). ChIP-on-Chip analysis has demonstrated that HNF4α binds to the regulatory regions of more than 1500 genes including *PXR*, *CYP1A2*, *CYP2B6*, *CYP2C8*, *CYP2D6*, and *CYP2E1* (Odom et al., 2004).

Kim and coworkers (Tirona et al., 2003) also demonstrated a role for HNF4α in PXR- and CAR-mediated transactivation of *CYP3A4*. Li and Chiang (2006) concluded that the competition between PXR and HNF4α for their coactivators PGC-1α and SRC-1 contributes to an interaction between these receptors. Our laboratory recently reported cross-talk between the proximal HNF4α and the upstream CAR/PXR sites of the *CYP2C9* promoter (Chen et al., 2005; Surapureddi et al., 2008). HNF4α and CAR/PXR synergistically activate the *CYP2C9* promoter in HepG2 cells in the presence of the CAR agonist 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime or the PXR agonist rifampicin. In addition, mutation of the HNF4α sites nearly abolishes CAR- or PXR/rifampicin-mediated induction of *CYP2C9* in HepG2 cells.

Although HNF4α has been shown to be capable of contributing to CAR/PXR-mediated *CYP2C9* transcription in HepG2 cells (Chen et al., 2005), the role of HNF4α in the regulation of *CYP2C8* by CAR or PXR has not been addressed because of the unavailability of an appropriate in vitro cell line. Unlike the *CYP2C9* promoter, the *CYP2C8* promoter is not up-regulated by CAR, PXR, or HNF4α in HepG2 cells (Ferguson et al., 2005). We recently used cultured primary human hepatocytes to identify a CAR/PXR site at -8.8 kb, which appeared to be solely responsible for the induction of *CYP2C8* by PXR ligands such as rifampicin as well as the CAR ligand 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (Ferguson et al., 2005). In the present study, we use primary cultures of human hepatocytes to address whether HNF4α sites in the *CYP2C8* promoter are also critically involved in the constitutive and CAR/PXR-mediated transcriptional activation of *CYP2C8*. We further confirmed the role of the HNF4α sites in the basal expression and rifampicin-PXR-mediated induction of CYP2C9 in primary cultures of human hepatocytes. We identified additional new putative HNF4α sites in both the *CYP2C8* and *CYP2C9* promoters and showed that these bind HNF4α in electrophoretic mobility shift assay (EMSA) studies. We mutated each of these HNF4α sites to determine their relative importance in the up-regulation of *CYP2C8* and *CYP2C9* in primary human hepatocytes by HNF4α and PXR/rifampicin. Finally, we used an adenovirus containing HNF4α-small interfering RNA (siHNF4) to examine the role of HNF4α in both the constitutive and the PXR/rifampicin-mediated promoter transactivation of the *CYP2C* promoters as well as CYP2C mRNA expression.

Materials and Methods

Promoter Constructs and Expression Plasmids. The pGL3-Basic constructs of wild-type *CYP2C8* (-8.9 to -8.5)-3 kb and *CYP2C9*-3 kb and the HNF4α site mutants *2C9*/150-mut and *2C9*/185-mut were described previously (Chen et al., 2004; Ferguson et al., 2005). Mutation of the new putative HNF4α sites at -181 bp in *CYP2C8* (-8.9 to -8.5)-3 kb and -211 bp in *CYP2C9*-3 kb was performed using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA). *CYP2C8*-1 kb and *CYP2C9*-1.9 kb/pdmut constructs were used as templates to generate the *2C8*/152-mut, *2C8*/181-mut, *2C8*/HNF4αdmut, and *2C9*/HNF4αtmut. The forward primers used for mutagenesis were as follows (hexamer half sites are indicated by bold capital letters and mutated nucleotides are underlined): -211 2C9 HNF4 site, 5′-TGTACAG**A**<u>**CACC**</u>**A**CAA**TGGAA**CGAAG-3′; -152 2C8 HNF4 site, 5′-CTATCCAT**GGGC**<u>**GT**</u>**AAGTC**<u>**GT**</u>CTCAGAAAAAAAGTATAAATTG-3′; and -181 2C8 HNF4 site: 5′-GAAGGAGTA**GGAC**<u>**TT**</u>**AAGAAG**<u>**T**</u>TTT-TATTTCTATCCATGGGC-3′. DNA sequencing was performed for all constructs to verify the mutation and to assure that no spurious mutations occurred.

Adenoviral Constructs and RNA Interference. Specific adenoviral constructs were produced by double recombination between a cotransformed adenoviral backbone plasmid (pAdEasy-1) and a linearized shuttle vector (pShuttle-HNF4α or pShuttle-lacZ) using the AdEasy XL Adenoviral Vector system (Stratagene). Positive clones were amplified by transformation into XL-10 gold cells according to the manufacturer's directions (QIAGEN, Valencia, CA). Plasmid DNA was used to transfect human embryonic kidney Ad-293 cells, the virus was harvested and amplified, and the titer was determined according to the manufacturer's instructions (Stratagene).

To silence the expression of HNF4α, specific small interfering RNAs (siRNAs) were prepared using an AdEasy XL Adenoviral Vector system. siRNA targets for HNF4α were identified using the GenScript (Piscataway, NJ) target finder. The following sequences were used to silence HNF4α: siHNF4-I at 248 bp, 5′-ACATGTACTCCTGCAGATTTA-3′; siHNF4-II at 387 bp, 5′-CACTCGAAGGTCAAGCTATGA-3′; and siHNF4-III at 822, 5′-CAATGAGTATGCCTACCTCAA-3′. The scrambled sequence 5′-GCGCTTCATAATATCTAACGT-3′ was used as a negative control. Double-stranded short hairpin RNA oligonucleotides designed with the construct builder were annealed and ligated to the MluI and XhoI sites of pRNAT-H1.1/Adeno (SD-1219) siRNA shuttle vector. The vector contains an H1 promoter to drive siRNA expression and a coral green fluorescent protein marker under the control of the cytomegalovirus promoter. The inserted sequences were confirmed by sequencing. Adenoviruses expressing each siRNA were prepared, purified using continuous cesium chloride gradient centrifugation, and stored in Tris-buffered sucrose (10 mM Tris, pH 8.0, 2 mM $MgCl_2$, and 4% sucrose). Primary human hepatocyte cells were routinely infected with 1000 viral particles/cell. The infection efficiency of the adenovirus was monitored by the expression of green fluorescent protein and typically reached 80 to 90% within 36 to 48 h.

Cell Culture and Transfections. HepG2 cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin-streptomycin at 37°C under 5% CO_2. Primary human hepatocytes from nine different donors were obtained from CellzDirect (division of Invitrogen, Carlsbad, CA) (Table 1) and maintained in Williams' E medium supplemented with ITS+1 (insulin, human transferrin, sodium selenite, bovine serum albumin, and linoleic acid in Earle's balanced salt solution; Sigma-Aldrich, St. Louis, MO), HEPES, L-glutamine, and 100 nM dexamethasone. None of the donors was a current smoker. Transfections were performed on freshly isolated nonoverlay human hepatocyte cells with Effectene transfection reagent (QIAGEN) using the manufacturer's procedures. Conditions were optimized for primary human hepatocytes. In brief, DNA and reagents were mixed and incubated at room temperature for 30 min. The reaction mix was diluted with the complete medium and added to the cells.

After 12 h, cells were infected with 1000 viral particles/cell in a serum-free medium each with adenovirus expressing LacZ, HNF4α, scrambled siRNA, or siHNF4-I. After 12 h, the medium was replaced with complete medium containing appropriate ligands (0.2% DMSO and 10 μM rifampicin). Twenty-four hours later, cells were assayed for promoter activity using a dual luciferase assay kit (Promega, Madison, WI). Luciferase values were normalized with *Renilla* luciferase values to calculate promoter activity.

RT-PCR. Induction of CYP2Cs was confirmed by using quantitative RT-PCR for all the transactivation assays performed in this study. RNA was isolated using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. RT-PCR analysis was performed as described previously (Ferguson et al., 2005). In brief, the RT reaction was performed using 200 ng of total RNA, 2 μl (40 units) of RNase inhibitor, 1× first strand buffer, 10 mM dithiothreitol, 0.5 mM dNTPs, and 1 μl (200 units)

TABLE 1

Donor information for the nine human hepatocyte donors

No.	Lot No.	Overlay/Nonoverlay	Sex	Age	Race	Smoking	Alcohol Use	Obese
				y				
1	Hu-0694	Overlay	F	54	W	No	No	
2	Hu-0714	Nonoverlay	M	68	W	No	No	
3	Hu-0747	Nonoverlay	F	64	W	No	Social	
4	Hu-0798	Nonoverlay	F	56	W	No	Rare	
5	Hu-0808	Both	F	43	W	Quit in 1996	2–3 Units every other day	
6	Hu-0813	Both	M	63	W	Quit in 1986	1–2 Beers/day	
7	Hu-0861	Nonoverlay	F	61	AA	No	No	
8	Hu-0999	Nonoverlay	F	35	W	No	No	Yes
9	Hu-1125	Nonoverlay	M	56	W	Quit in 1989	Once per week	Yes

F, female; M, male; W, white; AA, African American.

of SuperScript II (Invitrogen) to a total volume of 20 μl. Amplification reactions were performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900 HT Sequence Detection System using TaqMan probes (Applied Biosystems) for CYP2C8, CYP2C9, CYP2C19, CAR, PXR, HNF4α, PGC-1, SRC-1, retinoid X receptor, peroxisome proliferator-activated receptor-binding protein, and internal control TATA-binding protein. The relative quantity for each sample was normalized to the endogenous control gene (TATA-binding protein) content, calibrated to the respective experimental control, and calculated as $2^{-\Delta\Delta C_T}$.

Gel Shift Assays. Electrophoretic mobility shift assays were performed as described previously (Chen et al., 2005). In brief, human HNF4α was synthesized in vitro using the TNT Quick Coupled in Vitro Transcription/Translation System (Promega) following the manufacturer's protocol. Klenow fragment (New England Biolabs, Ipswich, MA) was used to incorporate [^{32}P]dCTP at the 5′ ends of the double-stranded oligonucleotides. Approximately 50,000 cpm of labeled probe was incubated with 2 μl of in vitro synthesized human HNF4α in a 10-μl binding reaction containing 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 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-Aldrich).

In competition experiments, specific cold competitors or a specific antibody to human HNF4α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the mixture before the addition of proteins. After incubation for 20 min at room temperature, 9.5 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. The following are the sequences of the oligonucleotides used as probes, wild-type, or mutated specific cold competitors (hexamer half-sites are indicated by bold capital letters and mutated nucleotides are underlined): *CYP2C8*-181HRE, 5′-ctagAGTA**GGACAAAAGAACA**TTTT-3′; *CYP2C8*-181HREMut, 5′-ctagAGTA**GGAC<u>TT</u>AAGAA<u>GT</u>**TTTT-3′; *CYP2C9*-211HRE, 5′-tgtACAG**AGTGGACAATGGA**ACGA-3′; *CYP2C9*-211HREMut, 5′-TGTACAGA<u>CACC</u>ACA<u>TACC</u>AACGAAG-3′; *CYP2C19*-213HRE, 5′-**TGTACAGAGTGG<u>G</u>CA<u>C</u>TGG<u>G</u>ACGAAG-3**′; APF-1 wild type, 5′-GCGC**TGGGCAAAGGTCA**CCTGC-3′; and APF-1Mut, 5′-GCGC**TGG<u>CG</u>A**AAGG**<u>AG</u>A**CCTGC-3′.

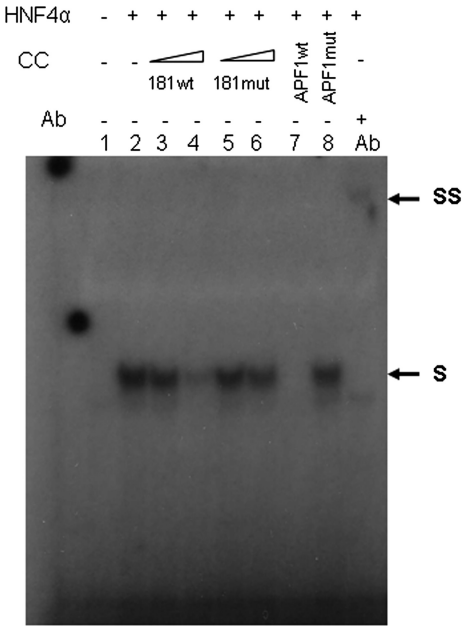
Western Blot Analysis. Whole-cell lysates were prepared from HepG2 cells infected with adenoviral constructs expressing scrambled control siRNA or siHNF4-I, II, and III using immunoprecipitation assay buffer (Promega). Nuclear extracts from primary human hepatocytes were prepared as described by Pascussi et al. (2000) and analyzed for protein expression. In briefly, 40 μg of samples were resuspended in SDS sample buffer containing 0.13 M dithiothreitol, separated on a 4 to 20% gradient gel, and transferred to a nitrocellulose membrane (Millipore Corporation, Billerica, MA). The membrane was blocked with 5% nonfat milk blocking buffer and probed with rabbit anti-HNF4α IgG (1:1000) (Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. After washing, the blot was incubated for 1 h with goat anti-rabbit horseradish peroxidase (Promega) as a secondary antibody. Detection was achieved using a SuperSignal West Femto kit (Pierce Biotechnology, Rockford, IL).

Statistical Analysis. For comparing activity of luciferase reporter constructs, statistical analysis was performed in SigmaStat (version 9.1; SAS Institute, Cary, NC) using nonparametric methods. Kruskal-Wallis analysis of variance was used to confirm differences in expression across luciferase constructs, For Figs. 3 to 5, Mann-Whitney tests were used to compare pairs of expression values, both between HNF4α and LacZ and between rifampicin and DMSO for each luciferase construct as well as between each mutant and wild-type constructs and to compare mRNA expression values.

Results

Identification of New HNF4α-Binding Sites within the *CYP2C8* and *CYP2C9* Promoters. We have previously identified a single HNF4α-binding site at −152 bp in the 5′-flanking region of the *CYP2C8* gene (Ferguson et al., 2005). To determine whether there are additional HNF4α-binding sites in the *CYP2C8* promoter, we scanned 3 kb of the *CYP2C8* promoter for the presence of putative motifs using SeqLab software and identified a second new DR1-like element −181 bp upstream of the translation start site for *CYP2C8*. An EMSA was performed with a ^{32}P-labeled probe covering the new putative HNF4α-binding site incubated with in vitro translated HNF4α (Fig. 1). A shift of the HNF4α-RE probe-protein complex was observed with in vitro translated HNF4α, which was effectively eliminated by the addition of an excess of wild-type cold competitors (Fig. 1, lanes 3 and 4) and by an APF-1 wild-type oligonucleotide encompassing a known HNF4α-binding element of the apolipoprotein CIII promoter (Jiang and Sladek, 1997) as a positive control (Fig. 1, lane 7). There was no competition by cold competitors containing a mutated −181 HNF4α-binding site (Fig. 1, lanes 5 and 6) or a mutated APF-1 oligonucleotide (Fig. 1, lane 8). Antibodies against HNF4α decreased the intensity of this complex, and a faint supershifted band appeared at the top of the gel (Fig. 1, lane 9), providing further evidence that the second DR1 motif at −181 in *CYP2C8* is an HNF4α-binding site.

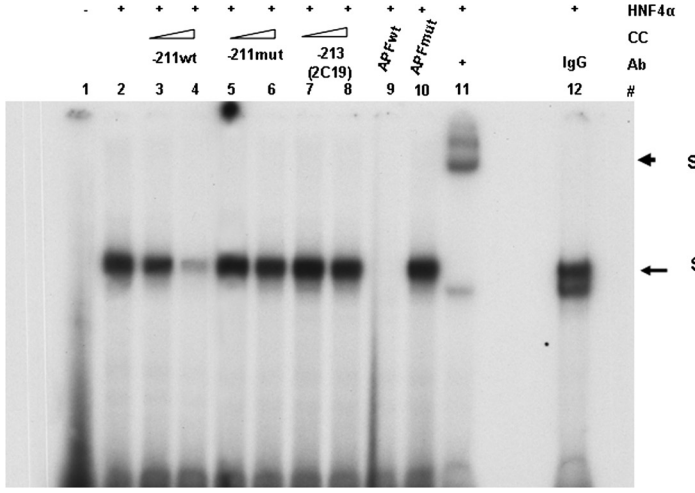
We previously identified two sites (located at −150 to −138 bp and −185 to −173 bp from the translation start site) that are essential for the activation of the *CYP2C9* promoter by HNF4α in HepG2 cells (Chen et al., 2005). However, additional follow-up studies indicated that the *CYP2C9* promoter with mutations in both the HNF4α sites (at −150 and −185 bp) was still significantly activated by HNF4α in HeLa cells. Using progressive deletions, we found that a short region between −181 and −219 bp appeared to be required for optimum HNF4α activation (Y. Chen, unpublished observations). By inspection of this region, we observed a DR-1 site in a reverse orientation at −211 to −199 bp that might represent a new putative HNF4α-responsive element. As shown in Fig. 2, gel shift assays verified that HNF4α protein efficiently bound to this new DR-1 site. The intensity of the DNA-protein complex was decreased by an unlabeled oligomer containing the wild-type sequence from the *CYP2C9* promoter or the APF-1 oligonucleotide but not by an unlabeled *CYP2C19* oligomer (the oligomer from *CYP2C19* aligns with the −211 oligomer of *CYP2C9,* but it does not bind HNF4α) or an APF-1 oligomer



1. Free Probe
2. HNF4α
3. HNF4α 10X cc
4. HNF4α 100X cc
5. HNF4α 10X mut cc
6. HNF4α 100X mut cc
7. APF1-wt
8. APF1-mut

Ab. HNF-4α Anti-HNF-4alpha

FIG. 1. Identification of a new HNF4α-binding site in the *CYP2C8* promoter region. EMSA demonstrates binding of the new putative HNF4α-binding site of *CYP2C8* at −181 bp to HNF4α. The ^{32}P-labeled probe containing the new putative HNF4α-binding site of *CYP2C8* was incubated with in vitro synthesized HNF4α at room temperature for 20 min. Excess (5× or 50×) wild-type (wt) or mutant (mut) cold competitors (CC) were added to the binding reactions for competition analysis. Antibody (Ab) against HNF4α (last lane) resulted in a supershifted band. S, shifted complex; SS, supershifted band.



1. Free Probe
2. HNF4α
3. HNF4α 5X cc
4. HNF4α 50X cc
5. HNF4α 5X mutcc
6. HNF4α 50X mutcc
7. HNF4α 5X mutcc
8. HNF4α 50X mutcc
9. APF1-wt
10. APF1-mut
11. HNF4α-Anti-HNF-4alpha
12. HNF4α-IgG

FIG. 2. EMSA demonstrates binding of HNF4α to the new putative HNF4α-binding site of the *CYP2C9* promoter at −211 bp. A ^{32}P-labeled oligonucleotide probe containing the new putative HNF4α-binding site of *CYP2C9* was incubated with in vitro synthesized HNF4α at room temperature for 20 min. Excess (5× or 50×) wild-type (wt) or mutant (mut) cold competitors (CC) (*CYP2C9* or APF-1 oligonucleotides containing an HNF4α site) was added to binding reactions for competition analysis. Antibody (Ab) against HNF4α resulted in a supershifted band (last lane) with the *CYP2C9* oligomer. S, shifted complex; SS, supershifted band.

containing mutations of the HNF4α sites. Although binding of the −211 oligomer of *CYP2C9* was less intense than that of oligomers containing the −150 and −185 bp sites (data not shown), addition of antibody to HNF4α resulted in a clear supershift of the −211 *CYP2C9* oligomer-HNF4α complex, confirming the presence of HNF4α in the complex.

Role of the Two HNF4α-Binding Sites in HNF4α Transactivation and PXR-Rifampicin Mediated Transactivation of *CYP2C8* in Primary Cultures of Human Hepatocytes. Because HNF4α does not activate *CYP2C8* promoter activity in HepG2 cells (Ferguson et al., 2005), we used primary human hepatocytes in the present study to assess the importance of the two HNF4α-binding sites in HNF4α and PXR-mediated rifampicin up-regulation of the *CYPC8* promoter. We first compared the ability of HNF4α to transactivate the wild-type *2C8* (−8.9 to −8.5)-3 kb construct versus constructs harboring mutations at HNF4α sites at −152 bp (*2C8*/152-mut), −181 bp (*2C8*/181-mut), or both sites (*2C8*/HNF4αdmut). Basal activity of the wild-type *CYP2C8* reporter construct was decreased by mutation of each of the HNF4α sites (Fig. 3, A and B) presumably because the effects of constitutive levels of HNF4α in primary hepatocytes were abolished. The wild-type reporter construct was further activated by adenoviral constructs containing exogenous HNF4α (5.6-fold) compared with the LacZ control. However, mutation of the HNF4α sites at −152 or −181 bp dramatically decreased transactivation by exogenous HNF4α (Fig. 3A). Moreover, the double mutation completely abolished HNF4α-mediated transactivation of the *CYP2C8* reporter construct.

We then assessed the importance of the two HNF4α-binding sites in PXR/rifampicin-mediated induction of the *CYPC8* promoter. We transfected wild-type *2C8* (−8.9 to −8.5)-3 kb and the three HNF4α mutant constructs (*2C8*/152-mut, *2C8*/181-mut, or *2C8*/HNF4αdmut) into primary cultures of human hepatocytes and treated them with 0.2% DMSO or 10 μM rifampicin. The PXR agonist rifampicin produced a 2-fold increase in activity of the wild-type *2C8* (−8.9 to −8.5)-3 kb promoter construct. Rifampicin produced a small but significant increase in luciferase activity for the *2C8*/152-mut, whereas rifampicin up-regulation was abolished by the mutation at −181 and by the double mutation (Fig. 3B). These data indicate that the two HNF4α sites in the *CYP2C8* promoter construct play a vital

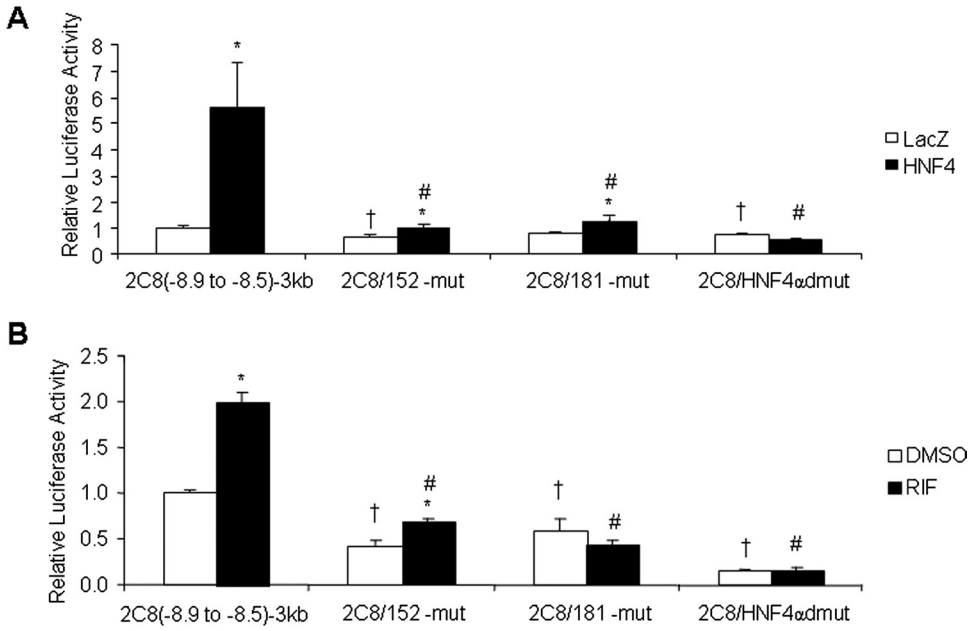


FIG. 3. Mutations in the HNF4α-binding sites at -152 or -181 bp significantly reduced transactivation of the *CYP2C8* promoter by exogenous HNF4α (A) and induction by rifampicin (RIF) in primary human hepatocytes (B). A, primary cultures of human hepatocytes from donor Hu-0861 were transfected with wild-type *CYP2C8* (-8.9 to -8.5)-3 kb or mutant constructs containing mutations at -152 bp (*2C8*/152-mut), -181 bp (*2C8*/181-mut), or both sites (*2C8*/dmut). After 12 h, cells were infected with 2.5×10^9 viral particles/ml AdHNF4α or LacZ as a control as described under *Materials and Methods*. After 48 h, luciferase activity was measured and normalized to the internal control pRL. Transfections were performed in triplicate, and values represent the mean $\pm$ S.E. of fold activation relative to that of the wild-type *CYP2C8* (-8.9 to -8.5)-3 kb promoter infected with LacZ. AdHNF4α significantly activated the wild-type and single mutant *CYP2C8* reporter constructs (*, $p < 0.05$) and the single mutants 2C8/152-mut, and 2C8/181-mut but not the double mutant. Activation of the *CYP2C8* mutant constructs (*2C8*/152-mut, *2C8*/181-mut, or *2C8*/HNF4αdmut) was significantly lower than of the wild-type *CYP2C8* reporter construct (#, $p < 0.05$, whereas the basal activity of those transfected with lacZ was significantly lower for the *2C8*/152-mut and *2C8*/HNF4αdmut than those of the wild-type construct (†, $p < 0.05$). B, effect of mutation of the HNF4α sites in the *CYP2C8* promoter on activation of *CYP2C8* by rifampicin. Primary cultures of human hepatocytes transfected with the wild-type *CYP2C8* (-8.9 to -8.5)-3 kb promoter or its HNF4α mutants were treated with 0.2% DMSO or 10 μM rifampicin for 24 h. Values for rifampicin-mediated transactivation are expressed relative to those of the wild-type *CYP2C8* promoter treated with the vehicle DMSO. Rifampicin significantly increased activity of the wild-type *CYP2C8* and *2C8*/152-mut reporter activity compared with the vehicle control (*, $p < 0.05$), whereas mutation of each of the HNF4α sites significantly decreased constitutive activity (†, $p < 0.05$) or the induction by rifampicin (#, $p < 0.05$).

role in PXR/rifampicin-mediated transactivation of *CYP2C8* in primary human hepatocytes.

Role of the Different HNF4α-Binding Sites in Constitutive and Rifampicin-Mediated Transactivation of *CYP2C9* in Cultured Primary Human Hepatocytes. In the present study, we also examined the functional relevance of the new HNF4α site as well as that of two previously identified HNF4α-binding sites in activation of the *CYP2C9* promoter by HNF4α and in induction by rifampicin in primary human hepatocytes. Primary hepatocytes were transiently transfected with wild-type *CYP2C9*-3 kb promoter or mutants (*2C9*/150-mut, *2C9*/185-mut, *2C9*/211-mut, and *2C9*/HNF4αtmut) and infected with adenoviral HNF4α or LacZ as a control (Fig. 4A). In cells infected with adenoviral HNF4α, the basal reporter activity of the wild-type *CYP2C9*-3 kb increased 2.4-fold. HNF4α activation was significantly decreased to 1.7-fold by the mutation at -150 bp and further decreased ($p < 0.05$) to 1.4-fold by the mutations at -185 and -211 bp. HNF4α activation was abolished by the triple mutation of all three responsive HNF4α elements. These results indicate that all three HNF4α response elements contribute to up-regulation of the *CYP2C9* gene by HNF4α.

To confirm our previous studies in HepG2 cells, which indicate that HNF4α has an important role in modulating rifampicin-PXR-mediated transactivation of *CYP2C9* (Chen et al., 2005), we transfected the wild-type *CYP2C9*-3 kb reporter construct and its HNF4α mutants into primary cultures of human hepatocytes and treated the cells with 10 μM rifampicin. Rifampicin increased the activity of the *CYP2C9*-3 kb reporter construct 2-fold (Fig. 4B). Activation of the *CYP2C9* promoter by rifampicin was significantly decreased ($p < 0.05$) to 1.2-, 1.3-, and 1.3-fold, respectively, by the mutations of HNF4α sites at -150, -185, and -211 bp and abolished by the triple mutation. These data indicate that the three HNF4α sites are required for maximum transactivation of the *CYP2C9* promoter by rifampicin. The basal activity of the *CYP2C9*-3 kb promoter was also significantly decreased by mutations of each of the HNF4α sites to a maximum of 35% by the triple mutation, indicating that endogenous levels of HNF4α affect promoter activity.

Silencing HNF4α Resulted in Almost Complete Loss of Rifampicin-Induced Transactivation of *CYP2C8* and *CYP2C9* in Cultured Primary Human Hepatocytes. We constructed three siRNA adenoviral constructs to silence HNF4α expression. Of the three siHNF4α constructs tested, siHNF4-I was most efficacious in reducing HNF4α mRNA in four separate lots of primary hepatocytes by $69 \pm 9\%$ ($p < 0.001$) and resulted in undetectable amounts of HNF4α protein in nuclear extracts of primary human hepatocytes by Western blot analysis (data not shown). Wild-type *2C8* (-8.9 to -8.5)-3 kb and *2C9*-3 kb were transiently transfected into primary cultures of human hepatocytes, and 12 h later the cells were infected with adenovirus expressing scrambled siRNA or siHNF4-I. Silencing HNF4α expression decreased the basal activity of both the *CYP2C8* and *CYP2C9* promoter reporters (48 and 43%, respectively), abolished the 2-fold rifampicin-mediated transactivation of the *CYP2C8* promoter, and decreased the activation of the *2C9* promoter from 2.1- to 1.2-fold (Fig. 5).

Silencing HNF4α Resulted in Decreased Basal and Rifampicin-Mediated Induction of CYP2C mRNA in Primary Human Hepatocyte Cultures. We also examined the effect of siHNF4 on CYP2C mRNA levels in cultured primary human hepatocytes. Cells from four

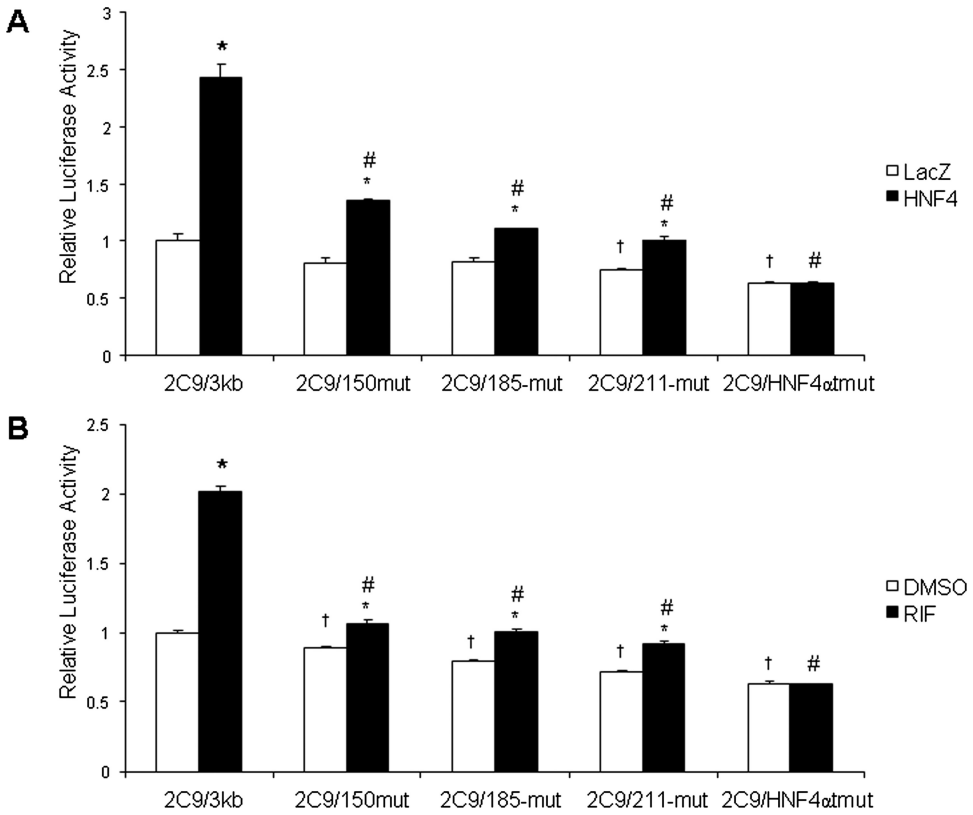


FIG. 4. Effect of mutation of different HNF4α-binding sites on *CYP2C9* promoter transactivation by HNF4α and rifampicin (RIF) in primary human hepatocytes. A, primary cultures of human hepatocytes from donor Hu-1125 were transfected with the wild-type *CYP2C9*-3 kb promoter or *CYP2C9* promoters containing mutations of the individual HNF4α-binding sites at -150 (*2C9*/150-mut), -185 (*2C9*/185-mut), or -211 bp (*2C9*/211-mut), or mutation of all three sites (*2C9*/HNF4αtmut) followed by infection with AdHNF4α or LacZ as a control. Luciferase activity assays were performed 48 h later. All transfections were performed in quadruplicate, and values represent the mean $\pm$ S.E. of the fold activation relative to that of the *CYP2C9*-3 kb promoter infected with LacZ. AdHNF4α significantly enhanced the activation of wild-type *CYP2C9* and single HNF4α mutants (*, $p < 0.05$) but not the triple mutation. Luciferase activity of the *CYP2C9* mutants transfected with LacZ (†, $p < 0.05$) or adenoviral HNF4α was significantly lower than that of the wild-type *CYP2C9* reporter construct (#, $p < 0.05$). B, effect of mutation of the HNF4α-binding sites on transactivation of the *CYP2C9* promoter by rifampicin (RIF). Cultures of primary human hepatocytes were transfected with the *CYP2C9*-3 kb promoter or its HNF4α mutants followed by treatment with 10 μM rifampicin for 24 h. Values for rifampicin-mediated transactivation are expressed as fold relative to the activity of the wild-type *CYP2C9* promoter construct treated with the vehicle DMSO. Rifampicin significantly increased activity of the wild-type *CYP2C9* promoter and promoter constructs containing single mutants compared with the vehicle control (*, $p < 0.05$). However, activation of the *CYP2C9* HNF4α promoter mutants by rifampicin was significantly lower than that of the wild-type (#, $p < 0.05$;), and the triple mutation abolished rifampicin activation. Constitutive activity of the mutants in the absence of rifampicin was significantly lower than that of wild type (†, $p < 0.05$).

different donors (Table 2) were infected with adenoviruses expressing either siHNF4 or control (scrambled) siRNA and treated with 0.2% DMSO or 10 μM rifampicin for 24 h. HNF4α mRNA was decreased to 31 $\pm$ 4%. We observed a variable 4- to 8-fold induction of CYP2C8 mRNA by rifampicin in primary human hepatocytes in different control donors (mean 6.6-fold) (Tables 2 and 3). When primary human hepatocytes were treated with siHNF4, the basal expression of CYP2C8 mRNA was down-regulated by ~53%, and the magnitude of rifampicin-mediated induction of CYP2C8 mRNA was significantly decreased (from 6.6- to 2.7-fold) ($p < 0.01$).

Rifampicin also induced CYP2C9 mRNA by 3-fold (Tables 2 and 3). Silencing HNF4α decreased the constitutive expression of CYP2C9 by 55% and the magnitude of the induction by rifampicin from 3- to 1.5-fold. We also observed a significant (1.8-fold) increase in CYP2C19 mRNA induction in cultured primary hepatocytes but no significant increase in cells treated with siHNF4 (1.1-fold). Because preliminary studies indicated that rifampicin produced little or no increase in *CYP2C19* promoter activation in primary hepatocytes, we were unable to study the effects of mutating the two HNF4α sites on promoter activity. Silencing HNF4α also significantly decreased expression of mRNA for the xenobiotic sensing receptors CAR and PXR by 60 and 40%, respectively (data not shown). However, silencing of HNF4α had no effect on the expression of RXRα mRNA, the closest homolog of HNF4α. mRNA for the cofactor PGC-1α was decreased by 50%, but SRC-1 mRNA was not affected.

Discussion

We previously reported that CAR/PXR directly regulates the *CYP2C8* and *CYP2C9* gene promoters via CAR/PXR response elements located at -8.8 kb (Ferguson et al., 2005) and -2897 and -1839 bp (Ferguson et al., 2002; Chen et al., 2005), respectively. Mutation of these sites abolishes rifampicin induction of the two genes. Our previous studies have also indicated that HNF4α sites are important for the CAR- and PXR-mediated up-regulation of *CYP2C9* transcription in HepG2 cells (Chen et al., 2005). However, these studies did not address the role of HNF4α in *CYP2C8* regulation, because neither CAR, PXR, nor HNF4 up-regulated *CYP2C8* in cell lines such as HepG2 cells (Ferguson et al., 2005). The present study used primary human hepatocytes as a more appropriate model to show that binding of HNF4α to multiple proximal HNF4α-binding sites in both the *CYP2C8* and *CYP2C9* promoters is vital for the rifampicin-PXR-mediated transactivation of these genes.

The regulatory regions of many of the P450s contain multiple HNF4α consensus DNA-binding sites, whereby HNF4α binds and enhances transcriptional activation (Akiyama and Gonzalez, 2003; Chen et al., 2005; Ferguson et al., 2005; Kawashima et al., 2006). As

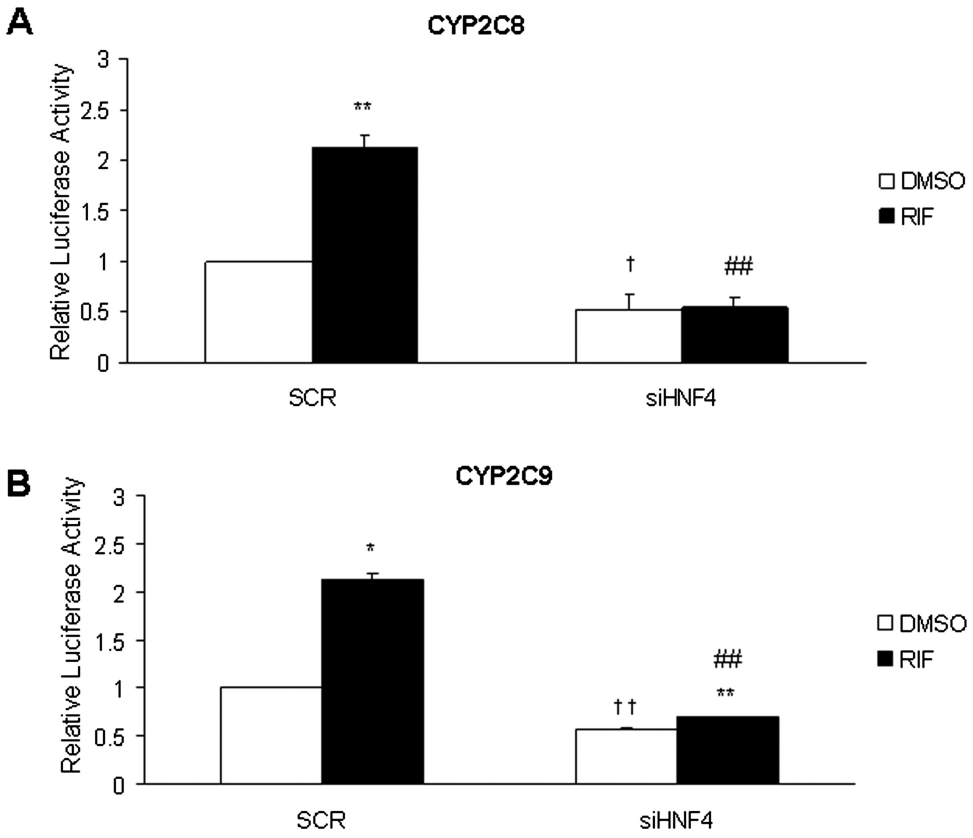


FIG. 5. Silencing HNF4α decreases basal *CYP2C8* and *CYP2C9* promoter activity and essentially abolishes rifampicin (RIF)-mediated transactivation in primary cultures of human hepatocytes. A, primary cultures of human hepatocytes were transfected with the wild-type *CYP2C8* (−8.9 to −8.5)-3 kb promoter reporter construct. After 12 h, cells were infected with siHNF4-I or scrambled (SCR) adenovirus for 1.5 h and then incubated at 37°C for 24 h. Transfected cells were then treated with 10 μM rifampicin or 0.2% DMSO for 24 h. Transfections were performed in triplicate, and values represent the mean ± S.E. in two donors (Hu-0747 and Hu-0808). Treatment with rifampicin resulted in a 2-fold increase in wild-type *CYP2C8* (−8.9 to −8.5)-3 kb promoter reporter activity (*, $p < 0.05$; **, $p < 0.01$). Silencing HNF4α resulted in significant down-regulation of the basal wild-type *CYP2C8* (−8.9 to −8.5)-3 kb reporter activity (†, $p < 0.05$; ††, $p < 0.01$) and abolished rifampicin-mediated transactivation (##, $p < 0.01$). B, silencing HNF4α reduced both the basal and rifampicin-mediated transactivation of the wild-type *CYP2C9*-3 kb reporter construct. All transfections were performed in triplicate samples from donors Hu-0798 and Hu-0813, and values represent the mean ± S.E. Treatment with rifampicin resulted in a significant 2-fold increase in wild-type *CYP2C9* promoter reporter activity compared with DMSO treatment and a small but significant increase after silencing HNF4α (*, $p < 0.01$; **, $p < 0.001$). Silencing HNF4α resulted in significant down-regulation of the constitutive wild-type *CYP2C9* reporter activity in the absence of rifampicin (†, $p < 0.05$; ††, $p < 0.01$) and significantly decreased transactivation by rifampicin (##, $p < 0.01$).

TABLE 2

Effects of rifampicin siHNF4 versus scrambled siRNA and rifampin on CYP2C8, CYP2C9, and CYP2C19 mRNA in primary human hepatocytes infected with scrambled siRNA or siHNF4-I

Expression of CYP2C8, CYP2C9, CYP2C19, and HNF4α mRNAs was evaluated in primary human hepatocytes in triplicate from four different donors after infection with adenovirus containing scrambled siRNA or siHNF4-I in the presence of 0.2% DMSO or 10 μM rifampicin. Data are expressed as fold induction over the DMSO controls and represent the mean ± S.D. of the four donors. Values were compared using Mann-Whitney tests.

Treatment	HNF4		2C8		2C9		2C19	
	DMSO	Rif	DMSO	Rif	DMSO	Rif	DMSO	Rif
SCR	1.00 ± 0.00	1.06 ± 0.11	1.00 ± 0.00	6.58 ± 1.79[a]	1.00 ± 0.00	3.00 ± 0.70[a]	1.00 ± 0.00	1.84 ± 0.31[a]
siHNF4	0.31 ± 0.09[b]	0.40 ± 0.10[a,b]	0.47 ± 0.06[b]	1.09 ± 0.45[a,b]	0.45 ± 0.14[b]	0.63 ± 0.14[b]	0.57 ± 0.15[b]	0.66 ± 0.24[b]

SCR, scrambled; Rif, rifampin.
[a] Values from cells treated with rifampicin significantly higher than those treated with DMSO for cells infected with scrambled siRNA or siHNF4-I, $p < 0.05$.
[b] Values from cells treated with siHNF4-I significantly lower than those treated with SCR siRNA for either DMSO or rifampicin controls, $p < 0.05$.

reviewed previously (Akiyama and Gonzalez, 2003), HNF4α has been reported to bind to direct repeats of AGGTCA separated by one base (DR1) or to the HPF-1 motif RRNCAAAGKNCANYY. It has been proposed that HNF4α functions by recruiting transcriptional coactivators with histone acetylase activity such as cAMP response element-binding protein binding protein (Yoshida et al., 1997), PGC-1α, and SRC-1 (Martinez-Jiménez et al., 2006), thereby facilitating gene activation by chromatin remodeling through relaxation of chromatin structure in the enhancer and promoter regions. We had previously identified two HNF4α-binding sites in the *CYP2C9* promoter (Chen et al., 2005) and one site in the *CYP2C8* promoter (Ferguson et al., 2005). In the present study, we identified additional HNF4α-binding sites in the *CYP2C8* (at −181 bp) and in the *CYP2C9* (at −211 bp) proximal promoters and assessed the role of each HNF4α site in HNF4α- and rifampicin-mediated up-regulation of the *CYP2C8* and *CYP2C9* promoters in human primary hepatocytes by mutational analysis. Mutation of either HNF4α site in the *CYP2C8* promoter markedly attenuated activation by HNF4α, and the double mutation abolished this response. The mutation of the new HNF4α-binding site at −181 bp abolished rifampicin-mediated transactivation of the

TABLE 3

Comparison of fold induction of CYP2C8, CYP2C9, and CYP2C19 mRNAs by rifampicin in cultured primary human hepatocytes after infecting cells with adenovirus expressing scrambled siRNA (controls) or siHNF4-I

Primary human hepatocytes from four different donors were treated with control (scrambled siRNA) or siHNF4-I in the presence or absence of rifampicin in triplicate and analyzed for mRNA expression of CYP2C8, CYP2C9, CYP2C19, and HNF4α. Data represent the mean ± S.D.

Fold Changes	HNF4α	2C8	2C9	2C19
Rif/control	1.06 ± 0.11 (N.S.)	6.57 ± 1.79[b,c]	3.00 ± 0.70[b,c]	1.84 ± 0.31[b,c]
siHNF4/Rif-siHNF4	1.31 ± 0.24[a]	2.68 ± 1.40[a]	1.53 ± 0.67	1.13 ± 0.21

Rif, rifampin; N.S., not significant.
[a] Fold change significantly greater than 1.00, $p < 0.05$.
[b] $p < 0.01$.
[c] Fold induction for Rif/control significantly greater than fold induction for siHNF4/Rif versus siHNF4, $p < 0.01$ in all cases.

CYP2C8 promoter, whereas mutation of the known site at −152 bp greatly diminished the response. The double mutation of both sites abolished the response to rifampicin. Mutation of any of the three *CYP2C9* HNF4-REs at −150, −185, and −211 bp greatly decreased up-regulation by HNF4α, although mutation of the site at −150 bp had a slightly smaller effect. All three mutations (*2C9*/150-mut, *2C9*/185-mut, and *2C9*/211-mut) dramatically decreased transactivation of the *CYP2C9* promoter in response to rifampicin, and mutation of all three sites completely abolished this transactivation. The cooperativity of the HNF4α sites in the *CYP2C8* and *CYP2C9* promoters is reminiscent of a study by Mellon and coworkers (Coss et al., 2005), which showed that mutation of any of three Smad-binding elements in close proximity to each other on the luteinizing hormone β-subunit gene promoter dramatically reduces induction by Smad3 cotransfection. Maximum induction of this gene by activin requires a homeobox element and the three Smad-binding elements. The authors proposed that multiple Smad-binding sites in close proximity to each other allow for cooperative binding. A similar mechanism could account for the cooperativity between the HNF4α sites in the *CYP2C9* promoter.

It should be noted that mutation of the two HNF4α sites in the *CYP2C9* promoter at −150 and −185 bp essentially abolished rifampicin induction in HepG2 cells but had no effect on dexamethasone induction via the glucocorticoid response element at −1697 bp (Chen et al., 2005). This finding suggests that these mutations do not exert a nonspecific effect on basal promoter structure that prevents up-regulation of the gene. Because glucocorticoids are needed for maintenance of CAR and PXR in primary culture of human hepatocytes, the effects of mutation of the HNF4 sites on dexamethasone induction could not be tested in this system.

Consistent with our mutational studies, silencing HNF4α expression in primary hepatocytes also abolished transactivation of the *CYP2C8* and *CYP2C9* promoters. Moreover, adenoviral constructs containing siHNF4 decreased both constitutive and rifampicin-induced expression of CYP2C8 and CYP2C9 mRNA in primary human hepatocytes. Our results are consistent with reports that HNF4α binds to the promoter regions of numerous genes including *CYP2C8* in hepatocytes freshly isolated from human liver using ChIP-on-Chip studies (Odom et al., 2004). In addition, Chiba and coworkers (Kawashima et al., 2006) used ChIP analysis to show binding of HNF4α to the area of the CYP2C9 promoter containing the HNF4α response elements in fresh human liver.

Our conclusions regarding the involvement of HNF4α in the PXR-mediated induction of CYP2C8 and CYP2C9 differ somewhat from those of another laboratory (Kamiyama et al., 2007), which reported that adenoviral constructs expressing HNF4α-siRNA had no effect on xenobiotic-mediated induction of CYP2C8 and CYP2C9 mRNA in cultures of primary hepatocytes. However, their interpretations were based on the observations that constitutive as well as xenobiotic-induced levels of CYP2C8 and CYP2C9 mRNA were decreased by HNF4α-siRNA; subsequently, the magnitude of the induction by CAR/PXR ligands remained unchanged in their hands. We also observed a decrease in constitutive levels of both CYP2C8 and CYP2C9 mRNA; however, our study found a difference in the magnitude of induction by rifampicin when expression of HNF4α was silenced. One difference between the two studies is that we used cultures of fresh human hepatocytes from four different donors, whereas the previous study used primary cultures from cryopreserved human hepatocytes from a single donor. Moreover, our conclusions are supported by our mutational analysis, which indicates that the HNF4α sites are necessary for the up-regulation of *CYP2C* promoter constructs by rifampicin. However, both studies agree that induction of CYP2C8 and CYP2C9 mRNA was not completely abolished by adenovirally expressed HNF4α-siRNA. This finding could indicate that HNF4α expression is not absolutely obligatory for rifampicin induction, or it could reflect incomplete silencing of HNF4α in all hepatocytes (down-regulation of HNF4α mRNA varied from 60 to 81% in different hepatocyte cultures in our studies).

Silencing HNF4α also decreased the basal levels of 2C19 mRNA (~50%) in cultured primary human hepatocytes in our studies and abolished the ~2-fold induction of 2C19 by rifampicin. Although Chiba and coworkers (Kawashima et al., 2006) could not detect binding of HNF4α in vivo to the HNF4α response elements in the *CYP2C19* promoter by ChIP analysis of human liver, our findings suggest the possibility that HNF4α may be involved in the regulation of this gene. Alternatively, the down-regulation of CYP2C19 mRNA could be secondary to down-regulation of receptors such as CAR and PXR. The regulation of CYP2C19 by HNF4α is in agreement with the conclusions of Wortham et al. (2007), who analyzed 20 human liver samples and found a significant correlation between the basal CYP2C19 expression and the expression of HNF4α. As described in previous studies, *CYP2C19* contains two HNF4α-binding sites identical to those found in *CYP2C9* (Kawashima et al., 2006). However, the newly identified HNF4α-binding site at −211 bp in the *CYP2C9* promoter is not present in the *CYP2C19* promoter, and the absence of this site could possibly contribute to reports of a lack of HNF4α transactivation of the *CYP2C19* promoter in cell lines (Kawashima et al., 2006).

PXR and CAR mRNA levels were moderately decreased (40 and 60%, respectively) by silencing HNF4α, as also noted by Yamazoe and coworkers (Kamiyama et al., 2007). Although this decrease in PXR could conceivably contribute to the decreased response of *CYP2C* genes to the PXR ligand rifampicin, their study showed that infection of primary human hepatocytes with adenoviral constructs containing PXR or CAR simultaneously with siHNF4α restored CAR/PXR levels but did not restore induction by xenobiotics (Kamiyama et al., 2007). Although mRNA levels of the coactivator PGC-1 were also decreased by 50% in our studies, those of SRC-1 and peroxisome proliferator-activated receptor-binding protein were not affected. However, silencing HNF4α could decrease expression of other nuclear transcription factors and coactivators.

The present studies support growing evidence that HNF4α is a master regulator, regulating receptors and coactivators such as CAR, PXR, and PGC-1α as well as many xenobiotic-metabolizing enzymes and drug transporters (Kamiya et al., 2003; Tirona et al., 2003; Ding et al., 2006). The results of our reporter studies show unequivocally that HNF4α sites are important for PXR-mediated induction of *CYP2C8* as well as *CYP2C9* in cultured primary human hepatocytes, because mutation of the HNF4α sites of either the *CYP2C8* and *CYP2C9* promoters or silencing HNF4α expression abolishes promoter activation by rifampicin. Consistent with these results, induction of CYP2C8 and CYP2C9 mRNA by rifampicin in primary human hepatocytes was markedly reduced by siHNF4α. In conclusion, our studies provide clear evidence that HNF4α transcriptionally is important for both the response of the human *CYP2C8* and *CYP2C9* genes to PXR agonists and the constitutive levels of these enzymes.

References

Akiyama TE and Gonzalez FJ (2003) Regulation of P450 genes by liver-enriched transcription factors and nuclear receptors. *Biochim Biophys Acta* **1619:**223–234.

Bort R, Gómez-Lechón MJ, Castell JV, and Jover R (2004) Role of hepatocyte nuclear factor 3γ in the expression of human CYP2C genes. *Arch Biochem Biophys* **426:**63–72.

Chen Y, Ferguson SS, Negishi M, and Goldstein JA (2003) Identification of constitutive androstane receptor and glucocorticoid receptor binding sites in the CYP2C19 promoter. *Mol Pharmacol* **64:**316–324.

Chen Y, Ferguson SS, Negishi M, and Goldstein JA (2004) Induction of human *CYP2C9* by rifampicin, hyperforin, and phenobarbital is mediated by the pregnane X receptor. *J Pharmacol Exp Ther* **308:**495–501.

Chen Y, Kissling G, Negishi M, and Goldstein JA (2005) The nuclear receptors constitutive androstane receptor and pregnane X receptor cross-talk with hepatic nuclear factor 4α to synergistically activate the human *CYP2C9* promoter. *J Pharmacol Exp Ther* **314:**1125–1133.

Coss D, Thackray VG, Deng CX, and Mellon PL (2005) Activin regulates luteinizing hormone β-subunit gene expression through Smad-binding and homeobox elements. *Mol Endocrinol* **19:**2610–2623.

Ding X, Lichti K, Kim I, Gonzalez FJ, and Staudinger JL (2006) Regulation of constitutive androstane receptor and its target genes by fasting, cAMP, hepatocyte nuclear factor α, and the coactivator peroxisome proliferator-activated receptor gamma coactivator-1α. *J Biol Chem* **281:**26540–26551.

Duncan SA, Manova K, Chen WS, Hoodless P, Weinstein DC, Bachvarova RF, and Darnell JE Jr (1994) Expression of transcription factor HNF-4 in the extraembryonic endoderm, gut, and nephrogenic tissue of the developing mouse embryo: HNF-4 is a marker for primary endoderm in the implanting blastocyst. *Proc Natl Acad Sci USA* **91:**7598–7602.

Ferguson SS, Chen Y, LeCluyse EL, Negishi M, and Goldstein JA (2005) Human CYP2C8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4α. *Mol Pharmacol* **68:**747–757.

Ferguson SS, LeCluyse EL, Negishi M, and Goldstein JA (2002) Regulation of human CYP2C9 by the constitutive androstane receptor: discovery of a new distal binding site. *Mol Pharmacol* **62:**737–746.

Gerbal-Chaloin S, Daujat M, Pascussi JM, Pichard-Garcia L, Vilarem MJ, and Maurel P (2002) Transcriptional regulation of CYP2C9 gene. Role of glucocorticoid receptor and constitutive androstane receptor. *J Biol Chem* **277:**209–217.

Goldstein JA (2001) Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. *Br J Clin Pharmacol* **52:**349–355.

Goldstein JA and de Morais SM (1994) Biochemistry and molecular biology of the human CYP2C subfamily. *Pharmacogenetics* **4:**285–299.

Hayhurst GP, Lee YH, Lambert G, Ward JM, and Gonzalez FJ (2001) Hepatocyte nuclear factor 4α (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol* **21:**1393–1403.

Inoue Y, Hayhurst GP, Inoue J, Mori M, and Gonzalez FJ (2002) Defective ureagenesis in mice carrying a liver-specific disruption of hepatocyte nuclear factor 4α (HNF4α). HNF4α regulates ornithine transcarbamylase in vivo. *J Biol Chem* **277:**25257–25265.

Jiang G and Sladek FM (1997) The DNA binding domain of hepatocyte nuclear factor 4 mediates cooperative, specific binding to DNA and heterodimerization with the retinoid X receptor α. *J Biol Chem* **272:**1218–1225.

Jover R, Bort R, Gómez-Lechón MJ, and Castell JV (1998) Re-expression of C/EBP α induces CYP2B6, CYP2C9 and CYP2D6 genes in HepG2 cells. *FEBS Lett* **431:**227–230.

Jover R, Bort R, Gómez-Lechón MJ, and Castell JV (2001) Cytochrome P450 regulation by hepatocyte nuclear factor 4 in human hepatocytes: a study using adenovirus-mediated antisense targeting. *Hepatology* **33:**668–675.

Kamiya A, Inoue Y, and Gonzalez FJ (2003) Role of the hepatocyte nuclear factor 4α in control of the pregnane X receptor during fetal liver development. *Hepatology* **37:**1375–1384.

Kamiyama Y, Matsubara T, Yoshinari K, Nagata K, Kamimura H, and Yamazoe Y (2007) Role of human hepatocyte nuclear factor 4α in the expression of drug-metabolizing enzymes and transporters in human hepatocytes assessed by use of small interfering RNA. *Drug Metab Pharmacokinet* **22:**287–298.

Kawashima S, Kobayashi K, Takama K, Higuchi T, Furihata T, Hosokawa M, and Chiba K (2006) Involvement of hepatocyte nuclear factor 4α in the different expression level between CYP2C9 and CYP2C19 in the human liver. *Drug Metab Dispos* **34:**1012–1018.

Komoroski BJ, Zhang S, Cai H, Hutzler JM, Frye R, Tracy TS, Strom SC, Lehmann T, Ang CY, Cui YY, and Venkataramanan R (2004) Induction and inhibition of cytochromes P450 by the St. John's wort constituent hyperforin in human hepatocyte cultures. *Drug Metab Dispos* **32:**512–518.

Krishna DR and Klotz U (1994) Extrahepatic metabolism of drugs in humans. *Clin Pharmacokinet* **26:**144–160.

Li J, Ning G, and Duncan SA (2000) Mammalian hepatocyte differentiation requires the transcription factor HNF-4α. *Genes Dev* **14:**464–474.

Li T and Chiang JY (2006) Rifampicin induction of CYP3A4 requires pregnane X receptor cross talk with hepatocyte nuclear factor 4α and coactivators, and suppression of small heterodimer partner gene expression. *Drug Metab Dispos* **34:**756–764.

Madan A, Graham RA, Carroll KM, Mudra DR, Burton LA, Krueger LA, Downey AD, Czerwinski M, Forster J, Ribadeneira MD, Gan LS, LeCluyse EL, Zech K, Robertson, P Jr, Koch P, Antonian L, Wagner G, Yu L, and Parkinson A (2003) Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. *Drug Metab Dispos* **31:**421–431.

Martínez-Jiménez CP, Castell JV, Gómez-Lechón MJ, and Jover R (2006) Transcriptional activation of CYP2C9, CYP1A1, and CYP1A2 by hepatocyte nuclear factor 4α requires coactivators peroxisomal proliferator activated receptor-γ coactivator 1alpha and steroid receptor coactivator 1. *Mol Pharmacol* **70:**1681–1692.

Niemi M, Backman JT, Neuvonen M, Neuvonen PJ, and Kivistö KT (2001) Effects of rifampin on the pharmacokinetics and pharmacodynamics of glyburide and glipizide. *Clin Pharmacol Ther* **69:**400–406.

Odom DT, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, Volkert TL, Schreiber J, Rolfe PA, Gifford DK, Fraenkel E, Bell GI, and Young RA (2004) Control of pancreas and liver gene expression by HNF transcription factors. *Science* **303:**1378–1381.

Raucy JL, Mueller L, Duan K, Allen SW, Strom S, and Lasker JM (2002) Expression and induction of CYP2C P450 enzymes in primary cultures of human hepatocytes. *J Pharmacol Exp Ther* **302:**475–482.

Stoffel M and Duncan SA (1997) The maturity-onset diabetes of the young (MODY1) transcription factor HNF4α regulates expression of genes required for glucose transport and metabolism. *Proc Natl Acad Sci USA* **94:**13209–13214.

Surapureddi S, Rana R, Reddy JK, and Goldstein JA (2008) Nuclear receptor coactivator 6 mediates the synergistic activation of human cytochrome P-450 2C9 by the constitutive androstane receptor and hepatic nuclear factor-4α. *Mol Pharmacol* **74:**913–923.

Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y, Gonzalez FJ, Schuetz EG, and Kim RB (2003) The orphan nuclear receptor HNF4α determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nat Med* **9:**220–224.

Williamson KM, Patterson JH, McQueen RH, Adams KF Jr, and Pieper JA (1998) Effects of erythromycin or rifampin on losartan pharmacokinetics in healthy volunteers. *Clin Pharmacol Ther* **63:**316–323.

Wiwi CA and Waxman DJ (2004) Role of hepatocyte nuclear factors in growth hormone-regulated, sexually dimorphic expression of liver cytochromes P450. *Growth Factors* **22:**79–88.

Wortham M, Czerwinski M, He L, Parkinson A, and Wan YJ (2007) Expression of constitutive androstane receptor, hepatic nuclear factor 4α, and P450 oxidoreductase genes determines interindividual variability in basal expression and activity of a broad scope of xenobiotic metabolism genes in the human liver. *Drug Metab Dispos* **35:**1700–1710.

Yoshida E, Aratani S, Itou H, Miyagishi M, Takiguchi M, Osumu T, Murakami K, and Fukamizu A (1997) Functional association between CBP and HNF4 in trans-activation. *Biochem Biophys Res Commun* **241:**664–669.

Zhou HH, Anthony LB, Wood AJ, and Wilkinson GR (1990) Induction of polymorphic 4′-hydroxylation of S-mephenytoin by rifampicin. *Br J Clin Pharmacol* **30:**471–475.

Address correspondence to: Dr. Joyce A. Goldstein, Laboratory of Pharmacology, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709. E-mail: goldste1@niehs.nih.gov