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Hepatocyte nuclear factor 4α regulates rifampicin-mediated induction of *CYP2C* genes in primary cultures of human hepatocytes

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Running Title: Regulation of CYP2Cs by HNF4α in primary human hepatocytes

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Abbreviations: CYP, Cytochrome P450; HNFα, Hepatocyte nuclear factor alpha; (siHNF4; HNF4α-small interfering RNA, CAR, Constitutive androstane receptor; RE, Response element; PXR, Pregnane X receptor; EMSA, Electrophoretic mobility shift assay; DMSO, Dimethyl sulfoxide; ANOVA, Analysis of variance; ITS+1, Insulin, human transferrin, sodium selenite, bovine serum albumin and linoleic acid in Earle's Balanced Salt Solution; CITCO, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime; CREB, cAMP response element-binding protein; SRC-1, steroid receptor coactivator-1; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1; CBP, cAMP response element-binding protein binding protein; qPCR, quantitative polymerase chain reaction; apoCIII, apolipoprotein CIII, TBP, TATA-binding protein.

Abstract

CYP2C enzymes are expressed constitutively and comprise ~20% of the total P450 in human liver. However, the factors influencing the transcriptional regulation of the *CYP2C* subfamily have only been recently addressed. In the present study, we used primary cultures of human hepatocytes to investigate the role of HNF4α in the rifampicin-PXR-mediated upregulation of *CYP2C8, CYP2C9* and *CYP2C19* gene expression. We first identified new proximal *cis*-acting HNF4α sites in the proximal *CYP2C8* promoter (at -181 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 identify 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 upregulation of the *CYP2C8* and *CYP2C9* promoters by rifampicin. Furthermore, silencing of HNF4α abolished the transactivation of the *CYP2C8* and *CYP2C9* promoters by rifampicin. Constitutive promoter activity was also decreased. qPCR analysis demonstrating that silencing HNF4α reduced the constitutive expression of CYP2C8 (53%), 2C9 (55%), and 2C19 (43%) mRNAs and significantly decreased the magnitude of the rifampicin-mediated induction of CYP2C8 (6.6 vs 2.7-fold), 2C9 (3 vs 1.5- fold), and 2C19 (1.8 vs 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 upregulation by the PXR agonist rifampicin.

Introduction

The human CYP2C subfamily of cytochrome P450 enzymes consists of four liver enzymes, CYP2C8, CYP2C9, CYP2C18 and CYP2C19, which metabolize ~20% of all clinically prescribed therapeutics 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 (C/EBPα) (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) upregulates 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; Chen et al., 2004; Ferguson et al., 2005). This contributes to inter-individual 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) 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 colinearity 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 CYPs (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*. Chiang and coworkers (Li and Chiang, 2006) concluded that the competition between PXR and HNF4α for their co-activators PGC-1α and SRC-1 contributes 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 CITCO or PXR agonist rifampicin. Also, 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 due to the unavailability of an appropriate *in vitro* cell line. Unlike the *CYP2C9* promoter, the *CYP2C8* promoter is not upregulated by CAR,

PXR, or HNF 4α in HepG2 cells (Ferguson et al., 2005). We recently used cultured primary human hepatocytes to identify a PXR/CAR 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 CITCO (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 HNF 4α 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 EMSA studies. We mutated each of these HNF4α sites to determine their relative importance in the upregulation 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 PXR/rifampicin-mediated promoter transactivation of the *CYP2C* promoters as well as CYP2C mRNA expression.

Methods

Promoter constructs and expression plasmids. The pGL3-Basic constructs of wild-type *CYP2C8* (-8.9 to -8.5)-3kb, *CYP2C9*-3kb 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*-3kb was performed using QuickChange Site-directed mutagenesis (Stratagene, La Jolla, CA). *CYP2C8*-1kb and *CYP2C9*-1.9kb/pdmut constructs were used as templates to generate the *2C8*/152-mut, *2C8*/181-mut, *2C8*/HNF4αdmut, and *2C9*/HNF4αtmut. The forward primers utilized 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**C**AATGGA**ACGAAG-3'

-152 2C8 HNF4 site: 5'-

CTATCCAT**GGGC<u>GT</u>**A**AGTC<u>GT</u>**CTCAGAAAAAAAGTATAAATTG-3'

-181 2C8 HNF4 site: 5'-

GAAGGAGTA**GGAC<u>TT</u>**A**AGAA<u>GT</u>**TTTTATTTCTATCCATGGGC- 3'

DNA sequencing was performed for all constructs to verify the mutation and to assure that no spurious mutations occurred.

Adenovirus constructs and RNA interference. Specific adenoviral constructs were produced by double recombination between a co-transformed adenoviral backbone plasmid (pAdEasy-1) and a linearized shuttle vector (pShuttle-HNF4α or pShuttle-lacZ) using the AdEasyTM XL Adenoviral Vector system (Stratagene, CA). Positive clones were amplified by transformation into XL-10 gold cells according to the manufacturer's directions (Qiagen, Hilden,

Germany). Plasmid DNA was used to transfect human embryonic kidney Ad-293 cells and the virus was harvested, 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 AdEasyTM XL Adenoviral Vector system (Stratagene, CA). siRNA targets for HNF4α were identified using Genscript's target finder. The following sequences were used to silence HNF4α: siHNF4-I @248 bp: 5'–ACATGTACTCCTGCAGATTTA-3'; siHNF4-II @387 bp: 5'- CACTCGAAGGTCAAGCTATGA-3'; siHNF4-III @ 822: 5'-CAATGAGTATGCCTACCTCAA- 3'. The scrambled sequence 5' - GCGCTTCATAATATCTAACGT 3' was used as a negative control. Double stranded shRNA oligos 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 cGFP marker under the control of the cytomegalovirus (CMV) 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 (10mM 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 (GFP) and typically reached 80-90% within 36-48 h.

Cell Culture and Transfections. HepG2 cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin-streptomycin at 37^{o}C under 5% CO_2. Primary human hepatocytes from nine diffferent donors were obtained from CellzDirect (Division of Invitrogen, Carlsbad, CA)

and maintained in William's E medium supplemented with ITS+1 (Sigma), HEPES, L-glutamine, and 100 nM dexamethasone. None of the donors was a current smoker. Transfections were performed on freshly isolated non-overlay human hepatocyte cells with Effectene transfection reagent (QIAGEN) using the manufacturer's procedures. Conditions were optimized for primary human hepatocytes. Briefly, 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 12h, cells were infected with 1000 VP/cell in a serum-free medium each with adenovirus expressing LacZ, HNF4α, scrambled siRNA, or siRNA for HNF4α (siHNF4-I). After 12h, the medium was replaced with complete medium containing appropriate ligands (0.2% DMSO & 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.

Reverse Transcription-PCR. Induction of CYP2Cs was confirmed by employing quantitative RT-PCR (qPCR) for all the transactivation assays performed in this study. RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA) 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) RNase inhibitor, 1X First strand buffer, 10mM dithiothreitol, 0.5 mM dNTPs and 1μl (200 units) of SuperScript II (Invitrogen, Carlesbad, CA) to a total volume of 20 μl. Amplification reactions were performed using Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA) on an Applied Biosystems 7900 HT Sequence Detection System using Taqman probes (ABI) for CYP2C8 (Cat# HS00426387_m1), CYP2C9 (Cat# HS00426397_m1), CYP2C19 (Cat#

HS00426380_m1), CAR (Cat# HS00231959_m1), PXR (Cat# HS01114267_m1), HNF4α (Cat# Hs00230853_m1), PGC-1 (Cat# HS00173304_m1), SRC-1(Cat# HS00186661_m1), RXR (Cat# HS00172565_m1), PBP (Cat# HS00191130_m1) and internal control TBP (Cat# 4333769F). The relative quantity for each sample was normalized to the endogenous control gene (TBP) 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). Briefly, 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, Beverly, MA) was employed 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 hHNF4α 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 DTT, 4% (v/v) glycerol, 50 mM NaCl and 1 μg of non-specific competitor poly (dI-dC) (Sigma, St. Louis, MO).

In competition experiments, specific cold competitors or a specific antibody to human HNF4α (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were added to the mixture before the addition of proteins. After incubating for 20 min at room temperature, 9.5 μl of the reaction mixture was loaded onto a 5% non-denaturing polyacrylamide gel for electrophoresis in 0.5X TBE buffer for 2 hours at 150V. 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**GGACAA**A**AGAACA**TTTT- 3'; *CYP2C8*-181HREMut: 5'-ctagAGTA**GGAC<u>TT</u>**A**AGAA<u>GT</u>**TTTT-3'; *CYP2C9*-211HRE: 5'-

tgtACAG**AGTGGA**C**AATGGA**ACGA-3'; *CYP2C9*-211HREMut: 5'-TGTACAGACACCACATACCAACGAAG 3'; *CYP2C19*-213HRE: 5'-**TGTACAGAGTGGGCACTGGGACGAAG**-3'; APF1 wt: 5'-GCGC**TGGGCA**A**AGGTCA**CCTGC-3'; and APF1Mut: 5'-GCGC**TGGCGA**A**AGGAGA**CCTGC-3'.

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

Statistical Analysis. For comparing activity of luciferase reporter constructs, statistical analysis was performed in SigmaStat version 9.1 (SAS Insitute, Cary, NC) using nonparametric methods.Krushal-Wallis analysis of variance was used to confirm differences in expression across luciferase constructs, For Figs 3-5, 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 wildtype constructs and to compare mRNA expression values.

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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 –181bp upstream of the translation start site for *CYP2C8*. An electrophoretic mobility shift assay (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 (lane 3 & 4, Fig. 1) and by an APF-1-wt(wild-type) oligonucleotide encompassing a known HNF4α binding element of the APOCIII promoter (Jiang and Sladek, 1997) as a positive control (lane 7, Fig. 1). There was no competition by cold competitors containing a mutated -181 HNF4α binding site (lane 5 and 6, Fig. 1) or a mutated APF-1 oligonucleotide (lane 8, Fig. 1). Antibodies against HNF4α decreased the intensity of this complex, and a faint supershifted band appeared at the top of the gel (lane 9, Fig. 1), 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) which 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 (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 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.

The role of the two HNF4α binding sites in HNF4α-transactivation and PXR-rifampicin mediated transactivation of *CYP2C8* in primary cultures of human hepatocytes. Since HNF4α does not activate the *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 HNF4 α and PXR-mediated rifampicin upregulation 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 vs. 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 constuct was decreased by mutation of each of the HNF4α sites (Fig 3A and B) presumably because the effects of constitutive levels of HNF4α in primary hepatocytes was abolished. The wild type reporter construct was further activated by adenoviral constructs

containing exogenous HNF4α (5.6-fold) compared to 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-mediated rifampicin 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) bp 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 actitvity 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, while rifampicin upregulation 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 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 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*-3kb 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

CYP2C9-3kb 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. 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 upregulation of the *CYP2C9* gene by HNF4α.

To confirm our previous studies in HepG2 cells which indicated that that HNF4α has an important role in modulating rifampicin-PXR-mediated transactivation of *CYP2C9* (Chen et al., 2005) we transfected the wild-type *CYP2C9*-3kb 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*-3kb 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 HNF4 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*-3kb promoter was also significantly decreased by mutations of each of the HNF4 sites to a maximum of 35% by the triple mutation, indicating 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 small interfering RNA (siRNA) adenoviral constructs to silence HNF4α expression. Of the three siHNF4α tested, siHNF4-I was most efficacious reducing HNF4α mRNA in four separate lots of primary hepatocytes by 69% ± 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 *2C9* 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 different donors (Table 2) were infected with adenoviruses expressing either siHNF4 or control (scrambled) and treated with 0.2% DMSO or 10 μM rifampicin for 24 h. HNF4 α mRNA was decreased to 31 ± 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 *CYP2C*8 mRNA was down-regulated by ~53%, and the magnitude of induction of CYP2C8 mRNA by rifampicin-mediated was significantly decreased from 6.6-fold 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-fold 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 rifampicin produced little or no increase in CYP2C19 promoter activation in primary hepatocytes we were unable to study the effects of mutating the two HNF4 sites on promoter activity. Silencing HNF4 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 homologue of HNF4α . mRNA for the cofactor PGC-1 was decreased by 50% but SRC-1 mRNA was not affected.

Discussion

We previously reported that PXR/CAR directly regulate the *CYP2C8* and *CYP2C9* gene promoters via PXR/CAR 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 upregulation of CYP2C9 in HepG2 cells (Chen et al., 2005). However, these studies did not address the role of HNF4α in *CYP2C8* regulation, since neither CAR, PXR, or HNF4 upregulate CYP2C8 in cell lines such as HepG2 cells (Ferguson et al., 2005). The present study uses 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 CYPs 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 reviewed previously (Akiyama and Gonzalez, 2003), HNF4α has been reported to bind to direct repeats of AGGTCA separated by one base (DR-1) or to the HPF-1 motif RRNCAAAGKNCANYY. It has been proposed that HNF4α functions by recruiting transcriptional coactivators with histone acetylase activity such as CBP (Yoshida et al., 1997), PGC-1α and SRC-1 (Martinez-Jimenez 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 *2C9* (at -211 bp) proximal promoters and assessed the role of each HNF4α site in HNF4α and rifampicin-mediated upregulation of the *CYP2C8* and *2C9* 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 *CYP2C8* promoter, while mutation of the known site at -152 bp greatly dimished 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 upregulation by HNF4α although mutation of the site at -150 bp had a slightly smaller effect. All three mutations (*2C9*/150-mut, *2C9*/185-mut, *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 which showed that mutation of any of three Smad-binding elements in close proximity to each other on the Luteninizing hormone β-submunit gene promoter dramatically reduces induction by Smad3 cotransfection (Coss et al., 2005). 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 bp 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 suggests that these mutations do not exert a nonspecific effect on basal promoter structure that prevents up regulation of the gene. Since 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 2C9 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 HNF4 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 2C9 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 PXR/CAR 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, while 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 upregulation of *CYP2C* promotor constructs by rifampicin. However, both studies agree that induction of CYP2C8 and CYP2C9 mRNA was not completely abolished by adenovirally expressed HNF4α-siRNA. This 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 HNF4 mRNA varied from 60-81% 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. Though 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 downregulation of CYP2C19 could be secondary to downregulation of receptors such as CAR and PXR. The regulation of CYP2C19 by HNF4α is in agreement with 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 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 si-HNF4α restored PXR /CAR 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 PBP (Peroxisome Proliferator-Activated Receptor Binding Protein) were not affected. However, silencng 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 *CYP2C8* as well as *CYP2C9* in cultured primary human hepatocytes, since mutation of the HNF4α sites of either the CYP2C8 and 2C9 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.

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Footnotes

[1] These two co-authors contributed equally toward this work.

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Figure legends

Figure 1. **Identification of a new HNF4α binding site in the *CYP2C8* promoter region. A.** 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 (5X or 50X) wild-type or mutant cold competitors (CC) were added to the binding reactions for competition analysis. Antibody against HNF4α (last lane) resulted in a supershifted band. S, shifted complex; SS, supershifted band.

Figure 2. **EMSA demonstrates the binding of HNF4α to the new putative HNF4α binding site in the *CYP2C9* promoter region.** 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 (5X or 50 X) wild-type or mutant cold competitors (CC) (*CYP2C9* or APF-1 oligonucleotides containing an HNF4 α site) was added to binding reactions for competition analysis. Antibody against HNF4α resulted in a supershifted band (last lane) with the *CYP2C9* oligomer. S, shifted complex; SS, supershifted band.

Figure 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 in primary human hepatocytes (B). A. Primary cultures of human hepatocytes from donor Hu0861 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 X 10^9 VP/ml of AdHNF4α or LacZ as a

control as described in Methods. After 48h, luciferase activity was measured and normalized to the internal control pRL. Transfections were performed in triplicate and values represent the mean ± 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 mut. 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., while the basal activity of those transfected with lacZ was significantly lower for the 2C8/152-mut and 2C8/HNF4αdmutnt 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 24h. 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 as compared to the vehicle control (*, p< 0.05), while mutation of each of the HNF4α sites significantly decreased constitutive activitiy (†, p< 0.05) or the induction by rifampicin (#, p<0.05).

Figure 4. Effect of mutation of different HNF4α binding sites on *CYP2C9* promoter transactivation by HNF4α and rifampicin in primary human hepatocytes. A. Primary cultures of human hepatocytes from donor Hu1125 were transfected with the wild-type *CYP2C9*-3kb 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 48h later. All transfections were performed in quadruplicate and values represent the mean ± S.E. of the fold activation relative to that of the *CYP2C9*-3kb promoter infected with LacZ. AdHNF4α significantly enhanced the activation of wild-type *CYP2C9* and single HNF4α mutants (*, <0.05), but not the triple mutation. Luciferase activity of the *CYP2C9* mutants transfected with LacZ (†, <0.05) or adenoviral HNF4α were significantly lower than that of the wild-type *CYP2C9* reporter construct (#, <0.05). **B**. Effect of mutation of the HNF4α binding sites on transactivation of the *CYP2C9* promoter by rifampicin. Cultures of primary human hepatocytes were transfected with the *CYP2C9*-3kb promoter or its HNF4α mutants followed by treatment with 10 μM rifampicin for 24h. 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 to the vehicle control (*, <0.05). However, activation of the CYP2C9 HNF4α promoter mutants by rifampicin was significantly lower than wild-type (#, <0.05;) and the triple mutation abolished rifampicin activation. Constitutive activity of the mutants in the absense of rifampicin was significantly lower than that of wild type with p values of (†, <0.05)

Figure 5. Silencing HNF4*α* decreases basal *CYP2C8* and *CYP2C9* promoter activity and essentially abolishes rifampicin-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)-3kb promoter reporter construct. After 12h, cells were infected with siHNF4-I or scrambled adenovirus for 1.5h and then incubated at 37^{o}C for 24h. Transfected cells were then treated with 10 μM rifampicin or 0.2% DMSO for 24h. Transfections were performed

in triplicate and values represent the means ± S.E in two donors (Hu0747 and Hu0808). Treatment with rifampicin resulted in a two-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 downregulation 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*-3kb reporter construct. All transfections were performed in triplicate samples from donors Hu0798 and Hu0813, and values represent the means ± S.E. Treatment with rifampicin resulted in a significant 2-fold increase in wild type *CYP2C9* promoter reporter activity as compared to DMSO treatment and a small but significant increase after silencing HNF4α ($p<0.01$, $p< 0.001$). Silencing HNF4α resulted in significant downregulation 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 1. Donor information for the nine human hepatocyte donors.

N L	ot#	overlay/Non-overlay	Sex	Age	Race	Smoking	Alcohol use	Obese
1 Hu	-0694	overlay	F	54	C	No	No	-
2 Hu	-0714	Non-overlay	M	68	C	No	No	-
3 Hu	-0747	Non-overlay	F	64	C	No	Social	-
4 Hu	-0798	Non-overlay	F	56	C	No	Rare	-
5	Hu-0808	Both	F	43	C	Quit in 1996	2-3 units/other day	-
6	Hu-0813	Both	M	63	C	Quit in 1986	1-2 beer/day	-
7 Hu	-0861	Non-overlay	F	61	AA	No	No	-
8 Hu	-0999	Non-overlay	F	35	C	No	No	Yes
9	Hu-1125	Non-overlay	M	56	C	Quit in 1989	Once per week	Yes

Table 2. Effects of rifampicin SiHNF4 vs SCR siRNA and Rif on CYP2C8, CYP2C9 and CYP2C19 mRNA in primary human hepatocytes infected with scrambled siRNA or SiHNF4-I.

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

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

[a] Values from cells treated with siHNF4-I significantly lower than those treated with SCR siRNa for either DMSO or rifampicin controls, $p < 0.05$;

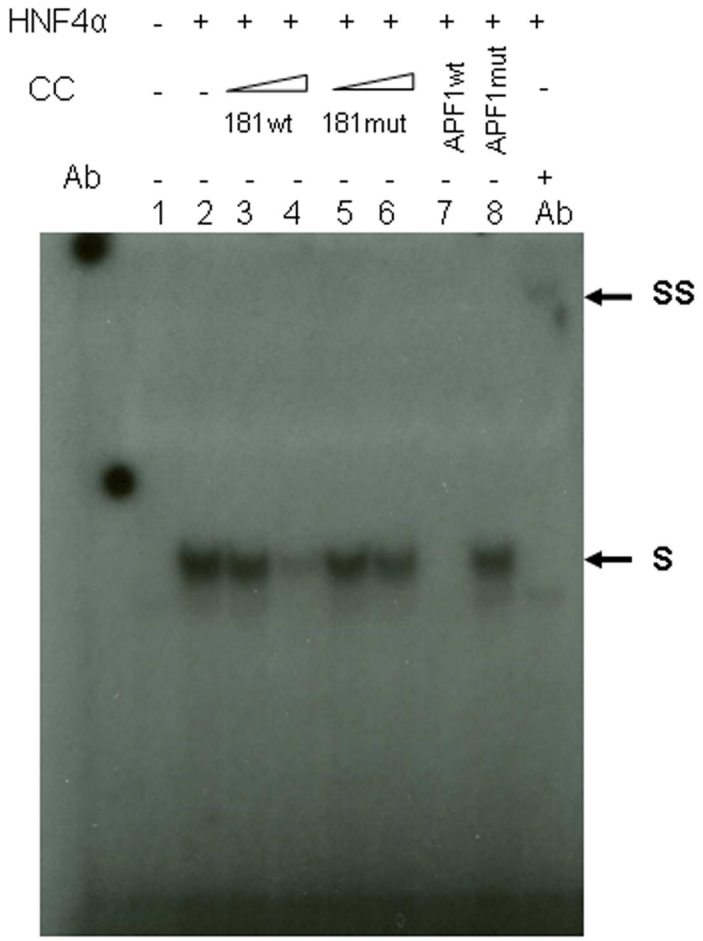
[b] Values from cells treated with rifampicin significantly higher than those treated with DMSO, $p < 0.05$ for cells infected with scrambled siRNA or or siHNF4-I.

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(SCR) siRNA (controls) or siHNF4-I.

Fold-changes	HNF4α	2C8	2C9	2C19
Rif:Control	1.06 ± 0.11(NS)	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

Primary human hepatocytes from 4 different donors were treated with control (SCR) or siHNF4-I in the presence or absence of rifampicin in triplicates and analyzed for mRNA expression of CYP2C8, CYP2C9, CYP2C19, and HNF4α. Data values represent Mean ± S.D.
Fold change significantly greater than 1.00, [a] $p<0.05$, [b] $p<0.01$(NS= not significantly different. [c]Fold induction for Rifampicin(Rif):control significantly greater than fold induction for Rif:si:HNF4 vs siHNF4, $p<0.01$ in all cases.

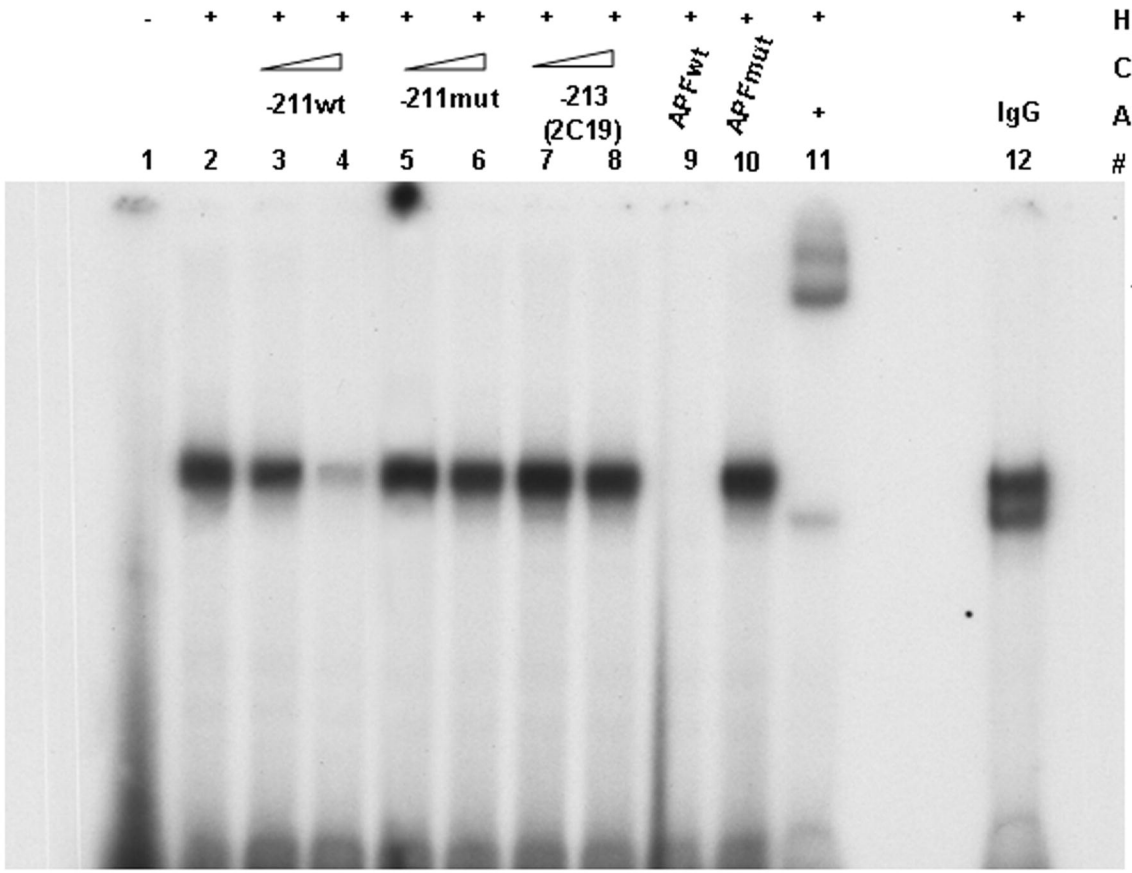
Figure 1



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

Figure 2



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

Figure 3

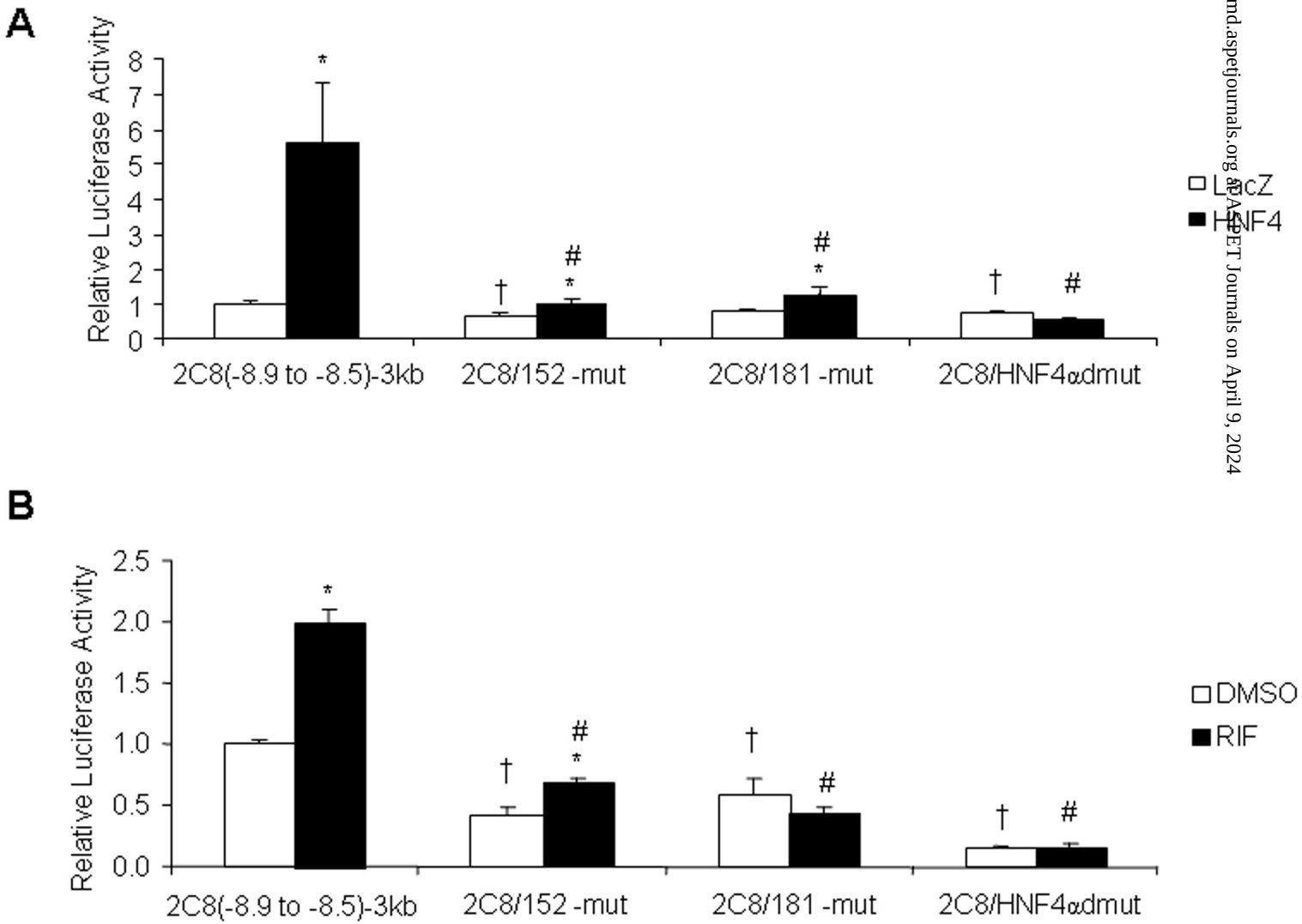


Figure 4

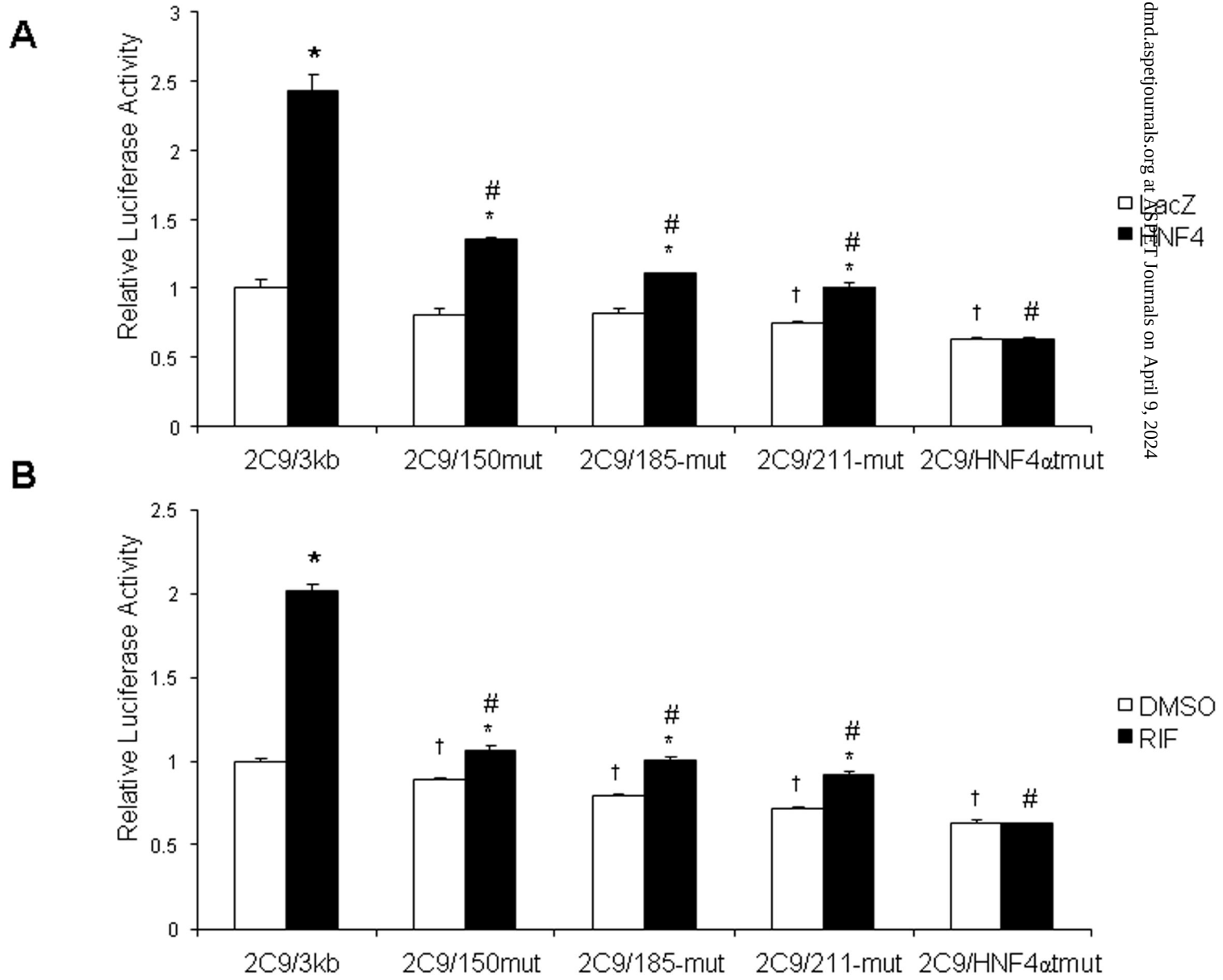


Figure 5