Positive and Negative Regulation of Human Hepatic Hydroxysteroid Sulfotransferase (SULT2A1) Gene Transcription by Rifampicin: Roles of Hepatocyte Nuclear Factor 4α and Pregnan X Receptor[S]

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Received April 19, 2007; accepted August 7, 2007

ABSTRACT

The effects of rifampicin treatment on SULT2A1 mRNA expression were evaluated in 23 preparations of primary cultured human hepatocytes. In contrast to the consistently occurring induction of CYP3A4, a prototypical pregnane X receptor (PXR) target gene, rifampicin treatment increased SULT2A1 mRNA levels in 12 of the hepatocyte preparations, but it produced little change or even suppression in the others. Transient transfection of HepG2 cells with a series of reporter constructs implicated two SULT2A1 5′-flanking regions as containing rifampicin-responsive information. Each of these regions contained a hepatocyte nuclear factor 4α (HNF4) binding site (at nucleotide [nt] −6160 and −54), as demonstrated by in vitro binding and site-directed mutagenesis. HNF4α bound to the HNF4−54 region of the endogenous SULT2A1 gene, as indicated by chromatin immunoprecipitation. Cotransfection of HepG2 cells with pregnane X receptor (PXR) dose-dependently suppressed reporter expression from SULT2A1 constructs containing the HNF4 sites, and rifampicin treatment augmented the suppression. Rifampicin treatment concentration-dependently suppressed SULT2A1 reporter expression at the same concentrations that progressively induced expression from a PXR-responsive CYP3A4 reporter, whereas higher rifampicin concentrations reversed the SULT2A1 suppression. The suppressive effect of rifampicin was diminished, whereas the activating effect was augmented, in HepG2 cells with RNA interference-mediated PXR knockdown. These results suggest that HNF4α plays a central role in the control of SULT2A1 transcription and that rifampicin-ligated PXR suppresses SULT2A1 expression by interfering with HNF4α activity. By contrast, the rifampicin-inducible SULT2A1 expression that occurs in many human hepatocyte preparations seems to be mediated through a PXR-independent mechanism.

As a major detoxicating enzyme in human liver, hydroxysteroid sulfotransferase (SULT2A1) is well poised to catalyze the sulfonation of biologically important endogenous and xenobiotic substrates, such as the androgen precursor hormone dehydroepiandrosterone, bile acids, and hydroxymethyl polycyclic aromatic hydrocarbon procarcinogens (Radominska et al., 1990; Falany et al., 1995). Recombinant human SULT2A1 also sulfonates the endocrine-disrupting agent t-octylphenol (Nishiyama et al., 2002), and it exhibits a low apparent Km toward triiodothyronine, the most potent form of thyroid hormone (Li and Anderson, 1999). These findings suggest that human SULT2A1 plays a role in the metabolism of environmental endocrine disruptors and also figures prom...
inently as a key factor in thyroid hormone metabolism. Because of its important role in hepatic metabolism, gaining a clearer picture of the molecular dynamics that regulate human hepatic SULT2A1 gene transcription has implications for understanding xenobiotic and hormone metabolism in humans.

In mice and rats, hepatic SULT2A transcription is transactivated by the PXR, in concert with its heterodimeric partner, RXR (Xu and Kong, 2005). The PXR-RXR transcription factor transactivates rodent SULT2A transcription by binding to an IR0 nuclear receptor motif (inverted repeat of A/GG/G/T/ACA with 0 intervening bases) in the 5'-flanking region of the gene (Runge-Morris et al., 1999; Sonoda et al., 2002). The regulation of rodent hepatic SULT2A by PXR plays an essential role in bile acid metabolism. Cholestatic liver disease is characterized by the accumulation of toxic hydrophobic secondary bile acids such as lithocholic acid (Sonoda et al., 2002). The activation of PXR by lithocholic acid transactivates murine hepatic SULT2A expression and protects the liver from the damaging effects of lithocholic acid retention (Sonoda et al., 2002; Kitada et al., 2003). PXR also controls the transcription of hepatic transporters that are required for the recognition and transport of sulfonated conjugates in the liver (Sonoda et al., 2002; Xu and Kong, 2005). As a wide spectrum “xenosensor,” PXR, along with other nuclear receptors, coordinates the transcriptional control of the xenobiotic-metabolizing and transporter networks in the liver that are essential for the detoxification of both endogenous and xenobiotic substrates (Mohan and Heyman, 2003; Klaassen and Slitt, 2005; Runge-Morris and Kocarek, 2005).

Our previous investigations in primary cultured rat hepatocytes suggested a dual role for PXR- and GR-mediated mechanisms in the transcriptional regulation of rat hepatic SULT2A expression (Runge-Morris et al., 1999). Pharmacological concentrations of dexamethasone were demonstrated to induce rat hepatic SULT2A expression via a PXR-mediated mechanism, whereas treatment with a GR-activating concentration of glucocorticoid transactivated murine SULT2A transcriptions indirectly, through intermediary steps involving GR-inducible C/EBP (Fang et al., 2005a).

In contrast to rodent systems, the role of PXR and other nuclear receptors in the transcriptional regulation of human SULT2A1 is just emerging. Activation of CAR, a mediator of phenobarbital-inducible gene expression, was shown to induce the expression of murine hepatic SULT2A, as well as of the Mrp4 bile acid transporter (Assem et al., 2004). In a microarray study, phenobarbital treatment of primary cultured human hepatocytes increased the expression of SULT2A1 mRNA, implicating a possible role for CAR in the regulation of human SULT2A1 (Assem et al., 2004).

Our recent studies indicate that important species differences pertain to the regulation of hepatic SULT2B2 by members of the nuclear receptor superfamily. Primary hepatocyte culture studies suggest that human, but not rat, hepatic SULT2A2 is transactivated by PPARα, acting through a peroxisome proliferator response element in the distal 5'-flanking region of the SULT2A1 gene (Fang et al., 2005b). Initial studies also suggested that PXR-activating concentrations of the potent glucocorticoid, dexamethasone, or of the prototypical human PXR ligand rifampicin are capable of inducing SULT2A1 gene expression (Duanmu et al., 2002; Fang et al., 2005b). The current study was undertaken to investigate the impact of PXR activation on human hepatic SULT2A1 expression and to identify the cis-acting sequences in the 5'-flanking region of the SULT2A1 gene that are essential for the modulation of human hepatic SULT2A1 transcription by PXR. Our investigation indicates that human hepatic SULT2A1 expression is subject to a balance of positively and negatively acting mechanisms. HNF4α is identified as a major positive regulator of human hepatic SULT2A1 transcription, and ligand-activated PXR is capable of suppressing HNF4α function. Data are also provided suggesting that the positive effects of rifampicin treatment on SULT2A1 transcription are mediated through a PXR-independent mechanism.

**Materials and Methods**

**Materials.** Rifampicin was purchased from Sigma-Aldrich (St. Louis, MO). Other supplies and reagents were purchased from the sources described previously (Fang et al., 2005a,b) or that are described below.

**Primary Cultured Human Hepatocytes.** Plated primary cultures of human hepatocytes were obtained from the Liver Tissue Procurement and Distribution System of the University of Minnesota (Minneapolis, MN), in collaboration with Dr. Stephen Strom (University of Pittsburgh, Pittsburgh, PA). Following hepatocyte preparation and overnight culture, the hepatocytes, in T25 flasks, were express shipped to our facility. Upon receipt, medium was replaced with our standard human hepatocyte medium, consisting of Williams’ medium E supplemented with 0.25 U/ml insulin, 0.1 μM triamcinolone acetonide, 50 μg/ml gentamicin, and 2.5 μg/ml amphotericin B, and the cells were maintained at 37°C under a humidified atmosphere of 95% air, 5% CO2. The following day, medium was replaced with standard medium containing 200 μg/ml Matrigel (BD Biosciences, San Jose, CA). The following day, the medium was replaced with standard medium containing 0.1% DMSO or 10 or 50 μM rifampicin (in DMSO). The cultures were either harvested after 24-h incubation, or they were retreated at 24 h and harvested after a total of 48-h incubation.

**TaqMan Real-Time Reverse Transcription-Polymerase Chain Reaction and Enzyme Activity Analyses.** Total RNA was prepared from individual T25 flasks of primary cultured human hepatocytes, by using the TaqMAY RNA kit (Ambion, The Woodlands, TX). Samples of total RNA were reverse transcribed, using either Superscript II (Invitrogen, Carlsbad, CA) or the Omniscript RT kit (Qiagen, Valencia, CA), according to the manufacturers’ instructions. The levels of SULT2A1 and CYP3A4 were measured, using TaqMan Gene Expression Assays Hs00430021_m1 and Hs00430021_m1, respectively (Applied Biosystems, Foster City, CA). Each PCR reaction included cDNA template, a primer/probe (5-carboxyfluorescein fluor, minor groove binder quencher), a primer-limited primer/probe (VIC-minor groove binder) set for 18S rRNA and Universal PCR Master Mix (Applied Biosystems), and amplifications were performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). Standard thermocycling parameters were 94°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Cycle threshold values were obtained using the SDS software package (Applied Biosystems). For each RNA sample, the target mRNA level was normalized to the 18S rRNA level. These normalized data were then expressed relative to the DMSO control group. The Wilcoxon sign-ranked test was used to compare median -fold changes against the hypothetical value of 1.0 (GraphPad Prism, version 5.0; GraphPad Software Inc., San Diego, CA). Correlation analysis was performed, using the nonparametric Spearman test (Prism).

For measurement of SULT2A1 activity, human hepatocyte monolayers (3 T25s per treatment group) were washed with ice-cold phos-
phate-buffered saline, scraped (replicates pooled) into Matrisperse (Collaborative Research, Bedford, MA), mixed end-over-end at 4°C for ~30 min to remove traces of Matrigel, and centrifuged. The cell pellets were homogenized by sonication in buffer (50 μl per flask of hepatocytes) consisting of 10 mM potassium phosphate, 0.15 M potassium chloride, 1 mM dithiothreitol, and 10% glycerol, pH 7.4. Homogenates were centrifuged at 20,000g at 4°C for 20 min, and supernatants were centrifuged at 105,000g at 4°C for 1 h. SULT2A1 enzyme activities were measured in the cytosolic fractions, as described previously (Fang et al., 2005b).

**Preparation of SULT2A1 Reporter Plasmids.** A luciferase reporter plasmid containing nt −492 to +48 (hereafter designated −492:48) of the SULT2A1 5′-flanking region has been described previously (Fang et al., 2005b). Deletion constructs containing nt −225:48, −191:48, −165:48, −141:48, and −107:48 of the SULT2A1 5′-flanking region were amplified by PCR, using −492:48 as template and the primers shown in Supplemental Table 1. These amplified fragments were ligated into the MluI and XhoI sites of pGL3-Basic (Promega, Madison, WI). Reporter constructs containing a series of upstream fragments (−1000 nt each) of the SULT2A1 5′-flanking region, extending to −8.8 kb upstream from the transcription start site, in tandem with the −492:48 promoter region, have been described previously (Fang et al., 2005b). To prepare reporter constructs containing this series of upstream fragments in tandem with the −107:48 promoter region, the upstream fragments were removed from the original constructs with KpnI and MluI and ligated into the corresponding sites of the −107:48 construct. In the same manner, fragments corresponding to SULT2A1 nt −6248:−5812, −6832:−6225, −6044:−5813, and −6248:−6025 were prepared by PCR and ligated into the MluI and KpnI sites of −107:48.

**Site-Directed Mutagenesis.** Candidate transcription factor binding sites were identified using MatInspector (Quandt et al., 1995). Mutations were introduced into core regions of predicted transcription factor binding sites using the QuikChange II XL site-directed mutagenesis kit, according to the manufacturer’s instructions (Stratagene, La Jolla, CA). The targeted sites and mutagenic primer pairs are shown in Supplemental Table 1. The sequences of all constructs were verified using the resources of the Applied Genomics Technology Center at Wayne State University (Detroit, MI).

**Transient Transfection Analysis.** Approximately 250,000 HepG2 cells were seeded into the wells of 12-well plates and cultured in 2 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% charcoal-stripped fetal bovine serum (Invitrogen), at 37°C under a humidified atmosphere of 95% air, 5% CO2. The following day, culture medium was replaced with 0.6 ml of Opti-MEM (Invitrogen) and plasmid DNA consisting of selected combinations of pRL-SV40 (to normalize for differences in transfection efficiency; Promega; and sufficient pBluescript II KS+ (Collaborative Research, Memphis, TN); 1.25 μg of pCMV-HNF4α (Stratagene) to provide 50,000 cpm of a labeled probe for 20 min at room temperature in buffer containing 12 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, and 1 μg of poly(dI-dC), in a total volume of 15 μl. The DNA-protein complexes were separated on native 4% polyacrylamide gels in 0.5× TBE running buffer, at 30 mA, for 1.5 h. For competition analysis, incubations included various amounts of unlabeled double-stranded oligonucleotides. For supershift analysis, 1 μl of an antibody directed against HNF4α (H-171), PXR (H-160), or RXR (D-20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the binding reaction.

**Chromatin Immunoprecipitation.** ChIP analysis was performed using the ChIP assay kit, according to the manufacturer’s instructions (Upstate Biotechnology, Chicago, IL). HepG2 cells were grown in 100-mm dishes to ~70 to 80% confluence in DMEM containing 10% charcoal-stripped fetal bovine serum. Some of the cultures were then transfected with 0.8 μg of pS5G-PXR, alone or in combination with 0.8 μg of pCMV-HNF4α, using Lipofectin Reagent (the total amount of DNA per transfection was adjusted to 5 μg by the addition of pBluescript II KS+). The following day, the cultures were incubated with either Opti-MEM alone or containing either 0.1% DMSO or rifampicin (at the concentrations indicated in the individual figure legends), and they were harvested 24 h later for the measurement of firefly and Renilla luciferase activities by using the Dual Luciferase Reporter Assay System (Promega) and a Dynex model MLX Luminometer. Data were statistically analyzed using one-way analysis of variance followed by either the Newman-Keuls multiple comparison test or Dunnett’s test (Prism).
Results

In a previous report, we observed an ~2-fold increase in SULT2A1 mRNA content when primary cultured human hepatocytes were treated with rifampicin, a prototypical ligand of the human PXR (Fang et al., 2005b). The reproducibility and concentration dependence of this observation were initially evaluated in three different preparations of human hepatocytes that were treated with 10 or 50 μM rifampicin (Fig. 1A). The effects of rifampicin treatment on SULT2A1 mRNA expression were compared with those on CYP3A4 expression, a well validated PXR target gene. Treatment with 50 μM rifampicin increased SULT2A1 mRNA content in each of the hepatocyte preparations, from ~1.8 to 4.1-fold relative to DMSO-treated controls, while increasing CYP3A4 mRNA levels from ~8.8 to 62-fold. The concentration-induction relationships for these two genes were somewhat different, in that the magnitude of SULT2A1 mRNA induction was always greater at 50 μM rifampicin than it was at 10 μM. By comparison, in two of the three hepatocyte preparations, CYP3A4 mRNA induction was maximal at 10 μM rifampicin.

To compare the effects of rifampicin treatment on SULT2A1 and CYP3A4 expression further, transcript levels were measured in 20 additional preparations of primary cultured human hepatocytes (Fig. 1B). Because our initial findings indicated that CYP3A4 mRNA induction was sometimes higher after treatment with 50 μM rifampicin than it was after treatment with 10 μM rifampicin, the additional cultures were treated with DMSO or 50 μM rifampicin. The data from these 20 preparations and the three preparations shown in Fig. 1A are shown sorted according to -fold induction of CYP3A4 mRNA, and they demonstrate that rifampicin treatment produced multifold (from 7.2 to 436-fold) increases in CYP3A4 mRNA, and they demonstrate that rifampicin treatment produced multifold (from 7.2 to 436-fold) increases in CYP3A4 mRNA content in all but one hepatocyte preparation and that it produced an average increase of ~1.7-fold in SULT2A1 mRNA content in the hepatocyte preparations. Although the SULT2A1 mRNA -fold increase was statistically significant, rifampicin treatment produced SULT2A1 mRNA induction of at least 1.5-fold in only 12 of the hepatocyte preparations, but it produced little change or even suppression in the other preparations. The rifampicin-mediated changes in CYP3A4 and SULT2A1 mRNA levels did not correlate (r = 0.0287; p = 0.897). The divergence of the effects of rifampicin on CYP3A4 and SULT2A1 expression is clearly illustrated by the two preparations exhibiting the greatest magnitudes of CYP3A4 induction (preparations 22 and 23), which also showed either one of the highest levels of SULT2A1 induction (preparation 22) or the greatest suppression (preparation 23). These results demonstrate that although CYP3A4 and SULT2A1 are both regulated by rifampicin treatment, the patterns of regulation are not the same, with SULT2A1 expression exhibiting induction, suppression, or little net change, depending on the hepatocyte preparation. The rifampicin-mediated changes in SULT2A1 mRNA expression did not significantly correlate with the basal levels of SULT2A1 mRNA that were present in the various hepatocyte preparations (r = 0.372; p = 0.0809).

The effects of rifampicin treatment on SULT2A1 enzymatic activity were also determined in one of the hepatocyte preparations. Activities of 1.14 and 2.28 pmol of dehydroepiandrosterone sulfate formed per minute per milligram of protein were measured in cytosols prepared from cultures treated with DMSO or 50 μM rifampicin, respectively. This rifampicin-mediated -fold increase in SULT2A1 enzymatic activity (2.0-fold) corresponded closely to the mRNA change that was measured in this hepatocyte preparation (1.7-fold).

To investigate possible mechanisms underlying the complex regulation of SULT2A1 expression by rifampicin, we performed a series of transient transfection experiments us-
ing reporter constructs containing various amounts of the 5’-flanking region of the SULT2A1 gene. HepG2 cells were used for these studies, because this human liver cell model was previously found to be useful for investigating SULT2A1 gene regulation by peroxisome proliferators (Fang et al., 2005b). In preliminary experiments, we evaluated rifampicin treatment effects on luciferase reporter expression from the series of constructs that was used in our earlier study (Fang et al., 2005b), which contained progressively upstream ~1000-nt regions of the SULT2A1 5’ gene (to ~8.8 kb from the transcription start site) linked to the first 492 nt of the SULT2A1 promoter, and we found that rifampicin treatment increased reporter expression from several of these constructs (data not shown). We therefore considered the possibility that information essential for mediating rifampicin-inducible SULT2A1 transcription might be contained within the first 492 nt of the SULT2A1 5’-flanking region that constituted the common SULT2A1 “core promoter” for the aforementioned reporter series (Fang et al., 2005b). This series of transfection experiments was performed using a reporter construct containing 492 nt of 5’-flanking sequence (SULT2A1 reporters are hereafter referred to by the 5’ and 3’ positions of the fragment, e.g., −492:48) and several deletion constructs (−225:48, −191:48, −165:48, −141:48, and −107:48), and it was done in HepG2 cells that either contained only the endogenous amount of PXR or that were supplemented by cotransfection of a human PXR expression vector (Fig. 2A). In control HepG2 cells (i.e., containing endogenous PXR and treated with DMSO), reporter expression was decreased significantly (by ~15%) in construct −225:48 compared with −492:48, suggesting that one or more cis-acting elements contained in the −492 to −225 region contributed to basal promoter activity. For example, several computer-predicted binding sites for liver-enriched transcription factors, HNF1, HNF4, HNF3B, and C/EBP are contained within this region. A significant decrease in promoter activity was also observed in construct −191:48 compared with −225:48 (49% decrease), and then in construct −141:48 compared with −165:48 (61% decrease). No further decrease in promoter activity was observed when the 5’-flanking region was shortened to 107 nt. A computer-predicted C/EBP binding site was present within the −165 to −141 region (CEBP-146), and mutation of this site within the context of construct −225:48 reduced promoter activity to the level seen for construct −107:48 (Fig. 2B), indicating a functional role for this C/EBP site, which is consistent with a recent report (Song et al., 2006).

Rifampicin treatment of HepG2 cells transfected with construct −492:48 produced a significant increase of 1.5-fold in reporter expression, relative to the respective DMSO-treated control (Fig. 2A). Significant rifampicin-mediated increases of 1.3- to 1.4-fold in promoter activity were also observed for constructs −225:48, −191:48, and −165:48, and increases of

![Fig. 2](https://jpet.aspetjournals.org/)

**Fig. 2.** Transient transfection analysis of the proximal SULT2A1 promoter for regions controlling basal activity and PXR and rifampicin responsiveness in HepG2 cells. A, HepG2 cells were transiently transfected with each of the indicated reporter plasmids together with either pSG5 (−PXR) or pSG5-PXR (+PXR), as described under Materials and Methods. Transfected cells were treated for 24 h with 0.1% DMSO or 50 μM rifampicin (Rif), and they were harvested for measurement of luciferase activities. For comparison, effects on expression from a PXR-responsive luciferase reporter construct (XREM-CYP3A4-Luc) are shown. Each bar represents the mean ± S.D. of normalized (firefly/Renilla) luciferase measurements (three wells per treatment group). *, **, and *** represent significantly different from the previous deletion construct (e.g., −225:48 versus −492:48, −191:48 versus −225:48, etc.) treated in the same manner. #, ##, and ### represent significantly different from the corresponding DMSO-treated group. The numbers adjacent to the # symbols indicate the Rif-induced -fold changes. +, ++, and ++++, significantly different from the corresponding pSG5-transfected group. Approximate positions of several computer-predicted liver-enriched transcription factor binding sites, including a C/EBP site at nt −146 and an HNF4 site at nt −54 relative to the transcription start site, are indicated. B, HepG2 cells were transfected with SULT2A1 luciferase reporter plasmid −107:48 or −224:48, or with −224:48(mutCEBP-146), in which the C/EBP-binding site at nt −146 was mutated. ***, Significantly different from the −107:48 construct treated in the same manner. For statistical symbols, one, two and three copies indicate p < 0.05, p < 0.01, and p < 0.001, respectively.
1.3-fold were still maintained for constructs −141:48 and −107:48, suggesting that the essential information for achieving rifampicin-mediated inducibility may be contained within the first 107 nt of the SULT2A1 promoter, and that other elements within the 492 region may function largely to amplify SULT2A1 expression levels. The −191 to −165 region of SULT2A1 contains an IR2 (inverted repeat with two intervening bases) motif that has recently been implicated as a vitamin D-response element (Song et al., 2006). There was no significant difference in the basal or rifampicin-inducible promoter activities obtained with constructs −191:48 and −165:48, suggesting that the IR2 motif does not play a functional role in rifampicin-mediated SULT2A1 promoter activation (Fig. 2A).

To evaluate the role of PXR in rifampicin-regulated SULT2A1 transcription, HepG2 cells were transiently transfected with human PXR (Fig. 2A). As a positive control for these experiments, reporter expression from the PXR-responsive XREM-CYP3A4-Luc construct was evaluated. Reporter expression from XREM-CYP3A4-Luc was increased 4.9-fold by rifampicin treatment in HepG2 cells containing only endogenous PXR, whereas in PXR-transfected cells, both basal and rifampicin-inducible reporter expression were increased, and the rifampicin/control -fold response was ~7.3-fold. By comparison, PXR transfection suppressed the promoter activities of the SULT2A1 constructs, relative to the respective activities that were measured in HepG2 cells containing only endogenous PXR. Moreover, unlike the positive effects of PXR supplementation on rifampicin-inducible expression from XREM-CYP3A4-Luc, PXR transfection decreased, or even abolished, the rifampicin-mediated increases in SULT2A1 promoter activity (Fig. 2A).

To evaluate the SULT2A1 5′-flanking region for additional upstream sequences that might contribute substantially to rifampicin-mediated SULT2A1 regulation, constructs containing the aforementioned −1000-nt regions were transiently transfected into HepG2 cells (Fig. 3A). For these experiments, the upstream fragments were re-engineered into reporter constructs containing the −107:48 fragment as core promoter, since the above-described studies suggested that −107:48 region was not only sufficient to confer rifampicin-mediated regulation but also demonstrated that the magnitude of expression from the −107:48 construct was relatively low, thereby facilitating our ability to detect upstream sequences with enhancer activity. Although most of the upstream regions provided little enhancement to SULT2A1 promoter activity, the presence of the −6832:−5812 region markedly increased basal promoter activities while maintaining −1.4-fold rifampicin-mediated induction. When the −6832:−5812 region was divided, all activity was contained within the downstream portion (−6248:−5812), and when this latter region was subdivided, the rifampicin-inducible activity was contained within the −6248:−6025 fragment (Fig. 3B).

Computational analysis of the responsive −6248:−6025 fragment failed to identify a consensus PXR-RXR-binding site (i.e., two PXR half sites arranged as a repeat), but it did indicate the presence of an HNF4 binding site at nt −6160 (Fig. 4A). To evaluate the ability of HNF4α to bind to this site, EMSA was performed using in vitro transcribed/translated HNF4α. As shown in Fig. 4B, HNF4α bound to both a consensus HNF4-RE probe and a probe containing the SULT2A1 HNF4-6160 site. Binding to both probes was quantitatively supershifted by the addition of an HNF4α antibody, and binding to the HNF4-6160 probe was reduced by the addition of either unlabeled HNF4-RE or HNF4-6160 competitor, but less effectively by HNF4-6160 containing a mutated HNF4 core sequence (Fig. 4B). Not surprisingly, the computational analysis also indicated the colocalization of a PXR/CAR half site with the HNF4-6160 site (Fig. 4A). Therefore, to consider the possibility that PXR may also bind to this site, EMSA was performed using in vitro transcribed/translated PXR and RXR. Figure 4C shows that PXR-RXR bound specifically to a consensus PXRE probe, as demonstrated by competitor and antibody incubations, but it showed only slight binding to the HNF4-6160 probe.

Computational analysis of the −107:48 region also suggested the possible presence of an HNF4α binding site at −54 (Fig. 5A), although this site was only identified when the search stringency was reduced somewhat from optimal (i.e., from matrix similarity > optimized matrix threshold to matrix similarity > optimized matrix threshold − 0.04). The
analysis also identified this site as a candidate PPARα binding site (Fig. 5A). As described above, EMSA was used to evaluate the ability of in vitro transcribed/translated HNF4α to bind to the HNF4-54 site (Fig. 5B), and it indicated that HNF4α bound specifically to both the consensus HNF4-RE probe and the HNF4-54 probe, with both bands completely supershifted by HNF4α antibody. Competition analysis revealed no difference in the abilities of unlabeled consensus HNF4-RE and HNF4-54 site to reduce the intensity of the HNF4-shifted band, whereas HNF4-54 competitor containing a mutated HNF4 core sequence was markedly less effective. EMSA was also used to evaluate the abilities of PPARα-RXR and PXR-RXR to bind to the HNF4-54 site (Fig. 5C), and it indicated that although both heterodimers were able to bind to probes containing their respective consensus motifs, PPARα-RXR, but not PXR-RXR, was able to bind to HNF4-54. ChIP analysis confirmed the ability of HNF4α to interact with the endogenous SULT2A1 gene in the region containing the HNF4-54 site (Fig. 6, top left, PCR product −164 to +66), but not a region predicted to be devoid of any nuclear receptor motifs (Fig. 6, top right, PCR product −11,748 to −11,605). HNF4α association with the HNF4-54 region was detected under several conditions of treatment (i.e., DMSO or 50 μM rifampicin) and transfection (i.e., PXR and HNF4α).

To evaluate the functional significance of the HNF4-54 and HNF-6160 sites in the regulation of SULT2A1 transcription, transient transfection experiments were performed using various SULT2A1 reporter constructs containing either intact or mutated HNF4-54 or HNF4-6160 sites (Fig. 7). In the −107:48 construct, containing only the HNF4-54 site, mutation of that site reduced reporter expression by ~80% (Fig. 7A). The simple linkage of the HNF4-6160 site to the −107:48 fragment produced a construct with ~2.4 times greater promoter activity than the −107:48 construct, and mutation of HNF4-54 in this construct again markedly suppressed reporter expression (Fig. 7A). When the two HNF4 sites were evaluated within the context of a longer SULT2A1 reporter construct, specifically the (−6832:−5812)(−107:48) construct that displayed the greatest activity in our earlier experiment (Fig. 3A), mutation of HNF4-54 caused a significant reduction in promoter activity, whereas mutation of HNF4-6160 had no significant effect, and the double mutation of HNF4-54 and HNF4-6160 did not cause any greater reduction than occurred following mutation of HNF-54 alone (Fig. 7A). Also in this experiment, the two (−6832:−5812)(−107:48) constructs containing an intact HNF-54 site showed significant increases in reporter expression following rifampicin treatment, whereas those containing a mutated HNF-54 site were not significantly increased by rifampicin (Fig. 7A). When the HNF4-6160 site was mutated within the context of the smaller −6248:−6025 fragment, which retained substantial constitutive and rifampicin-inducible activity (Fig. 3B), a significant reduction of promoter activity occurred that was further reduced when the
a mutated core sequence (54m). The indicated samples also contained in vitro transcribed/translated HNF4, PPAR reactions were supplemented with an antibody directed against HNF4 oligonucleotide corresponding to the consensus HNF4-RE (HNF4), 54 or 54 with mutated HNF4 core sequence (54m). For supershift experiments, transcribed/translated pcDNA3.1 empty vector (V). For competition experiments, reactions also contained the indicated amounts of unlabeled amounts of unprecipitated chromatin DNA are also shown (input).

For comparison, amplifications derived from equivalent HNF4 site). Ethidium bromide-stained agarose gels of the PCR products (containing HNF4-54) or with a primer pair targeting the SULT2A1 gene region

Fig. 5. EMSA analysis of HNF4α, PPARα, RXR, or PXR-RXR binding to a candidate HNF4 binding site located 54 nt upstream of the SULT2A1 transcription start site. A, sequence of a computer-predicted HNF4-binding site at nt −54 of the SULT2A1 5′-flanking region is indicated, together with the matched TRANSFAC matrices for HNF4 and PPARα. Capitalized letters in the matrices indicate core nt. n, A, T, G, or C; r, A or G; k, G or T, and w, A or T. B, in vitro binding reactions contained 32P-labeled double-stranded oligonucleotide probe corresponding either to a consensus HNF4-RE or the predicted HNF4-binding site at nt −54 (54) and in vitro-transcribed/translated HNF4α or no protein (−) or protein from in vitro transcribed/translated pcDNA3.1 empty vector (V). For competition experiments, reactions also contained the indicated amounts of unlabeled oligonucleotide corresponding to the consensus HNF4-RE (HNF4), 54 or 54 with mutated HNF4 core sequence (54m). For supershift experiments, reactions were supplemented with an antibody directed against HNF4α (+). Positions of free probe, HNF4-shifted bands and supershifted bands are indicated. C, binding reactions contained 32P-labeled double-stranded oligonucleotide probe corresponding either to a consensus HNF4-RE (C under HNF4), a consensus PXRE (C under PXR), the predicted HNF4-binding site at nt −54 (54) or 54 with a mutated core sequence (54m). The indicated samples also contained in vitro transcribed/translated HNF4, PPARα and RXR, or PXR and RXR. Positions of free probe and shifted bands are indicated.

Fig. 6. Chromatin immunoprecipitation analysis of HNF4α association with the SULT2A1 5′-flanking region containing HNF4-54 in HepG2 cells. HepG2 cells were either not transfected or were transfected with pSG5-PXR alone or in combination with pCMV-HNF4α. Each transfection group was then treated for 24 h with either 0.1% DMSO (−) or 50 µM rifampicin (+). Cultures were fixed and harvested for the preparation of sheared chromatin, and immunoprecipitations were performed using an antibody directed against HNF4α, or with nonspecific IgG, as a negative control. Following immunoprecipitation, associated DNA was amplified with a primer pair targeting the SULT2A1 gene region −164 to +66 (containing HNF4-54) or −11,748 to −11,605 (containing no predicted HNF4 site). Ethidium bromide-stained agarose gels of the PCR products are shown. For comparison, amplifications derived from equivalent amounts of unprecipitated chromatin DNA are also shown (input).

HNF4-54 site was also mutated (Fig. 7B). Overall, these results indicate that the HNF4-54 site is functionally important for the control of basal SULT2A1 transcription, and may also play a role in rifampicin-mediated regulation. Although the HNF4-6160 site is also functional, other information contained within the −6832:−5812 region contributes substantially to SULT2A1 transcription.

To gain further insight into the interacting roles of PXR and HNF4 in regulating SULT2A1 transcription, additional studies were performed in which HepG2 cells were transfected with the simple reporter construct containing only the HNF4-6160 element and the −107:48 core promoter (i.e., construct (HNF4-6160)(−107:48)), in combination with expression constructs for HNF4α and/or PXR, with or without rifampicin treatment (Fig. 8). In the experiment that is shown, rifampicin treatment increased reporter expression, although such a clear increase was not always evident when this simple construct was used. Transfection with HNF4α alone increased promoter activity, and rifampicin treatment had no further effect (Fig. 8A). PXR transfection significantly reduced promoter activity, and rifampicin treatment augmented this reduction (Fig. 8A). When cotransfected with HNF4α, PXR significantly reduced promoter activity relative to that seen in cells transfected with HNF4α alone, and again, rifampicin treatment augmented the reduction (Fig. 8A).

To characterize the suppressive effect of PXR transfection/rifampicin treatment further, HepG2 cells were transfected with various amounts of PXR expression plasmid and treated with DMSO or rifampicin. As shown in Fig. 8B, PXR transfection produced a dose-dependent suppression of promoter activity, and the suppressive effects were further augmented by rifampicin treatment. When the effects of PXR transfection/rifampicin treatment were evaluated in the context of the longer (−6832:−5812)(−107:48) construct, as before, rifampicin treatment increased reporter expression in cells
containing the endogenous amount of PXR (i.e., transfected with empty vector, pSG5), and the increase was blunted when the HNF4-54 site was mutated (Fig. 8C). However, PXR transfection suppressed reporter expression, and rifampicin treatment augmented the suppression, even when the HNF4-6160 and HNF4-54 sites were mutated (Fig. 8C).

In additional experiments, the concentration-dependent effects of rifampicin treatment on reporter expression from the SULT2A1 (HNF4-6160(-107:48)) construct were evaluated in comparison to effects on the known PXR-responsive construct, XREM-CYP3A4-Luc (Fig. 9). For the CYP3A4 construct, rifampicin maximally increased reporter expression at a concentration of 3 μM, and expression was always augmented when the cells were supplemented with additional PXR. For the SULT2A1 construct, rifampicin treatment produced a progressively suppressive effect up to a concentration of 3 μM, and the suppression was always augmented by PXR transfection. However, treatment with higher concentrations of rifampicin caused progressive reversal of the SULT2A1 suppression. These data suggest that the major PXR-mediated effect of rifampicin on SULT2A1 transcription is suppression, with SULT2A1 induction only occurring at rifampicin concentrations higher than those sufficient for obtaining maximal PXR activation.

To evaluate the possibility that rifampicin treatment produces PXR-dependent suppression, but PXR-independent activation, of SULT2A1 promoter activity, RNA interference was used to prepare HepG2 cells with stably suppressed PXR expression. The above-described rifampicin concentration-response analysis was repeated in the PXR knockdown cells, in comparison with HepG2 cells expressing a nontargeting siRNA (Fig. 10A). As seen in standard HepG2 cells (Fig. 9), rifampicin treatment produced a concentration-dependent activation of the XREM-CYP3A4-Luc reporter in the HepG2 cells expressing the nontargeting siRNA. This effect was abolished in the PXR knockdown cells. Also as seen in standard HepG2 cells, rifampicin treatment produced a concentration-dependent suppression of the SULT2A1 (HNF4-6160(-107:48)-Luc reporter, which was significant at rifampicin concentrations of 3 and 10 μM but progressively reversed at higher concentrations. In the PXR knockdown cells, the suppressive component was diminished (i.e., no longer significant), whereas the activating component was enhanced (i.e., 50 μM rifampicin treatment now produced a significant activation of this reporter construct). Rifampicin (50 μM)-mediated activation was also retained when the PXR knockdown HepG2 cells were transfected with the longer SULT2A1 (-6832:5812)-Luc reporter construct (Fig. 10, B and C), but it was lost when the HNF4-54 and HNF4-6160 sites were mutated (Fig. 10C). However, transfection of PXR into the PXR knockdown cells produced a suppressive effect on SULT2A1 reporter expression that was augmented by rifampicin treatment, irrespective of whether the HNF4 sites were intact or mutated (Fig. 10C), as was seen in the above-described experiment (Fig. 8C).

Discussion

Increasing evidence suggests that the net effect of xenobiotic exposure on target gene transcription is a consequence of both transcription factor dynamics at critical cis-acting regulatory sites and superimposed “cross talk” mechanisms. The present study suggests a combined role for two liver-enriched nuclear receptors, HNF4α and PXR, in the transcriptional regulation of human hepatic SULT2A1.

PXR is activated by a wide spectrum of xenobiotic and endogenous ligands (Kliever et al., 1998; Goodwin et al., 1999), and liganded PXR transactivates CYP3A4 at proximal and distal PXREs in the 5′-flanking region of the gene (Bar-
three copies indicate and PXR and treated with DMSO. For statistical symbols, one, two and three copies indicate significantly different from the group transfected with the same reporter and pSG5 and treated with DMSO. #, ##, ###, significantly different from the group transfected with either the empty vector, pSG5 (open bars), or with pSG5-PXR (closed bars) in combination with either the PXR-responsive luciferase reporter construct XREM-CYP3A4-Luc (top) or (HNF4-6160)(-107:48)-Luc (bottom). Transfected cells were treated for 24 h with either 0.1% DMSO or rifampicin at concentrations ranging from 0.1 to 50 μM and harvested for measurement of luciferase activities. Each bar represents the mean ± S.D. of normalized (firefly/Renilla) luciferase measurements (three wells per treatment group). ***, **, significantly different from the group transfected with the same expression plasmid and treated with DMSO. +, ***, significantly different from the corresponding DMSO-treated group. xxx, significantly different from the corresponding group (i.e., same treatment) transfected with pSG5. For statistical symbols, one, two and three copies indicate p < 0.05, p < 0.01, and p < 0.001, respectively.

Fig. 8. Effects of HNF4α and/or PXR cotransfection on basal and rifampicin-regulated reporter expression from SULT2A1 constructs containing intact or mutated HNF4-54 and HNF4-6160 sites. A, HepG2 cells were transiently transfected with (HNF4-6160)(-107:48)-Luc (reporter containing the HNF4-6160 site and the first 107 nt of the SULT2A1 promoter with intact pDNA) (pcDNA, empty vector control for HNF4), 50 ng of pCMV-HNF4α, 100 ng of pSG5 (empty vector control for PXR), 100 ng of pSG5-PXR, or 50 ng of pCMV-HNF4α, and 100 ng of pSG5-PXR in combination. Transfected cells were treated for 24 h with 0.1% DMSO or 50 μM rifampicin and harvested for measurement of luciferase activities. Each bar represents the mean ± S.D. of normalized (firefly/Renilla) luciferase measurements (3 wells per treatment group). ***, **, significantly different from the corresponding pDNA-transfected, DMSO-treated group. ++, ***, significantly different from the corresponding pSG5-transfected, DMSO-treated group. +++, significantly different from the corresponding DMSO-treated group. xxx, significantly different from corresponding HNF4α-transfected group. B, HepG2 cells were transiently transfected with (HNF4-6160)(-107:48)-Luc together with amounts of pSG5-PXR ranging from 0 to 160 ng (the amount of expression plasmid in each transfection was kept constant at 160 ng by the addition of pSG5). Transfected cells were treated for 24 h with 0.1% DMSO or 50 μM rifampicin, and cells were harvested for measurement of luciferase activities. Each bar represents the mean ± S.D. of normalized (firefly/Renilla) luciferase measurements (3 wells per treatment group). ***, **, significantly different from the corresponding pDNA-transfected, DMSO-treated group. ++, ***, significantly different from the corresponding pSG5-transfected, DMSO-treated group. +++, significantly different from the corresponding DMSO-treated group. xxx, significantly different from corresponding HNF4α-transfected group. The integrated roles of nuclear receptors in gene regulation are complex and likely to contribute to interindividual differences in drug metabolism. CAR also partners with RXR to regulate transcription of xenobiotic-metabolizing enzyme genes, and the same regulatory motifs in target genes can often bind PXR or CAR (Xie et al., 2000; Wang et al., 2003). In contrast to PXR and CAR, HNF4α is a homodimer, and it is not known to be ligand-activated (Dhe-Paganon et al., 2002). HNF4α contains fatty acid as a structural component of its ligand-binding pocket (Dhe-Paganon et al., 2002), and mutations in the HNF4α gene have been linked to maturity onset diabetes of the young (Yamagata et al., 1996; Dhe-Paganon et al., 2002).

The integrated roles of nuclear receptors in gene regulation are complex and likely to contribute to interindividual differences in drug metabolism. The transactivation of CYP2C8 is mediated by separate cis-acting sites that confer PXR/CAR, GR, and HNF4α binding activity (Ferguson et al., 2005). Inducible CYP2C9 transcription is synergistically activated by proximal HNF4α binding and distal PXR/CAR binding to the CYP2C9 promoter (Chen et al., 2005). The cotransfection
of HNF4α and PXR into cells produced a synergistic increase in CYP3A4 reporter activity, and mice with a conditional deletion of hepatic HNF4α lost both basal and PXR/CAR-inducible CYP3A expression (Tirona et al., 2003). The induction of CYP3A4 transcription by activated PXR was shown to require both the suppression of small heterodimer partner and the recruitment of HNF4α and coregulators to the CYP3A4 promoter (Li and Chiang, 2006).

By comparison with CYP3A4, CYP2C8, and CYP2C9, the genes encoding the bile acid-biosynthesizing cytochromes P450 CYP7A1 and CYP8B1 are negatively regulated by PXR (Bhalla et al., 2004; Li and Chiang, 2005). Current evidence indicates that these suppressive effects occur as a consequence of PXR interactions with HNF4α. In one study (Bhalla et al., 2004), competition between HNF4α and ligand-activated PXR for recruitment of the PGC-1 coactivator was demonstrated. Through this “squelching” mechanism, PXR activation would be expected to suppress the expression of multiple HNF4α-regulated genes, and indeed suppression of phosphoenolpyruvate carboxykinase was demonstrated (Bhalla et al., 2004). In another study, the cis-acting site controlling rifampicin-repressible CYP7A1 transcription was localized to a motif, termed BARE-I, in a proximal region of the CYP7A1 promoter (Li and Chiang, 2005). EMSA studies suggested that PXR-RXR binds to BARE-I, whereas HNF4α binds to a more distal BARE-II motif (Li and Chiang, 2005). Mammalian two-hybrid and coimmunoprecipitation analyses suggested that rifampicin treatment suppresses CYP7A1 transcription by causing PXR to disengage from BARE-I in favor of protein-protein interaction with BARE-II-bound HNF4α (Li and Chiang, 2005). These ligand-induced nuclear receptor interactions block HNF4α interaction with PGC-1α, leading to suppression of CYP7A1 transcription (Li and Chiang, 2005).

We began the current investigation by examining the effects of rifampicin treatment on SULT2A1 expression in primary cultured human hepatocytes, as this system represents the only authentic cell culture model of the normal human hepatocyte that is currently available. Inextractable from this model is the same heterogeneity that exists among humans, which includes genetic polymorphisms and environmental influences that exert durable effects on hepatocellular phenotype. When the effects of rifampicin treatment were evaluated in several preparations of human hepatocytes, CYP3A4 expression was consistently enhanced multifold, consistent with the well described model of PXR-RXR activation. By comparison, the effects of rifampicin treatment on SULT2A1 expression were complicated, in that induction, suppression, or little change was seen, depending on the hepatocyte preparation. The rifampicin-mediated effects on CYP3A4 and SULT2A1 expression were not correlated, suggesting that the mechanisms underlying these effects are not identical. These findings indicate that SULT2A1 transcription is regulated by both positive- and negative-acting factors, both of which are sensitive to rifampicin treatment.

Based on these initial findings, we conducted transfection studies in HepG2 cells, with the goal of identifying the factors conferring the positive and/or negative effects of rifampicin on SULT2A1 expression. The results indicated that several elements located within the first 492 nt of the SULT2A1 promoter contribute to basal SULT2A1 transcription. In addition, multiple elements contained within upstream regions, particularly the −6832−5812 region, enhance SULT2A1 expression. Although some of the tested constructs displayed increased reporter expression in response to 50 μM rifampicin treatment, none of the responsive constructs contained a typical PXR binding site, and rifampicin-mediated induction, albeit somewhat variable among experiments, was retained in a reporter construct containing only the first 107 nt of the SULT2A1 promoter. This region contained a high-affinity and functional HNF4-binding site, as determined by EMSA and site-directed mutagenesis. Likewise, the −6832−5812 region contained an HNF4-binding site, although the requirement of this site for maintaining a high level of transcription was less apparent.

Simple SULT2A1 reporter constructs, containing only the
proximal 107 nt or the upstream HNF4-G610 site in cis with the 107 nt promoter, were used to evaluate the roles of PXR and HNF4α in rifampicin-mediated transcriptional regulation. Rifampicin treatment caused progressive suppression of reporter expression from the SULT2A1 reporters at the same concentrations that caused progressive activation of a known PXR-responsive reporter. Rifampicin-mediated suppression of SULT2A1 promoter activity was reversed only at rifampicin concentrations higher than necessary to achieve maximal CYP3A4 reporter induction. Thus, the SULT2A1 “induction” that was seen at 50 μM rifampicin was actually the net ability of the positive-acting effect of rifampicin to counteract its suppressive effect. In addition to the concentration-response analysis, the finding that PXR supplementation augmented rifampicin-mediated suppression of SULT2A1 reporter expression, while producing the expected enhancement of rifampicin-inducible CYP3A4 reporter expression, suggests that the primary PXR-mediated effect on SULT2A1 transcription is suppression, and that the positive effect on SULT2A1 transcription that occurs at higher concentrations of rifampicin is mediated through a PXR-independent mechanism. Most importantly, the suppressive effect of rifampicin was diminished in HepG2 cells with knocked down PXR expression, whereas the activating effect remained intact. The involvement of both PXR-mediated and PXR-independent mechanisms in SULT2A1 regulation provides a plausible explanation for the lack of correlation between rifampicin-mediated effects on CYP3A4 and SULT2A1 expression that was observed among human hepatocyte preparations. As noted above, rifampicin-regulated SULT2A1 promoter constructs contained HNF4α, but not PXR-, binding sites. For the PXR-dependent suppressive effect, we hypothesize that, as for CYP7A1, rifampicin-activated PXR engenders protein-protein interactions that disrupt the transactivation of SULT2A1 transcription by HNF4α, possibly through the impaired recruitment of one or more coactivators. Although PXR overexpression, without or with rifampicin treatment, was able to cause suppression of SULT2A1 promoter activity in constructs with intact or mutated HNF4 binding sites (Figs. 8 and 10), we suspect that the mutations reduced, but did not abolish the ability of HNF4α to bind (the mutants retained some ability to compete for probe binding in EMSAs) and that the residual transactivation could be suppressed by PXR overexpression. Although the above-described PXR-mediated mechanism provides a plausible explanation for the finding that rifampicin treatment suppressed SULT2A1 expression in some human hepatocyte cultures, the mechanism that mediates the rifampicin-inducible SULT2A1 expression that occurs in many human hepatocyte preparations is not yet known. Rifampicin is not known to bind to any nuclear receptor other than PXR. Our data, demonstrating the retention of rifampicin-mediated SULT2A1 promoter activation in HepG2 cells lacking PXR expression but the loss of induction when HNF4-binding sites are mutated, suggest that the activating effect of rifampicin, like the suppressive effect, is transduced through HNF4α.

Regulation of gene expression by nuclear receptors is gaining appreciation for its shades of complexity that are likely to translate into interindividual differences in xenobiotic and endogenous metabolism. For example, the impact on human health of natural PXR variants that lack DNA-binding and transactivation capacity is unknown (Koyano et al., 2004). It has also been proposed that the relative cellular contents of nuclear receptors, and not just the absolute amounts, are important determinants of CYP3A inducibility (Swales et al., 2003). Future investigations will focus on the interactive dynamics that occur between cis-acting regions, transcription factors, and coregulator complexes to orchestrate the net regulation of human SULT2A1 gene expression. Extensions of these studies will also shed light on the physiological implications of PXR interactions with liver-enriched transcription factors, such as HNF4α, thereby strengthening the mechanistic bridge that links the chemical environment with the cellular physiology of the human hepatocyte.

References

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