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Dehydroepiandrosterone Sulfotransferase Is a Target for Transcriptional Induction by the Vitamin D Receptor

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ABSTRACT

Dehydroepiandrosterone sulfotransferase (SULT2A1) is a cytosolic enzyme that mediates sulfo-conjugation of endogenous hydroxysteroids (dehydroepiandrosterone, testosterone, bile acids), and diverse xenobiotic compounds. Upon sulfonation, SULT2A1 substrates become polar and water-soluble and thus suitable for rapid excretion. SULT2A1 is abundantly expressed in the liver and intestine. Recent evidence has shown that the ligand-activated vitamin D receptor (VDR) can transcriptionally induce the xenobiotic-metabolizing cytochrome P450 enzymes. Herein, we report that VDR also targets *SULT2A1* for transcriptional activation. Vitamin D stimulated endogenous SULT2A1 expression and induced transfected human, mouse, and rat *SULT2A1* promoters in liver and intestinal cells upon cotransfection with VDR. An inverted repeat DNA element (IR0), located within −191 to −168 positions of mouse and rat *Sult2A1*, mediates VDR induction of *Sult2A1*. DNase1 footprinting, competition EMSA, and antibody supershift assay showed that the IR0 is a binding site for the RXR-α/VDR heterodimer. Point mutations within the IR0 prevented RXR/VDR binding and abolished VDR-mediated *Sult2A1* induction. The IR0 element conferred VDR responsiveness on a thymidine kinase promoter. Thus, VDR-mediated nuclear signaling may be important in the phase II metabolism involving Sult2A1. The rodent *Sult2A1* gene is also induced by the farnesoid X receptor (FXR) and pregnane X receptor (PXR) through the same IR0. In competition transfections, FXR or PXR inhibited VDR induction of the IR0. Competitive functional interactions among VDR, PXR, and FXR suggest that the intracellular hormonal and metabolic milieu may determine the extent to which a specific nuclear receptor pathway would influence steroid/xenobiotic metabolism using dehydroepiandrosterone sulfotransferase.

Sulfonation of small, bioactive endogenous or exogenous molecules catalyzed by cytosolic sulfotransferases (SULTs) is a commonly used phase II mechanism for the inactivation, detoxification, and disposition of hormones, biogenic amines, and structurally diverse drugs and other xenobiotics (Weinshilboum et al., 1997; Strott, 2002). Dehydroepiandrosterone (DHEA) sulfotransferase (SULT2A1) is a member of the SULT2 subfamily that acts exclusively on several endogenous hydroxysteroids, namely DHEA, testosterone, estrogen, pregnenolone, and bile acids (Falany, 1997; Song et al., 2001; Strott, 2002). SULT2A1 substrates also include environmental xenoestrogens and various drugs, including hydroxytamoxifen, a breast cancer chemopreventive agent, and budesonide, a synthetic glucocorticoid useful in inflammatory diseases such as asthma and Crohn's disease (Shibutani et al., 1998; Meloche et al., 2002; Pai et al., 2002). SULT2A1 is expressed abundantly in the liver and intestine, the two first-pass metabolic tissues, and in the adrenal cortex, a major steroidogenic tissue (Falany, 1997). Other tissues such as the kidney and brain produce this enzyme at low levels (Shimada et al., 2001). The role of SULT2A1 in the cellular protection against the harmful build up of endogenous/exogenous substrates may be further heightened in cases in which phase I cytochrome P450 (P450) enzymes or other phase II conjugating transferases are functionally impaired because of genetic, dietary, and environmental factors.

A subset of orphan nuclear receptors, specifically the bile acid activated farnesoid X receptor (FXR) and steroid- and xenobiotic-activated pregnane X receptor (PXR), are tran-

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ABBREVIATIONS: SULT, sulfotransferase; DHEA, dehydroepiandrosterone; P450, cytochrome P450; FXR, farnesoid X receptor; PXR, pregnane X receptor; CAR, constitutive androstane receptor; VDR, vitamin D receptor; LCA, lithocholic acid; RT, reverse transcription; PCR, polymerase chain reaction; vit D_3, 1α,25-dihydroxyvitamin D_3; VDRE, vitamin D responsive element; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; CDCA, chenodeoxycholic acid.

scriptional inducers of *Sult2A1* in the liver and intestine (Runge-Morris et al., 1999; Song et al., 2001; Echchgadda et al., 2002; Sonoda et al., 2002). A corresponding increase in the enzyme activity has also been observed. The constitutive androstane receptor (CAR), a second xenobiotic-activated nuclear receptor, can induce human SULT2A1 gene transcription (Echchgadda et al., 2003). A cis element (GGGTCATGAACT), configured as a spacerless imperfect inverted repeat (IR0) with a half site resembling the consensus (A/G)G(G/T)TCA half-site binding sequence for nonsteroid nuclear receptors, directs the bile acid- and xenobiotic-mediated induction of *Sult2A1* (Song et al., 2001; Sonoda et al., 2002). CYP2 and CYP3, which promote oxidative metabolism of hydrophobic hormones, drugs, and other foreign chemicals, are also transcriptionally stimulated by PXR and CAR (Schuetz et al., 2001; Goodwin et al., 2002). The cis elements directing the PXR- and CAR- mediated *P450* induction configure either as direct repeats with three or four spacer nucleotides (DR3, DR4) or as an everted repeat with six spacer nucleotides (ER6) (Honkakoski et al., 2003).

The vitamin D receptor (VDR) in its ligand-activated form also stimulates *CYP2* and *CYP3* gene transcription in the liver and enteric tract by inducing the same DR3/ER6 elements that mediate the PXR/CAR induction of these genes (Schmiedlin-Ren et al., 2001; Thummel et al., 2001; Makishima et al., 2002; Thompsonet al., 2002). In its classic endocrine role, the VDR regulates calcium and phosphate ion homeostasis in tissues such as the bone, intestine, and kidney (Norman, 1990). Vitamin D is also chemoprotective against epithelial cell cancers, including colon cancer, presumably because of its inhibitory effect on cell proliferation and its ability to induce cellular differentiation and apoptosis (Guyton et al., 2001). A part of the anticancer activity of vitamin D may stem from its ability to stimulate CYP2/CYP3 expression, which would facilitate the metabolism and excretion of toxic compounds. The importance of the VDR pathway in toxin clearance is further underscored by the finding that lithocholic acid (LCA), a hepatotoxic secondary bile acid and a potential intestinal carcinogen, can bind and activate this nuclear receptor to induce *CYP3* transcription (Makishima et al., 2002). As a bile acid sensor, VDR is thought to protect enteric cells against LCA-induced DNA damage that may otherwise cause excessive proliferation of the colonic cells, leading to colon cancer.

Herein, we report that the ligand-activated VDR can stimulate endogenous *SULT2A1* gene transcription and induce mouse, rat, and human *SULT2A1* promoters in transfected HepG2 hepatoma and Caco-2 intestinal cells. In the context of the mouse and rat promoters, we have identified an IR0 element from −191 to −168 positions that binds to the RXRα/VDR complex and mediates VDR responsiveness. This IR0 in rodent promoters was previously identified as a PXR- and FXR-responsive element. We further show that PXR and FXR use the IR0 to compete with VDR for mediating *Sult2A1* induction. Thus, phase II conjugation by SULT2A1 is regulated by multiple xenobiotic-sensing nuclear receptors, and the intracellular hormonal and metabolic environment presumably dictates which of these receptors would be the dominant player in regulating the sulfonation pathway to steroid/drug metabolism involving SULT2A1.

Materials and Methods

Analysis of Endogenous SULT2A1 Expression. The endogenous SULT2A1 mRNA and protein in HepG2 (human hepatoma) cells and in Caco-2 (human colonic adenocarcinoma) cells were assayed by semiquantitative RT-polymerase chain reaction (PCR) and Western blot, respectively. Cells (seeded in six-well flasks in DMEM supplemented with 5% charcoal-stripped serum) were transfected with the expression plasmids for human VDR (hVDR; 100 ng) and human RXR-α (hRXR-α; 10 ng) using Fugene (Roche Diagnostics, Indianapolis, IN) and at 2 h after transfection, 10 nM 1α,25-dihydroxyvitamin D_3 (vit D_3; BIOMOL Research Laboratories, Plymouth Meeting, PA) or ethanol (ETOH) was added to the culture medium. After 24 h, total RNAs were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). PCR was conducted for 28 cycles at 95°C for 30 s; 55°C for 30 s; and 72°C for 30 s. For RT-PCR of human SULT2A1 mRNAs, the oligonucleotides 5′-GTATACAGCACTCAGTGA-3′ and 5′-CCCAGGAATTGACAGATC-3′ were used as the sense and antisense primers, respectively (Otterness et al., 1992). The oligonucleotides 5′-GACAGGATGCAGAAGGAGAT-3′ (sense) and 5′-TTGCTGATCCACATCTGCTG-3′ (antisense) served as the β-actin specific PCR primers (Chatelain et al., 1995). The intensity of each band was quantified using UN-SCAN-IT gel automated digitizing system (Silk Scientific, Orem, UT) and the fold induction by vitamin D_3 was normalized to constant β-actin expression.

For Western blot, HepG2 cells in charcoal-stripped serum supplemented DMEM were seeded in 100-mm culture dishes and were cotransfected with hVDR (300 ng), and the hRXR-α (30 ng) expression plasmids and treated with vit D_3 or ethanol as described above. The cells were lysed (2% SDS, 5% β-mercaptoethanol, 125 mM Tris-HCl, pH 6.8, and 20% glycerol) and 10 μg of total cellular proteins were run on 10% SDS-polyacrylamide gels. For immunodetection of human SULT2A1, the polyclonal anti-human SULT2A1 (ST 17; Oxford Biomedical Research, Oxford, MI) was used as the primary antibody. The monoclonal anti–β-actin (Abcam, Cambridgeshire, UK) served as the primary antibody for the detection of β-actin. Using the peroxidase-conjugated secondary antibody, the antigen/antibody complex was detected via chemiluminescence (PerkerElmer Life and Analytical Sciences, Boston, MA). The band intensities corresponding to SULT2A1 and β-actin were quantified as described above for RT-PCR, and the fold induction of SULT2A1 normalized to constant β-actin expression was calculated.

DNase 1 Footprinting and Electrophoretic Gel Mobility Shift Assay. The probe for DNase 1 footprinting containing a radiolabeled coding strand was generated by PCR amplification of the mouse *Sult2A1* promoter using a ^{32}P-end-labeled sense primer (at −292 of the *Sult2A1* promoter) and an unlabeled antisense primer sequence from within the vector (pGL3b). The conditions for footprinting assay and preparation of the mouse liver nuclear extract were same as described previously (Song et al., 1998). In competition footprinting, the unlabeled homologous or heterologous oligonucleotide duplex was added as the competitor at 300-fold molar excess. The heterologous competitors were as follows: VDRE from the rat osteocalcin gene promoter at −449 (5′-GCACTGGGTGAATGAGGACATTACT; Jurukuta et al., 2002); the proximal ER6 at −173 of the human *CYP3A4* gene promoter that is known to bind to RXR/VDR and confer VDR responsiveness (Drocourt et al., 2002; Makishima et al., 2002), and a consensus HNF1-α element (TGTGGTTAATGATCTACAGTTA).

Electrophoretic mobility shift assay (EMSA) was performed with the baculovirus-expressed recombinant human VDR (BIOMOL) and the bacterially expressed glutathione *S*-transferase fusion of RXR-α (GST-RXR-α; gift from Nuttawut Saelim and Dr. James Lechleiter, University of Texas Health Science Center, San Antonio, Texas). The unlabeled oligonucleotide competitors and antibody samples were added to the preincubation reactions in competition EMSA and antibody supershift assay, respectively. Antibodies to FXR (C-20),

RXR-α (ΔN 197), and VDR (D-6) were from Santa Cruz Biotech. (Santa Cruz, CA).

SULT2A1 Promoter-Reporter Constructs and Mutagenesis. Based on the rat *Sult2A1* promoter sequence (Song et al., 1990), we designed sense and antisense primers to isolate the mouse *Sult2A1* promoter (−292 to +42) from mouse genomic DNA by PCR. Similarly, the human *SULT2A1* promoter from −1070 to +42 was isolated by PCR amplification of human genomic DNA from HepG2 cells using the high-fidelity platinum Pfx polymerase (Invitrogen) and sense/antisense primers whose sequences were chosen based on the DNA sequence of the genomic human SULT2A1 (Otterness et al., 1992). For PCR isolation of the mouse Sult2A1 promoter, we used the sense primer (5′-TGCATATTTAAAATCATTCTG-3′) corresponding to the rat Sult2A1 promoter from −291 to −271, and the antisense primer (5′-GGTTCTCTTAGGATTCCAGC-3′) corresponding to the mouse Sult2A1 cDNA from +42 to +21 (Kong and Fei, 1994). The gel-purified PCR product was subcloned into pCR-BluntII-TOPO (Invitrogen) and analyzed for DNA sequence. The EcoRI-digested promoter insert was then cloned 5′ to the luciferase cDNA in pGL3b (Promega, WI). For cloning convenience, we added an EcoR1 site within the multiple cloning region of pGL3b. The transcription start site of the mouse promoter, identified by primer extension analysis (Song and Chatterjee, unpublished observations) is at a position similar to that of the rat *Sult2A1* promoter. A shorter mouse *Sult2A1* promoter (−157 to +42) was isolated from the −292 to +42 mouse promoter by PCR. The reporter constructs (−215 rat *Sult2A1*-CAT and −158 rat *Sult2A1*-CAT) were described previously (Song et al., 1998). The −1070 to +42 human *SULT2A1* promoter was cloned into pGL3b to prepare −1070 human *SULT2A1*-Luc construct.

The mouse *Sult2A1* promoter from −191 to −168 containing the IR0 element was inserted as a three-copy tandem repeat in front of the tk promoter in tk-Luc to produce $(IR0)_3$-tk-Luc, using MluI and BglII sites. Oligonucleotides with appropriate restriction sites were custom-synthesized. The mutant construct $(IR0\text{-}mt)_3$-tk-Luc was prepared by ligating an oligonucleotide containing three copies of the mutant −191/−168 sequence. The mutant oligonucleotide contains three-base substitutions (CAT → ACC) within the IR0 of −191/−168. All constructs were sequence-verified. The plasmid 3A4-PXRE-Luc (kindly provided by Rommel Tirona, Vanderbilt University) contains the xenobiotic enhancer module (−7836 to −7208) and the proximal −362 to +53 of the human CYP3A4 promoter.

Cell Transfection and Reporter Assay. HepG2 and Caco-2 cell lines were purchased from the American Type Culture Collection (Manassas, VA) and maintained in minimum essential medium with 10% fetal bovine serum. Cells (seeded in 24-well flasks at 10^4cells/well and suspended in medium containing 5% charcoal-stripped serum) were transiently transfected with 50 ng of the human VDR-encoding plasmid pSG5-hVDR, the reporter construct (300 ng) and the *Renilla reniformis* luciferase control plasmid pRSVL (5 ng; Promega, Madison, WI) using Fugene. One to two hours after DNA transfer, vit D_3 at final concentration of 50 nM or ethanol (vehicle) was added to the culture medium and cells were harvested ~40 h after transfection. We cloned the full-length human VDR cDNA (American Type Culture Collection) in the pSG5 expression vector (Stratagene). For the VDR/FXR competition transfection, cells were cotransfected with the reporter construct (300 ng), a constant amount of the VDR-encoding plasmid (50 ng), and increasing amounts of the rat FXR plasmid (CMX-FXR; a generous gift from Dr. David Mangelsdorf, Southwestern Medical School, Dallas, TX). For the VDR/PXR competition assay, the cells were cotransfected with the reporter construct (300 ng), VDR (50 ng), RXR-α (10 ng), and increasing amounts of the human PXR (pSG5-hPXR; kindly provided by Dr. Steven Kliewer, Southwestern Medical School). Cotransfection of the constitutively active pRSVL (5 ng) was used for the normalization of transfection efficiency in all assays. Total DNA amounts were kept constant (500 ng) using an empty vector (CMX for the VDR/FXR competition and pSG5 for VDR/PXR competition). The cells were then treated with ethanol, 50 nM vitamin D_3, or 25 μM CDCA for the VDR/FXR cotransfected cells. The VDR/PXR cotransfected cells were treated with one of the following: ethanol (vehicle for vitamin D_3), dimethyl sulfoxide (vehicle for rifampicin), 50 nM vitamin D_3 or 10 μM rifampicin. Cell extracts were assayed for firefly luciferase and *R. reniformis* luciferase, using a dual luciferase assay kit (Promega), and reporter expression was normalized to constant *R. reniformis* luciferase expression. Results were computed based on three independent transfections, each conducted in duplicate. Histograms without error bars represent averages from two independent transfections, each conducted in duplicate.

Results

Induction of SULT2A1 Expression in Cells Treated with Vitamin D. The endogenous expression of human *SULT2A1*, normalized to constant β-actin expression, increased 6-fold at the mRNA level and 3-fold at the protein level in response to vitamin D treatment of the VDR-transfected HepG2 cells (Fig. 1) and Caco-2 cells (data not shown). Vitamin D_3 was used as the hormonally active vitamin D and the autoradiographic results from two independent experiments are shown. The discrepancy in the fold induction for the mRNA and protein levels (6-fold versus 3-fold) was most probably caused by the higher sensitivity of the RT-PCR over Western blot assay. On the other hand, post-transcriptional regulation of SULT2A1 expression may also explain the difference in the extent of induction between the mRNA and protein levels. Whether the increased mRNA expression was caused by transcriptional regulation was subsequently determined by assessing the effect of vitamin D/VDR on the SULT2A1 promoter function.

VDR Induction of the *SULT2A1* Promoter. Treatment of VDR-transfected HepG2 and Caco-2 cells with vitamin D_3 caused induction of the human, mouse, and rat *SULT2A1* promoters by severalfold (Fig. 2). Without VDR cotransfec-

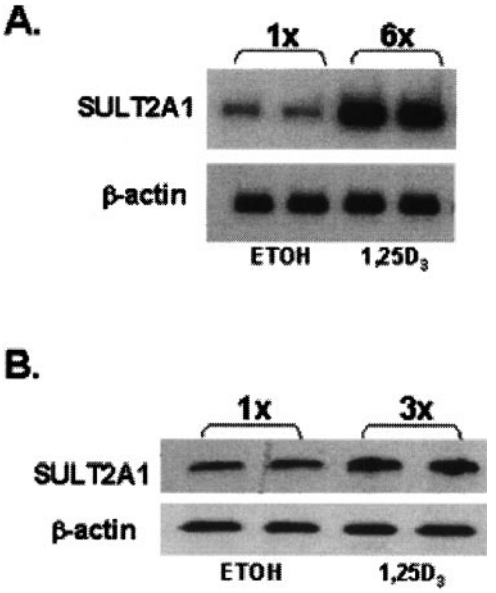

Fig. 1. Stimulation of SULT2A1 mRNA and protein expression by vitamin D in HepG2 cells. The hVDR and hRXR-α cotransfected cells were treated with vitamin D_3 (10 nM) or ethanol and then analyzed for SULT2A1 mRNA levels by RT-PCR (A) and for the immunoreactive SULT2A1 by Western blot (B). The autoradiograms show results from two different experiments. The β-actin mRNA and protein expression served as invariant controls. The fold induction by vit D_3 shown here is the average of the normalized values from two independent data points.

tion, vitamin D did not mediate induction of any of these promoters (data not shown). In parallel transfections, we used the reporter construct 3A4-PXRE containing the human CYP3A4 promoter, a known VDR target. The robust induction of 3A4-PXRE (>200-fold in Caco-2 cells and ~50-fold in HepG2 cells) is caused by a xenobiotic-responsive enhancer element (located in the natural CYP3A4 promoter at ~7 kilobase pairs upstream) that is juxtaposed to the proximal CYP3A4 promoter (−362 to +53). Cotransfection of RXR-α, the heterodimer partner of VDR, caused no additional induction of the SULT2A1 and CYP3A4 promoters, presumably because of the sufficient endogenous expression of RXR-α in these cells. Thus, we conclude that VDR is a transcriptional activator of *SULT2A1*, and human and rodent promoters are equally effective in responding to the VDR/vitamin D signaling. The cis regulatory element mediating VDR responsiveness in the mouse and rat *Sult2A1* promoters was subsequently identified and characterized as described below.

DNase1 Footprinting of the *Sult2A1* Promoter by the RXR-α/VDR Complex and the Role of the Footprinted Sequence in VDR Responsiveness. To determine whether VDR targets the *Sult2A1* promoter directly for transactivation by binding to a VDR-responsive cis element, we conducted DNase 1 footprinting analysis on the −292 mouse Sult2A1 promoter. Figure 3 shows that in the combined presence of recombinant VDR and RXR-α, a distinct nuclease-protected footprint was produced from −192 to −170 positions (Fig. 3, compare lanes 1 and 2), whereas no footprint was formed when the assay reaction included VDR or RXR-α alone (Fig. 3, lanes 7 and 8). In competition assay, the footprint from −192 to −170 disappeared in the presence of a homologous (−191 to −168) sequence (Fig. 3, lane 3) but not a heterologous HNF1-binding sequence (Fig. 3, lane 4). Importantly, the footprint was also abolished in the presence of the two well characterized RXR/VDR binding sites, namely the functional VDRE from the rat osteocalcin promoter and the ER6 element from the human CYP3A4 promoter (Fig. 3, lanes 5 and 6). Thus the footprinting result of Fig. 3 strongly suggests that the −192 to −170 region of the mouse *Sult2A1* promoter constitutes a specific RXR/VDR binding site.

The −192 to −170 region of the mouse *Sult2A1* promoter shows 84% sequence identity with the corresponding region of the rat *Sult2A1* promoter (Fig. 4A) and the GGGTCA TGAACT sequence within this region (identical for rat and mouse promoters) conforms to an imperfect IR0 arrangement for the consensus (A/G)G(G/T)TCA half-site. This consensus sequence is the typical half-site binding element for all class II nuclear receptors. Because the −192 to −170 region binds to the RXR/VDR complex in vitro, as shown by the footprinting data above, we examined whether the same region would also confer VDR responsiveness to the *Sult2A1* promoter. Indeed, Fig. 4B shows that removal of the −192/−170 sequence through 5′ deletion caused loss of VDR responsiveness for both the mouse and rat *Sult2A1* promoters. Thus, the RXR/VDR-binding IR0 site plays a functional role in the VDR-mediated activation of the rodent *Sult2A1* promoter.

Characterization of the IR0 Element As a VDR-Responsive cis-Regulator of *Sult2A1*. The functional role of the IR0 element in the VDR responsiveness of mouse *Sult2A1* is shown in Fig. 5. Point mutations identical to those present in the IR0-mt oligonucleotide (see also Fig. 6, top) prevented induction of the −292 promoter by the vitamin D activated VDR (Fig. 5A). The IR0 also confers VDR responsiveness upon a heterologous tk promoter (Fig. 5B). HepG2 cells cotransfected with VDR and treated with vitamin D showed a 14-fold stimulation of luciferase expression from the plasmid $(IR0)_3$-tk-Luc but not from tk-Luc. Similar results were also observed in Caco-2 cells (data not shown). VDR responsiveness was abrogated for $(IR0mt)_3$-tk-Luc that contains a mutated IR0 motif.

EMSA performed in the presence of the recombinant VDR and RXR-α showed that the RXR/VDR complex binds specifically to the IR0 element within the footprinted region (Fig. 6A). The gel-shifted DNA-protein complex (marked with an arrow) containing the radiolabeled double-stranded DNA probe (−191 to −168) and VDR plus RXR-α was competed out by the unlabeled homologous oligonucleotide (Fig. 6A, lanes 4 and 5) but not by the HNF1 binding element (Fig. 6A, lane 8). The IR0-mt oligonucleotide, which has a disrupted IR0 motif because of changes in two bases (Fig. 6, top) failed

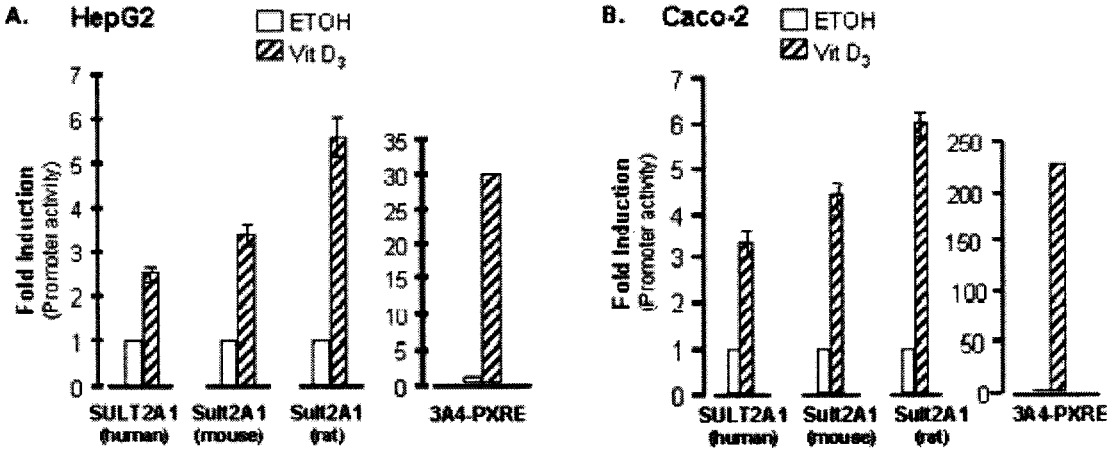

Fig. 2. Induction of the human, mouse and rat Sult2A1 promoters by the vitamin D_3-activated VDR in transfected HepG2 (A) and Caco-2 (B) cells. The cells were cotransfected with a VDR expression plasmid, a promoter-reporter construct (human −1070/+42 *SULT2A1*-Luc, mouse −292/+42 *Sult2A1*-Luc, or rat −215/+38 *Sult2A1*-CAT) along with pRSVL and then treated with either ethanol (vehicle) or vit D_3 for 40 h before analysis. Fold induction (normalized to constant *R. reniformis* luciferase expression) indicates reporter activity (luciferase or chloramphenicol acetyl transferase) in vit D_3-treated cells relative to the vehicle-treated control cells. The reporter construct 3A4-PXRE-Luc contains the proximal promoter of the human CYP3A4-linked to a distally located enhancer from the same promoter. The histograms for the *Sult2A1* promoter activity are averages ± S.D. (standard deviation) from three independent transfections. For CYP3A4 promoter, the average of two different transfection results is shown.

to compete out the labeled probe. On the other hand, the mutB oligonucleotide with five point mutations—all outside the IR0 element—was an effective competitor (Fig. 6A, lane 7). Conversely, mutB oligonucleotide but not IR0-mt oligonucleotide was able to form a specific complex in the presence of VDR and RXR-α (data not shown). Thus the IR0 element of the natural promoters for mouse and rat *Sult2A1* is a binding site for the RXR/VDR complex, and this binding occurs independently of the sequence outside the IR0 site. The gel shifted DNA-protein complex is specific for VDR and RXR-α, because the complex is supershifted by the anti-VDR antibody (Fig. 6A, lane 10; the shifted position shown as an asterisk), and its formation is disrupted in the presence of the anti-RXR-α antibody (Fig. 6A, lane 9). The anti-FXR antibody and the nonimmune serum did not affect this EMSA complex (Fig. 6A, lanes 11 and 12).

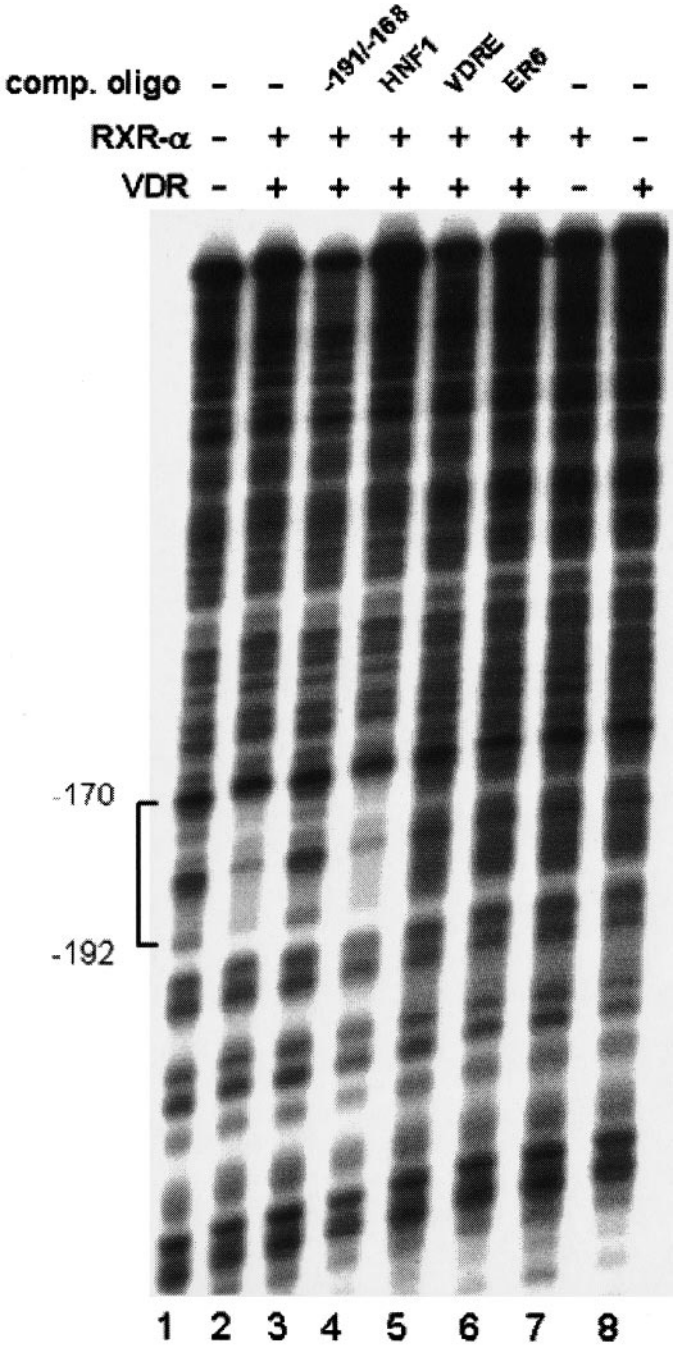

Fig. 3. DNase1 footprinting of a segment of the −292 mouse *Sult2A1* promoter produced by the heterodimeric RXR-α/VDR complex and competition assay for assessing the footprint specificity. DNase1 digestion was performed after incubation of the labeled DNA probe with recombinant GST-RXR-α and VDR (lanes 2–6), VDR alone (lane 7), RXR-α alone (lane 8), and bovine serum albumin (lane 1). The bracketed sequence indicates the footprint. Competition was conducted with various unlabeled duplex oligonucleotides as shown. VDRE is the VDR binding element in the rat osteocalcin promoter (30). The proximal ER6 element is from the human CYP3A4 promoter (at −173 position).

The IR0 element from the rodent *Sult2A1* promoter can compete with the VDRE from the osteocalcin promoter for binding to the RXR/VDR complex (Fig. 6B), because the complex containing VDRE, RXR, and VDR (Fig. 6B, lane 3) was competed out by both the −191/−168 sequence (Fig. 6B, lanes 4 and 5) and by mutB (Fig. 6B, lanes 8 and 9), but not by IR0-mt (Fig. 6B, lanes 6 and 7). As expected, the EMSA complex was also competed out by the homologous VDRE (lane 12) and by ER6 (Fig. 6B, lanes 10 and 11), which is another VDR-responsive element present in the CYP3A4 promoter.

IR0 also binds to RXR/FXR and RXR/PXR heterodimers and mediates induction of the tk promoter by the activated FXR and PXR (Fig. 7). These results are similar to those reported previously (Song et al., 2001; Sonoda et al., 2002). Thus, as shown earlier for the rat promoter, IR0 from the mouse *Sult2A1* promoter binds specifically to FXR/RXR-α, evident from antibody supershifts and competition EMSAs (Fig. 7A) and confers FXR responsiveness upon the heterologous tk promoter (Fig. 7B). Cotransfection of HepG2 cells with increasing amounts of the constitutively activated FXR (expressed from the VP-FXR plasmid containing the transactivation domain of the herpes simplex VP-16 protein), caused a dose-dependent progressive increase in the stimulation of luciferase expression (up to 40-fold) from $(IR0)_3$-tk-Luc relative to the luciferase expression in VP-CMX (vector)-transfected cells. FXR-directed stimulation was much higher (up to ~350-fold) in Caco-2 cells (not shown). Similar dose-dependent responses were also observed with the CDCA activated FXR (not shown). The tk promoter alone, in the absence of IR0, was induced by neither the VP-FXR nor CDCA-activated FXR. The same IR0 element also mediates PXR responsiveness (Figs. 7, C and D). To demonstrate specific binding of the RXR/PXR heterodimer to the IR0 element (Fig. 7C), we used a longer EMSA probe (−220 to −170) instead of the −191/−168 sequence so that the PXR/RXR-α containing EMSA bands (two arrows) could be electrophoretically separated from the background bands that were produced by the unprogrammed reticulocyte lysate. Cotransfection of VP-PXR in HepG2 cells caused more than a 12-fold stimulation of luciferase expression from $(IR0)_3$-tk-Luc (Fig. 7D) but no stimulation of the tk-Luc construct. The IR0-dependent induction of the tk promoter was at least an order of magnitude higher in Caco-2 cells (data not shown). Based on the results presented in Figs. 5 to 7, we conclude that the IR0 element is the focal point for the transcriptional response of *Sult2A1* to three different nuclear receptors (i.e., VDR, FXR, and PXR).

We also examined whether CAR, the constitutively activated xenobiotic receptor that is abundantly expressed in the enterohepatic tissues, regulates the promoter activity of *Sult2A1*. Cotransfection of a CAR expression plasmid (VP-

CAR) in HepG2 and Caco-2 cells caused only a marginal induction (~1.5-fold or less) of the promoters of mouse Sult2A1 (up to −292 bases) and rat Sult2A1 (up to −1000 bases) (I. Echchgadda and B. Chatterjee, unpublished data). In contrast, we observed a strong induction of a proximal human *SULT2A1* promoter containing 270 bases of the upstream sequence by CAR, and a CAR-responsive region within this promoter fragment has been mapped (Echchgadda et al., 2003; C. S. Song, I. Echchgadda, T. Oh, S. A. Kim, S. Y. Ko, L. H. Shi, and B. Chatterjee, manuscript in preparation). Lack of any significant CAR responsiveness of the mouse and rat *Sult2A1* promoters suggests that either CAR is not a significant regulator of rodent Sult2A1 or a CAR responsive cis regulatory element in the rodent promoter lies farther upstream. In

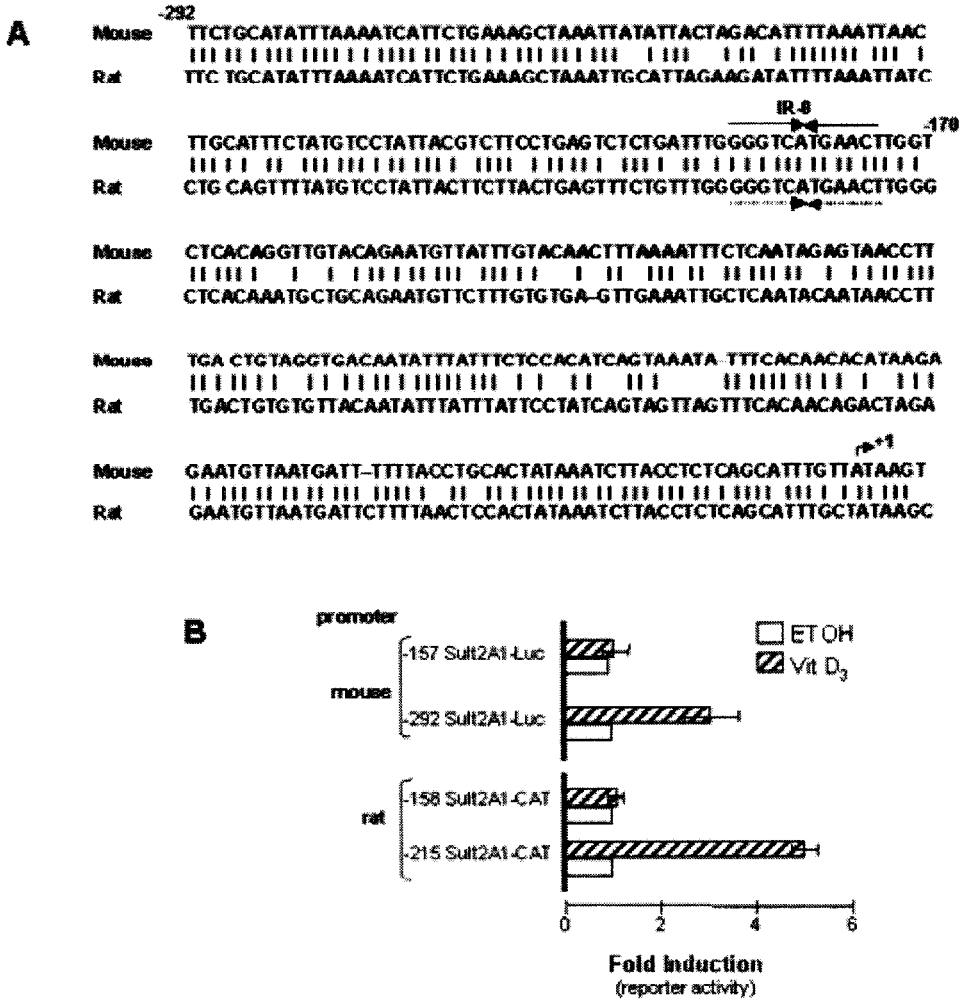

Fig. 4. The IR0 sequence in the rodent Sult2A1 promoter and its role in the promoter responsiveness to VDR. A, sequence alignment for the mouse and rat *Sult2A1* promoters and location of the conserved IR0 motif. B, functional assay for mapping of the VDR responsive region. IR0-containing *Sult2A1* promoters and the corresponding 5′-end deleted fragments lacking the IR0 sequence were analyzed for induction of the promoter activity in VDR-transfected cells treated with vit D_3. Normalized fold inductions of the reporter activity in vit D_3-treated cells relative to the vehicle (ethanol)-treated cells are shown as the average ± S.D. from three independent transfections.

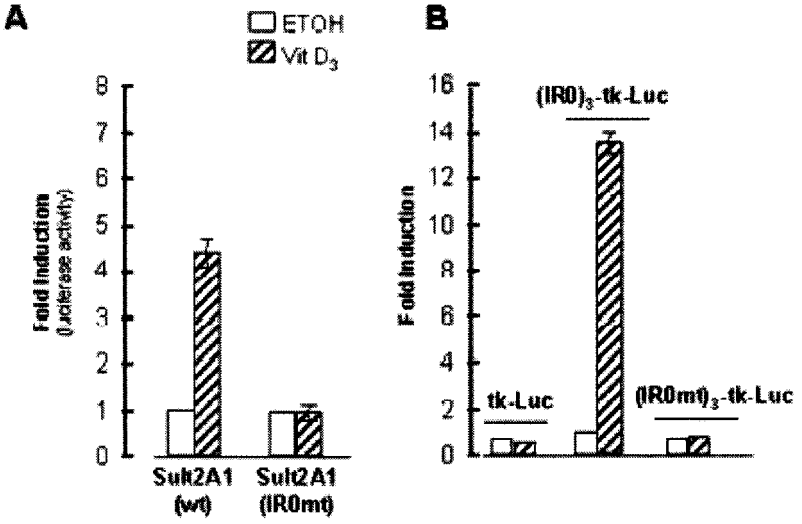

Fig. 5. VDR responsiveness of the natural promoter for mouse *Sult2A1* and thymidine kinase (tk) promoter directed by the IR0 of the mouse promoter. HepG2 cells were cotransfected with the VDR expression plasmid and the promoter-reporter constructs as indicated. Fold stimulations of the normalized luciferase activity in vit D_3-treated cells relative to vehicle treatment are shown. The histograms are average values from three different transfections (each conducted in duplicate) ± S.D.

addition, we explored the effect of the liver X receptor (LXR-α), another RXR-α partner with an important role in cholesterol metabolism (Peet et al., 1998). In a cell-based reporter assay, we observed no effect of LXR-α on the activity of the rodent and human Sult2A1 promoters.

Inhibition of the VDR-Induced Transcriptional Response Caused by Competitive Interactions Involving FXR and PXR at the Common IR0 Element. Because the VDR-responsive IR0 element is also activated by FXR and PXR, we sought to determine whether FXR and PXR would inhibit the VDR signaling at the IR0. Fig. 8A shows competition of FXR with VDR for the IR0 site. HepG2 cells were cotransfected with a fixed amount of VDR (50 ng), increasing amounts of FXR (from 0 to 100 ng), and the reporter construct $(IR0)_3$-tk-Luc (300 ng). The transfected cells were subsequently treated with vitamin D or CDCA or ethanol (vehicle). Cotransfection of increasingly higher amounts of FXR caused a progressive decline in the vitamin D responsiveness of the $(IR0)_3$-tk promoter and a concomitant enhancement in the CDCA-mediated induction of this promoter. The IR0 is a stronger responder to FXR relative to VDR, because at a VDR-to-FXR ratio of 5:1, the vitamin D induction of the $(IR0)_3$-tk-Luc was reduced from 5-fold (in the absence of any exogenous FXR) to 3-fold; at a VDR-to-FXR ratio of 1:1, the vitamin D-mediated induction decreased to 1.3-fold. The vitamin D response was further inhibited (to 1-fold) when the VDR-to-FXR ratio was changed to 1:2. Similar competition at the IR0 element was observed between PXR and VDR (Fig. 8B). The VDR-mediated 5-fold induction of the $(IR0)_3$-tk promoter, observed in the absence of exogenous PXR expression, declined with increasing amounts of PXR cotransfection, and at a VDR-to-PXR ratio of 1:2, the VDR-mediated induction dropped to less than 2-fold. The decline in the promoter response to VDR/vitamin D signaling was accompanied by a concomitant increase in the induction of this promoter by the rifampicin-activated PXR (Fig. 8B). Compared with FXR, PXR was less efficient in inhibiting the VDR-mediated transactivation at the IR0 element.

Discussion

The cellular uptake of nutrients, drugs, and other xenobiotics, their phase I/phase II metabolism and subsequent efflux and excretion require the basal and induced expression of several classes of metabolic proteins and enzymes. These include transporters with import/export functions, the CYP2/CYP3 monooxygenases, and various conjugating transferases, including the members of the sulfotransferase family (Mulder and Jakoby, 1990; Klaassen and Boles, 1997; Willson and Kliewer, 2002). The xenobiotic-sensing nuclear receptors PXR and CAR, and to some extent the glucocorticoid receptor, mediate the activation of the corresponding genes in the liver and intestine, the two primary tissues involved in first-pass metabolism. Recent studies have revealed that VDR is yet another nuclear receptor, which upon activation by vitamin D is able to induce the *CYP2* and *CYP3* genes in the enterohepatic tissues (Drocourt et al., 2002; Makishima et al., 2002). In addition, LCA and its major metabolite 3-ke-

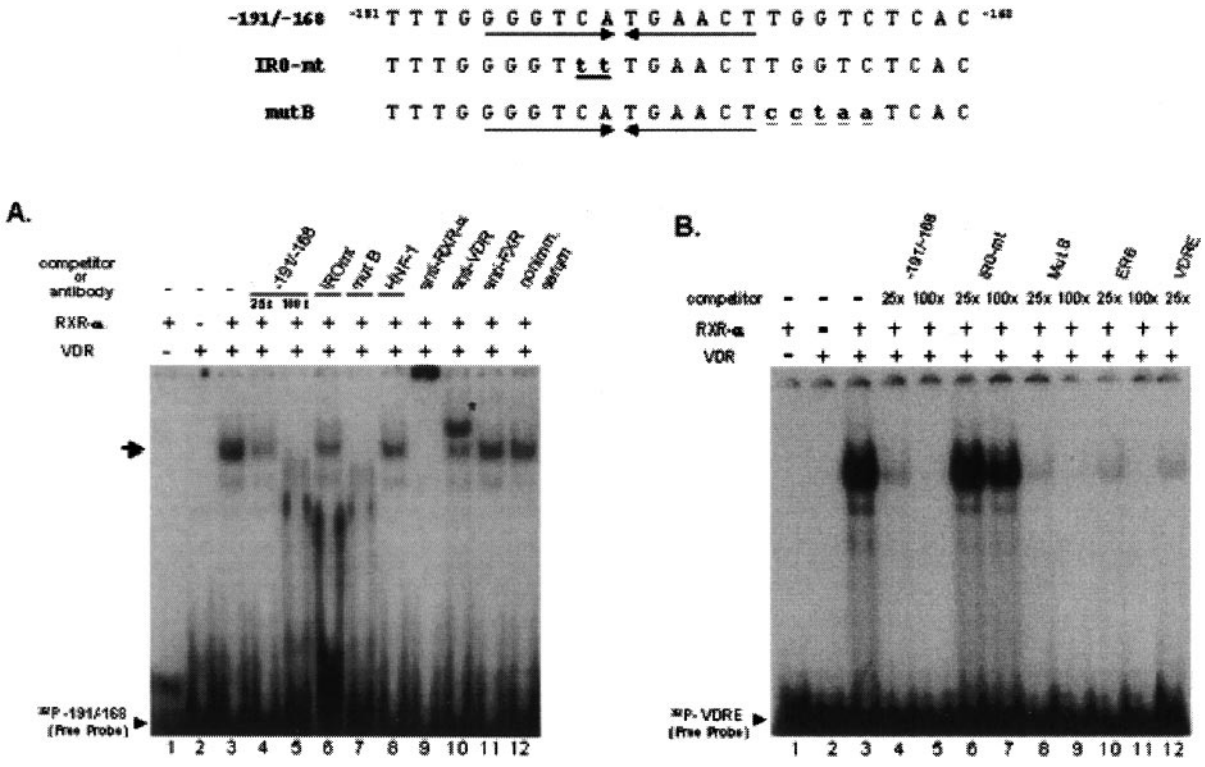

Fig. 6. The IR0 element as the binding site for the RXR-α/VDR heterodimer. Specific binding of RXR/VDR heterodimer to the IR0 sequence of the mouse *Sult2A1* promoter (A) and to the VDRE of the rat osteocalcin promoter (B) are shown using competition EMSA and antibody supershift. The baculovirus-expressed VDR and bacterially expressed RXR-α as a GST-RXR-α fusion were used in the assay. Double-stranded ^{32}P-labeled oligonucleotides from the mouse *Sult2A1* promoter (−191 to −168; A) and rat osteocalcin promoter (−449 to −425; B) were used as the DNA probes. Sequences of competitor oligonucleotides are shown at the top. IR0mt contains point mutations within the IR0 motif; mut B oligonucleotide contains point mutations outside the IR0 motif. The consensus sequence for the HNF1-binding element (HNF1) and the VDRE sequence from the rat osteocalcin promoter are described under *Materials and Methods*.

to-LCA bind and activate VDR at micromolar concentrations. The LCA activated VDR induces *CYP3* gene expression in the liver and intestine. The induced P450 activity in turn catalyzes oxidation of LCA to the polar 6α- and 6β-hydroxyl derivatives, which are easily solubilized and thus rapidly excreted. Importantly, VDR binds LCA more avidly than the bile acid receptor FXR; as an efficient sensor of secondary bile acids, VDR is likely to play a vital role in the metabolic clearance of LCA (Makishima et al., 2002). The finding on the VDR regulation of the CYP system opens up the possibility that other enzymes in the xenobiotic pathway may be similarly regulated.

In the present study, we show that SULT2A1 is a target for transcriptional induction by VDR in liver (HepG2) and intestinal (Caco-2) cells. The endogenous SULT2A1 mRNA and protein were induced in these cells in response to treatment with 10 nM 1α,25-dihydroxy vitamin D_3. The stimulated SULT2A1 expression reflected transcriptional regulation because the corresponding human, mouse and rat promoters were induced >5-fold in HepG2 and Caco-2 cells that were cotransfected with VDR and treated with vitamin D_3. An imperfect IR0 element (GGGTCATGAACT) within the first 200 bases of the upstream mouse and rat *Sult2A1* promoters specifically bound to RXR/VDR heterodimer, and this IR0 rendered the heterologous tk promoter responsive to transcriptional induction by VDR in the presence of vitamin D. The VDR-responsive human *SULT2A1* promoter fragment (−1070 to +42) does not contain a similar IR0 element. Identification and characterization of the VDR-responsive DNA element in the human *SULT2A1* promoter is an area of ongoing investigation in our laboratory.

Despite the earlier findings that the IR0 element in the rodent *Sult2A1* promoter is a functional FXR- and PXR-responsive element (Song et al., 2001; Sonoda et al., 2002), we conclude that the *Sult2A1* promoter is directly induced by the VDR via the IR0 element, because neither FXR nor PXR is activated by vitamin D, and the VDR/vitamin D pathway does not stimulate PXR expression (Schmiedlin-Ren et al., 2001). The classic VDRE in VDR target genes is primarily configured as a DR3 motif; however, alternate arrangements such as DR4, DR6, IR9, and ER6 can also mediate VDR induction of several vita-

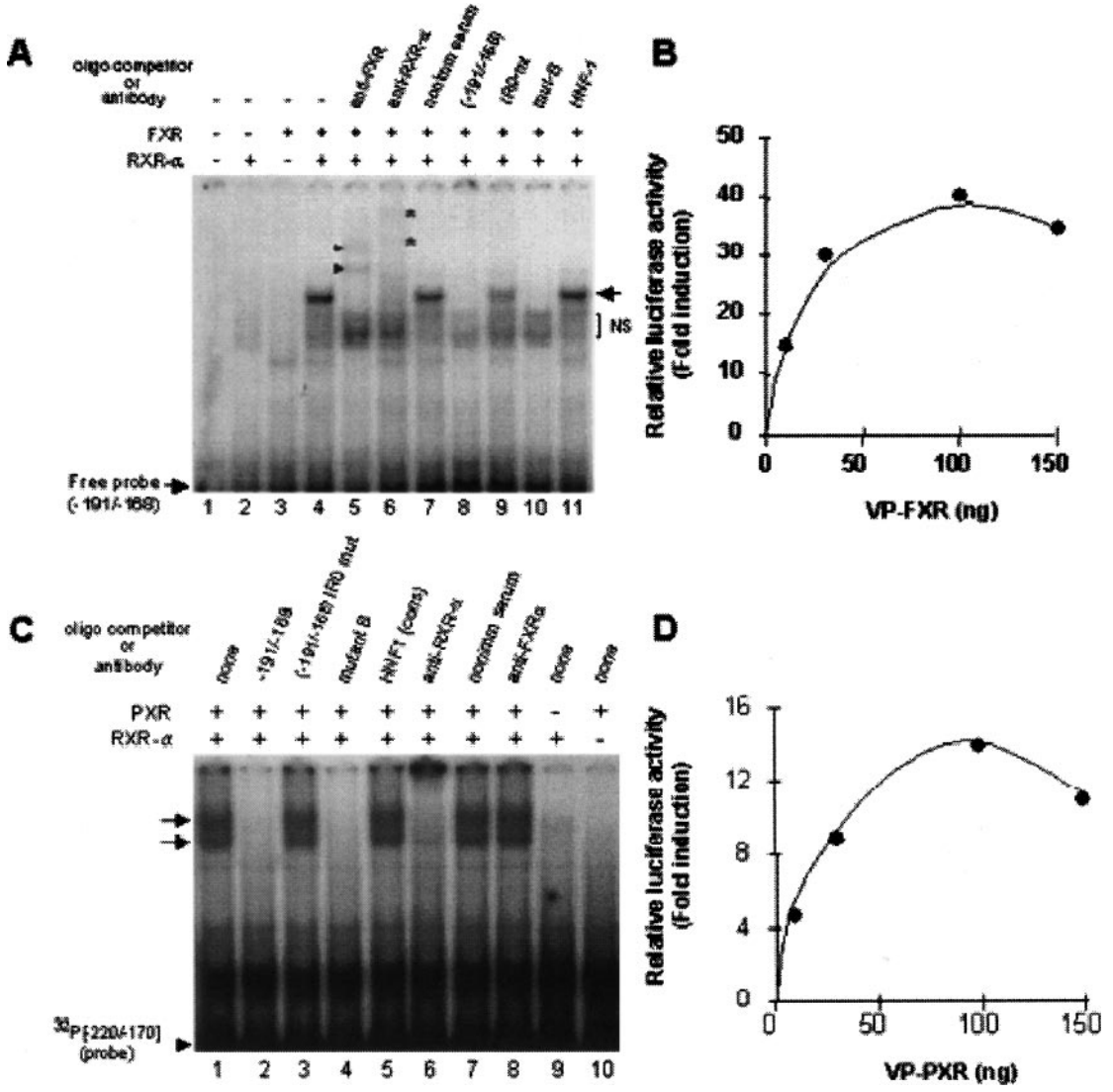

Fig. 7. IR0 as a FXR responsive and PXR responsive element. A and C, specific binding of FXR/RXR-α (A) and PXR/RXR-α (C) revealed by competitive EMSA and antibody supershift of the EMSA complex. B and D, induction of the reporter construct $(IR0)_3$-tk-Luc by increasing amounts of the constitutively active VP-FXR (B) and VP-PXR (D). Fold induction is relative to the basal luciferase activity in cells cotransfected with the control vector (VP-CMX). All values are normalized to constant *R. reniformis* luciferase expression. Each point is the average from two independent transfection assays.

min D-responsive genes (Toell et al., 2000). Our result reveals IR0 as yet another *cis*-element that functions as a VDRE in the context of a natural gene. The ability of the same receptor complex (in this case, the VDR/RXR heterodimer) to bind and induce DNA elements with such varying spacer lengths separating the two consensus half sites is thought to be a result of the conformational flexibility of these sequence motifs as well as the ability of the DNA-bound receptor to assume multiple configurations (Drocourt et al., 2002).

The concentration at which vitamin D_3 was effective in inducing endogenous *SULT2A1* and the corresponding transfected promoter in HepG2 and Caco-2 cells (10 to 50 nM) falls within the normal range of the vitamin D level in adult blood (19 to 190 nM; Drocourt et al., 2002). Thus, it is likely that the basal SULT2A1 expression in the enterohepatic tissues is at least partly controlled by VDR. Recently it has been reported that the female mice that are null-mutated for FXR or PXR show higher basal expression of Sult2A1 in the liver than that shown by the wild-type counterpart (Kitada et al., 2003). This increase in Sult2A1 expression may functionally compensate for the reduced expression of various FXR- and PXR-regulated transporters, including Bsep, Mrp2, and Mdr1. It is likely that VDR and possibly CAR mediate increased Sult2A1 expression in FXR-null, PXR-null, and combined FXR-/PXR-null mice. Furthermore, because VDR is present at a relatively high level in the intestine, its role in stimulating SULT2A1 expression in the enteric cells may be particularly significant. A high-fat, Western-type diet is thought to contribute to colon cancer in part by causing elevated production of LCA in the enteric tract (Makishima et al., 2002, and references therein). The primary metabolite of LCA is its 3α-sulfate form, which, because of poor reabsorption into the enterohepatic circulation, is readily eliminated through fecal excretion (Cowen et al., 1975; Hofmann, 1994). SULT2A1 is the exclusive sulfating enzyme for LCA (Falany, 1997). We currently have no direct evidence for the induction of SULT2A1 by the LCA-activated VDR. Nevertheless, LCA is reported to induce *CYP3A4* through VDR-mediated transactivation (Makishima et al., 2002), and SULT2A1 may be similarly transactivated. It is reasonable to propose that enhanced *SULT2A1* expression by the accumulated LCA would facilitate sulfation of this secondary bile acid, thus providing for a protective mechanism against the fat-induced intestinal buildup of LCA. In addition, in the event that the liver encounters pathologic levels of LCA caused by specific metabolic disorders, increased SULT2A1 expression by the LCA-activated VDR can help shield the hepatobiliary system from LCA-induced injury. The VDR pathway in this case is particularly important given that LCA is an FXR antagonist (Yu et al., 2002); thus, LCA should not activate SULT2A1 expression via FXR signaling. Indeed, a protective role of Sult2A1 against LCA-induced toxicity has been demonstrated in the mouse liver (Kitada et al., 2003).

The present study shows that the induction of the IR0 element by the vitamin D_3-activated VDR was inhibited by the cotransfected FXR or PXR. Competition of FXR for VDR signaling was more efficient than that of PXR, because with a constant level of VDR, 50 ng of FXR cotransfection resulted in ~75% inhibition of the VDR function compared with the ~52% inhibition rendered by 50 ng of the cotransfected PXR (Fig. 8, A and B). Functional interference of PXR and CAR with the VDR/vitamin D signaling on the *CYP3A4* gene involving common response elements was reported previously (Drocourt et al., 2002). It has been speculated that the reduction in the serum vitamin D level in healthy persons associated with prolonged rifampicin treatment (Brodie et al., 1982) and that impaired bone mineralization caused by abnormal vitamin D me-

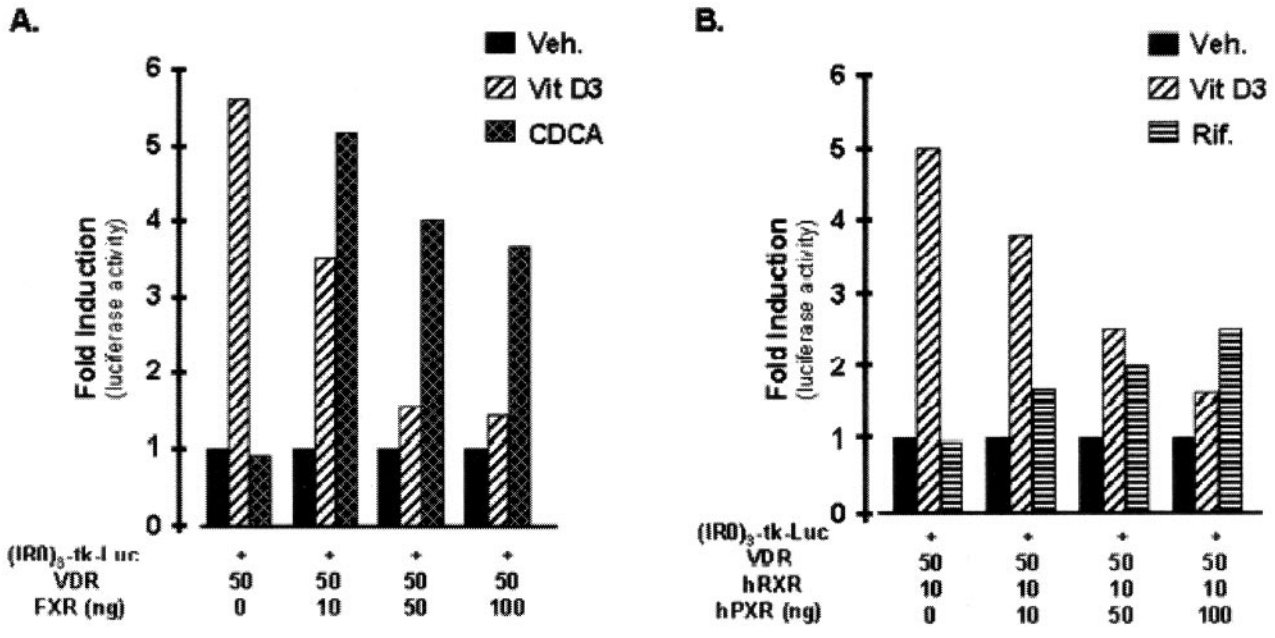

Fig. 8. Competitive functional interactions of FXR and PXR with VDR for the VDR-induced transcriptional responses at the IR0 element. A, interactions between VDR and FXR; B, interactions between VDR and PXR. HepG2 cells were cotransfected with the $(IR0)_3$-tk-Luc and pRSVL, a constant amount of either the VDR expression plasmid (A) or VDR expression plasmid plus RXR-α expression plasmid (B) along with increasing amounts of either the rat FXR-expression plasmid (A) or the human PXR expression plasmid (B). DNAs were normalized to constant amounts using the empty vector. The transfected cells were treated with vit D_3, CDCA (FXR ligand), or ethanol (for the experiments in A) and vitD3, rifampicin (a ligand for human PXR), or ethanol (for the experiments in B). The ligand-dependent fold stimulation of the normalized luciferase activity was determined relative to the vehicle treatment. Histograms are averages of two independent transfections, each conducted in duplicate.

tabolism/function associated with the clinical use of rifampicin and phenobarbital (D'Erasmo et al., 1998) may be related to the functional antagonism involving PXR/CAR and VDR. In the case of Sult2A1, if the observed negative cross-talk involving VDR, FXR, and PXR holds true in vivo, it would imply that at a given physiological state, the hormonal and metabolic milieu of the cell should determine which of the competing nuclear receptors will assume a dominant role in activating the sulfonation pathway via stimulated SULT2A1 expression.

In summary, the present work demonstrates that SULT2A1 is transcriptionally induced by the vitamin D_3-activated VDR. In the case of the mouse and rat Sult2A1 promoters, the induction is mediated by an IR0 element, which in previous reports was established to be a functional FXR- and PXR-responsive element. We show that FXR and PXR inhibit VDR-mediated transactivation because of competitive interactions at the IR0 element. It has been reported that genetic polymorphism for SULT2A1 can cause reduced expression and activity of this enzyme (Thomae et al., 2002). We are currently investigating the role of VDR in basal and regulated Sult2A1 expression in the enterohepatic tissues in mice in response to various endobiotic and xenobiotic challenges. These in vivo studies should provide insights into the molecular basis for the interindividual differences in the efficiency at which the sulfonation pathway is used to metabolize diverse SULT2A1 substrates, such as steroids, bile acids, therapeutic drugs, and many other xenobiotics, including environmental estrogens.

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This article is dedicated to the memory of Dr. Arun K. Roy.

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