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Expression of *CYP3A4*, *CYP2B6*, and *CYP2C9* Is Regulated by the Vitamin D Receptor Pathway in Primary Human Hepatocytes*

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The fully active dihydroxylated metabolite of vitamin D_3 induces the expression of *CYP3A4* and, to a lesser extent, *CYP2B6* and *CYP2C9* genes in normal differentiated primary human hepatocytes. Electrophoretic mobility shift assays and cotransfection in HepG2 cells using wild-type and mutated oligonucleotides revealed that the vitamin D receptor (VDR) binds and transactivates those xenobiotic-responsive elements (ER6, DR3, and DR4) previously identified in *CYP3A4*, *CYP2B6*, and *CYP2C9* promoters and shown to be targeted by the pregnane X receptor (PXR) and/or the constitutive androstane receptor (CAR). Full VDR response of various *CYP3A4* heterologous/homologous promoter-reporter constructs requires both the proximal ER6 and the distal DR3 motifs, as observed previously with rifampicin-activated PXR. Cotransfection of a *CYP3A4* homologous promoter-reporter construct (including distal and proximal PXR-binding motifs) and of PXR or CAR expression vectors in HepG2 cells revealed the ability of these receptors to compete with VDR for transcriptional regulation of *CYP3A4*. In conclusion, this work suggests that VDR, PXR, and CAR control the basal and inducible expression of several *CYP* genes through competitive interaction with the same battery of responsive elements.

Cytochrome P450 (CYP)[1] enzymes are mainly expressed in the liver and catalyze the metabolic conversion of xenobiotics, including environmental pollutants and drugs, to more polar and easily disposable derivatives (2, 3). *CYP* genes from the *CYP2* and *CYP3* families are inducible by many xenobiotics, notably including barbiturates and rifampicin. Two nuclear receptors, the pregnane X receptor (PXR; NR1I2) and the constitutive androstane receptor (CAR; NR1I3), have recently been shown to mediate *CYP2* and *CYP3* gene induction in animals and man (4–6). Both PXR and CAR form heterodimers with the retinoid X receptor (RXR; NR2B1). PXR is activated by a wide spectrum of xenobiotics and steroids (4, 7, 8) and controls *CYP3A4* and *CYP3A7* induction by targeting two specific responsive elements present in the regulatory region of these genes (4, 7–12). The first of these is the proximal PXR-responsive element, located at –160. It consists of an everted repeat of the nuclear receptor half-site AGGTCA separated by 6 nucleotides (ER6); this element is necessary but not sufficient for full transactivation of the *CYP3A4* promoter. Indeed, full PXR-mediated induction requires the presence of a second distal xenobiotic-responsive element (dPXRE), located between –7800 and −7200 (9). This element is composite and consists of two direct repeats separated by 3 nucleotides (DR3), encompassing an ER6 motif. In contrast to PXR, CAR is sequestered in the cytoplasm and translocates into the nucleus upon activation, notably in response to phenobarbital (6, 13). Several groups have identified a complex phenobarbital-responsive element module that consists of two nuclear receptor-binding sites (termed NR1 and NR2) and one nuclear factor 1 binding site (12, 14). Both NR1 and NR2 are imperfect DR4 motifs and essential for phenobarbital induction of *CYP2B* genes. In human *CYP2B6*, the phenobarbital-responsive element module is located between –1684 and –1733 and has been shown to bind to and be transactivated by CAR and by PXR (12, 15).

Previous reports revealed that 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$), the most active metabolite of vitamin D_3, behaves as a transcriptional inducer of *CYP3A4* in the colic carcinoma Caco-2 cell line and in the human intestinal LS180 cell line (16, 17). Vitamin D_3 function is mediated through the vitamin D receptor (VDR; NR1I1), which, after binding 1α,25-$(OH)_2D_3$ with high affinity, forms heterodimers with RXR (18–20). The heterodimer then binds to and transactivates the vitamin D receptor-responsive elements (VDREs) present in the regulatory region of target genes (21). The classical VDREs consist of a direct repeat of nuclear receptor half-sites separated by 3 nucleotides (DR3) (18). In the classical vitamin D-responsive organs, including the intestine, bone, kidney, and parathyroid gland, vitamin D_3-activated VDR plays a central role in the regulation of calcium and phosphate homeostasis, bone mineralization and resorption, inhibition of cell growth, and parathyroid hormone synthesis (22). VDR is also expressed in many other non-classical vitamin D-responsive organs, including the liver, muscle, skin, immune system, pancreas, and brain, and in cancer cells (23), in which it controls a number of biological processes, including immunomodulation, tissue regeneration, inhibition of cell growth and apoptosis, and cell differentiation (24–26).

In an exploratory part of this work, we found that 1α,25-$(OH)_2D_3$ is an inducer of *CYP3A4* in human hepatocytes, as previously observed by others in intestinal cell lines (16, 17).

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[1] The abbreviations used are: CYP, cytochrome P450; ER, everted repeat (prefixes "p" and "d" indicate proximal and distal, respectively); DR, direct repeat; NR, nuclear receptor; dPXRE, distal pregnane X-responsive element; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; VDRE, vitamin D-responsive element; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; rANF, rat atrial natriuretic factor. The official nomenclature system for the nuclear receptor superfamily has been used in this work (1): PXR, pregnane X receptor (NR1I2); CAR, constitutive androstane receptor (NR1I3); RXR, retinoid X receptor (NR2B1); VDR, vitamin D receptor (NR1I1).

We therefore thought that VDR might be able to target PXR- and/or CAR-responsive elements of *CYP3A4*. We further reasoned that, if the hypothesis is correct, 1α,25-$(OH)_2D_3$ could be an inducer of other *CYP* genes controlled by these receptors. The data presented here show that 1α,25-$(OH)_2D_3$ induces not only *CYP3A4*, but also *CYP2B6* and *CYP2C9* in primary human hepatocytes. In addition, we show that VDR is able to bind and transactivate different motifs recognized by xenobiotic-activated PXR and CAR in the promoters of these *CYP* genes.

EXPERIMENTAL PROCEDURES

Materials—Ham's F-12 medium, Williams' medium E, 1α,25-$(OH)_2D_3$, vitamins, hormones, collagenase (type IV), dimethyl sulfoxide, and dexamethasone were purchased from Sigma. Collagen-coated culture dishes were obtained from Corning (Iwaki, Japan). [α-^{32}P]dCTP, [α-^{32}P]UTP, and ECL developing reagents were purchased from Amersham Biosciences.

Plasmids—The ΔATG-hPXR expression plasmid was generated by PCR amplification of cDNA encoding amino acids 1–434 of human PXR (kindly provided by Dr. S. Kliewer, Glaxo Wellcome) using oligonucleotides 5′-gggtgtggggaattcaccaccatggaggtgagacccaaagaaagc and 5′-gggtgtgggggatcctcagctacctgtgatgccg and insertion into pSG5 (Stratagene, La Jolla, CA) digested with *Eco*RI/*Bam*HI. The mouse CAR expression vector (pCR3-mCAR) was kindly provided by Dr. M. Negishi (NIEHS, Research Triangle Park, NC).

Homologous Construct Plasmids—The CYP3A4 5′-flanking fragment (−262/+11) containing the proximal ER6 (pER6) element was generated by PCR from a previously isolated genomic clone (27) used as a template and from oligonucleotides that create artificial cloning sites for KpnI (5′ end of the oligonucleotide) and SmaI (3′ end of the oligonucleotide). This fragment was cloned into pGL3-basic (Promega, Madison, WI) upstream of a luciferase reporter gene to generate the homologous construct plasmid p(3A4-pER6)-LUC. Plasmids p(3A4–5′dDR3/dER6/3′dDR3/pER6)-LUC and p(3A4–5′dDR3/dER6/pER6)-LUC were generated by inserting the −7800/−7200 or −7800/−7600 region of CYP3A4 (9), respectively, amplified by PCR from human genomic DNA into plasmid p(3A4-pER6)-LUC digested with *Kpn*I.

Heterologous Construct Plasmids—Plasmids p(3A4–5′dDR3/dER6/3′dDR3)-tk-LUC and p(3A4–5′dDR3/dER6)-tk-LUC were constructed as indicated above for the homologous constructs, except that the amplified regions were cloned in pGL3-basic upstream of the luciferase reporter gene driven by the thymidine kinase promoter. Plasmid p(2C9-$(DR4)_4$)-SV40-LUC was generated by cloning four copies of the oligonucleotide 2C9-DR4 (28) upstream of a luciferase reporter gene driven by the SV40 promoter in the pGL3 vector. Plasmid p(2B6-$(NR1)_3$)-tk-LUC was generated by cloning three copies of the NR1 (2B6–3′DR4) motif of human *CYP2B6* (12) upstream of a luciferase reporter gene driven by the thymidine kinase promoter in the pGL3 vector. Plasmids p(3A4-$(dDR3)_3$)-tk-LUC and p(3A4-$(pER6)_3$)-tk-LUC were generated by cloning three copies of the respective motif of human *CYP3A4* (9) upstream of a luciferase reporter gene driven by the thymidine kinase promoter in the pGL3 vector.

Cell Culture and Transfections—Human hepatocarcinoma HepG2 cells (purchased from the European Collection of Cell Cultures, Salisbury, England) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Transfection of plasmid DNA was performed in single batches with FuGENE 6 (Roche Molecular Biochemicals) as recommended by the manufacturer. Transfections were performed using 100,000 cells, 250 ng of reporter plasmid, and 50 ng of pSG5-hVDR expression vector (provided by Dr. P. Balaguer, INSERM U439, Montpellier, France). For competition experiments, we used 500 ng of reporter plasmid and 100 ng of pSG5-hVDR expression vector. Cotransfection of human PXR or mouse CAR was performed using increasing concentrations (10, 50, 100, and 300 ng) of both expression vectors, and pSG5 empty vector was added to normalize the total concentration of transfected plasmid DNA. As an internal control of transfection, 25 ng of pSV-β-galactosidase (Promega) was used in all experiments. After 16 h, the medium was changed, and fresh medium containing 0.1% dimethyl sulfoxide or inducers was added. Cells were harvested in reporter lysis buffer (Promega) after 24 h of incubation, and cell extracts were analyzed for luciferase and β-galactosidase activities as described (11).

Liver Samples and Hepatocyte Cultures—Hepatocytes were prepared from liver lobectomy segments resected from adult patients for medically required purposes unrelated to our research program. Three different cultures from three different liver donors were made in this work: FT181 (a 51-year-old male who became an organ donor after a car accident; the liver was not transplanted because of the presence of a kidney tumor), FT187 (a 67-year-old male who underwent a liver lobectomy for metastasis of a colon tumor), and FT189 (a 48-year-old male who underwent a liver lobectomy for metastasis of a sigmoid colon tumor). Hepatocytes were prepared and cultured according to the previously published procedure (29, 30). The cells were plated onto 100-mm plastic dishes precoated with collagen at 10×10^6 cells/plate in a total volume of 6 ml of a hormonally and chemically defined medium consisting of a mixture of Williams' medium E and Ham's F-12 medium (1:1 by volume). Forty-eight hours after plating, cells were cultured in the presence of the indicated concentrations of 1α,25-$(OH)_2D_3$ for an additional 24 h. Total RNA and protein were isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions.

RT-PCR Experiments—Reverse transcription was performed with 1 μg of mRNA using the Moloney murine leukemia virus reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. One-tenth of the RT reaction was then subjected to PCR.

Quantitative PCR—Quantification of CYP3A4, CYP2B6, CYP2C9, and GAPDH mRNAs was performed using the Roche Molecular Biochemicals Light Cycler apparatus. For CYP3A4, CYP2B6, and GAPDH, the following program was used: denaturation at 95 °C for 8 min and 40 cycles of PCR consisting of denaturation at 95 °C for 15 s, annealing at 70 °C for 6 s, and extension at 72 °C for 12 s. In all cases, the quality of the PCR product was assessed by monitoring a fusion step. For CYP2C9, the same program was used, except for the annealing, which was performed at 60 °C. Forward and reverse primers were as follows, respectively: CYP3A4, 5′-CACAAACCGGAGGCCTTTTG-3′ and 5′-ATCCATGCTGTAGGCCCCAA-3′; GAPDH, 5′-GGTCGGAGTCAACGGATTTGGTCG-3′ and 5′-CAAAGTTGTCATGGATGACC-3′; CYP2B6, 5′-GGCCATACGGGAGGCCCTTG-3′ and 5′-AGGGCCCCTTGGATTTCCG-3′; CYP2C9, 5′-TCCTATCATTGATTACTTCCCG-3′ and 5′-AACTGCAGTGTTTTCCAAGC-3′; and fructose-1,6-bisphosphatase, 5′-CCCCGCGCTCTACCCGGTTCA-3′ and 5′-TGTGTGAGACAAAAGGTCCA-3′ (31).

In Vitro Translation and Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were performed using VDR and RXR prepared by *in vitro* translation using a coupled transcription-translation system (Promega). Proteins were incubated for 20 min at room temperature with 50,000 cpm of T4 polynucleotide kinase-labeled oligonucleotides in 10 mM Tris (pH 8), 6% glycerol, 1 mM dithiothreitol, 1 μg/μl poly(dI-dC) (Amersham Biosciences). The mixture was then submitted to electrophoresis on a 4% polyacrylamide gel in 0.5× buffer containing 45 mM Tris base, 45 mM boric acid, and 1 mM EDTA. The following oligonucleotides were used either as radiolabeled probes or as competitors (the sense strand is shown, with hexanucleotides in boldface): CYP3A4-pER6, 5′-TAGAATA**TGAACT**CAAAGG**AGGTCA**GTGAGT-3′; CYP3A-5′dDR3, 5′-GAA**TGAACT**TGC**TGACCC**TCT-3′; CYP3A4–5′dDR3mutant, 5′-GAA**TCCCCA**TGC**TAATCT**TCT-3′; CYP3A4-dER6, 5′-CCCT**TGAAAT**CATGTC**GGTTCA**AGCA-3′; CYP2B6-DR4, 3′-AC**TGTACT**TTCC**TGACCC**TGAAGA-5′; CYP2C9-DR4, 5′-AAC**CAAACT**CTTC**TGACCT**CTCAATCTAGTCAACTGGG-3′; and rat atrial natriuretic factor (rANF) VDRE, 5′-GTCAG**AGGTCA**TGA**AGGACA**TTACA-3′ (32). Anti-RXRα antibody (N197, sc 774X, Santa Cruz Biotechnology) was used for the "supershift" assays. Autoradiography was carried out by exposing the dried gel to Kodak X-AR film.

RESULTS

Induction of CYP Genes by 1α,25-$(OH)_2D_3$ in Human Hepatocytes—Forty-eight hours after plating, hepatocytes were treated either with increasing concentrations (1–100 nM) of 1α,25-$(OH)_2D_3$ or, in parallel, with 10 μM rifampicin for 24 h. CYP mRNAs were then analyzed by both classical and real-time quantitative RT-PCR using the Light Cycler apparatus. In a preliminary series of experiments, we verified that hepatocytes responded as expected to 1α,25-$(OH)_2D_3$ in our culture model. For this purpose, the expression of the fructose-1,6-bisphosphatase gene, a gene known to be induced through VDR activation (31), was evaluated in response to increasing concentrations of 1α,25-$(OH)_2D_3$. The results are shown in Fig. 1. Expression of fructose-1,6-bisphosphatase mRNA as assessed by RT-PCR analysis was induced as expected. Analysis of the same RNA samples revealed that 1α,25-$(OH)_2D_3$ was a potent

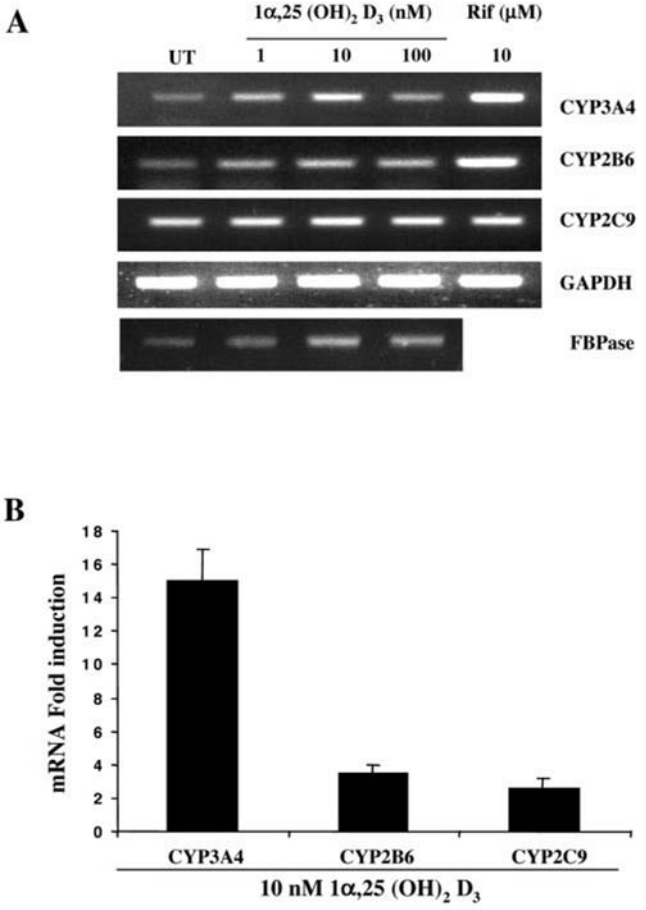

FIG. 1. **Induction of CYP3A4, CYP2B6, CYP2C9, and fructose-1,6-bisphosphatase mRNAs by 1α,25-$(OH)_2D_3$ in human hepatocytes.** Forty-eight hours after plating, hepatocytes were untreated (*UT*) or treated with increasing concentrations of 1α,25-$(OH)_2D_3$ (1–100 nM). Twenty-four hours later, total RNA was extracted and analyzed by RT-PCR. *A*, shown are the results obtained with culture FT187. Expression of CYP3A4, CYP2B6, CYP2C9, GAPDH, and fructose-1,6-bisphosphatase (*FBPase*) mRNAs was assessed by semiquantitative RT-PCR as described under "Experimental Procedures." PCR products exhibited the expected size and were analyzed on agarose gel after exposition to 1% ethidium bromide. *B*, CYP3A4, CYP2B6, CYP2C9, and GAPDH mRNAs were quantified by real-time RT-PCR analysis using the Light Cycler apparatus, and the quality of the PCR products was controlled through fusion step analysis at the end of each PCR run. Data presented are means (from three different cultures from three different liver donors, FT181, FT187, and FT189) of the ratio of mRNA levels in vitamin D-treated cells to corresponding levels in untreated cells, normalized with respect to GAPDH mRNA levels, which themselves exhibited no significant variation. *Rif*, rifampicin.

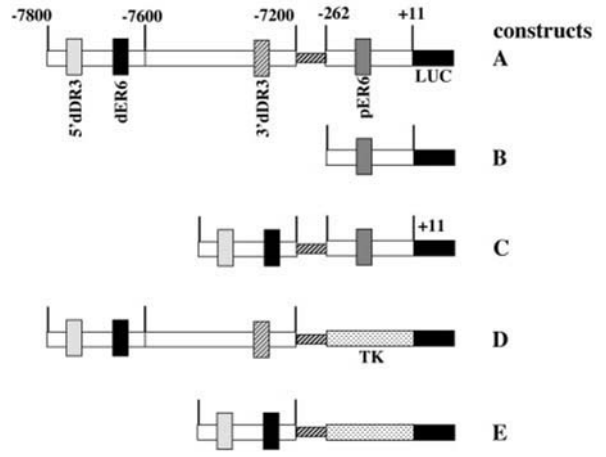

FIG. 2. **PXR-responsive elements present in the *CYP3A4* gene.** Shown is a schematic representation of the *CYP3A4* constructs used in this work. Constructs A–C are homologous constructs, and constructs D and E are heterologous constructs with the thymidine kinase (*TK*) gene promoter upstream of the luciferase reporter gene (*LUC*).

and concentration-dependent inducer of CYP3A4 mRNA and a modest inducer of CYP2B6 and CYP2C9 mRNAs, the maximum accumulation being reached at 10 nM (Fig. 1**)**. Next, real-time quantitative RT-PCR was used to evaluate the induction ratios (mRNA levels in treated cells compared with control cells) obtained from the analysis of three different cultures from three different liver donors. Induction ratios were as follows: 15 ± 2 for CYP3A4 mRNA, 3.5 ± 1 for CYP2B6 mRNA, and 2.6 ± 1 for CYP2C9 mRNA. In comparison, rifampicin induction ratios were 50 ± 15 for CYP3A4 mRNA, 10 ± 3 for CYP2B6 mRNA, and 3.3 ± 1.5 for CYP2C9 mRNA. This last gene was recently shown to be positively regulated by rifampicin and phenobarbital through PXR/CAR activation (28, 33). GAPDH mRNA levels used as quality controls of RNA preparations were not affected significantly by 1α,25-$(OH)_2D_3$. The finding that 1α,25-$(OH)_2D_3$ induced CYP mRNAs within the nanomolar concentration range suggested a classical vitamin D_3 receptor-mediated mechanism of induction. Although the consensus VDRE is a DR3 motif, this nuclear receptor has been shown to bind other motifs, including DR4, DR6, and inverted palindromes (32, 34). We therefore suspected that *CYP2B6*, *CYP2C9*, and *CYP3A4* induction by 1α,25-$(OH)_2D_3$ could be mediated by VDR through the previously identified PXR- and CAR-responsive elements.

VDR-RXR Heterodimer Binds the PXR-responsive Elements of the CYP3A4 Promoter—The PXR-responsive elements of *CYP3A4* consist of pER6 (−160), hereafter referred to as 3A4-pER6, and a distal enhancer (−7800/−7200) containing three nuclear receptor motifs, referred to hereafter as 3A4–5′dDR3, 3A4-dER6, and 3A4–3′dDR3 (Fig. 2). These elements correspond to dNR1, dNR2, and dNR3, identified by Goodwin *et al.* (9), respectively. dNR1 and dNR3 have been reported to be the key elements conferring enhancer activity. Gel mobility shift assays were performed to determine whether VDR interacts with these elements.

First, we checked the binding of the *in vitro* translated VDR-RXR heterodimer to a consensus VDRE oligonucleotide (rANF-DR3) by gel shift assay as shown in Fig. 3*A*. As expected, a retarded band was observed when both VDR and RXR were incubated with the target oligonucleotide (*lane 4*), but not when these receptors were incubated alone (*lanes 2* and *3*). Anti-RXR antibodies produced a supershift (*lane 5*), whereas an excess of unlabeled rANF-DR3 oligonucleotide suppressed the retarded band (data not shown). In addition, the specific VDR·RXR·DNA complex was suppressed in a dose-dependent manner when incubated in the presence of a 5- or 50-molar excess of unlabeled 3A4–5′dDR3 (*lanes 8* and *9*) or 3A4-pER6 (*lanes 10* and *11*). This suggests that these elements can be targeted by the VDR-RXR heterodimer. In contrast, an excess of the 3A4-dER6 oligonucleotide did not produce any suppression of the VDR·RXR·VDRE complex (*lanes 6* and *7*).

In the next series of experiments, we used the same assay to investigate the binding of VDR to both the 3A4–5′dDR3 and 3A4-pER6 oligonucleotides. As expected, no complex was observed when the probes were incubated with VDR or RXR alone (Fig. 3, *B*, *lanes 2* and *3*; and *C*, *lane 1*). In agreement with the data presented in Fig. 3*A*, a retarded band was observed when 3A4–5′dDR3 (Fig. 3*B*, *lane 4*) or 3A4-pER6 (Fig. 3*C*, *lane 2*) was incubated in the presence of the VDR·RXR complex, and anti-RXR antibodies produced a supershift of the band (Fig. 3, *B*, *lane 5*; and *C*, *lane 3*). The specificity of the interaction was confirmed by competition experiments using 10- and 100-fold

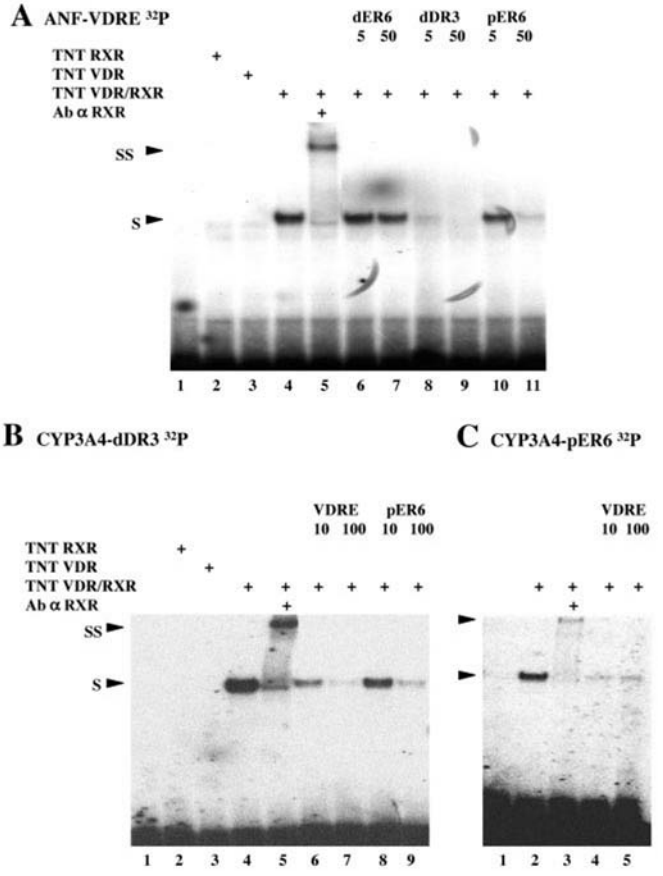

FIG. 3. **Analysis of *CYP3A4* xenobiotic-responsive element binding to VDR by electrophoretic mobility shift assay.** *A*, analysis of rANF-VDRE binding to VDR. Radiolabeled rANF-VDRE oligonucleotide (50,000 cpm of ^{32}P) was incubated in the absence (*lane 1*) or presence of RXR (*lane 2*), VDR (*lane 3*), or both proteins (*lane 4*) produced by an *in vitro* coupled transcription-translation system before loading onto the gel. In parallel experiments, incubation was performed in the presence of anti-RXR antibodies (*Ab α RXR*; 1 μg) (*lane 5*) or of a 5- or 50-fold molar excess of unlabeled 3A4-dER6 (*lanes 6* and *7*), 3A4–5′dDR3 (*lanes 8* and *9*), or 3A4-pER6 (*lanes 10* and *11*) oligonucleotide (see Fig. 2). *B*, analysis of 3A4-dDR3 binding to VDR. Radiolabeled dDR3 oligonucleotide (50,000 cpm of ^{32}P) was incubated as described in *A* for rANF-VDRE (*lanes 1–5*). In parallel experiments, incubation was performed in the presence of a 10- or 100-fold molar excess of unlabeled ANF-VDRE (*lanes 6* and *7*) or unlabeled pER6 (*lanes 8* and *9*). *C*, analysis of 3A4-pER6 binding to VDR. Radiolabeled pER6 oligonucleotide (50,000 cpm of ^{32}P) was incubated as described in *A* (*lanes 1–3*). In parallel experiments, incubation was performed in the presence of a 10- or 100-fold molar excess of unlabeled rANF-VDRE (*lanes 4* and *5*). *S*, shift; *SS*, supershift.

molar excesses of unlabeled oligonucleotides, including consensus rANF-DR3 and 3A4-pER6 (Fig. 3, *B*, *lanes 6* and *7* and *lanes 8* and *9*, respectively; and *C*, *lanes 4* and *5*).

VDR-RXR Heterodimer Binds the PXR/CAR-responsive Elements of the CYP2B6 and CYP2C9 Promoters—A 51-bp sequence termed the phenobarbital-responsive element has been shown to be necessary and sufficient for phenobarbital induction of the mouse *Cyp2B10* gene (35–37). Sequence analysis of various *CYP2B* phenobarbital-responsive elements revealed the presence of two conserved imperfect DR4 motifs (NR1 and NR2) that appear to be essential for a full response to phenobarbital. In the human *CYP2B6* gene, these elements are oriented in opposite directions with respect to those in the mouse and rat genes and are located in the –1733/–1684 region (12). They are hereafter referred to as 2B6–3′DR4 and 2B6–5′DR4, respectively. Recently, we identified a functional CAR-responsive element in the –1856/–1783 region of human *CYP2C9* (28). Sequence analysis revealed the presence of an imperfect

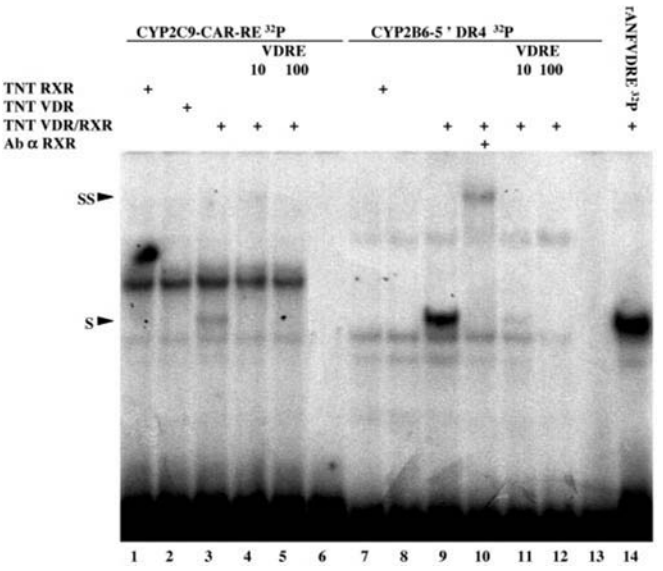

FIG. 4. **Analysis of *CYP2B6* and *CYP2C9* xenobiotic-responsive element binding to VDR by electrophoretic mobility shift assay.** Radiolabeled 2B6–5′DR4 and 2C9-CAR-RE (where is CAR-RE is CAR-responsive element) oligonucleotides (50,000 cpm of ^{32}P) were incubated in the absence or presence of RXR (*lanes 1* and *7*), VDR (*lanes 2* and *8*), or both proteins (*lanes 3* and *9*) prepared by *in vitro* translation using a coupled transcription-translation system before loading onto the gel. In parallel experiments, incubation was performed in the presence of anti-RXR antibodies (*Ab α RXR*; 1 μg) (*lane 10*) or of a 10- or 100-molar excess of unlabeled rANF-VDRE (*lanes 4*, *5*, *11*, and *12*). *Lane 14* is the same assay as *lane 4* in Fig. 3*A*.

DR4 motif, hereafter referred to as 2C9-DR4. This element was shown to bind to and be transactivated by CAR as well as by PXR, albeit to a lower extent.

As shown in Fig. 4, the VDR-RXR heterodimer efficiently bound both the 2C9-DR4 and 2B6–3′DR4 motifs as assessed by gel mobility shift assay. This binding was observed only in the presence of the heterodimerization partner RXR, and the specificity of the interaction was confirmed by competition experiments using 10- and 100-fold molar excesses of unlabeled consensus rANF-DR3 oligonucleotide (Fig. 4, *lanes 4*, *5*, *11*, and *12*). Note, however, that the binding of the VDR-RXR heterodimer to 2C9-DR4 seems to be of much lower affinity compared with the binding to the other *CYP3A4* and *CYP2B6* PXR/CAR elements. In sum, these observations show that the VDR-RXR heterodimer binds to the major PXR/CAR-responsive elements of *CYP3A4*, *CYP2B6*, and *CYP2C9*.

VDR Transactivates the PXR-responsive Elements of CYP3A4—Transactivation of the PXR-responsive elements of *CYP3A4* (shown to bind to the VDR-RXR heterodimer) by 1α,25-$(OH)_2D_3$-activated VDR was analyzed by transient transfection assays in HepG2 cells. Cells were cotransfected with the various *CYP3A4*-specific heterologous and homologous promoter-reporter plasmids and with the VDR expression plasmid or the empty expression plasmid as a control. Cells were then treated with increasing concentrations of 1α,25-$(OH)_2D_3$ for 24 h, and reporter gene activities were measured. The results are shown in Fig. 5. 1α,25-$(OH)_2D_3$ strongly increased the transcriptional activity of the heterologous promoter constructs containing 3A4–5′dDR3 and 3A4-pER6 (by factors of 12 and 40, respectively) in a concentration-dependent manner (the maximum being reached at 1 nM) (Fig. 5, *A* and *B*). This effect was observed only in cells cotransfected with the VDR expression vector. In the absence of VDR, the transcriptional activity of these elements was only modestly increased by 1α,25-$(OH)_2D_3$ (by factors of 2 and 5, respectively), suggesting weak VDR expression in HepG2 cells. Note that 1α,25-

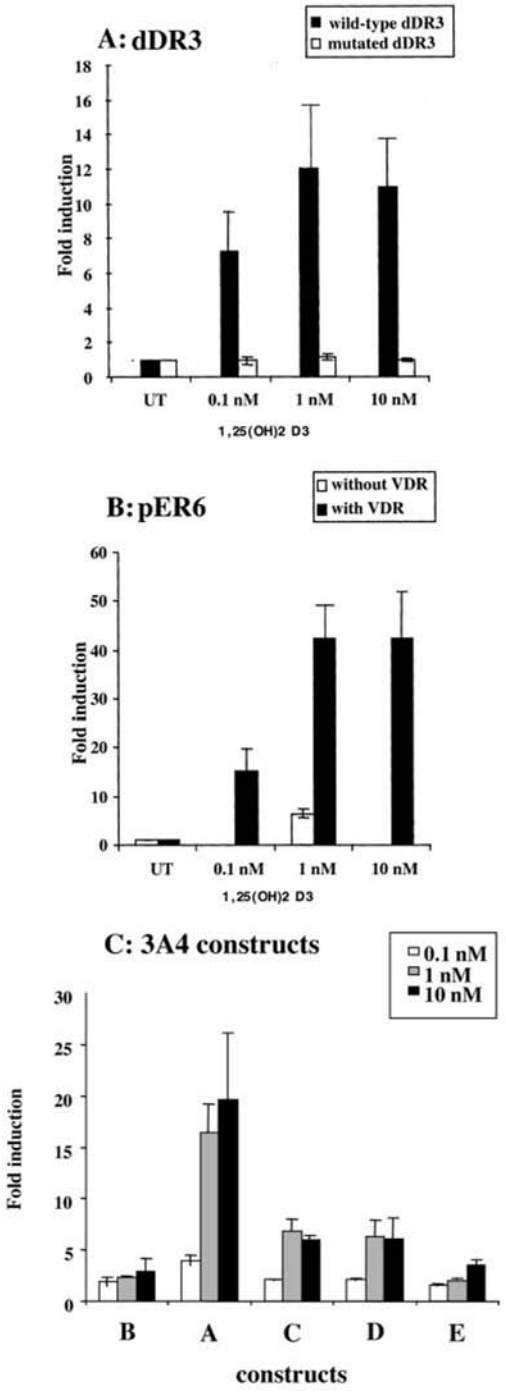

FIG. 5. **Transactivation of the xenobiotic-responsive elements of *CYP3A4* in heterologous and homologous promoter constructs by the VDR-RXR heterodimer.** HepG2 cells were cotransfected with the various *CYP3A4* heterologous and homologous promoter-reporter constructs (see Fig. 2) and with the VDR expression plasmid or the empty expression plasmid and the pSV-β-galactosidase expression vector as controls. Cells were then treated with increasing concentrations of 1α,25-$(OH)_2D_3$ for 24 h, and reporter gene activities were measured. The mean luciferase induction (expressed as the ratio of activity in vitamin D-treated cells to activity in untreated cells, normalized to the β-galactosidase signal) determined in triplicate independent experiments is presented. *A*, 3A4–5′dDR3 (wild-type and mutated element) in plasmid p(3A4-$(dDR3)_3$)-tk-LUC; *B*, 3A4-pER6 in plasmid p(3A4-$(pER6)_3$)-tk-LUC; *C*, *CYP3A4* homologous and heterologous promoter constructs. The −7800/−7200 region of *CYP3A4* (harboring 5′dDR3, dER6, and 3′dDR3; see Fig. 2) was fused upstream of the –262/+11 region (harboring pER6) in front of the luciferase reporter gene. Several deletions of this construct were then generated (see Fig. 2), and their transcriptional activity was measured in response to 1α,25-$(OH)_2D_3$-activated VDR. *UT*, untreated.

$(OH)_2D_3$ had no significant effect on the transcriptional activity of the mutated 3A4–5′dDR3 element (Fig. 5*A*) or of 3A4-dER6 or the pGL3 reporter (data not shown). Similar experiments were then carried out with different *CYP3A4* homologous promoter-reporter constructs with the aim of comparing the pattern of transcriptional activity of these constructs in response to VDR with that observed in response to PXR. For this purpose, the −7800/−7200 region (harboring the 5′dDR3, dER6, and 3′dDR3 elements) was fused upstream of the –262/+11 region of *CYP3A4* (harboring the pER6 element) in front of the luciferase reporter gene (Fig. 2). This construct (CYP3A4–5′dDR3/dER6/3′dDR3/pER6-LUC, construct A) has been shown to be fully responsive to PXR (9), and this was confirmed in this work (see Fig. 7*A*). Several deletions of this construct (constructs B and C in Fig. 2) were then generated, and their transcriptional activity was measured in response to 1α,25-$(OH)_2D_3$-activated VDR. The results are presented in Fig. 5*C*. VDR strongly transactivated (by factors of 15–20) construct A in a 1α,25-$(OH)_2D_3$ concentration-dependent manner. In contrast, all other constructs exhibited only a modest transcriptional activity. The absence of 3′dDR3 resulted in a >60% inhibition of transcriptional activity (construct C), whereas the proximal promoter containing pER6 alone was only slightly affected by VDR (by factors of 2–3, construct B). Interestingly, when the proximal promoter of *CYP3A4* (−262/+11) was replaced by a minimal thymidine kinase promoter (corresponding to the loss of pER6), the transcriptional activity of 5′dDR3/dER6/3′dDR3 (construct D) and of 5′dDR3/dER6 (construct E) was <50% of that measured with construct A, suggesting a cooperative interaction between the dPXRE region and the pER6 element, as previously reported for PXR-mediated transactivation of these elements (9). Finally, in control experiments, neither PXR nor CAR was activated by 1α,25-$(OH)_2D_3$ (data not shown). In sum, these results show that both the proximal region containing pER6 and the distal enhancer dPXRE containing the dDR3 motifs are necessary to confer full VDR response and that, in the context of the *CYP3A4* homologous promoter, transactivation by 1α,25-$(OH)_2D_3$-activated VDR parallels transactivation by xenobiotic-activated PXR.

VDR Transactivates the PXR/CAR-responsive Elements of CYP2B6 and CYP2C9—Similar experiments were carried out with the PXR/CAR-responsive elements identified in *CYP2B6* and *CYP2C9*. The results are shown in Fig. 6. A modest but significant and reproducible activation of both 2B6–3′DR4 and 2C9-DR4 constructs was observed in the presence of 1α,25-$(OH)_2D_3$-activated VDR. Indeed, VDR-mediated transactivation of the major *CYP3A4* responsive elements was much greater than the activation observed here. This is consistent with the finding that, in primary hepatocytes, the induction ratio of CYP3A4 mRNA in response to 1α,25-$(OH)_2D_3$ is much greater than that of both CYP2B6 and CYP2C9 mRNAs (Fig. 1*B*).

Competition of VDR-mediated CYP3A4 Transactivation by PXR and CAR—Because VDR binds and transactivates PXR- and CAR-responsive elements, the next step of our investigation was to determine whether PXR and CAR compete with VDR. For this purpose, plasmid p(3A4-dPXRE/pER6)-LUC (construct A in Fig. 2) was transfected in HepG2 cells in the presence of a fixed amount of VDR expression vector (100 ng) and in the absence or presence of increasing amounts of PXR or CAR expression vectors (10–300 or 10–100 ng, respectively). Cells were then cultured for 24 h in the absence or presence of (i) 1 nM 1α,25-$(OH)_2D_3$, 10 μM rifampicin (PXR activator), or a mixture of both or (ii) 1 nM 1α,25-$(OH)_2D_3$, 5 μM androstenol (mouse CAR deactivator) (10), or a mixture of both; and reporter gene activities were measured.

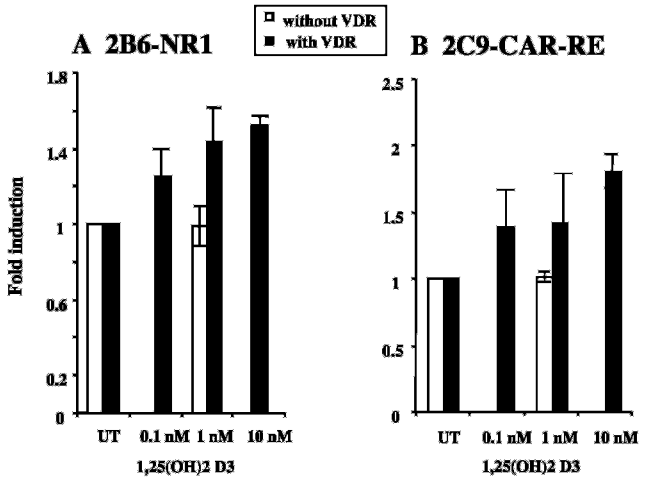

FIG. 6. **Transactivation of the xenobiotic-responsive elements of *CYP2B6* and *CYP2C9* by the VDR-RXR heterodimer.** HepG2 cells were cotransfected with the p(2B6-$(NR1)_3$)-tk-LUC (*A*) or p(2C9-$(DR4)_4$)-SV40-LUC (*B*) construct and with the VDR expression plasmid or the empty expression plasmid and the pSV-β-galactosidase expression vector as controls. Cells were then treated with increasing concentrations of 1α,25-$(OH)_2D_3$ for 24 h, and reporter gene activities were measured. The mean luciferase induction (expressed as the ratio of activity in vitamin D-treated cells to activity in untreated (*UT*) cells, normalized to the β-galactosidase signal) determined in triplicate independent experiments is presented. *CAR-RE*, CAR-responsive element.

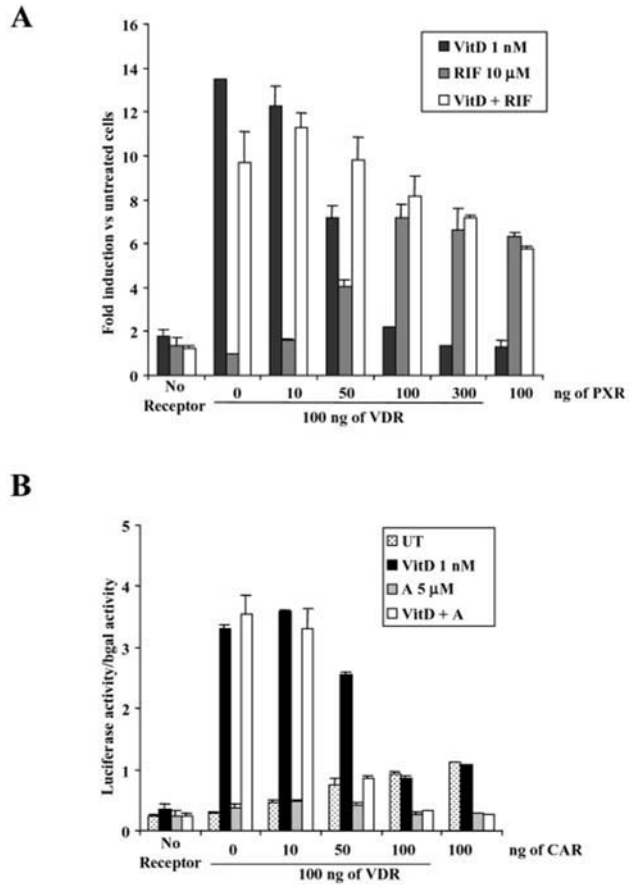

FIG. 7. **Competitive effect of PXR and CAR on the transactivation of the *CYP3A4* homologous promoter by VDR.** HepG2 cells were transfected with 500 ng of construct A (see Fig. 2), various concentrations of PXR or CAR, and pSV-β-galactosidase vectors as described under "Experimental Procedures." The amount of PXR or CAR varied from 0 to 300 ng depending on experiments, with the total amount of expression vector being kept constant by addition of corresponding amounts of empty vectors (pSG5 (PXR) or pCR3 (CAR)), whereas the amount of VDR expression vector was constant in all experiments. Twenty-four hours after transfection, cell were cultured without fetal calf serum for 16 h before determination of luciferase and β-galactosidase activities. Luciferase activity was normalized to β-galactosidase activity. *A*, effect of PXR in the absence or presence of 10 μM rifampicin (*RIF*), 1 nM 1α,25-$(OH)_2D_3$, or 10 μM rifampicin + 1 nM 1α,25-$(OH)_2D_3$. The results are presented as -fold induction (ratio of luciferase activity in vitamin D (*VitD*)- or xenobiotic-treated cells to corresponding levels in untreated cells (*UT*)) and are the mean values of triplicate transfections from two independent experiments. *B*, effect of CAR in the absence or presence of 5 μM androstenol (*A*), 1 nM 1α,25-$(OH)_2D_3$, or 5 μM androstenol +1 nM 1α,25-$(OH)_2D_3$. The results are presented as absolute values of luciferase activity normalized to β-galactosidase (*bgal*) activity to evaluate CAR basal transactivation and are the mean values of triplicate transfections from two independent experiments.

Data on the PXR/VDR competition are shown in Fig. 7*A*. The -fold induction ratios are presented here because no significant change in reporter gene activity was observed in cells cultured in the absence of inducers (untreated cells). A weak transactivation (<2) was observed in the absence of receptor; this might reflect the low endogenous level of receptors in HepG2 cells. VDR was not activated by rifampicin (*0 bars*), and PXR was not activated by 1α,25-$(OH)_2D_3$ (*last bar*). Transactivation of the dPXRE/pER6 construct by the PXR/VDR combinations in the presence of 1 nM 1α,25-$(OH)_2D_3$ alone decreased from 14-fold (no PXR) to ~1-fold, as observed with PXR alone, as the amount of transfected PXR increased. In contrast, in the same experiment, transactivation of the construct in the presence of rifampicin alone increased from 1-fold (no PXR) to 7-fold, as observed with PXR alone, as the dose of PXR increased. When cells were treated with both rifampicin and 1α,25-$(OH)_2D_3$, transactivation of the construct varied from a "pure" vitamin D/VDR response to a pure rifampicin/PXR response, *i.e.* from 11-fold (the maximum observed with VDR alone) to 7-fold (the maximum observed with PXR alone). These results suggest a competition between PXR and VDR for the *CYP3A4* promoter elements.

Data on the VDR/CAR competition are shown in Fig. 7*B*. The results are presented here as luciferase activity (normalized to β-galactosidase activity) to emphasize the increase in basal transactivation of the dPXRE/pER6 construct (~4-fold) as the amount of CAR increased in the absence of any ligand (untreated (*UT*)). This reflects the well established fact that CAR is constitutively active when transfected in cell lines such as HepG2. These results also show the androstenol-mediated inhibition of CAR (untreated *versus* androstenol), as previously described (10). In addition, the results show that the transcriptional activity of VDR and CAR was not affected by androstenol and 1α,25-$(OH)_2D_3$, respectively. Transactivation of the dPXRE/pER6 construct by the VDR/CAR combinations in the presence of 1 nM 1α,25-$(OH)_2D_3$ alone decreased from ~3.5 (no CAR) to ~1 luciferase activity (arbitrary units), as observed with CAR alone, as the amount of transfected CAR increased. When cells were treated with both androstenol and 1α,25-$(OH)_2D_3$, transactivation of the construct varied from a pure vitamin D/VDR response to a pure androstenol/CAR response, *i.e.* the maximum activity (~3.5) observed with VDR alone to the minimum activity (~0.25) observed with CAR alone in the presence of androstenol. The reason for this observation is that, in the presence of androstane, CAR is inactivated because the coactivator recruitment is blocked, but it is still able to bind to its responsive element. These results suggest a competition between CAR and VDR for the *CYP3A4* promoter elements. Finally, these results are in agreement with the gel shift experiments showing that VDR can bind to PXR-responsive (Fig. 3) and CAR-responsive (Fig. 4) elements and therefore confirm that, in the context of the *CYP3A4* homologous promoter, the

sites targeted by VDR overlap with those recognized by PXR and CAR.

DISCUSSION

In this study, we have shown that 1α,25-$(OH)_2D_3$ induces the expression of the *CYP3A4* gene in normal differentiated primary human hepatocytes and, to a lesser extent, *CYP2B6* and *CYP2C9*. Data obtained from electrophoretic mobility shift assays, cotransfection experiments with various oligonucleotides and heterologous/homologous promoter-reporter constructs, and competition experiments between nuclear receptors suggest that 1α,25-$(OH)_2D_3$-activated VDR is responsible for this induction by transactivating those responsive elements previously identified in the promoters of these genes and shown to be targeted by PXR and/or CAR in response to xenobiotics.

Vitamin D (vitamins D_2 and D_3) is a provitamin that requires a two-step biotransformation for full activation, including a first hydroxylation step at position 25 occurring mainly in the liver through mitochondrial CYP27A and a second hydroxylation step at position 1α occurring mainly in the kidney through mitochondrial CYP27B (22). This leads to the production of 1α,25-$(OH)_2D_3$, the most biologically active form of vitamin D. This metabolite is then catabolized mainly in the kidney through hydroxylation at position 24 by CYP24 as well as by another minor pathway involving the formation of a lactone derivative (22). Thus, although our culture medium contained significant amounts of vitamin D_2 (~250 nM), 1α,25-$(OH)_2D_3$ could not be produced or catabolized in our cultured hepatocytes because the kidney biotransformation pathways obviously are missing. Therefore, in this work, cells were treated with a range of concentrations of 1α,25-$(OH)_2D_3$ (0.1–100 nM) reflecting the blood level in the normal adult (19–190 nM). Although it was considered in the past that VDR could be absent or expressed at very low level in the liver, it was recently demonstrated that this receptor is present in fetal, neonatal, and adult rat liver by RT-PCR and immunohistochemistry (38). Control experiments using the inducible expression of fructose-1,6-bisphosphatase, previously shown to be controlled by VDR (31), have clearly shown that VDR was expressed and activated in our cultures after treatment with 1α,25-$(OH)_2D_3$.

Although each nuclear receptor binds preferentially to a specific DNA sequence (1, 39, 40), there have recently been indications that a given receptor (whatever the family it belongs to) may bind to and transactivate different responsive elements. Thus, for example, the steroid hormone receptors (NR3C subfamily) bind classically and almost exclusively as homodimers to palindromic sequences separated by 3 nucleotides. However, the glucocorticoid (NR3C1) and estrogen receptors have been shown to bind to direct repeats with different spacings between half-sites (including DR2, DR5, DR6, and DR9) as well as to ER9, although binding to these motifs is weaker than to the palindrome (41). Zhou *et al.* (42) reported that the androgen receptor may bind to a DR1 motif in addition to the classical palindrome. On the other hand, VDR, PXR, and CAR belong to the NR1I subfamily and form heterodimers with RXR. Their responsive motifs consist of a hexanucleotide consensus sequence (AGGTCA), which can be configured into different motifs, including direct repeats, everted repeats, and inverted repeats. Several authors have reported that CAR and PXR can transactivate *CYP2* or *CYP3* genes via the same responsive elements in a xenobiotic-dependent manner. Thus, for example, PXR is able to transactivate *CYP2B* genes via recognition of the phenobarbital-responsive DR4 element (43), and reciprocally, CAR is able to transactivate human *CYP3A4* through the PXR-responsive elements pER6 and dDR3 (15). The existence of a possible cross-talk between these two nuclear receptor signaling pathways has accordingly been suggested. This apparent versatility in the ability of a given nuclear receptor to target similar but distinct DNA sequences is believed to result from the flexibility of either the ligand- and/or DNA-binding domains, the intervening linker region, or the DNA template itself.

The results presented here suggesting that VDR binds to and transactivates DR4 and ER6 motifs in addition to the more classical DR3 elements are therefore not surprising and clearly offer another example of this nuclear receptor versatility. Indeed, other VDRE motifs have been previously identified, including DR4 (for which VDR exhibited a higher affinity than for DR3), DR6, and the inverted palindrome IP9 (32, 44). In addition, sequence comparison with other members of the nuclear receptor family shows that VDR and PXR isoforms share the greatest similarity (64%) in their DNA-binding domains (4). The versatility of these nuclear receptors in their DNA-binding capacity stands in contrast to their distinct specificity in ligand binding. Indeed, VDR was not activated by rifampicin or by phenobarbital, and neither PXR nor CAR was activated by 1α,25-$(OH)_2D_3$; this is consistent with the finding that the similarity in the ligand-binding domains of VDR and PXR is only 37%. On the other hand, the extent of induction of CYP3A4, CYP2B6, and CYP2C9 mRNA expression in response to 1α,25-$(OH)_2D_3$ correlated with the relative binding to and transactivation of the respective PXR- and CAR-responsive elements by VDR (compare Fig. 1 and Figs. 5*A* and 6). This most likely reflects the fact that deletion or insertion of a single (or several) base pair(s) in the nuclear receptor half-site spacer region is expected to alter both the distance and the rotation angle between the half-sites, thus altering both the binding affinity of the receptor heterodimer and its ability to interact with the different transcription factors and/or the various coactivators or corepressors.

Recently, we have shown that the expression of PXR, RXR, and CAR is under the control of the glucocorticoid receptor in primary human hepatocytes (45–47). Whether VDR expression is controlled by this receptor as well is not known. Thus, a fully activated glucocorticoid receptor is a prerequisite for maximum *CYP2*/*CYP3* induction by xenobiotics. We observed in the same model that interleukin-6 decreases the expression of PXR and CAR (48), thus leading to a decrease in *CYP2* and *CYP3* gene expression. The present and previous results (16, 17) showing that vitamin D affects *CYP* gene expression increase the list of those physiological compounds able to interfere with the metabolism of xenobiotics. Actually, our results suggest that, in the absence of xenobiotic, the basal expression of *CYP2* and *CYP3* genes may be, at least in part, controlled through VDR activation. In the presence of xenobiotics able to activate either PXR or CAR, these receptors will then compete efficiently with VDR (see Fig. 7) on *CYP* gene promoter responsive elements. In this respect, it has to be noted that the extent of CYP3A4 and CYP2B6 mRNA induction in primary human hepatocytes was much greater in response to rifampicin than in response to 1α,25-$(OH)_2D_3$. Finally, although the results presented here suggest that the effect of VDR on *CYP3A4* basal expression is substantial at physiological concentrations of vitamin D, its effect on *CYP2C9* and *CYP2B6* appears to be relatively modest, so the physiological significance of vitamin D effects on these genes is less clear. In this respect, it is worth emphasizing that *CYP2C9* appears to be a primary glucocorticoid receptor-responsive gene (28), the expression of which, under normal physiological conditions, is maintained at a substantial level, and this may account for the fact that xenobiotic- and vitamin D-mediated induction of this gene is modest.

Vitamin D can be obtained from different sources (22). A few

dietary components, including fish oils, egg yolks, milk, and liver, contain naturally significant amounts of vitamin D_3, whereas some plants contain vitamin D_2. Many other foods are fortified with these vitamins. Another source is the skin, in which ultraviolet light induces the photoconversion of 7-dehydrocholesterol to previtamin D_3, followed by thermal isomerization to vitamin D_3. It is therefore possible that interindividual differences in dietary and/or light exposure habits may partly account for interindividual variations in *CYP2*/*CYP3* basal expression and related processes such as drug and xenobiotic metabolism as well as prodrug and procarcinogen activation. These considerations provide another reasonable basis for the occurrence of xenobiotic-dietary compound interactions.

Finally, the reason why these genes are controlled by VDR is unclear. CYP2B6, CYP2C9, and CYP3A4 have not been shown to be involved in the metabolism of vitamin D (49–51). However, it has been observed that prolonged therapy with rifampicin can cause vitamin D deficiency (52). In eight healthy subjects, rifampicin treatment reduced circulating levels of 25-hydroxyvitamin D and 1α,25-$(OH)_2D_3$ by 34 and 23%, respectively. In addition, rifampicin and phenobarbital are two of the drugs most frequently associated with osteomalacia, a metabolic bone disease characterized by a defect of bone mineralization frequently due to an alteration of vitamin D metabolism (53). This suggests that CAR and/or PXR might be involved in the control of genes involved in vitamin D synthesis or catabolism.

In conclusion, this work suggests that VDR, PXR, and CAR control the basal and inducible expression of several *CYP* genes through competitive interaction with the same battery of responsive elements (ER6, DR3, and DR4). In consequence, we suggest that the expression of VDR-controlled genes might be affected by xenobiotics such as rifampicin through the PXR and/or CAR pathway. This possibility is under current evaluation in our laboratory.

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