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Transcriptional Control of Intestinal Cytochrome P-4503A by 1α,25-Dihydroxy Vitamin D_3

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ABSTRACT

It was previously shown that CYP3A4 is induced in the human intestinal Caco-2 cell model by treatment with 1α,25-dihydroxy vitamin D_3 (1,25-D_3). We demonstrate the vitamin D analog, 19-nor-1α,25-dihydroxy vitamin D_2, is also an effective inducer of CYP3A4 in Caco-2 cells, but with half the potency of 1,25-D_3. We report that treatment of LS180 cells, a human intestinal cell line, with 1 to 10 nM 1,25-D_3 dose dependently increased CYP3A4 protein and CYP3A4 mRNA expression. *CYP3A4*- and *CYP3A23*-promoter-Luciferase reporter constructs transiently transfected into LS180 cells were transcriptionally activated in a dose-dependent manner by 1,25-D_3, whereas mutation of the nuclear hormone receptor binding motif (ER6) in the *CYP3A4* promoter abrogated 1,25-D_3 activation of *CYP3A4*. Although the *CYP3A4* ER6 promoter element has been shown to bind the pregnane X receptor (PXR), this receptor does not mediate 1,25-D_3 induction of CYP3A4 because a) PXR is not expressed in Caco-2 cells; b) PXR mRNA expression is not induced by 1,25-D_3 treatment of LS180 cells; and c) the ligand binding domain of human PXR was not activated by 1,25-D_3. 1,25-D_3 uses the vitamin D receptor to induce CYP3A4 because a) the vitamin D receptor (VDR)-retinoid X receptor (RXR) heterodimer binds specifically to the *CYP3A4* ER6; b) selective mutation of the *CYP3A4* ER6 disrupted the binding of VDR-RXR; and c) reporter constructs containing only three copies of the CYP3A4 ER6 linked to a TK-CAT reporter were activated by 1,25-D_3 only in cells cotransfected with a human VDR expression plasmid. These data support the hypothesis that 1,25-D_3 and VDR induce expression of intestinal CYP3A by binding of the activated VDR-RXR heterodimer to the *CYP3A* PXR response element and promoting gene transcription.

The CYP3A subfamily of enzymes catalyzes the metabolic transformation of numerous drugs and some endogenous molecules. Although CYP3A can be detected in several tissues of the body (Wrighton and Thummel, 2000), the highest specific content is found in parenchymal cells of the liver and in mature enterocytes lining mucosal villi of the small intestine (Kolars et al., 1994b). Accordingly, CYP3As have been implicated directly in the first-pass metabolism of cyclosporine (Kolars et al., 1991) and midazolam (MDZ) (Pine et al., 1996) and probably contribute to the low oral bioavailability of these and several other CYP3A substrates, including lovastatin, felodipine, saquinavir, and buspirone.

One of the most confounding and poorly understood aspects of CYP3A-dependent drug metabolism, is the significant interindividual variability in hepatic and intestinal CYP3A content observed in the human population. The two dominant isoforms in adults, CYP3A4 and CYP3A5, can vary 10-fold or more (Lown et al., 1994; Shimada et al., 1994; Paine et al., 1997), even when obvious causes of high (induction) or low (degradation) specific content are excluded. Although interindividual differences in observed CYP3A levels may be attributed to variable rates of CYP3A degradation, particularly after the ingestion of certain drugs or dietary products (Wrighton and Thummel, 2000), recent research suggests that constitutive differences can arise from variable mRNA levels and translation (for review, see Guengerich, 1999).

Interindividual differences in hepatic and intestinal *CYP3A5* mRNA levels and polymorphic enzyme expression have recently been attributed to an intronic point mutation that leads to aberrant splicing and production of a truncated protein (Kuehl et al., 2001). Differences in hepatic *CYP3A4* mRNA levels have been attributed to multiple factors, including variable expression and activation of the pregnane X receptor/steroid and xenobiotic receptor (PXR/SXR) by en-

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ABBREVIATIONS: PXR, pregnane X receptor; hPXR, human pregnane X receptor; SXR, steroid and xenobiotic sensor; RXR, retinoid X receptor; 1,25-D_3, 1α,25-dihydroxy vitamin D_3; 19-nor-1,25-D_2, 19-nor-1α,25-dihydroxy vitamin D_2; VDRE, vitamin D receptor binding element; PXRE, pregnane X receptor response element; FBS, fetal bovine serum; bp, base pair(s); MDZ, midazolam; hVDR, human vitamin D receptor; SV40, simian virus 40; TK, thymidine kinase.

dogenous and exogenous ligands, and variable expression of P-glycoprotein (Schuetz et al., 1996b; Blumberg et al., 1998; Lehmann et al., 1998). Human PXR is found preferentially in liver and intestine (Blumberg et al., 1998; Lehmann et al., 1998), as is CYP3A4 (Wrighton and Thummel, 2000). Moreover, expression of constitutively active human PXR in transgenic mouse liver leads to an up-regulation of hepatic CYP3A (Xie et al., 2000). Although several steroids bind to the receptor (Blumberg et al., 1998; Lehmann et al., 1998), the exact nature of the endogenous, high-affinity PXR ligand is unknown.

Although it is plausible to assume that PXR will also mediate constitutive intestinal CYP3A4 expression, several lines of evidence suggest an additional regulatory mechanism in this organ. First, hepatic and intestinal CYP3A4 specific contents are not concordant (Lown et al., 1994; Thummel et al., 1996; Paine et al., 1997; Gorski et al., 1998). A patient may exhibit high levels of hepatic CYP3A and low intestinal CYP3A levels and vice versa. Moreover, the relative level of PXR mRNA in the colon is greater than or equal to the level found in small intestine (Bertilsson et al., 1998; Lehmann et al., 1998), yet CYP3A4 expression is greatest in mucosa of the proximal small intestine (Paine et al., 1997) and very low in the colon (DeWaziers et al., 1990; Gervot et al., 1996).

A second nuclear hormone, the vitamin D receptor (VDR) is found at higher levels in the intestine than in the liver (Berger et al., 1988; Stumpf, 1995). Intestinal VDR plays a major role in calcium absorption and systemic calcium homeostasis (Schellens et al., 1988). In the rat, there seems to be a higher level of VDR in differentiated villus cells compared with cells of the Crypts of Lierberhün (Chan and Atkins, 1984), and ligand binding studies indicate a higher number of receptors in the proximal small intestinal mucosa, than in the colonic mucosa (Feldman et al., 1979). These patterns of heterogenous VDR expression mirror the heterogenous expression of CYP3A4 in human intestine (DeWaziers et al., 1990; Paine et al., 1997).

The regulation of intestinal CYP3A4 expression by VDR and 1α,25-dihydroxy vitamin D_3 was first suggested by studies showing that treatment of Caco-2 cells with vitamin D analogs led to significant enhancement of CYP3A4 mRNA and protein levels and increased midazolam hydroxylation activity (Schmiedlin-Ren et al., 1997). Moreover, the rank order of CYP3A4 induction by vitamin D analogs (1α,25-dihydroxy vitamin D_3 > 25-hydroxy vitamin D_3 > unhydroxylated vitamin D_3) was consistent with a mechanism of increased *CYP3A4* gene transcription that involved hormone binding to the vitamin D receptor. Thus, it was suggested that intestinal expression of CYP3A4 in vivo might also be mediated by a VDR signaling pathway (Schmiedlin-Ren et al., 1997). However, the *CYP3A4* gene lacks a vitamin D receptor binding element (VDRE) consensus sequence—an imperfect direct repeat of a core hexanucleotide sequence, [G/A] GGT[G/C]A, with a three-nucleotide spacer (i.e., DR3). Therefore, we hypothesized that if VDR affects *CYP3A4* transcription, it must be mediated through cross-talk with a different response motif such as the PXR response element (PXRE) (i.e., ER6). In this article, we report on results from in vitro and in vivo experiments that support this hypothesis.

Experimental Procedures

Cell Lines. Cos-7 monkey kidney cells, human embryonic kidney 293 cells, and LS180 cells [American Type Culture Collection (ATCC), Manassas, VA] were cultured in ATCC-recommended medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and 1% Pen/Strep solution and maintained at 37°C in a humidified incubator. Caco-2 cells (American Type Culture Collection) were seeded onto semipermeable laminin-coated inserts at 10^5 cells/cm^2 and cultured as described previously (Schmiedlin-Ren et al., 1997). After reaching confluence, cells were fed for 1 to 3 weeks with Dulbecco's modified Eagle's medium containing 0.1 mM nonessential amino acids, 100 U/ml sodium penicillin, 100 μg/ml streptomycin, 0.1 M sodium selenite, 3 μM zinc sulfate, 45 nM DL-α-tocopherol, 5% heat-inactivated FBS, and 1α,25-dihydroxy vitamin D_3 (calcitriol) or 19-nor-1α,25-dihydroxy vitamin D_2 (paricalcitol) as indicated for individual experiments. Calcitriol and paricalcitol were kindly provided by Abbott Laboratories (Chicago, IL).

Plasmids. The hVDR expression plasmid was a gift from Dr. J. Wesley Pike (University of Cincinnati, OH). pSG5-hPXRΔATG and *CYP3A4*-(*ER6*)$_3$-TK-CAT were generously provided by Dr. Steve Kliewer (GlaxoSmithKline, Research Triangle Park, NC; Lehmann et al., 1998). pCMX-GAL-L-SXR and tk(MH100)$_4$-LUC (Blumberg et al., 1998) were generously provided by Dr. Bruce Blumberg (University of California, Irvine). *CYP3AP1*-LUC [bp −266 to +17 relative to the start site of transcription in *CYP3AP1* and previously thought to be the *CYP3A5* promoter and that contains a PXRE that cannot bind PXR (Blumberg et al., 1998)] was described previously (Schuetz et al., 1996c). *CYP3A23*-TK-LUC (bp −220 to −56 relative to the start site of *CYP3A23*) contained both the proximal DR3 (bp −134 to −120, TGAACTtcaTGAACT) and distal ER6 (bp −166 to −149, TTAACTcaaaggAGGTCA) overlapping a DR4 (bp −164 to −149, AACTCAaaggAGGTCA). *CYP3A4*-SV40-LUC (bp −220 and −56, relative to the start site of *CYP3A4* transcription) contained the ER6 (TGAACTCAAAGGAGGTCA). Mutant (mtCYP3A4-SV40-LUC) with a mutated ER6 (TGAACTCAAAGGA**ATA**CA; underlined bases indicate receptor binding sites and bold bases were changed to disrupt nuclear hormone receptor binding) was created directly from *CYP3A4*-SV40-LUC by PCR with a mutant primer and the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutant was sequenced to confirm base changes.

Midazolam Hydroxylation Activity in Caco-2 Cells Treated with 1,25-D_3 or 19-nor-1,25-D_3. Caco-2 cells (passage 23) were seeded on laminin-coated polyethylene terephthalate inserts (0.9 cm^2). Upon reaching confluence, cells were cultured in differentiation medium supplemented with vehicle (ethanol, ~ 1%) or 0.05, 0.1, 0.25, 0.5, or 1.0 μM 1,25-D_3. Medium was changed every other day. On day 15, medium was changed again (FBS excluded), and cells were incubated with midazolam (2 μM applied to the apical compartment) for 60 min (n = 3 inserts per dose group) to determine 1′-hydroxymidazolam formation activity. Media from the apical and basolateral compartments and cultured cells were collected and stored at −80°C for analysis.

For a second experiment, confluent cells were cultured in differentiation medium supplemented with vehicle (ethanol, ~ 1%), 0.1, or 1.0 μM of either 1,25-D_3 or 19-nor-1,25-D_2. Medium was changed every other day. On days 3, 7, 15, and 21, medium was changed again (FBS excluded), and cells were incubated with midazolam (2 μM) for 60 min to determine 1′-hydroxymidazolam formation activity (n = 3 inserts per group). Media from the apical and basolateral compartments and cultured cells were collected and stored at −80°C for analysis.

To assess CYP3A4 catalytic activity in Caco-2 cell culture, the extent of 1′-hydroxymidazolam (MDZ) formation was monitored using liquid chromatography combined with tandem mass spectrometry. Analysis was performed on a Micromass Quattro II tandem quadruple mass spectrometer (Micromass Ltd., Manchester, UK) coupled to a Shimadzu LD-10AD solvent delivery system and SIL-

10AD autosampler (Shimadzu Scientific Instruments, Inc., Columbia, MD). Separations were achieved on an XBD-C8, 5-μm, 2.1- $\times$ 50-mm column (Zorbax; Agilent Technologies, Palo Alto, CA) using a water-methanol gradient that contained 0.1% acetic acid. The column effluent was subjected to mass spectral analysis by positive electrospray ionization. Using multiple reaction monitoring the transitions of m/z 341.9 to 202.9 and m/z 345.9 to 206.9, corresponding to a common fragment loss for the ^{35}Cl-d_0-1′-OH MDZ and ^{37}Cl-d_2-1′-OH MDZ (internal standard), respectively, were monitored. Peak area ratios of (1′-OH MDZ/d_2-1′-OH MD) for samples were compared with those of the standard curve and unknown concentrations were interpolated. Statistical analysis of results from the different treatment regimens was performed using analysis of variance, followed by Bonferroni's pair-wise comparisons.

Immunoblot Analysis of CYP3A4 and Pgp in LS180 Cells. LS180 cells growing at high density on tissue culture plastic were treated with various concentrations of 1,25-D_3 for 48 h and then removed from the plate by scraping into phosphate-buffered saline. The cells were pelleted at 10,000*g*, and resuspended in storage buffer (100 mM potassium phosphate, pH 7.4, 1.0 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 20 μM butylated hydroxytoluene, and 2 mM phenylmethylsulfonyl fluoride), and cell lysates were generated by sonication. Cell lysates (50 or 100 μg) were resolved on 6% Laemmli polyacrylamide gels for Pgp and CYP3A4, respectively, immunoblotted to nitrocellulose and developed with antibodies to human CYP3As and Pgp (Schuetz et al., 1996a) and appropriate secondary antibodies and developed with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Northern Blot Analysis. Total RNA was extracted from LS180 cells (Schuetz et al., 1988) and 20 μg was analyzed by Northern blot (Schuetz et al., 1996c). Equivalent loading of RNA samples and uniform transfer was assessed by analysis of ethidium bromide staining of the 18S and 28S ribosomal RNAs before and after transfer to membrane. The membrane was hybridized with a *CYP3A* cDNA (Schuetz et al., 1996a)

Electrophoretic Mobility Shift Assay. Double-stranded, ^{32}P-labeled oligonucleotides representing the CYP3A4 PXR DNA binding sequence, an everted repeat with 6-bp spacer (hereafter referred to as CYP3A4-ER6) 5′-GATCAATATGAACTCAAAGGAGGTCAGTG-3′ or a mutant CYP3A4-ER6 5′-GATCAATATGAACTCAAAGG**AATA**CAGTG-3′ (bolded bases disrupt PXR binding half-site) or a consensus VDRE 5′-GGCAGGTCATGGAGGTCAGTTC-3′ or mutated VDRE 5′-GGCAGAACATGGAGAACAGTTC-3′ was incubated with vitamin D receptor nuclear extracts in the presence or absence of VDR-specific antibody or approximately 200-fold molar excess of unlabeled oligonucleotides, according to the manufacturer's instructions. Electrophoretic mobility shift assay reagents were purchased from Geneka (Montreal, Quebec, Canada). Complexes were resolved by electrophoresis through a nondenaturing 4% polyacrylamide gel and analyzed using a Storm 860 PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Transient Transfection Studies. LS180 cells (120 $\times$ 10^5 cells per 1.6-cm well, plated on day 1) were transfected on day 2 by calcium phosphate coprecipitation with 2 μg of *CYP3A* reporter plasmids. Cos-7 cells (300 $\times$ 10^5 cells per 2.2-cm well, plated on day 1) were cotransfected on day 2 by LipofectAMINE (Invitrogen) (4 μl/well) with 600 ng of TK(MH100)$_4$-LUC and 400 ng of vector or pCMX-GAL-L-SXR plasmid, or were transfected with 200 ng of *CYP3A4*(ER6)$_3$-TK-CAT, 100 ng TK-LUC, and 66 ng of pCMV-hVDR or pcDNA3 vector. 293 cells (200 $\times$ 10^5 cells per 1.6-cm well, plated on day 1) were cotransfected on day 2 by calcium phosphate coprecipitation with 500 ng of *CYP3A4*(ER6)$_3$-TK-CAT, 100 ng TK-LUC, and 150 ng of pCMV-hVDR or pcDNA3 vector reporter plasmids. All cells were washed and incubated with fresh medium with and without 1,25-D_3 18 h after transfection. Reporter activities were determined (Thottassery et al., 1999) 24 h later and normalized to either TK-LUC or cell protein. CAT activities were normalized to luciferase activity or cell protein.

Reverse Transcription-PCR: hPXR and hVDR. Total RNA (5 μg) from LS180 cells treated for 48 h and Caco-2 cells treated for 2 weeks with or without 0.25 μM 1,25-D_3 was reverse transcribed according to the manufacturer's instructions (Invitrogen). hPXR cDNA was amplified from first-strand cDNA by using oligonucleotides hPXR-U 5′-CAAGCGGAAGAAAAGTGAACG-3′ and hPXR-L 5′-CTGGTCCTCGATGGGCAAGTC-3′ under conditions described previously (Dotzlaw et al., 1999). hVDR cDNA was amplified using primers from bp 310 to 764 (S) 5′-CGGGAGATGATCCTGAAGCG-3′ and (AS) 5′-GTGAGGTCTCTGAATCCTGG-3′. The pSG5-hPXRΔATG cDNA and hVDR cDNA served as positive controls for amplification.

Results

Concentration-Dependent Induction of CYP3A4 in LS180 and Caco-2 Cells. Two types of immortalized human colon carcinoma cell lines were used for these studies. Treatment of LS180 cells with 1,25-D_3 for 2 days resulted in a concentration-dependent increase in cellular CYP3A4 protein (Fig. 1a) and *CYP3A4* mRNA (Fig. 1b). The inductive

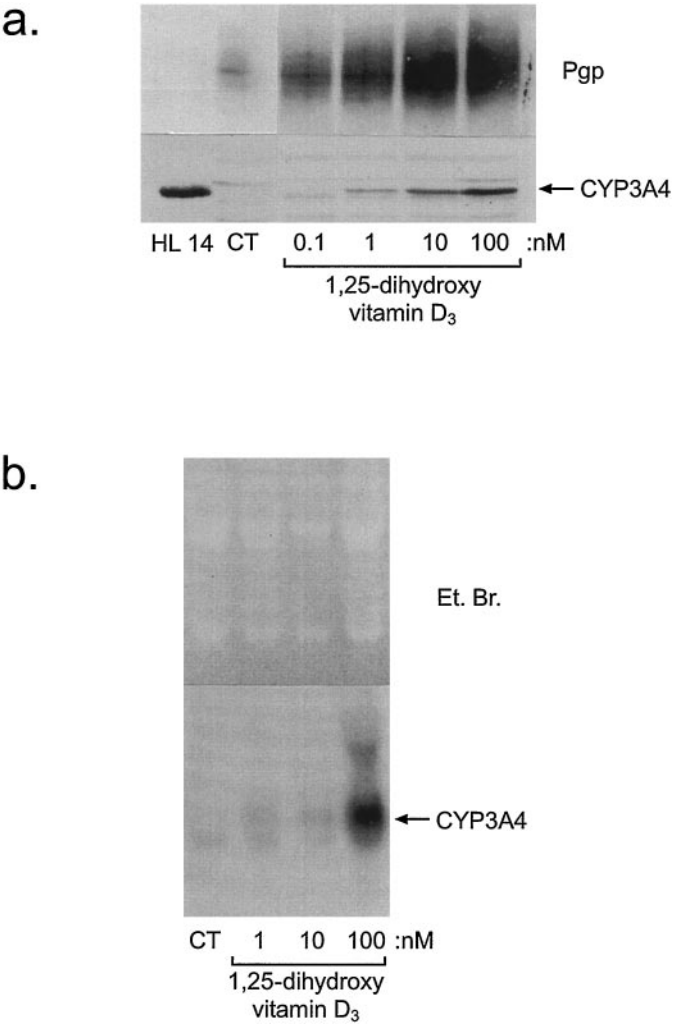

Fig. 1. Induction of CYP3A4 protein and mRNA in LS180 cells by 1,25-D_3. LS180 cells were treated for 48 h with 1 to 100 nM 1,25-D_3. a, immunoblot: 50 or 100 μg of total cell lysate was analyzed by immunoblot for Pgp or CYP3A4, respectively. Microsomes (1 μg) from an adult human liver (HL14) were loaded as a CYP3A4 control. b, Northern blot: Total RNA was isolated and 20 μg resolved on formaldehyde denaturing agarose gels, blotted to membrane and ethidium bromide stained or hybridized to a ^{32}P-labeled *CYP3A* cDNA.

response was detectable with 1 nM 1,25-D_3, and this effect increased up to the maximum concentration employed—100 nM. Treatment of Caco-2 cells with 50 to 1000 nM 1,25-D_3 for 2 weeks gave a similar hormone concentration-dependent response with a parallel increase in CYP3A4 protein (not shown) and in situ midazolam 1′-hydroxylation activity. An apparent maximum inductive effect was observed with 250 nM 1,25-D_3 (Table 1). Interestingly, immunochemical detection of P-glycoprotein in LS180 cells (Fig. 1a) and in Caco-2 cells (not shown) increased in parallel to the change in CYP3A4 over the same respective 1,25-D_3 concentration range.

Time-Course of Effect of Paricalcitol and Calcitriol on CYP3A4 in Caco-2 Cells. 1,25-D_3 binds to the vitamin D receptor with a higher (~3-fold) affinity than 19-nor-1,25-D_2 (Takahashi et al., 1997). Caco-2 monolayers were cultured with 0.1 and 1 μM 1,25-D_3 or 19-nor-1,25-D_2 for 3, 7, 15, and 21 days, and then midazolam 1′-hydroxylation activity was measured. Results presented in Table 2 demonstrate induction of CYP3A catalytic activity by both hormones, and that 1,25-D_3 was approximately 2-fold more effective than 19-nor-1,25-D_2 at the 0.1 μM concentration but was equally effective at the higher 1 μM concentration. The inductive effect after treatment with 0.1 μM hormone seemed to be maximal by week 2 but was still increasing at week 3 with the 1 μM dose.

Transcriptional Activation of CYP3A-Luciferase Constructs by 1,25-D_3. LS180 cells were transiently transfected with *CYP3A* promoter-Luciferase, or SV-40-LUC or TK-LUC control plasmids. The *CYP3A4* promoter contains a proximal ER-6 element (Barwick et al., 1996). The *CYP3A23* promoter contains both ER-6 and DR-3 elements (Barwick et al., 1996). The *CYP3AP1* promoter [previously believed to represent the *CYP3A5* promoter (GenBank S74700 and S74699)] (Finta and Zaphiropoulos, 2000), contains a PXRE with several nucleotide substitutions that render it incapable of binding PXR (Blumberg et al., 1998). Treatment of *CYP3A4* and *CYP3A23* promoter constructs with 1,25-D_3 for 18 h resulted in 5- and 8-fold increases in luciferase activity above that of untreated cells (Fig. 2a). In contrast, the same 1,25-D_3 treatment produced no change in luciferase activity above untreated controls in cells containing either the *CYP3AP1* promoter construct containing the mutant PXRE, the SV40 construct, or the TK construct. Transcriptional activation of the *CYP3A4* promoter construct was 1,25-D_3 concentration-dependent with a significant 25% increase detected after incubation of 1 nM 1,25-D_3 and a maximal 225% increase in activity observed after treatment with 10 nM 1,25-D_3 (Fig. 2b). Selective mutation of three sequential bases within the proximal ER-6 element of *CYP3A4* (Fig. 2b, wild-type, TGAACT CAAAGG AGGTCA → mutant, TGAACT CAAAGG A*ATA*CA) completely abrogated the inductive effect of 1,25-D_3 (Fig. 2c).

1,25-D_3 Does Not Induce CYP3A by Modulating PXR Expression and VDR Is Expressed in LS180 and Caco-2 Cells. Because the *CYP3A4* ER6 promoter element has been shown to bind the PXR, we determined whether the effect of 1,25-D_3 on *CYP3A4* transcription could be mediated indirectly by 1,25-D_3 inducing PXR expression. PXR expression was compared in Caco-2 and LS180 cells cultured in the presence or absence of 1,25-D_3. PXR mRNA was readily detected in LS180 cells, but its expression was unchanged by treatment with 1,25-D_3 (Fig. 3a). PXR mRNA was undetectable in Caco-2 cells, regardless of whether the cells had been treated with hormone (Fig. 3a). In addition, a combined 2-week treatment of Caco-2 cells with 0.1 μM dexamethasone, a concentration shown to up-regulate the expression of PXR in primary human hepatocytes, and 10 μM rifampin, a potent activator of PXR, had no effect on midazolam 1′-hydroxylation activity (106 ± 5.6 and 99 ± 3.2%, respectively). We simultaneously examined VDR expression in RNA from these same cells and found that LS180 cells expressed at least 2-fold more VDR than Caco-2 cells, and that the amount of VDR mRNA was unchanged by treatment with 1,25-D_3 in either cell line (Fig. 3b).

1,25-D_3 Does Not Activate PXR. To further rule out the possibility that 1,25-D_3 was inducing CYP3A4 in LS180 cells by a non–VDR-dependent mechanism involving PXR, we determined whether this hormone could ligand activate hPXR.

TABLE 1
Induction of midazolam hydroxylation activity by 1,25-D_3 in Caco-2 cells

1,25-D_3 Dose[a]	1′-OH-MDZ Formation Rate[b]
nM	*pmol/h/insert*
0	Bld
50	18.2 ± 2.01
100	32.1 ± 3.56
250	41.5 ± 11.0
500	36.1 ± 3.83
1000	38.8 ± 11.6

[a] Dose concentration applied to the apical compartment every other day for 14 days.
[b] Product formation rate (mean ± S.D. for triplicate incubations) following application of a single 2 μM dose of midazolam to the apical compartment.
Bld, below limit of detection.

TABLE 2
Time course of induction of midazolam hydroxylation activity in Caco-2 cells after treatment with 19-nor-1,25-D_2 or 1,25-D_3

Treatment	1′-OH-MDZ Formation Rate (pmol/hr/insert)[a]			
	19-nor-1,25-D_2 (0.1 μM)	1,25-D_3 (0.1 μM)	19-nor-1,25-D_2 (1.0 μM)	1,25-D_3 (1.0 μM)
day				
0	Bld	Bld	Bld	Bld
3	Bld	1.34[b]	8.00[b]	7.90 ± 3.20
7	2.44 ± 1.66	5.25 ± 0.39	24.1 ± 3.6	21.3 ± 1.46
15	10.0 ± 1.46[c]	18.7 ± 3.0	54.6 ± 11.1	44.9 ± 4.70
21	9.52 ± 0.40[c]	21.4 ± 1.13	66.6 ± 14.0	70.5 ± 5.90

[a] Product formation rate (mean ± SD for triplicate incubations) following application of a single 2 μM dose of midazolam to the apical compartment. 1′-OH MDZ formation was below limit of detection in cells treated with vehicle for 3 to 21 days.
[b] Quantifiable rate for one of three incubations.
[c] Significantly different from 1,25-D_3 effect at equivalent dose, $p < 0.001$.
Bld, below limit of detection.

We cotransfected Cos-7 cells with a GAL-PXR/SXR construct containing only the ligand binding domain of PXR/SXR fused to the DNA binding domain of the yeast transcription factor GAL-4, along with a reporter containing the GAL4 response element fused to a luciferase reporter. 1,25-D_3, at concentrations ranging from 0.1 to 100 nM, was incapable of ligand activating PXR and transcriptionally activating the CYP3A4 PXREs, whereas 10 μM rifampin readily increased *CYP3A4* transcriptional activity in a PXR-dependent fashion (Fig. 4).

Specific Binding of the VDR-RXR Heterodimer to the *CYP3A4* PXRE-ER6. To determine whether the VDR bound directly to the *CYP3A4* PXRE-ER6, we performed electrophoretic mobility shift experiments. ^{32}P-labeled oligonucleotides containing either the consensus or mutated VDRE or wild-type or mutated PXR response element (*CYP3A4*-ER6) were incubated with nuclear extracts containing VDR and RXR in the absence or presence of competitor unlabeled oligonucleotides. VDR formed a specific complex with the labeled *CYP3A4* PXRE-ER6 and with labeled consensus VDRE, but not with the labeled mutant *CYP3A4*-ER6 or mutant VDRE (Fig. 5). Unlabeled oligonucleotides for the consensus VDRE and *CYP3A4* PXRE-ER6, but not mutant VDR-response element and mutant *CYP3A4* PXRE-ER6, reduced the intensity of labeled PXRE-VDR-RXR and VDRE-VDR-RXR complexes. Anti-VDR antibody produced a supershift of the *CYP3A4*- ER6-VDR-RXR complex, but had no effect on the nonspecific banding pattern associated with labeled mutated PXRE and VDR nuclear extracts (Fig. 5).

Cotransfection of VDR and *CYP3A4* PXRE-Luc in Cos-7 or K293 Cells. Because Cos-7 cells (a derivative of CV-1 cells) and K293 cells express RXRα, but are deficient in other nuclear hormone receptors, they are useful for studies of nuclear hormone receptor ligands (Lu et al., 2000). These cells were cotransfected with hVDR or pcDNA3 and a *CYP3A4* $(ER6)_3$-CAT reporter containing three copies of the *CYP3A4* ER6 and no other *CYP3A4* promoter sequences. Addition of 10 or 100 nM 1,25-D_3 but not 20 μM rifampin to transfected Cos-7 cells produced a 70-fold increase in *CYP3A4* $(ER6)_3$-CAT reporter activity, but only in those cells cotransfected with the human vitamin D receptor plasmid

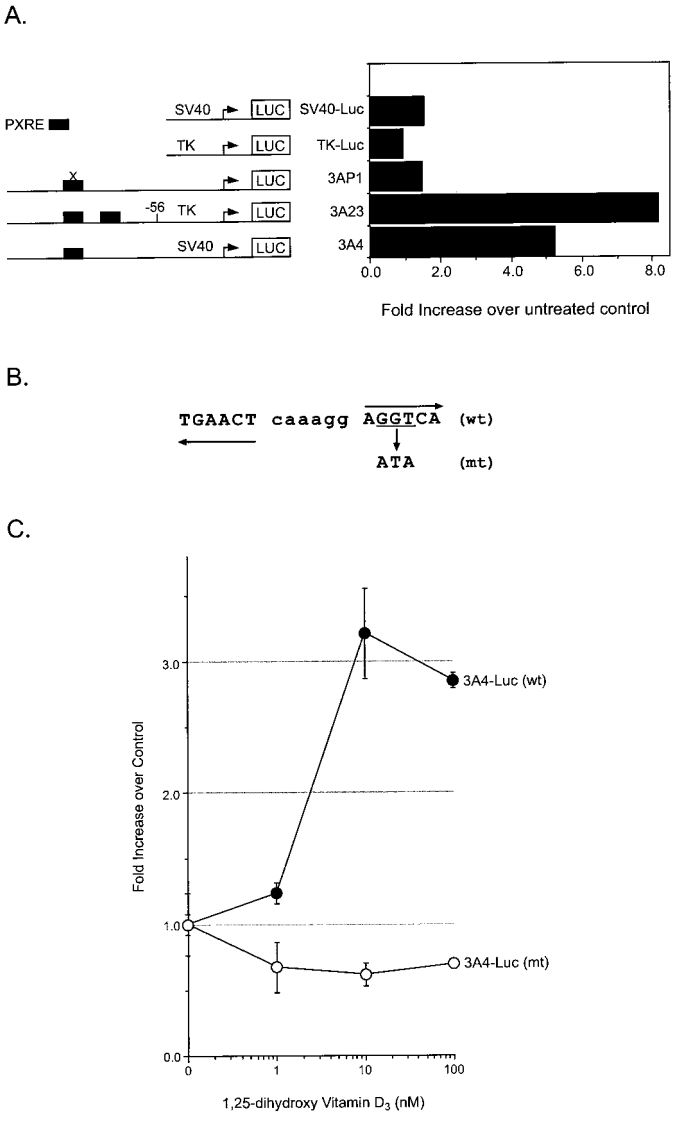

Fig. 2. Transcriptional activation of the *CYP3A* promoters by 1,25-D_3 in transiently transfected LS180 cells. a, luciferase activity for *CYP3A4*, *CYP3A23*, and *CYP3AP1* promoter constructs that contain ER-6, ER-6 + DR3, and nonfunctional ER-6, respectively. b, nucleotide sequence for wild type (wt) and site-directed mutant (mt) of the *CYP3A4* ER-6 promoter. c, luciferase activity from wt and mt CYP3A4 ER-6 constructs after treatment with increasing concentration of 1,25-D_3. Cell extracts were assayed for firefly luciferase and normalized to cellular protein. The treated LUC activity was then divided by nontreated LUC activity and the results expressed as -fold increase over untreated controls. Each error bar (c) indicates the mean of three replicate determinations from a single experiment and is representative of results from at least three independent experiments.

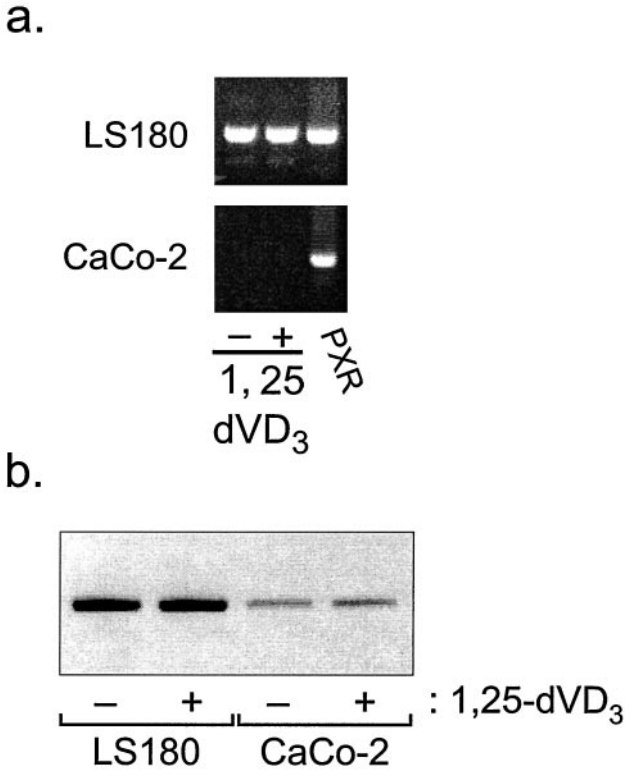

Fig. 3. Expression of PXR and VDR mRNAs in human intestinal cells. First strand cDNA was reverse transcribed from total RNA prepared from LS180 cells or Caco-2 cells cultured in the absence (−) or presence (+) of 0.25 μM 1,25-D_3. hPXR (a) and hVDR (b) were amplified from the RT products, with the PXR or VDR cDNA serving as positive controls, respectively. Forty microliters of PCR product was resolved on an agarose gel and visualized by ethidium bromide.

(Fig. 6a). A similar requirement of the VDR for 1,25-D_3 to induce the *CYP3A4* $(ER6)_3$-CAT reporter was seen in transfected K293 cells (Fig. 6b) with a significant effect (60%) observed with 1 nM 1,25-D_3, and maximal induction (390%) after incubation with 10 nM the hormone.

Discussion

The specific content of CYP3A4 in both liver and small intestine is subject to considerable interindividual variability. In addition, interorgan expression of CYP3A4 is discordant. The cause of these apparent interindividual and interorgan differences in CYP3A4 regulation is largely unknown. Our studies demonstrate that the vitamin D nuclear receptor is an important signaling pathway for expression of CYP3A4 in mucosal villi of the small intestine. Several lines of evidence implicate the VDR as necessary for 1,25-D_3 to induce CYP3A4. First, 1,25-D_3 treatment was shown to increase *CYP3A4* mRNA content, CYP3A4 protein content and CYP3A-dependent midazolam hydroxylation in two immortalized cell lines that are accepted surrogate models for the human enterocyte. Moreover, CYP3A4 induction in LS180 cells was observed at total extracellular concentrations of 1,25-D_3 (1–10 nM) similar to that needed to induce calcium transport in cell culture (Giuliano et al., 1991) although somewhat higher than the ~0.1 nM concentration of 1,25-D_3 that might be expected in vivo in arterial blood perfusing the small intestine (Bikle et al., 1984; Ebeling et al., 1992).

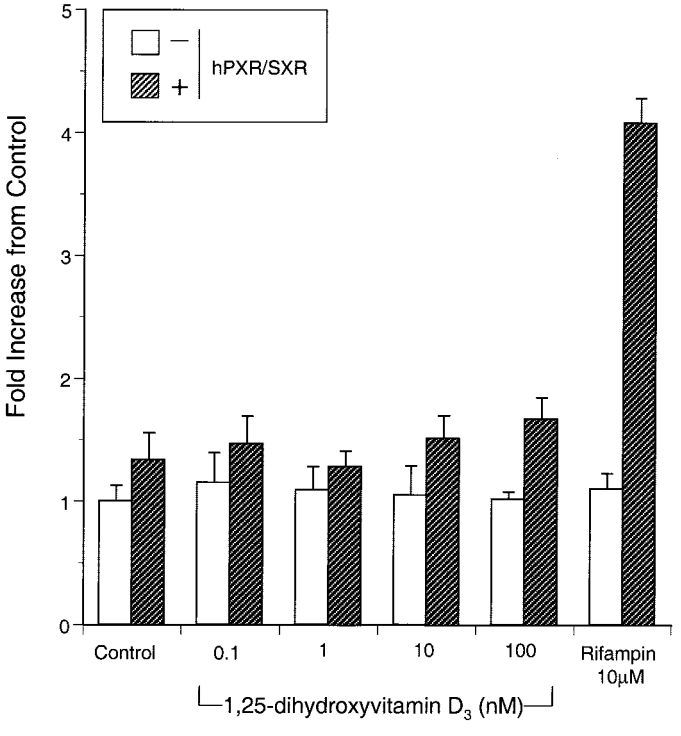

Fig. 4. 1,25-D_3 does not activate human PXR/SXR. Cos-7 cells were cotransfected with $TK(MH100)_4$-LUC and (+) pCMX-GAL-L-SXR plasmid or vector (−hPXR/SXR). Cells were cultured in the absence or presence of rifampin (10 μM) or various concentrations (0.1–100 nM) of 1,25-D_3. Cell extracts were assayed for firefly luciferase and normalized to cellular protein. The treated LUC activity was then divided by non-treated LUC activity and the results expressed as -fold increase over untreated controls. Each error bar indicates the mean of three replicate determinations from a single experiment and is representative of results from at least three independent experiments.

Second, there is good correlation between the biologic potency of 1,25-D_3 to induce CYP3A4 transcription, mRNA and protein production in LS180 cells, and its reported capacity to bind to the VDR. In addition, 1,25-D_3 and 19-nor-1,25-D_2 induced CYP3A in Caco-2 cells with potencies directly related to their affinities for the VDR (affinity of 19-nor-1,25-D_2 is one-third that of 1,25-D_3) (Takahashi et al., 1997). Third, results from electrophoretic mobility shift assay and *CYP3A4* promoter transfection experiments demonstrated the specific binding of VDR-RXR to an ER-6 domain (nuclear hormone response element) of the *CYP3A4* promoter and that this signal activated gene transcription. An additional line of evidence implicating the VDR as necessary for 1,25-D_3 to induce CYP3A comes from transient transfection experiments of Cos-7 and 293 cells. These experiments demonstrated an absolute requirement for the hVDR for 1,25-D_3 to transcriptionally activate the *CYP3A4* ER6 elements. Again, a significant, stimulatory effect was detected at a relevant hormone concentration shown to increase VDR-mediated calcium uptake in cell culture (Giuliano et al., 1991).

These studies conclusively ruled out any role for PXR in mediating 1,25-D_3 induction of CYP3A4 because, first, Caco-2 cells lack PXR and are resistant to the PXR inducing effects of dexamethasone, yet respond to 1,25-D_3 with induction of CYP3A. Second, up-regulation of PXR by 1,25-D_3 cannot be indirectly activating CYP3A4 expression in LS180 cells because 1,25-D_3 had no effect on PXR protein expression in these cells. Third, 1,25-D_3 was incapable of activating the *CYP3A4* PXRE reporter in cells cotransfected with PXR.

Caco-2 cells were clearly less sensitive to the inductive effect of 1,25-D_3 in comparison with LS180 cells. This discrepancy may reflect the dramatic difference in the number of 1,25-D_3 binding sites within the two cell types. Indeed, VDR message levels were dramatically lower in the Caco-2

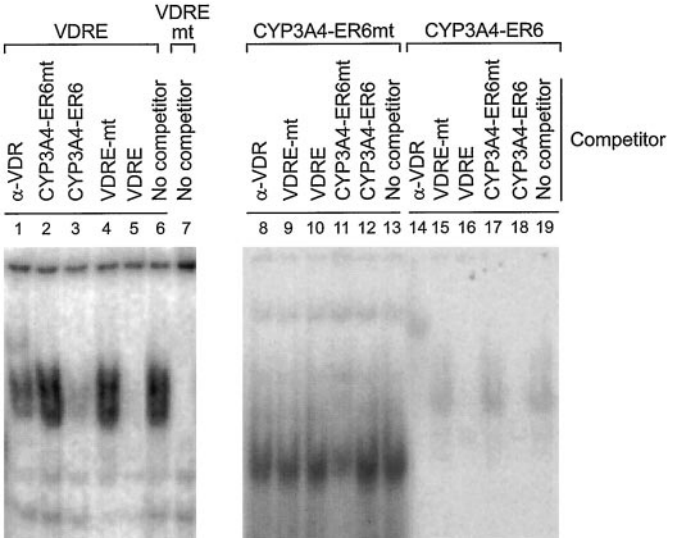

Fig. 5. Electrophoretic mobility shift assay for VDR-*CYP3A4* promoter complexes. ^{32}P-labeled oligonucleotides representing the consensus or mutant vitamin D response element (VDRE or VDRE-mt, respectively) or a wild-type or mutated *CYP3A4* PXRE (*CYP3A4*-ER6 or *CYP3A4*-ER6 mt, respectively) were incubated with nuclear extracts containing vitamin D receptor. Reactions were incubated in the absence (no competitor) or presence of 200-fold molar excess of unlabeled VDRE, mutant VDRE, PXRE, or mutant-PXRE binding sites as indicated. In some assays, an anti-VDR antibody (α-VDR) was also added as indicated.

cells compared with the LS180 cells (2-fold higher band intensity in LS180 cells). Thus, in Caco-2 cells, the VDR B_{max} value at low 1,25-D_3 concentrations may be insufficient to yield a detectable signal (increased transcription of CYP3A4 protein and activity). By analogy, the magnitude of glucocorticoid receptor transcriptional activation is directly proportional to the number of receptor molecules per cell (Vanderbilt et al., 1987). In addition, Caco-2 cells may show the lag in CYP3A induction (relative to LS180) because they require several days of 1,25-D_3 treatment to fully differentiate into mature enterocytes. Indeed, the maximum number of 1,25-D_3 binding sites is 2.6-fold greater in differentiated Caco-2 cells compared with undifferentiated ones (Giuliano et al., 1991) and increased vitamin D receptor levels enhance 1,25-D_3-mediated gene expression and calcium transport in these cells (Shao et al., 2001). Because cells interpret signals in a context-dependent fashion, the observed cell-dependent dose response to 1,25-D_3 may also be the result of a difference in expression of a critical coactivator or corepressor protein, such as the YY1 transcription factor shown to modulate the genomic response to 1,25-D_3 (Raval-Pandya et al., 2001). It is also possible that the induction response in LS180s at high concentrations and some of the induction in Caco-2 cells involves nongenomic effects of 1,25-D_3 (Dusso and Brown, 1998). Nevertheless, the rapid transcriptional activation of the endogenous CYP3A4 gene by near physiological concentrations of 1,25-D_3 in LS180 cells strongly supports direct transactivation by VDR as the basis for increased CYP3A4 observed in vivo (Takahashi et al., 1997).

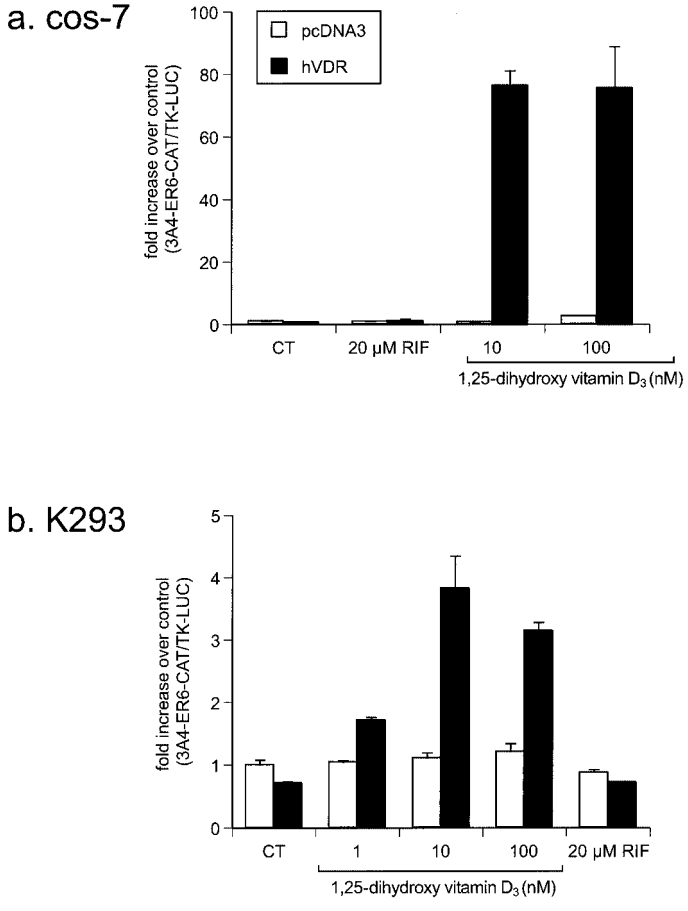

Fig. 6. 1,25-D_3 uses the hVDR to activate the *CYP3A4* PXRE. Cos-7 or 293 cells were cotransfected with *CYP3A4*-$(ER6)_3$-CAT and hVDR expression plasmid or pcDNA3 vector and TK-LUC. Eighteen hours later, cells were treated with various concentrations of 1,25-D_3 or 20 μM rifampin, and CAT activity was measured 24 h later, normalized to TK-luciferase activity and expressed as the -fold increase in activity of treated group compared with activity in control cells. Each bar indicates the average of triplicate determinations from a single experiment and is representative of results from at least three independent experiments.

Our data demonstrate that the VDR-RXR complex binds to the *CYP3A4* PXRE (ER6) to induce *CYP3A* transcription. Although the most common VDR binding sites are direct repeats of AGGTCA separated by three nucleotides (DR3), there is precedence for the VDR-RXR heterodimer to bind to a DR6 (Kahlen and Carlberg, 1994). Alternatively, the VDR-RXR heterodimer could be recognizing the *CYP3A4* PXRE as an imperfect DR4. Although the *CYP3A4* PXRE is an imperfect ER6 (TGAACT CAAAGG AGGTCA), it is also an imperfect DR4 (AACTCA AAGG AGGTCA), and VDR-RXR heterodimers bind even more avidly to DR4s than two DR3s (Quack and Carlberg, 2000). In addition, although we tested only the potential for the VDR-RXR complex to bind to the *CYP3A4* proximal ER6, there is a distal enhancer module containing nuclear hormone receptor sites including a DR3 and adjacent ER6 at nucleotides −7273/−7286, −7733/−7719, and −7689/7672, respectively, in the *CYP3A4* gene (Goodwin et al., 1999) that probably cooperates in mediating maximal VDR induction of *CYP3A4*.

The proposed regulation of intestinal CYP3A4 by VDR is complementary to the apparent role of PXR in regulating hepatic CYP3A4. VDR expression is more prominent in the human and rat small intestine, compared with liver (Berger et al., 1988; Stumpf, 1995), and the converse is true for PXR expression (Blumberg et al., 1998; Lehmann et al., 1998). Indeed, PXR was not involved in activation of the *CYP3A4* promoter after treatment of Cos-7 and 293 cells with 1,25-D_3, or in the 1,25-D_3 induction of CYP3A4 expression in Caco-2 cells that lacked PXR. Moreover, our results demonstrate that 1,25-D_3 does not ligand activate PXR.

The absence of PXR in Caco-2 cells helps explain the previously observed failure to induce CYP3A4 after treatment with rifampin and other known CYP3A4 inducers (Schmiedlin-Ren et al., 1997). Rifampin did not activate hVDR and CYP3A4 transcription in cotransfected Cos-7 and 293 cells (Fig. 4); thus, no effect should be expected. The deficiency of PXR in Caco-2 cells limits their use for studying induction phenomena, including other PXR targets such as MDR-1 (Synold et al., 2001). However, our data from LS180 and Caco-2 cell culture experiments (Fig. 1; Table 1) demonstrates that the MDR-1 gene and production of P-glycoprotein is also induced by 1,25-D_3 treatment. Presumably, transcriptional activation occurs through the binding of the VDR-RXR complex with PXR response elements in the 5′-flanking sequences of MDR-1 (Geick et al., 2001).

Our results demonstrate conclusively that formation of a 1,25-D_3–VDR complex is sufficient for activation of *CYP3A4* transcription and, thus, identify the vitamin D receptor as potentially important regulator of intestinal CYP3A4 expression. Therefore, interindividual differences in the expression of intestinal CYP3A4 could arise from known mutations in the vitamin D receptor (Hughes et al., 1988) and from differences in intestinal VDR content and circulating unbound 1,25-D_3. Why does the vitamin D receptor induce CYP3A4 in intestinal cells? CYP3A4 is not involved in the metabolism of

vitamin D and thus should not participate in an autoregulatory process. Perhaps it contributes to a protective enzymatic barrier to the influx of potentially toxic xenobiotic molecules. Indeed, vitamin D has been shown to enhance differentiation of intestinal cells (Halline et al., 1994). Moreover, one typically thinks of induction of CYP3A as a mechanism that has evolved to protect the organism from toxic chemicals, including toxins in the diet, by enhancing their elimination. For example, it has been speculated that enterocyte metabolism of aflatoxin-B1 by CYP3A4 may actually protect from dietary aflatoxins (Kolars et al., 1994a). An inverse association has been identified between intake of vitamin D and risk of colorectal cancer (Martinez et al., 1996). Thus, the role of vitamin D in protection from colorectal cancer could include the induction of CYP3A4 and the accelerated detoxification of toxins found in the diet.

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