

# The *SLCO1A2* Gene, Encoding Human Organic Anion-Transporting Polypeptide 1A2, Is Transactivated by the Vitamin D Receptor

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## ABSTRACT

Organic anion-transporting polypeptide 1A2 (OATP1A2) (gene symbol, *SLCO1A2*) mediates cellular uptake of a wide range of endogenous substrates, as well as drugs and xenobiotics. OATP1A2 is expressed in several tissues, including apical membranes of small intestinal epithelial cells. Given its role in intestinal drug absorption, a detailed analysis of the mechanisms that regulate *SLCO1A2* gene expression is potentially of great pharmacological relevance. We show here that treatment of human intestine-derived Caco-2 cells with vitamin D<sub>3</sub> markedly increased endogenous OATP1A2 mRNA and protein levels. Suppression of endogenous vitamin D receptor (VDR) expression with siRNAs significantly reduced this induction. Two alternative promoter regions exist in genomic databases for the *SLCO1A2* gene. One putative VDR response element (VDRE) that was predicted to interact efficiently with VDR-retinoid X

receptor  $\alpha$  (RXR $\alpha$ ) was identified in silico within *SLCO1A2* promoter variant 1. This VDRE served as a strong binding site for the recombinant VDR-RXR $\alpha$  heterodimers in vitro and was potentially activated by VDR in the presence of vitamin D<sub>3</sub> in heterologous promoter assays. In reporter assays using native promoter constructs, *SLCO1A2* promoter variant 1 was strongly induced by VDR, and site-directed mutagenesis of a single VDRE within this region abolished this activation. Native VDR-RXR $\alpha$  also interacted with this element both in vitro and in living cells. We showed that expression of the *SLCO1A2* gene is induced by vitamin D<sub>3</sub> at the transcriptional level through the VDR. Our results suggest that pharmacological administration of vitamin D<sub>3</sub> may allow modulation of intestinal absorption of OATP1A2 transport substrates.

## Introduction

Organic anion-transporting polypeptides (OATPs) are 12-transmembrane domain transporter proteins encoded by the *SLCO* gene superfamily (Hagenbuch and Meier, 2004). Eleven OATP members have been identified in humans, and they can be classified into six families according to the amino acid sequence similarities between them. OATP proteins are expressed in a number of epithelial tissues throughout the body and transport a wide range of mainly amphipathic molecules in a polyspecific and sodium-independent manner. In addition to being essential for drug disposition,

certain OATPs seem to be dysregulated in several cancers and may prove to be important targets for anticancer therapy (Obaidat et al., 2012). Among the OATPs, members of the OATP1 and OATP2 families, many of which play crucial roles in the main metabolic organs (liver and intestine), are functionally best characterized to date. OATP1B1 (gene symbol, *SLCO1B1*) and OATP1B3 (*SLCO1B3*) are expressed at the sinusoidal membranes of hepatocytes, whereas OATP2B1 (*SLCO2B1*) is expressed at the apical membrane of enterocytes and the sinusoidal membrane of hepatocytes. Another OATP, namely, OATP1A2, is expressed in the intestine. The OATP1A2 protein (*SLCO1A2*) is located at the apical membranes of enterocytes (Glaeser et al., 2007), although mRNA levels for OATP1A2 are low in all regions of the intestine (Meier et al., 2007; Wojtal et al., 2009). Several other tissues also express OATP1A2, including the blood-brain barrier and cholangiocytes (Gao et al., 2000; Lee et al., 2005). OATP1A2 was the first human OATP

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**ABBREVIATIONS:** OATP, organic anion-transporting polypeptide; VDR, vitamin D receptor; VDRE, vitamin D response element; RXR, retinoid X receptor; LCA, lithocholic acid; DR-3, direct repeat-3; PCFT, proton-coupled folate transporter; siRNA, short interfering RNA; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; MRP, multidrug resistance-associated protein; PXR, pregnane X receptor; PBS, phosphate-buffered saline; PBS-T, PBS/Tween 20; PCR, polymerase chain reaction; BQ-123, cyclo(D-Trp,D-Asp,L-Pro,D-Val,L-Leu); CRC-220, 4-methoxy-2,3,6-trimethylphenylsulfonyl-L-aspartyl-D-4-amidinophenylalanyl-piperidine.

to be cloned (Kullak-Ublick et al., 1995), and it mediates the uptake of a variety of endogenous amphipathic substrates, as well as drugs and xenobiotics. Transport substrates include bile acids, conjugated sex steroids, thyroid hormones, prostaglandin E<sub>2</sub>, the endothelin receptor antagonist cyclo (D-Trp,D-Asp,L-Pro,D-Val,L-Leu) (BQ-123), the thrombin inhibitor 4-methoxy-2,3,6-trimethylphenylsulfonyl-L-aspartyl-D-4- amidinophenylalanyl-piperidide (CRC-220), the opioid receptor agonists [D-Pen<sup>2</sup>,D-Pen<sup>5</sup>]-enkephalin and deltorphin II, magnetic resonance imaging contrast agents, ouabain, and the cyanobacterial toxin microcystin (Kullak-Ublick et al., 1995; Hagenbuch and Meier, 2004). *SLCO1B1* (Jung and Kullak-Ublick, 2003) and *SLCO1B3* (Jung et al., 2002) genes have been shown to be regulated by the nuclear receptor for bile acids, the farnesoid X receptor (*NR1H4*), indirectly and directly, respectively. Relatively little was known previously about the gene regulation of *SLCO1A2*. Since the initial characterization of the *SLCO1A2* promoter (now termed variant 2) (Kullak-Ublick et al., 1997), the genomic databases have been updated to identify another *SLCO1A2* promoter variant 1, 60 kilobase pairs away from the original promoter variant 2.

In addition to its established roles in the regulation of calcium homeostasis, blood pressure, and electrolyte levels, the biologically active metabolite of vitamin D [i.e., 1,25-dihydroxyvitamin D<sub>3</sub> (vitamin D<sub>3</sub>)] exhibits many other important functions (Demay, 2006; Bouillon et al., 2008). In particular, its roles as a crucial regulator of the differentiation and proliferation of enterocytes and in mucosal immunity of the gastrointestinal tract have attracted increasing attention. For example, vitamin D<sub>3</sub> was shown to preserve the healing capacity in the colonic epithelium (Kong et al., 2008). Most effects of vitamin D<sub>3</sub> are mediated through its action as an agonistic ligand for the vitamin D receptor (VDR) (gene symbol, *NR1H1*), a nearly ubiquitously expressed member of the nuclear receptor family of transcription factors (Dusso et al., 2005). We note, however, that nongenomic actions by vitamin D<sub>3</sub> have become increasingly well recognized (Christakos et al., 2003; Dusso et al., 2005). In addition to using its classic ligand vitamin D<sub>3</sub>, VDR can function as an intestinal bile acid sensor by using the secondary bile acid lithocholic acid (LCA) as its agonistic ligand, thus inducing CYP3A expression (Makishima et al., 2002). Given the potential intestinal carcinogenic nature of LCA, this feature may contribute to the proposed protective effects of VDR against colon cancer (Thorne and Campbell, 2008). VDR typically regulates gene expression by interacting directly with direct repeat-3 (DR-3)-like DNA motifs (variants of a direct repeat of [A/G]G[G/T]TCA-like hexamers separated by three bases, the last of which is preferably purine) within the target promoters, as a heterodimer with another nuclear receptor, retinoid X receptor- $\alpha$  (RXR $\alpha$ ) (gene symbol, *NR2B1*) (Haussler et al., 1997).

It was reported that a number of genes encoding intestinal membrane transporters for both endogenous substances and drugs and xenobiotics are regulated by vitamin D<sub>3</sub> and VDR (Eloranta et al., 2009; Fan et al., 2009; Tachibana et al., 2009; Chow et al., 2010; Maeng et al., 2011). We have investigated whether vitamin D<sub>3</sub> and VDR regulate the expression of the *SLCO1A2* gene, which encodes an important membrane transporter for intestinal absorption of numerous drugs.

## Materials and Methods

**Chemicals.** [ $\alpha$ -<sup>32</sup>P]dATP (6000 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Restriction enzymes were obtained from Roche Diagnostics (Basel, Switzerland) and the T4 DNA ligase from Promega (Dübsdorf, Switzerland). The oligonucleotides were synthesized at Microsynth (Balgach, Switzerland). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise.

**Cell Culture.** Caco-2 cells (LGC Promochem, Molsheim, Switzerland), derived from human colon carcinoma, were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were grown at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Isolation of RNA, Reverse Transcription, and Quantitative Real-Time PCR.** Total RNAs from 80% confluent Caco-2 cells grown on 12-well plates were isolated with TRIzol reagent (Invitrogen). RNA levels were quantified spectrophotometrically at 260 nm (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, MA), and 2  $\mu$ g of total RNAs were reverse-transcribed by using random primers and a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNAs were diluted to a final volume of 100  $\mu$ l with nuclease-free water (Applied Biosystems). For quantitative real-time PCR assays, 2  $\mu$ l of diluted cDNAs were used for each reaction. TaqMan gene expression assays Hs00245360\_m1, Hs00611081\_m1, and Hs01045844\_m1 (Applied Biosystems) were used to measure OATP1A2, proton-coupled folate transporter (PCFT), and VDR cDNA levels, respectively, by using an ABI Prism 7900HT fast real-time PCR system (Applied Biosystems). Human  $\beta$ -actin cDNA levels (TaqMan gene expression assay 4310881E; Applied Biosystems) were measured for normalization of relative OATP1A2, PCFT, and VDR expression levels, which were calculated by using the comparative threshold cycle method. All PCR tests were performed in triplicate.

**Preparation of Whole-Cell Protein Extracts and Immunoblotting.** For preparation of whole-cell protein extracts, cells on 12-well plates were washed with ice-cold PBS and lysed with a 5-min incubation in 250  $\mu$ l of ice-cold lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Igepal CA-630, 0.5% (w/v) sodium deoxycholate, 1 mM EDTA, 0.1% (w/v) SDS, 10% (v/v) glycerol] supplemented with complete protease inhibitors (Roche Diagnostics). Debris was removed through centrifugation at 20000g for 30 min at 4°C. Protein concentrations were determined with the bicinchoninic acid protein assay (Thermo Fisher Scientific), and the samples were stored at -80°C until use. Ten micrograms of protein extracts were separated on 12% sodium dodecyl sulfate-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Hybond ECL; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Membranes were blocked for 1 h in 5% (w/v) nonfat milk in PBS-T [PBS with 0.1% (v/v) Tween 20]. After this, the membranes were probed overnight with a rabbit polyclonal antibody against OATP1A2 (LS-C40428; LabForce, Nunningen, Switzerland), at a concentration of 0.25  $\mu$ g/ml in 5% (w/v) nonfat milk/PBS-T. After three washes with 5% (w/v) nonfat milk/PBS-T, horseradish peroxidase-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific), at a concentration of 10 ng/ml in 5% (w/v) nonfat milk/PBS-T, was added for 1 h. Blots were then washed three times with 5% (w/v) nonfat milk/PBS-T and twice with PBS, followed by detection with the SuperSignal West Femto maximal sensitivity substrate (Thermo Fisher Scientific) and exposure on Hyperfilm ECL (GE Healthcare). To verify equal loading of the protein samples, the blots were stripped with Restore Western blot stripping solution (Thermo Fisher Scientific), reblocked, and reprobed for constitutively expressed  $\beta$ -actin protein. The  $\beta$ -actin probing and detection were performed as described above, except that the polyclonal anti-rabbit  $\beta$ -actin antibody (ab8227; Abcam, Cambridge, UK) was used at a concentration of 100 ng/ml and was added to the blots for 1 h.

**Transfections with Short Interfering RNAs.** Nearly confluent Caco-2 cells grown on 12-well plates were transfected with 40 nM final concentrations of ON-TARGETplus SMARTpool siRNAs designed to target VDR (L-003448-00; Thermo Fisher Scientific) or siGENOME nontargeting siRNA 1 (D-001210-01; Thermo Fisher Scientific), which is not known to target any human gene, by using the transfection reagent siLentFect (Bio-Rad Laboratories S.A., Reinach, Switzerland). The transfections were repeated identically after 24 h, and RNAs were harvested after 48 h of total transfection time. It was necessary to transfect the same cells twice with siRNAs to achieve efficient silencing of gene expression.

**Electrophoretic Mobility Shift Assays.** Electrophoretic mobility shift assay (EMSA) binding reactions and gel analyses were performed as described previously (Eloranta et al., 2009). The top strands of the EMSA oligonucleotide probes are listed in Table 1. We designed overhangs of 5'-AGCT (top strand) and 5'-GATC (bottom strand) to be present in all annealed EMSA oligonucleotides, which allowed their radioactive labeling with [ $\alpha$ - $^{32}$ P]dATP in filling-in reactions using MultiScribe reverse transcriptase (Applied Biosystems). Recombinant VDR and RXR $\alpha$  proteins were synthesized with the TNT T7 coupled reticulocyte lysate system (Promega), using the plasmids pCMX-VDR and pCMX-RXR $\alpha$  as templates. Caco-2 nuclear protein extracts were prepared from cells at 70 to 80% confluence by using a NE-PER kit (Perbio Science, Lausanne, Switzerland). Protein concentrations of nuclear extracts were determined by using a bicinchoninic acid assay kit (Perbio Science). In competition EMSA experiments, the unlabeled oligonucleotides were added immediately before the radioactive probes. In antibody supershift experiments, 1  $\mu$ g of anti-VDR antibody (C-20X; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added and the reactions were incubated at 4°C for 1 h before addition of the radioactive probes.

**Chromatin Immunoprecipitation Assays.** Caco-2 cells were grown to 80% confluence on two 10-cm plates per culture condition, after which they were treated with either 500 nM vitamin D $_3$  or the vehicle (ethanol). Twenty-four hours later, the cells were harvested through crosslinking with 1% methanol-free formaldehyde (Perbio Science) and were processed through chromatin immunoprecipitation by using a ChIP-IT Express kit (Active Motif, Carlsbad, CA). Shearing of the chromatin was achieved with five pulses of sonication, with 30-s pauses on ice between the pulses, by using a Branson digital sonifier (Branson Ultrasonics, Danbury, CT) at a power setting of 25%. For the immunoprecipitation steps, aliquots from both test conditions were incubated without any antibody, with 1  $\mu$ g of negative-control mouse IgG1 antiserum (X0931; Dako A/S, Baar, Switzerland) or with 1  $\mu$ g of one of the two antibodies raised against VDR (C-20X or H-81; Santa Cruz Biotechnology). The anti-VDR antibodies were selected on the basis of their ability to immunoprecipitate in vitro-translated, radiolabeled, VDR protein under ChIP conditions (data not shown). Two amplicons were assayed for immunoprecipitation tests, by using PuReTaq Ready-To-Go PCR beads (GE Healthcare) and the oligonucleotide primers listed in Table 1 [i.e., *SLCO1A2*(+83/+334) (primer locations indicated in Fig. 3) containing the *SLCO1A2*(+202/+216) element from promoter variant 1 and *SLCO1A2*(-3431/-3182) located within *SLCO1A2* promoter variant 2]. After the initial stage of denaturation at 94°C for 3 min, the PCR cycling conditions were 94°C for 20 s, 60°C for 30 s, and 72°C for 1 min. After 35 and 40 cycles, 10  $\mu$ l of each PCR product were resolved on 1.5% agarose gels and detected with SYBR Safe DNA gel stain (Invitrogen).

**DNA Constructs.** By using a pool of human genomic DNA (Clontech, Mountain View, CA) as the template and PuReTaq Ready-To-Go PCR beads (GE Healthcare), the -227/+347 region of human *SLCO1A2* promoter variant 1 (National Center for Biotechnology Information reference sequence, NT\_009714.17) was obtained through PCR using the oligonucleotide primers listed in Table 1. The PCR product *SLCO1A2*(-227/+347) was cloned into the pGEM-T vector (Promega) and then subcloned into the MluI/SmaI-digested pGL3basic luciferase reporter vector (Promega) by using the engi-

TABLE 1

Sequences of oligonucleotides used for cloning, site-directed mutagenesis, and EMSAs

Only the top strands are shown for oligonucleotides used in mutagenesis and EMSAs. Where applicable, restriction sites introduced are underlined and the corresponding enzymes used are given in parentheses. The DR-3 motifs are in *italics*, and mutated nucleotides within the *SLCO1A2*(+202/+216) DR-3 element are indicated in *bold*.

Oligonucleotide	Sequence (5'-3')	Purpose
<i>SLCO1A2</i> (-227/fwd)	ACGCGTCTGGGAGACCCACTGCTCTCTTC (MluI)	Cloning of <i>SLCO1A2</i> (-227/+347)luc
<i>SLCO1A2</i> (+347/rev)	GATATCTGCACTTTCATCTGAGTACCGGTTTCATCTC (EcoRV)	Cloning of <i>SLCO1A2</i> (-227/+347)luc
<i>SLCO1A2</i> (+202/+216)sdm_top	CTTTGACTCAGAAAGGGAACCTCCCTG <b>GC</b> ACCTTGAGCTCCCAAG	Mutagenesis of <i>SLCO1A2</i> (+202/+216) VDRE consensus EMSA probe; cloning of HSV- $\theta$ -luc constructs
<i>VDRE</i> con EMSA_top	AGCTGCAGGGGAGGTCACAGAGGTACACACTAGTA	VDRE consensus EMSA probe; cloning of HSV- $\theta$ -luc constructs
<i>SLCO1A2</i> (+202/+216)wt EMSA_top	AGCTTCAGAAAGGGAACCTCCCTGAGCCCTTGAGCT	<i>SLCO1A2</i> (+202/+216) EMSA probe/competitor; cloning of HSV- $\theta$ -luc constructs
<i>SLCO1A2</i> (+202/+216)mut EMSA_top	AGCTTCAGAAAGGGAACCTCCCTG <b>GC</b> ACCTTGAGC	<i>SLCO1A2</i> (+202/+216) EMSA probe/competitor
<i>SLCO1A2</i> (-101/-87)EMSA_top	AGCTAGCAGGACGGCTCTCTTGAGCTGCTGGTG	<i>SLCO1A2</i> (-101/-87) EMSA probe; cloning of HSV- $\theta$ -luc constructs
<i>SLCO1A2</i> (+83/+334)CHIP_fwd	CTGAGGTACCGGTTTCATCTCAC	ChIP assay
<i>SLCO1A2</i> (+83/+334)CHIP_rev	GTGGGGATATAATCTGTGGCGCG	ChIP assay
<i>SLCO1A2</i> var2(-3431/-3182)CHIP_fwd	CTGTGTTAGGGGAATGGTTG	ChIP assay
<i>SLCO1A2</i> (-3431/-3182)CHIP_rev	CAAGAGTCAGGATCTCTCC	ChIP assay



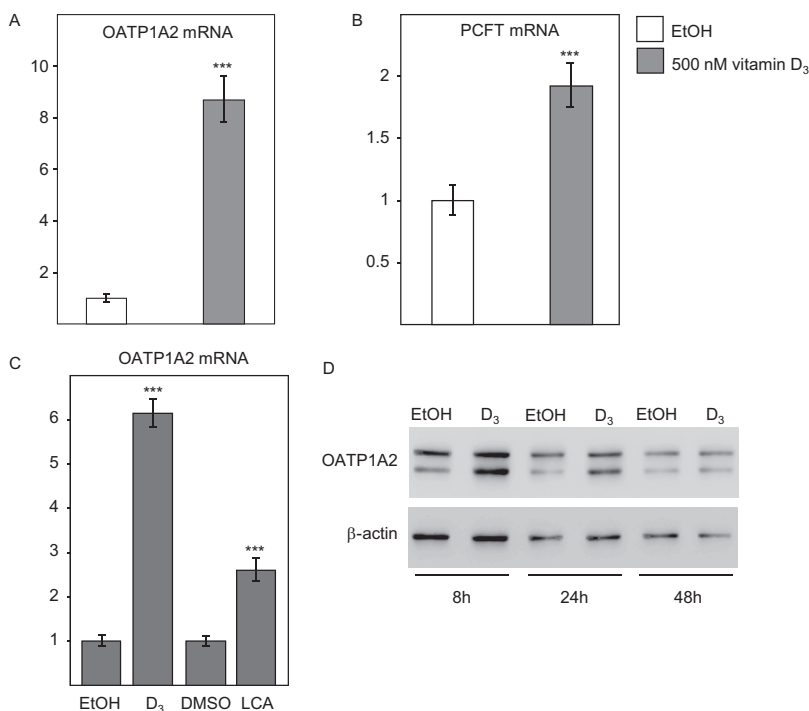
needed recognition sites for the restriction enzymes MluI and EcoRV. Construction of the luciferase reporter plasmid containing ~1600 proximal base pairs of *SLCO1A2* promoter variant 2 was described previously (Kullak-Ublick et al., 1997). Point mutations within the *SLCO1A2*(+202/+216) element were created in the *SLCO1A2*(-227/+347)luc construct by using a QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and oligonucleotides shown in Table 1. To create heterologous promoter constructs containing either the consensus VDRE, the wild-type *SLCO1A2*(+202/+216) element, or a mutated version of that element, single-stranded oligonucleotides (Table 1) containing HindIII and BamHI overhangs were annealed and then cloned into the HSV-*tk*-luc vector. The correct identities of all constructs were verified through DNA sequencing (Microsynth). The expression plasmids for the human VDR (pCMX-VDR) and the human RXR $\alpha$  (pCMX-RXR $\alpha$ ) were kindly donated by Dr. David Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX).

**Plasmid Transfections and Dual-Luciferase Reporter Assays.** Caco-2 cells were seeded on 48-well plates, at a confluence of 70%, 1 day before transfections. Cells were transiently transfected with 400 ng of the firefly luciferase constructs together with 200 ng of VDR and/or RXR $\alpha$  expression plasmids by using FuGENE HD reagent (Roche Diagnostics, Basel, Switzerland). For normalization of the amount of cytomegalovirus promoter-containing expression constructs transfected, an appropriate amount of the pcDNA3.1(+) vector (Invitrogen) was included in the transfection mixtures. To control for variations in transfection efficiency, 100 ng of the pHG-TK *Renilla reniformis* luciferase reporter plasmid (Promega) was cotransfected in each well. Twelve hours after transfection, cells were treated with the ligands 500 nM vitamin D<sub>3</sub> and/or 1  $\mu$ M 9-*cis*-retinoic acid (Sigma-Aldrich) or the vehicles ethanol and/or dimethylsulfoxide, respectively. Twenty-four hours after addition of the ligands, cells were harvested in 1 $\times$  passive lysis buffer (Promega) and luciferase activities were measured by using a GloMax-Multi detection system (Promega). Relative promoter activities were obtained by normalizing firefly luciferase activities to *R. reniformis* luciferase activities. The control condition values were set to 1, and all other results are shown relative to those values. Triplicate wells were measured for each transfection test.

**Statistical Analysis.** All experiments shown were repeated two or three times. Statistical analyses were performed with Prism (GraphPad Software, Inc., San Diego, CA). Error bars represent S.D. of the mean values. For luciferase and real-time PCR experiments, one-way analyses of variance, followed by post hoc Tukey's tests, were performed to determine statistical significance.

## Results

**Vitamin D<sub>3</sub> Induction of OATP1A2 mRNA and Protein Expression in Human Intestine-Derived Caco-2 Cells.** Human, well polarized, enterocyte-derived Caco-2 cells exhibit many characteristics associated with differentiated enterocytes and were used here for investigation of the effects of vitamin D<sub>3</sub> on *SLCO1A2* gene expression. Nearly confluent Caco-2 cells were treated with 500 nM vitamin D<sub>3</sub> for 24 h, after which endogenous OATP1A2 mRNA expression was measured with quantitative real-time PCR. As shown in Fig. 1A, OATP1A2 mRNA levels were induced ~9-fold under these conditions, compared with vehicle-treated cells. As a positive control, we measured the effects of vitamin D<sub>3</sub> treatment on a known target gene, *SLC46A1* (Eloranta et al., 2009), which encodes the PCFT, in parallel. PCFT mRNA expression was induced ~2-fold by vitamin D<sub>3</sub> treatment (Fig. 1B), which indicates how potent the effect on OATP1A2 expression is. We tested whether the hydrophobic bile acid LCA, which is known to function as an agonistic VDR ligand (Makishima et al., 2002), could affect OATP1A2 mRNA expression. Treatment of Caco-2 cells with 50  $\mu$ M LCA for 24 h led to significant induction of OATP1A2 mRNA, although to a lesser degree than with vitamin D<sub>3</sub> treatment (Fig. 1C). In addition to mRNA expression, we studied whether vitamin D<sub>3</sub> treatment of Caco-2 cells increased OATP1A2 protein expression. In agreement with the mRNA results, OATP1A2 protein levels were clearly increased with vitamin D<sub>3</sub> treatment for 24 h (Fig. 1D). This increase was



**Fig. 1.** Induction of OATP1A2 mRNA and protein expression by agonistic ligands for VDR in Caco-2 cells. A and B, treatment of cells with 500 nM vitamin D<sub>3</sub> (D<sub>3</sub>) for 24 h increased the mRNA expression of OATP1A2 (A) and PCFT (B). C, the VDR agonist LCA (50  $\mu$ M) similarly increased OATP1A2 mRNA expression. D, OATP1A2 protein expression was enhanced by the treatment of cells with vitamin D<sub>3</sub> for 8 or 24 h, after which (48 h) induction was attenuated. \*\*\*,  $P < 0.001$ . EtOH, ethanol; DMSO, dimethylsulfoxide.

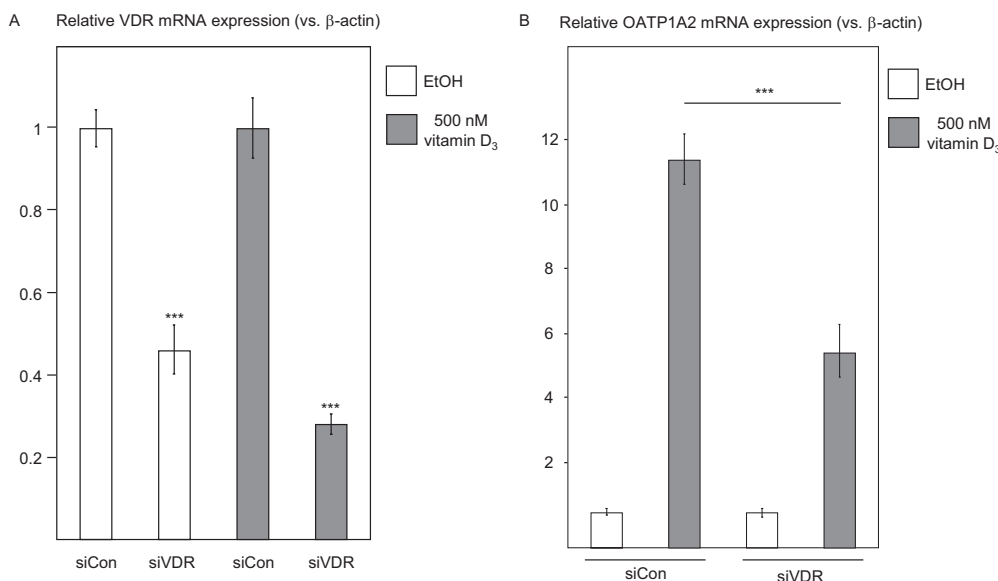
already visible at 8 h after the beginning of treatment but was clearly beyond its peak at 48 h.

**Short Interfering RNA-Mediated Reduction of VDR Expression and Suppression of Vitamin D<sub>3</sub>-Mediated Induction of OATP1A2 mRNA Expression.** We next transfected a pool of four siRNAs specifically targeting the nuclear receptor for vitamin D<sub>3</sub>, VDR, into Caco-2 cells in parallel with control siRNAs that are not known to target any human genes. As shown in Fig. 2A, VDR siRNA transfection led to significant (to 50–70%) reduction in VDR mRNA expression in both vehicle- and vitamin D<sub>3</sub>-treated cells (Fig. 2A). The VDR siRNA-transfected cells exhibited significantly reduced induction of OATP1A2 mRNA expression with vitamin D<sub>3</sub> treatment (Fig. 2B), which indicates that the effect of vitamin D<sub>3</sub> is mediated by its nuclear receptor, VDR.

**In Silico Analysis of *SLCO1A2* Promoter Regions.** Having shown the VDR-dependent induction of OATP1A2 expression by vitamin D<sub>3</sub>, we performed an in silico analysis of the *SLCO1A2* gene, to locate putative VDR-responsive elements (VDREs) of the DR-3-like configuration. The *SLCO1A2* gene is located in chromosome 12, and we used the genomic contig GRCh37.p5 primary assembly as our sequence of analysis (National Center for Biotechnology Information reference sequence, NT\_009714.17). Two transcript variants encoding identical OATP1A2 protein products are listed within this contig, with their transcription start sites separated by ~60 kilobase pairs. We analyzed 2 kilobase pairs upstream of each transcription start site plus the noncoding exon 1 (variant 1) or the exon 1 region up to the translational start site (variant 2) in silico with the algorithms MatInspector (Cartharius et al., 2005) and NubiSCAN (Podvinec et al., 2002), as well as through visual inspection. Within these regions, we identified one putative DR-3-like motif (GGGTCAGGGAGTTCC) predicted to function as a VDRE (Colnot et al., 1995) at position +202/+216 on the minus strand downstream of the transcription start site of variant 1 (Fig. 3). No near-consensus VDREs were predicted within the analyzed segment of *SLCO1A2* promoter variant 2.

### Direct Interaction of VDR-RXR $\alpha$ Heterodimers with the *SLCO1A2*(+202/+216) DR-3-Like Motif In Vitro.

Next, we studied whether the VDR-RXR $\alpha$  heterodimers interacted directly with the +202/+216 DR-3-like motif on the *SLCO1A2* promoter in vitro, by using EMSAs. When both in vitro-translated recombinant VDR and RXR $\alpha$  were mixed with the double-stranded, radioactively labeled, *SLCO1A2* (+202/+216) probe in the presence of 500 nM vitamin D<sub>3</sub> and 1  $\mu$ M retinoic acid, a distinct protein-DNA complex with mobility identical to that of the complex formed with the VDRE consensus probe was formed, although to a somewhat lesser degree than with the consensus probe (Fig. 4A). This complex formed only when both recombinant VDR and RXR $\alpha$  were included in the binding reactions, which confirmed that VDR binds to the *SLCO1A2*(+202/+216) element as a heterodimer with RXR $\alpha$ , instead of a homodimer or a monomer. As a negative control, we used an oligonucleotide from *SLCO1A2* promoter variant 1 at position -101/-87, relative to the transcription start site. This sequence loosely conforms to the DR-3-like motif but contains several base substitutions predicted to prevent VDR-RXR $\alpha$  binding (Colnot et al., 1995). The latter oligonucleotide probe could not form a complex with recombinant VDR and RXR $\alpha$ . As confirmation of the specific nature of the DNA binding of VDR-RXR $\alpha$  to the *SLCO1A2*(+202/+216) element, we performed EMSA competition experiments. When present in molar excess, unlabeled double-stranded oligonucleotides containing the wild-type *SLCO1A2*(+202/+216) region were efficient at competing with endogenous proteins present in Caco-2 nuclear extracts that were capable of binding to the radiolabeled VDRE consensus probe. A 10-fold molar excess of the wild-type oligonucleotide was sufficient to reduce complex formation, and this effect was enhanced stepwise when 50-fold and 100-fold excesses of this oligonucleotide were added to the DNA binding reactions (Fig. 4B). When the mutant version of the same element, containing two base changes predicted to disrupt complex formation with VDR-RXR $\alpha$ , was used as the competing oligonucleotide, it could not notably compete for protein complex formation with the VDRE consensus element. When an antibody raised against VDR was added to the binding reac-



**Fig. 2.** Transfection of siRNAs specifically targeting VDR (siVDR) and attenuation of OATP1A2 mRNA induction by vitamin D<sub>3</sub> in Caco-2 cells, compared with control siRNA (siCon)-transfected cells. A, VDR-specific siRNAs significantly suppressed endogenous VDR mRNA expression in both ethanol (EtOH)- and vitamin D<sub>3</sub>-treated cells. B, VDR-specific siRNAs significantly suppressed OATP1A2 mRNA induction by vitamin D<sub>3</sub>. VDR and OATP1A2 mRNA expression levels were normalized to those of the housekeeping gene  $\beta$ -actin. \*\*\*,  $P < 0.001$ .

A NT\_009714.17: *SLCO1A2* promoter variant 1 (transcript variant 1; NM\_134431.3; 7695 bp)

tgtccagctgctgctgggagaccactgctctcttcaaagctgtcagacagggacatttaagtctgcagaggttactgctgtcttttgt  
 ttgtctgtgcccctgccccagaggtggagcctacagagcagcagcagcctcttctgagctgtgggtccaccagcttcagagcttcacag  
 ctgctttgtttacctaatacaagcctgggcaatggcgggcccctccccagctctgctgctgcttgcagttgatctcagactgctgtg  
 cttagcaatcagcgagactccatggcgtaggacctccgagccaggtgctgggataatacttctgtggcgccgttttttaagcctgttga  
 agagcgcagtagtattcggtgggagtgaccgattttccaggtgcgtccgtcacccttcttcttgaactcagaaag  
 gagcttcccaagttaggcaatgcctcaccctgcttggcttgcgcacggtgcgcgacccactgacctgcgccattgtctggcactccct  
 agtgagatgaacccgggtacctcagatggaaatgcagaaatc... ~60.8 kbp ...acctgaagagcaacATGggagaaactgagaaaagaa  
 pos. 14247705  
 ChIP fwd  
 ChIP rev

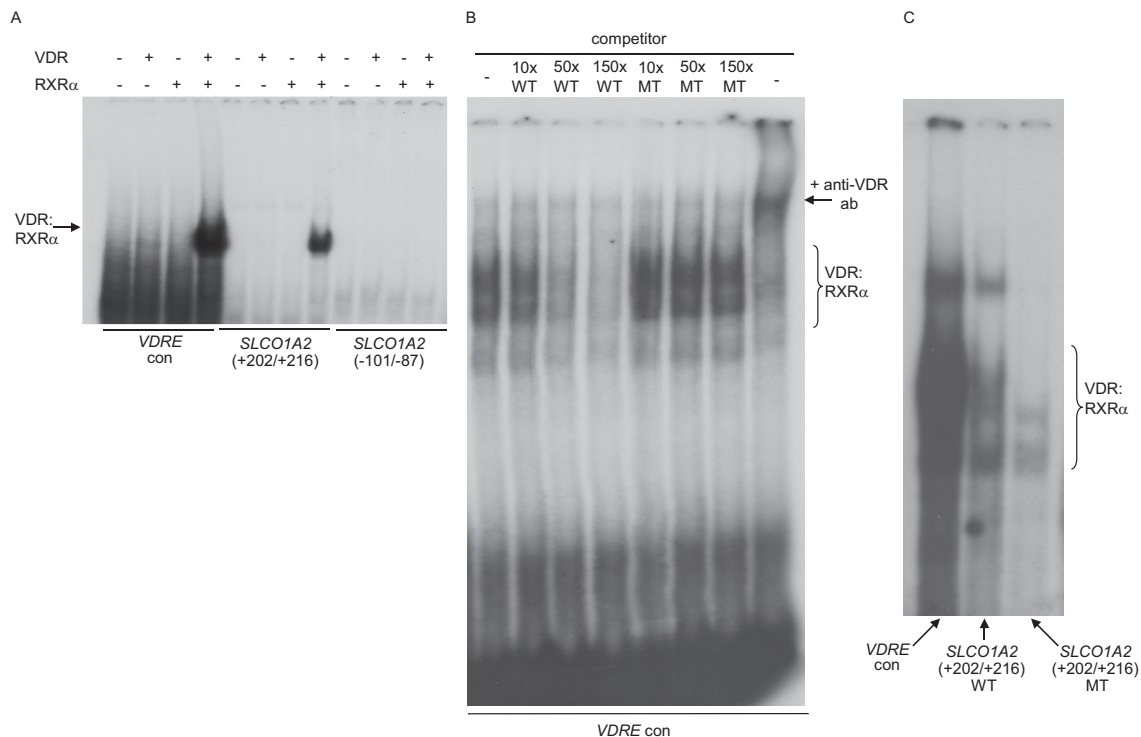
B NT\_009714.17: *SLCO1A2* promoter variant 2 (transcript variant 2; NM\_021094.3; 7225 bp)

tcatgattaaaattccgtgtctccccaggtttccatgtatcagcagtgccccagaaagtgttcccttctgagactctaggaagccttg  
 gaatgcaataacttgaaaagagagcctggccaaaaaaagatttttgagccttggtagggttagtgaagcaggttttttagtacaacacaa  
 ctcaaagtatgtgtgtattaaaagttagctattttcatggaagtcttttggtagctatttggtagtagaagtagaaagcctttctttt  
 aaccatgtgaccattattttgcaagatcatacaacaatttctaatacttttaactcactaaagagtcactaaagtattttgatgtacct  
 tttgaaatagaaaaccaacaactaaattcttcttaacaccatgatttttctaactgtattaaagcgttccagggtatttttgtaatgcaatg  
 gatttcaattatgttggcttcttctcagcacttacttcaaaagaatgatgctttacaattttgtagtttaatttaacataattttttgtttgc  
 ttgaaagggtagaagtaagacatatcaagatttaaacataaatagcttggaaacccctgaagagcaacATGggagaaactgagaaaagaa  
 pos. 14247956

**Fig. 3.** Proximal promoter sequences of *SLCO1A2* gene variants. Transcription start sites (+1) are indicated with bold letters and arrows and the common translation start site (ATG) for both variants with bold capital letters. Numbering of the base positions is according to that for chromosome 12 genomic contig NT\_009714.17 (<http://www.ncbi.nlm.nih.gov>). A, *SLCO1A2* promoter variant 1. The location of the +202/+216 DR-3-like element is indicated with arrows and framing. The locations of the ChIP oligonucleotide primers are underlined. B, *SLCO1A2* promoter variant 2.

tion before the probe, a supershift of the complexes formed with the radioactive probes was observed, which confirmed the identity of the VDR-containing complexes. In a reverse

experiment, we used the wild-type and mutant *SLCO1A2*(+202/+216) sequence-containing elements as radioactive probes. As shown in Fig. 4C, the radioactive wild-



**Fig. 4.** Specific binding of VDR-RXR $\alpha$  heterodimers to the +202/+216 DR-3-like motif of *SLCO1A2* promoter variant 1 in EMSAs. A, recombinant VDR and RXR $\alpha$  bound to the *SLCO1A2*(+202/+216) sequence as obligate heterodimers. In vitro-translated proteins added to the binding reactions are indicated above the lanes, and the double-stranded oligonucleotides used as radiolabeled EMSA probes are indicated below the lanes. B, EMSA competition studies confirmed the specific DNA binding of endogenous VDR-RXR $\alpha$  heterodimers present in Caco-2 cell nuclear extracts to the +202/+216 DR-3-like element of *SLCO1A2* promoter variant 1. The consensus VDRE was used as the radiolabeled EMSA probe in all tests, and the identities and molar excesses of the unlabeled competitor oligonucleotides are shown above the lanes. The VDR-specific antibody added to the binding reaction produced a supershift (indicated with an arrow), which confirmed the identity of the endogenous complexes formed with the radiolabeled probe. C, two critical point mutations within the *SLCO1A2*(+202/+216) DR-3-like element strongly reduced the binding of endogenous VDR-RXR $\alpha$  in Caco-2 nuclear extracts. Double-stranded oligonucleotides used as radiolabeled EMSA probes are indicated below the lanes. In all EMSA tests, 500 nM vitamin D<sub>3</sub> was included in the binding reactions. WT, wild-type; MT, mutant; con, consensus; ab, antibody.



type *SLCO1A2* probe was capable of binding endogenous proteins in Caco-2 nuclear extracts that had the same mobility as the complexes assembled with the VDRE consensus probe, but the mutant version had a significantly reduced ability to do so.

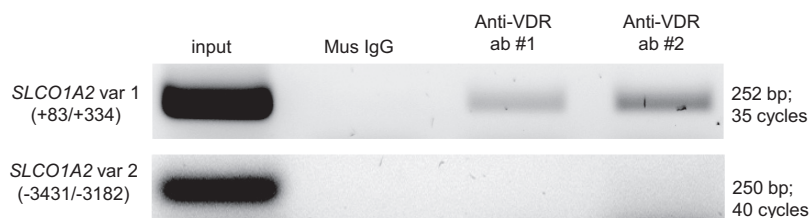
**VDR Interaction with *SLCO1A2* Gene Region Containing +202/+216 Motif in Living Cells.** To study whether there was a direct interaction between the *SLCO1A2* (+202/+216) DR-3 element and VDR in the context of living cells, we performed chromatin immunoprecipitation analyses. Caco-2 cells were treated with 500 nM vitamin D<sub>3</sub> or vehicle for 24 h, after which proteins were cross-linked to DNA in vivo by using formaldehyde and the cells were lysed. After shearing the genomic DNA into fragments of 300 to 600 bp, we performed immunoprecipitations with two different antibodies raised against VDR. As shown in Fig. 5, both anti-VDR antibodies successfully precipitated the 252-bp region (+83/+334) of the endogenous *SLCO1A2* gene, whereas the nonspecific mouse IgG antibodies failed to precipitate this gene region. As an additional specificity control, we amplified a 250-bp region (−3431/−3182) from promoter variant 2 of the *SLCO1A2* gene, using the same ChIP samples as templates. Neither anti-VDR antibody was able to precipitate this region of the *SLCO1A2* gene (Fig. 5).

***SLCO1A2*(+202/+216) Element as Functional Mediator of VDR-RXR $\alpha$ -Induced Transactivation of *SLCO1A2* Gene.** Having established that the DR-3 element present in the +202/+216 region of the human *SLCO1A2* promoter could specifically bind both recombinant and endogenous VDR-RXR $\alpha$  complexes, we verified that this element could functionally mediate *SLCO1A2* promoter activation in response to vitamin D<sub>3</sub>. To test this in a heterologous promoter context, we annealed single-stranded oligonucleotides containing *SLCO1A2*(+202/+216) and cloned the double-stranded oligonucleotides upstream of the herpes simplex virus thymidine kinase gene core promoter and firefly luciferase reporter gene. The oligonucleotides were identical to those used in the EMSA experiments described above. The heterologous promoter constructs were transfected into Caco-2 cells either with or without the expression plasmids for VDR and RXR $\alpha$ . As shown in Fig. 6A, the *SLCO1A2*(+202/+216)-containing construct was highly responsive to exogenous VDR-RXR $\alpha$  expression in the presence of their ligands, to the same degree as the heterologous promoter construct with the consensus VDRE sequence. This demonstrates that the *SLCO1A2*(+202/+216) DR-3-like motif can function as an independent VDR-responsive element. As a negative control, we used a heterologous promoter construct containing the −101/−87 region of *SLCO1A2* promoter variant 1, which could not form a complex with VDR-RXR $\alpha$  in EMSAs (Fig. 4A). In agreement with the EMSA results, this construct failed to respond to VDR and vitamin D<sub>3</sub>. We next tested the responsiveness of the two native *SLCO1A2* promoter variants to VDR and its ligand. To investigate whether the nuclear

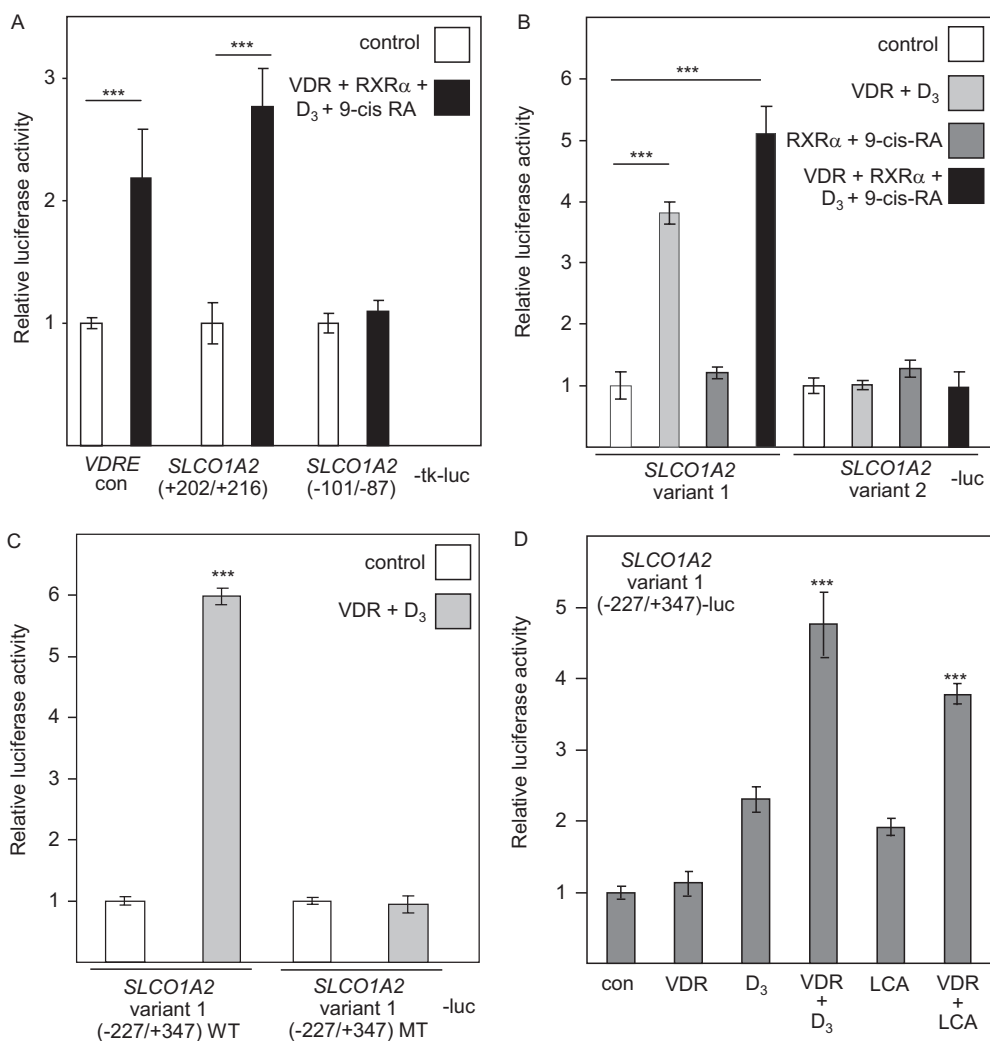
receptors VDR and RXR $\alpha$  directly regulate expression of the human *SLCO1A2* gene, the region between nucleotides −227 and +347 of *SLCO1A2* promoter variant 1 (with numbering relative to the transcriptional start site) was cloned upstream of the luciferase reporter gene, and Caco-2 cells were transiently cotransfected with the resulting reporter construct, in the presence or absence of expression constructs for VDR and/or RXR $\alpha$  and their respective ligands (vitamin D<sub>3</sub> and 9-*cis*-retinoic acid). In parallel, we cotransfected the construct containing the ~1600-bp promoter region of *SLCO1A2* promoter variant 2 (Kullak-Ublick et al., 1997). In agreement with our in silico results, *SLCO1A2* promoter variant 2 did not respond to overexpression of VDR-RXR $\alpha$  and treatment with their ligands (Fig. 6B). However, *SLCO1A2* promoter variant 1 was strongly induced by exogenous expression of VDR in the presence of vitamin D<sub>3</sub>. Addition of RXR $\alpha$  and its ligand slightly enhanced the VDR-mediated induction of promoter activity, whereas RXR $\alpha$  alone with 9-*cis*-retinoic acid had no influence on *SLCO1A2*(−227/+347) promoter activity. The relative baseline promoter activities, compared with parental reporter vector pGL3basic values, were  $4.22 \pm 0.95$  for promoter variant 1 and  $4.43 \pm 0.55$  for promoter variant 2. It is interesting to note that the two promoter variants seemed to be comparably active in Caco-2 cells, although only promoter variant 1 was responsive to VDR. We next mutated the same two bases within the +202/+216 element that abolished VDR-RXR $\alpha$  binding in in vitro assays, in the context of the native promoter construct *SLCO1A2*(−227/+347). The mutated construct completely lost its responsiveness to VDR and vitamin D<sub>3</sub> (Fig. 6C). Finally, having shown that OATP1A2 mRNA expression could be induced by the bile acid ligand for VDR (i.e., LCA), we tested whether LCA could activate *SLCO1A2* promoter variant 1 in a VDR-dependent manner. In agreement with the real-time PCR results, LCA could significantly increase the activity of the *SLCO1A2* promoter variant 1 region (−227/+347), in parallel with vitamin D<sub>3</sub> (Fig. 6C). We noted that treatment with either ligand alone, without exogenous expression of VDR, also could enhance *SLCO1A2* promoter activity, albeit in a more modest manner. This is probably attributable to endogenous expression of VDR and RXR $\alpha$  in Caco-2 cells.

## Discussion

Here we showed that VDR is a ligand-dependent transactivator of the human *SLCO1A2* gene, which encodes a vital membrane transporter protein for intestinal absorption of numerous drugs and endogenous substances. No other *SLCO* gene family member was shown previously to be under the control of the VDR nuclear receptor. Endogenous OATP1A2 mRNA and protein levels were potently induced by vitamin D<sub>3</sub> treatment in Caco-2 cells (Fig. 1). The bile acid ligand for VDR, LCA, was also



**Fig. 5.** VDR interaction with the vitamin D<sub>3</sub>-responsive region of the *SLCO1A2* promoter within Caco-2 cells. Cells were treated with 500 nM vitamin D<sub>3</sub> for 24 h, after which the ChIP assays were performed. Two different VDR-specific antibodies were efficient in precipitating the *SLCO1A2*(+83/+334) promoter variant 1 region containing the +202/+216 motif in cells treated with the VDR ligand, whereas no signal was obtained for a *SLCO1A2*(−3431/−3182) region located in *SLCO1A2* promoter variant 2.



**Fig. 6.** Activation of *SLCO1A2* promoter variant 1 by VDR and vitamin D<sub>3</sub> (D<sub>3</sub>) in Caco-2 cells through the +202/+216 DR-3-like motif. A, in heterologous promoter-reporter assays, the +202/+216 element mediated a strong response to VDR. B, *SLCO1A2* promoter variant 1 but not promoter variant 2 was responsive to VDR and its ligand. C, site-directed mutagenesis of the +202/+216 element abolished the VDR-mediated promoter activation of *SLCO1A2* variant 1. D, the *SLCO1A2*(-227/+347) promoter was responsive to the bile acid ligand of VDR, LCA, in addition to vitamin D<sub>3</sub>. \*\*\*,  $P < 0.001$ .

efficient in significantly elevating OATP1A2 mRNA levels. siRNA-mediated knockdown of endogenous VDR expression in Caco-2 cells led to clear suppression of vitamin D<sub>3</sub>-mediated induction of OATP1A2 mRNA expression (Fig. 2), which confirmed that the vitamin D<sub>3</sub> effect occurred through its nuclear receptor. Through in silico computational analyses and visual inspection, a promising candidate for a VDRE potentially mediating the ligand-dependent VDR activation was identified at position +202/+216 within the *SLCO1A2* promoter variant 1 (Fig. 3). This element was capable of directly and specifically interacting with both recombinant and endogenous VDR-RXR $\alpha$  heterodimers in EMSA experiments (Fig. 4). We noted that, in EMSAs, two complexes, both of which could be supershifted with antibodies raised against VDR, formed with radiolabeled VDRE-containing oligonucleotides when nuclear extracts from Caco-2 cells were used. We propose that this is attributable to more than one VDR isoform being expressed endogenously in these cells. More than one VDR isoform was reported previously to exist (Sunn et al., 2001). Consistent with the EMSA results, the region harboring the *SLCO1A2* promoter variant 1 element (+202/+216) interacted with endogenous VDRs within living cells (Fig. 5). Functional promoter-reporter assays using both heterologous and native promoter constructs verified the responsiveness of *SLCO1A2* promoter variant 1 to ligand-activated VDR and confirmed that the +202/+216 VDRE motif

mediated direct transactivation by VDR. Our study on VDR regulation of OATP1A2 expression adds to the previous report that *SLCO1A2* promoter variant 2 is regulated by a related nuclear receptor, pregnane X receptor (PXR) (*NRI12*), in breast cancer cells, through a direct interaction of PXR with its DR-4 response element within a distal region of *SLCO1A2* promoter variant 2 (Meyer zu Schwabedissen et al., 2008). The latter finding is in agreement with another study that showed that OATP1A2 and PXR expression levels closely correlated in breast carcinoma (Miki et al., 2006). It should be noted that membrane transporters are frequently regulated at the post-transcriptional level. In the case of OATP1A2, protein kinase C regulates its internalization, thus modulating its transport function (Zhou et al., 2011).

Before the current study, the expression of a number of intestinal membrane transporter genes was reported to be under the control of vitamin D<sub>3</sub> and VDR. We reported that the chief uptake system for dietary folates and for antifolate drugs at the intestinal epithelium [i.e., PCFT (*SLC46A1*)] is a direct target for ligand-dependent activation by VDR (Eloranta et al., 2009). Another study showed that mRNA levels for multidrug resistance protein 1 (*ABCB1*) and multidrug resistance-associated protein (MRP) 2 (*ABCC2*), as well as protein levels for MRP4 (*ABCC4*), were significantly increased with vitamin D<sub>3</sub> treatment of Caco-2 cells, whereas expression of the apical sodium-



dependent bile acid transporter (*SLC10A2*), oligopeptide transporter 1 (*SLC15A1*), and MRP3 (*ABCC3*) were not affected (Fan et al., 2009). Tachibana et al. (2009) verified that the induction of multidrug resistance protein 1 expression is VDR-dependent. Given that human *ABCB1* gene expression is also induced by the other two nuclear receptors, PXR (*NRI2*) and constitutive androstane receptor (*NRI3*) (Geick et al., 2001; Burk et al., 2005), the three members of the *NRI1* nuclear receptor subfamily seem to carry out overlapping functions in intestinal drug transport. The regulation of transporter genes by vitamin D<sub>3</sub> seems species-specific, because the compound has been reported not to induce *Abcb1* gene expression in the rat intestine (Chow et al., 2010). Because in the case of the *SLCO1A2* gene no direct homolog to the human gene can be identified in rodents, species conservation is not a relevant issue.

On the basis of our findings in Caco-2 cell cultures, we propose a role for VDR in the regulation of *SLCO1A2* gene expression in intestinal epithelial cells, and we suggest that, through this mechanism, vitamin D<sub>3</sub> enhances the intestinal absorption of OATP1A2 substrates. OATP1A2 was originally cloned from human liver cDNA on the basis of homology to rat Oatp1a1 (Kullak-Ublick et al., 1995). However, its level of expression in human hepatocytes has proved to be low. Likewise, human hepatocytes express very low levels of VDR (Gascon-Barré et al., 2003) (data not shown), which suggests that VDR-mediated regulation of *SLCO1A2* gene expression may not occur within hepatocytes. We note that both OATP1A2 (Lee et al., 2005) and VDR (Gascon-Barré et al., 2003) are more notably expressed in cholangiocytes, which form the bile duct epithelium, and it will be of interest to study whether VDR-dependent regulation occurs in these cells.

Our current results emphasize the emerging role for VDR in the regulation of intestinal membrane transport, drug disposition, and enteric compound absorption, in parallel with PXR, constitutive androstane receptor, and the bile acid receptor farnesoid X receptor (*NRIH4*), which have well established roles in these processes (Eloranta and Kullak-Ublick, 2005). The second-generation, histamine H<sub>1</sub> receptor antagonist fexofenadine was shown to be a substrate of OATP1A2, OATP2B1, and OATP1B3 (Shimizu et al., 2005). On the basis of the tissue distribution and substrate specificity of different OATPs, it can be assumed that OATP1A2 and/or OATP2B1 mediate the uptake of fexofenadine in the small intestine. To gain in vivo evidence to support our findings on the regulation of *SLCO1A2* gene expression by vitamin D<sub>3</sub>, we are currently designing a follow-up study with human volunteers, in which vitamin D<sub>3</sub> administration will be followed by measurements of fexofenadine pharmacokinetic parameters and gastrointestinal endoscopy for duodenal biopsies to measure OATP1A2 expression levels. Our current study should warrant an investigation into possible interactions between pharmacologically administered OATP1A2 transport substrates and vitamin D<sub>3</sub>.

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#### Authorship Contributions

Participated in research design: Eloranta and Kullak-Ublick.  
Conducted experiments: Eloranta, Hiller, and Jüttner.

Performed data analysis: Eloranta, Hiller, and Jüttner.

Wrote or contributed to the writing of the manuscript: Eloranta, Hiller, and Kullak-Ublick.

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