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The *SLCO1A2* gene, encoding the human organic anion transporting polypeptide 1A2 (OATP1A2), is transactivated by the vitamin D receptor (VDR)

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Running title: VDR regulates *SLCO1A2* gene expression

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ABBREVIATIONS: OATP, organic anion transporting polypeptide; SLC, solute carrier; VDR, vitamin D receptor; VDRE, vitamin D response element; RXR, retinoid X receptor; FXR, farnesoid 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; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; PXR, pregnane X receptor; CAR, constitutive androstane 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 of 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_3 markedly increased endogenous OATP1A2 mRNA and protein levels. Suppressing endogenous vitamin D receptor (VDR) expression by siRNAs significantly reduced this induction. Two alternative promoter regions exist in genomic databases for the *SLCO1A2* gene. One putative VDR response elements (*VDRE*s) predicted to efficiently interact with VDR:RXRα was identified *in silico* within the *SLCO1A2* promoter variant 1. This *VDRE* served as a strong binding site for the recombinant VDR:RXRα heterodimers *in vitro*, and was potently activated by VDR in the presence of vitamin D_3 in heterologous promoter assays. In reporter assays employing native promoter constructs, the *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:RXRα also interacted with this element both *in vitro* and within living cells. We have shown that the expression of the *SLCO1A2* gene is induced by vitamin D_3 at the transcriptional level via VDR. Our results suggest that pharmacological administration of vitamin D_3 may allow modulation of intestinal absorption of OATP1A2 transport substrates.

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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 appear dysregulated in several cancers, and they may prove to be important targets for anticancer therapy (Obaidat et al., 2011). Amongst the OATPs, the members of the OATP1 and OATP2 families, many of which play crucial roles in the main metabolic organs liver and intestine, are so far functionally best-characterized. OATP1B1 (gene symbol *SLCO1B1*) and OATP1B3 (*SLCO1B3*) are expressed at sinusoidal membranes of hepatocytes, whereas OATP2B1 (*SLCO2B1*) is expressed at the apical membrane of enterocytes and sinusoidal membrane of hepatocytes. One other OATP is expressed in the intestine, namely OATP1A2. The OATP1A2 (*SLCO1A2*) protein is located at the apical membranes of enterocytes (Glaeser et al., 2007), although mRNA levels of 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 to be cloned (Kullak-Ublick et al., 1995) and it mediates the uptake of a variety of endogenous amphipathic substrates as well as pharmacological drugs and xenobiotics. Transport substrates include bile acids, conjugated sex steroids, thyroid hormones, prostaglandin E2, the endothelin receptor antagonist BQ-123, the thrombin inhibitor CRC-220, the opioid receptor agonists DPDPE and deltorphin II, magnetic resonance imaging contrast agents, ouabain, and the cyanobacterial toxin microcystin (Kullak-Ublick et al., 1995; Hagenbuch and Meier, 2004).

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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, FXR (*NR1H4*), indirectly and directly, respectively. Relatively little is previously known about the gene regulation of *SLCO1A2*. Following the initial promoter characterization of the *SLCO1A2* promoter (now termed variant 2) (Kullak-Ublick et al., 1997), the genomic databases have been since updated to identify another *SLCO1A2* promoter variant 1, 60 kbp apart from the original promoter variant 2.

In addition to its established roles in regulation of calcium homeostasis, as well blood pressure and electrolyte regulation, the biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 (vitamin D_3), executes many other important functions (Demay, 2006; Bouillon et al., 2008). Recently, its roles particularly as a crucial regulator of differentiation and proliferation of enterocytes, as well as in mucosal immunity of the gastrointestinal tract, have attracted increasing attention. For example, vitamin D_3 has been shown to preserve healing capacity in the colonic epithelium (Kong et al., 2008). Most effects of vitamin D_3 are mediated via its action as an agonistic ligand for the vitamin D receptor (VDR; gene symbol *NR1I1*), a nearly ubiquitously expressed member of the nuclear receptor family of transcription factors (Dusso et al., 2005). We note, however, that non-genomic actions by vitamin D_3 have also become increasingly recognized (Christakos et al., 2003; Dusso et al., 2005). In addition to its classical ligand vitamin D_3, VDR can also function as an intestinal bile acid sensor by utilizing the secondary bile acid lithocholic acid (LCA) as its agonistic ligand, thus inducing CYP3A expression (Makishima et al., 2002). Given the potential intestinal carcinogen 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 directly interacting with so-called direct repeat-3-like (*DR-3*-like; variants of a direct repeat of [A/G]G[G/T]TCA-like hexamers separated by three bases, the last of

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which is preferably purine) DNA motifs within the target promoters, as a heterodimer with another nuclear receptor, retinoid X receptor-α (RXRα; gene symbol *NR2B1*) (Haussler et al., 1997).

Recently, it has been reported that a number of genes encoding intestinal membrane transporters for both endogenous substances and drugs and xenobiotics are regulated by vitamin D_3 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_3 and VDR regulate the expression of the *SLCO1A2* gene, encoding an important membrane transporter for intestinal absorption of numerous drugs.

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Materials and Methods

Chemicals. Deoxyadenosine 5'-[α-^{32}P]-triphosphate (6000 Ci/mmol) was purchased from Perkin Elmer (Schwerzenbach, Switzerland). Restriction enzymes were from Roche Diagnostics (Rotkreuz, Switzerland), and the T4 DNA Ligase from Promega (Dübendorf, Switzerland). The oligonucleotides were synthesized at Microsynth (Balgach, Switzerland). All other chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland), unless otherwise stated.

Cell Culture. Caco-2 cells (LGC Promochem, Molsheim Cedex, Switzerland), derived from human colon carcinoma, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Buchs, Switzerland), supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Zug, Switzerland). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

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). RNAs were quantified spectrophotometrically at 260 nm (NanoDrop ND-1000; Thermo Fisher Scientific, Wilmington, Delaware), and 2 μg of total RNAs were reverse transcribed using random primers and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rotkreuz, Switzerland). Complementary DNAs (cDNAs) were diluted to a final volume of 100 μl with nuclease-free water (Applied Biosystems). For quantitative real-time PCR reactions, 2 μl of diluted cDNAs were used per reaction. TaqMan Gene Expression Assays Hs00245360_m1, Hs00611081_m1, and Hs01045844_m1 (Applied Biosystems) were used to measure OATP1A2, PCFT, and VDR cDNAs, respectively, using the ABI Prism 7900HT Fast Real-Time PCR system (Applied Biosystems). Human β-actin cDNA (TaqMan Gene Expression Assay 4310881E ; Applied

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Biosystems) was measured to normalize the relative OATP1A2, PCFT, and VDR expression levels, which were calculated using the comparative threshold cycle method ($\Delta\Delta C_T$). All PCR tests were performed in triplicate.

Preparation of Whole Cell Protein Extracts and Immunoblotting. To prepare whole cell protein extracts, cells on 12-well plates were washed with ice-cold PBS, and lysed by a 5 minute incubation in 250 µl 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] Na-deoxycholate; 1 mM EDTA; 0.1% [w/v] SDS; 10% [v/v] glycerol), supplemented with Complete protease inhibitors (Roche Diagnostics). The debris were removed by centrifugation at 20000 x g for 30 minutes at +4°C. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay (Pierce) and the samples stored at -80°C until usage. Ten µg of protein extracts were separated on 12% SDS polyacrylamide gels and electroblotted onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences Europe). Membranes were blocked for one hour in 5% (w/v) non-fat milk in PBS-T (0.1% (v/v) Tween-20 in PBS). After this, the membranes were probed with a rabbit polyclonal antibody against OATP1A2 (LS-C40428; LabForce, Nunningen, Switzerland) at a concentration of 0.25 µg/ml in 5% (w/v) non-fat milk/PBS-T, for overnight. After three washes with 5% (w/v) non-fat milk/PBS-T, the horseradish peroxidase-conjugated goat anti-rabbit antibody (Pierce) was added at a concentration of 10 ng/ml in 5% (w/v) non-fat milk/PBS-T for 1 hour. Blots were then washed three times with 5% (w/v) non-fat milk/PBS-T and twice with PBS, followed by detection with the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and exposure on Hyperfilm ECL (GE Healthcare, Glattbrugg, Switzerland). To verify equal loading of the protein samples, the blots were stripped with Restore Western Blot Stripping Solution (Pierce), reblocked, and reprobed for constitutively expressed β-actin protein. The β-actin probing and detection were performed as above, except

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that the polyclonal anti-rabbit β-actin antibody (ab8227; Abcam, Cambridge, UK) was used at a concentration of 100 ng/ml, and it was added to the blots for one hour.

Transfections with Short Interfering RNAs. Near-confluent Caco-2 cells grown on 12-well plates were transfected with the final concentration of 40 nM of ON-TARGETplus SMARTpool siRNAs designed to target VDR (L-003448-00; Thermo Scientific Dharmacon, Wohlen, Switzerland) or 40 nM of siGENOME Non-Targeting siRNA #1 (D-001210-01; Thermo Scientific Dharmacon) not known to target any human gene, using the transfection reagent siLentFect (Bio-Rad, Reinach, Switzerland). The transfections were repeated identically after 24 hours, and RNAs harvested after 48 of total transfection time. It was necessary to transfect the same cells twice with siRNAs, in order to achieve efficient silencing of gene expression.

Electromobility Shift Assays (EMSAs). EMSA binding reactions and gel runs 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 5'-AGCT (top strand) and 5'-GATC (bottom strand) to be present in all annealed EMSA oligonucleotides, allowing their radioactive labelling with α-[^{32}P]-dATP in filling-in reactions using MultiScribe Reverse Transcriptase (Applied Biosystems). Recombinant proteins VDR and RXRα were synthesized with the TNT T7 Coupled Reticulocyte Lysate System (Promega) and using the plasmids pCMX-VDR and pCMX-RXRα as templates. Caco-2 nuclear protein extracts were prepared from cells at 70-80% confluence using the NE-PER kit (Perbio Science, Lausanne, Switzerland). Protein concentrations of nuclear extracts were determined using the bicinchoninic acid (BCA) kit (Perbio Science). In competition EMSA experiments the unlabelled oligonucleotides were added immediately prior to the radioactive probes. In antibody supershift experiments, 1 μg of the VDR antibody (C-20X; Santa Cruz

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Biotechnology, LabForce, Nunningen, Switzerland) was added and the reactions incubated at +4 °C for one hour prior to the addition of the radioactive probes.

Chromatin Immunoprecipitation (ChIP) Assays. Caco-2 cells were grown on two 10 cm plates per culture condition to 80% confluence, after which they were treated with either 500 nM vitamin D_3 or the respective vehicle ethanol. Twenty-four hours later the cells were harvested by crosslinking with 1% methanol-free formaldehyde (Perbio Science) and processed through chromatin immunoprecipitations using the ChIP-IT Express kit (Active Motif, Rixensart, Belgium). Shearing of the chromatin was achieved by five pulses of sonication with 30 second pauses on ice between each pulse, using the Branson Digital Sonifier (Branson Ultrasonics, Danbury, CT) at power setting 25%. For the immunoprecipitation steps, aliquots from both test conditions were incubated without any antibody, with 1 μg negative control antiserum mouse IgG1 (X0931, DAKO A/S, Baar, Switzerland), or with 1 μg of one of the two antibodies raised against VDR (C-20X, VDR ab #1, and H-81, VDR ab #2; Santa Cruz Biotechnology). The VDR antibodies were selected based on their ability to immunoprecipitate *in vitro* translated radiolabelled VDR protein in ChIP conditions (data not shown). Two amplicons were assayed for immunoprecipitation tests, using PuRe *Taq* Ready-To-Go PCR beads (GE Healthcare) and the oligonucleotide primers listed in Table 1: *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 the *SLCO1A2* promoter variant 2. After the initial denaturation stage at 94^{o}C for 3 minutes, the PCR cycling conditions were 94^{o}C for 20 seconds, 60^{o}C for 30 seconds, and 72^{o}C for 1 minute. After 35 and 40 cycles, ten microliters of each PCR product were resolved on 1.5% agarose gels and detected with SYBR Safe DNA gel stain (Invitrogen).

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DNA Constructs. Using a pool of human genomic DNAs (Clontech, Saint-Germain-en-Laye, France) as the template and PureTaq Ready-To-Go PCR beads (GE Healthcare) the region (-227/+347) of the human *SLCO1A2* promoter variant 1 (according to the NCBI Reference Sequence: *NT_009714.17*) was obtained by PCR using the oligonucleotide primers listed in Table 1. The PCR product *SLCO1A2*(-227/+347) was first cloned into the pGEM-T vector (Promega, Dübendorf, Switzerland), and further subcloned into the MluI-SmaI-digested pGL3basic luciferase reporter vector (Promega) using the engineered recognition sites for the restriction enzymes MluI and EcoRV. The construction of the luciferase reporter plasmid containing ~1600 proximal base pairs of the *SLCO1A2* promoter variant 2 has been previously described (Kullak-Ublick et al., 1997). Point mutations within the *SLCO1A2*(+202/+216) element were created in the *SLCO1A2*(-227/+347)luc construct using the QuikChange II site-directed mutagenesis kit (Stratagene, Agilent Technologies, Basel, Switzerland) and oligonucleotides shown in Table 1. In order to create the heterologous promoter constructs containing either the consensus *VDRE*, the wild-type SLCO1A2 (+202/+216) element, or a mutated version of it, 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 by DNA sequencing (Microsynth, Balgach, Switzerland). The expression plasmids for the human VDR (pCMX-VDR) and the human RXRα (pCMX-RXRα) 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-wells at a confluence of 70% one day before transfections. Cells were transiently transfected with 400 ng of the firefly luciferase constructs together with 200 ng of VDR and/or RXRα expression plasmids, using the FuGENE HD reagent (Roche Diagnostics, Rotkreuz,

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Switzerland). To normalize the amount of CMV promoter-containing expression constructs transfected, an appropriate amount of the pcDNA3.1(+) vector (Invitrogen) was included in transfections mixes. To control for variations in transfection efficiency, 100 ng of the phRG-TK renilla luciferase reporter plasmid (Promega) was cotransfected in each well. Twelve hours after transfections cells were treated with the ligands 500 nM vitamin D_3 and/or 1 μM 9-cis retinoic acid (Sigma-Aldrich) or the vehicles ethanol and/or DMSO, respectively. Twenty-four hours after adding the ligands, cells were harvested in 1 x Passive Lysis Buffer (Promega) and luciferase activities measured using a GloMax-Multi Detection System (Promega). Relative promoter activities were obtained by normalizing firefly luciferase activities to renilla luciferase activities. The control conditions were set to 1, and all other results are shown relative to these. Triplicate wells were measured for each transfection tests.

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

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Results

Vitamin D_3 Induces OATP1A2 mRNA and Protein Expression in Human Intestine-Derived Caco-2 Cells. The human well-polarized enterocyte-derived Caco-2 cells exhibit many characteristics associated with differentiated enterocytes and were used here to investigate the effects of vitamin D_3 on *SLCO1A2* gene expression. Near-confluent Caco-2 cells were treated with 500 nM of vitamin D_3 for 24 hours, after which the endogenous OATP1A2 mRNA expression was measured by quantitative real-time PCR. As shown in Fig.1A, under these conditions OATP1A2 mRNA levels were induced approximately nine-fold, when compared to the vehicle-treated cells. As a positive control, we measured the effects of vitamin D_3 treatment on a known target gene *SLC46A1* (Eloranta et al., 2009), encoding the proton-coupled folate transporter (PCFT), in parallel. PCFT mRNA expression was induced by vitamin D_3 treatment approximately two-fold (Fig. 1B), indicating how potent the effect on OATP1A2 expression is. We further tested whether the hydrophobic bile acid LCA, also known to function as an agonistic ligand for VDR (Makishima et al., 2002), can affect OATP1A2 mRNA expression. Indeed, treating Caco-2 cells with 50 μM LCA for 24 hours led to significant induction of OATP1A2 mRNA, although to a lesser degree than following vitamin D_3 treatment (Fig. 1C). In addition to mRNA expression, we studied whether vitamin D_3 treatment of Caco-2 cells also results in an increase in OATP1A2 protein expression. In agreement with the mRNA results, OATP1A2 protein was clearly increased upon vitamin D_3 treatment for 24 hours (Fig. 1D). This increase was already visible at time point eight hours after the beginning of treatment, but was clearly beyond its peak at time point 48 hours.

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Short Interfering RNA-Mediated Reduction of VDR Expression Suppressed Vitamin D_3-Mediated Induction of OATP1A2 mRNA Expression. We next transfected a pool of four siRNAs specifically targeting the nuclear receptor for vitamin D_3, VDR, into Caco-2 cells in parallel with control siRNAs 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_3-treated cells (Fig. 2A). The VDR siRNA-transfected cells exhibited significantly reduced induction of OATP1A2 mRNA expression upon vitamin D_3 treatment (Fig. 2B), indicating that the vitamin D_3 effect is mediated by its nuclear receptor VDR.

***In Silico* Analysis of the *SLCO1A2* Promoter Regions.** Having shown the VDR-dependent induction of OATP1A2 expression by vitamin D_3, we performed an *in silico* analysis of the *SLCO1A2* gene in order to locate putative VDR-responsive elements (*VDRE*s) of the *DR-3*-like configuration. The *SLCO1A2* gene is located in chromosome 12, and we employed the genomic contig GRCh37.p5 Primary Assembly as our sequence of analysis (NCBI Reference Sequence: NT_009714.17; *www.ncbi.nih.gov*). Two transcript variants encoding an identical OATP1A2 protein product are listed within this contig, with their transcription start sites separated by approximately 60 kbp. We analyzed 2 kbp upstream of each transcription start site, plus the non-coding exon 1 (variant 1) or the exon 1 region up to the translational start site (variant 2) *in silico*, by the algorithms MatInspector (Cartharius et al., 2005) and NubiSCAN (Podvinec et al., 2002), as well as by 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 shortly downstream of the transcription start site of variant 1 (Fig. 3). No near-consensus *VDRE*s were predicted within the analyzed segment of the *SLCO1A2* promoter variant 2.

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VDR:RXRα Heterodimers Interact Directly with the *SLCO1A2*(+202/+216) *DR-3*-Like Motif *In Vitro*. Next, we studied whether the VDR:RXRα heterodimers directly interact with the (+202/+216) *DR-3*-like motif on the *SLCO1A2* promoter *in vitro*, using electrophoretic mobility shift assays (EMSAs). When both *in vitro* translated recombinant VDR and RXRα were mixed with the double-stranded radioactively labelled *SLCO1A2*(+201/+216) probe in the presence of 500 nM vitamin D_3 and 1 μM retinoic acid, a distinct protein-DNA complex with an identical mobility to the complex forming on the *VDRE* consensus probe was formed, although to a somewhat lesser degree than on the consensus probe (Fig. 4A). This complex only formed when both recombinant VDR and RXRα were included the binding reactions, confirming that VDR binds to the *SLCO1A2*(+202/+216) element as a heterodimer with RXRα, instead of a homodimer or a monomer. As a negative control, we employed an oligonucleotide from the *SLCO1A2* promoter variant 1 at position (-101/-87) relative to the transcription start site. This sequence also loosely conforms to the *DR-3*-like motif, but contains several base substitutions predicted to prevent VDR:RXRα binding (Colnot et al., 1995). Indeed, the latter oligonucleotide probe could not form a complex with recombinant VDR and RXRα. As a confirmation of the specific nature of the DNA-binding by VDR:RXRα to the *SLCO1A2*(+202/+216) element, we performed EMSA competition experiments. When in molar excess, unlabelled double-stranded oligonucleotides containing the wild-type *SLCO1A2*(+202/+216) region were efficient at competing off endogenous proteins present in Caco-2 nuclear extracts that were capable of binding to the radiolabelled *VDRE* consensus probe when. Ten-fold molear excess of the wild-type oligonucleotide was already sufficient to reduce complex formation, and this effect was enhanced stepwise when 50-fold and 100-fold excesses of this oligo were added to the DNA-binding reactions (Fig.

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4B). When the mutant version of the same element, containing two base changes predicted to disrupt complex formation with VDR:RXRα, was used as the competing oligonucleotide, it could not notably compete for the protein complex formation on the *VDRE* consensus element. When an antibody raised against VDR was added into the binding reaction prior to the probe, a supershift of the complexes forming on the radioactive probes was observed, confirming the identity of the VDR-containing complexes. In a reverse experiment, we employed the wild-type and mutant *SLCO1A2*(+202/+216) sequence-containing element as radioactive probes. As shown in Fig. 4C, the radioactive wild-type *SLCO1A2* probe was capable of binding endogenous proteins in Caco-2 nuclear extracts that had the same mobility as the complexes assembling on the *VDRE* consensus probe, but the mutant version had a significantly reduced ability to do so.

VDR interacts with the region of the *SLCO1A2* gene containing the (+202/+216) motif within living cells. To study, whether there is 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_3 or the vehicle for 24 hours, after which proteins were cross-linked to DNA *in vivo* using formaldehyde, and the cells lysed. After shearing the genomic DNA into fragments of 300-600 bp, we performed immunoprecipitations using two different antibodies raised against VDR. As shown in Figure 5, both VDR antibodies successfully precipitated the 252 bp region (+83/+334) of the endogenous *SLCO1A2* gene, while the non-specific mouse IgG antibodies failed to precipitate this gene region. As an additional specificity control, we amplified a 250 bp region (-3431/-3182) from the promoter variant 2 of the *SLCO1A2* gene, using the same ChIP samples as templates. Neither VDR antibody was able to precipitate this region of the *SLCO1A2* gene (Fig. 5).

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The *SLCO1A2*(+202/+216) Element Is a Functional Mediator of the VDR:RXRα-Induced Transactivation of the *SLCO1A2* Gene. Having established that the *DR-3* element present in the (+202/+216) region of the human *SLCO1A2* promoter can specifically bind both recombinant and endogenous VDR:RXRα complexes, we verified that this element can also functionally mediate *SLCO1A2* promoter activation in response to vitamin D_3. To test this hypothesis in a heterologous promoter context, we annealed single-stranded oligonucleotides containing the *SLCO1A2*(+202/+216) and cloned the double-stranded oligonucleotides upstream of the herpes simplex virus thymidine kinase (HSV-*TK*) gene core promoter and firefly luciferase reporter gene. The oligonucleotides were identical to those used in EMSA experiments above. The heterologous promoter constructs were transfected into Caco-2 cells either together or without the expression plasmids for VDR and RXRα. As shown in Fig. 6A, the *SLCO1A2*(+202/+216)-containing construct was highly responsive to exogenous VDR:RXRα expression in the presence of their ligands, to the same degree as the heterologous promoter construct harbouring 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 employed a heterologous promoter construct containing the (-101/-87) region of the *SLCO1A2* promoter variant 1, which could not form a complex with VDR:RXRα in EMSAs (Fig. 4A). In agreement with the EMSA result, this construct failed to respond to VDR and vitamin D_3. 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 RXRα directly regulate the expression of the human *SLCO1A2* gene, the region between nucleotides -227/+347 of the *SLCO1A2* promoter variant 1 (numbering relative to the transcriptional start site) was cloned upstream

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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 RXRα and their respective ligands, vitamin D_3 and or 9-cis-retinoic acid. In parallel, we cotransfected the construct containing the ~1600 bp promoter region of the *SLCO1A2* promoter variant 2 (described in Kullak-Ublick et al., 1997). In agreement with our *in silico* results, the *SLCO1A2* promoter variant 2 did not respond to overexpression of VDR:RXRα and treatment with their ligands (Fig. 6B). On the other hand, the *SLCO1A2* promoter variant 1 was strongly induced by exogenous expression of VDR in the presence of vitamin D_3. Addition of RXRα and its ligand slightly enhanced the VDR-mediated induction of promoter activity, whereas RXRα alone together with 9-cis retinoic acid had no influence on *SLCO1A2*(-227/+347) promoter activity. The relative baseline promoter activities, compared to the parental reporter vector pGL3basic, were 4.22+/-0.95 for the promoter variant 1, and 4.43+/-0.55 for the promoter variant 2, and it is thus interesting to note that both promoter variants appear to be comparably active in Caco-2 cells, even if only promoter variant 1 is responsive to VDR. We next mutated the same two bases that in *in vitro* assays abolished VDR:RXRα binding within the (+202/+216) element in the context of the native promoter construct *SLCO1A2*(-227/+347). The mutated construct completely lost its responsiveness to VDR and vitamin D_3 (Fig. 6C). Finally, having shown that OATP1A2 mRNA expression can also be induced by the bile acid ligand for VDR, LCA, we tested whether LCA can also activate the *SLCO1A2* promoter variant 1 in a VDR-dependent manner. In agreement with the real-time PCR results, LCA could significantly increase the activity of *SLCO1A2* promoter variant 1 region (-227/+347), in parallel with vitamin D_3 (Fig. 6C). We note that treatment with either ligand alone, without exogenous expression of VDR

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could also enhance *SLCO1A2* promoter activity, albeit in a more modest manner. This is likely to be due to endogenous expression of VDR and RXRα in Caco-2 cells.

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Discussion

Here we have shown that VDR is a ligand-dependent transactivator of the human *SLCO1A2* gene, encoding a vital membrane transporter protein for intestinal absorption of numerous drugs and endogenous substances. No other *SLCO* gene family member has previously been shown to be under the control of the VDR nuclear receptor. Endogenous OATP1A2 mRNA and protein levels were potently induced by vitamin D_3 treatment in Caco-2 cells (Fig. 1). The bile acid ligand of VDR, LCA, was also efficient in significantly elevating OATP1A2 mRNA levels. SiRNA-mediated knockdown of endogenous VDR expression in Caco-2 cells led to a clear suppression of vitamin D_3-mediated induction of OATP1A2 mRNA expression (Fig. 2), confirming that the vitamin D_3 effect takes place via its nuclear receptor. In *in silico* computational 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:RXRα heterodimers in EMSA experiments (Fig. 4). We note that in EMSAs, two complexes, both of which can be supershifted with antibodies raised against VDR, form on radiolabelled *VDRE*-containing oligonucleotides when using nuclear extracts from Caco-2 cells. We propose that this is due to more than one VDR isoform endogenously being expressed in these cells. Indeed, more than one VDR isoform have previously been reported to exist (Sunn et al., 2001). Consistent with the EMSAs, the region harbouring the *SLCO1A2* promoter variant 1 element (+202/+216) interacted with endogenous VDR within living cells (Fig. 5). Functional promoter-reporter assays, employing 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 the direct

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transactivation by VDR. Our study on VDR regulation of OATP1A2 expression adds to the previous report that the *SLCO1A2* promoter variant 2 is regulated by a related nuclear receptor pregnane X receptor (PXR; *NRI12*) in breast cancer cells, via a direct interaction by 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 showing that OATP1A2 and PXR expression levels closely correlate in breast carcinoma (Miki et al., 2006). It should also be noted that membrane transporters are frequently also 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).

Previous to our current study, the expression of a number of intestinal membrane transporter genes have recently been reported to be under the control of vitamin D_3 and VDR. We have reported that the chief uptake system for dietary folates as well as for antifolate drugs, at the intestinal epithelium proton-coupled folate transporter (PCFT, *SLC46A1*) is a direct target for ligand-dependent activation by VDR (Eloranta et al., 2009). Another study showed that the mRNA levels of the multidrug resistance protein 1 (MDR1; *ABCB1*) and multidrug resistance-associated protein 2 (MRP2; *ABCC2*), as well protein levels of MRP4 (*ABCC4*), were significantly elevated by vitamin D_3 treatment of Caco-2 cells, while the expression of the apical sodium-dependent bile acid transporter (ASBT; *SLC10A2*), oligopeptide transporter 1 (PEPT1; *SLC15A1*), and MRP3 (*ABCC3*) were not affected (Fan et al., 2009). Tachibana et al. (2009) verified that the induction of MDR1 expression is VDR-dependent. Given that the human *ABCB1* gene expression is also induced by the other two nuclear receptors pregnane X receptor (PXR; *NRI12*) and constitutive androstane receptor (CAR; *NRI13*) (Geick et al., 2001; Burk et al., 2005), it appears that the three members of the *NRI1* nuclear receptor subfamily carry out overlapping functions in intestinal drug transport. The regulation of transporter genes by vitamin D_3 seems species-specific, as the compound

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has been reported not to induce *Abcb1* gene expression in the rat intestine (Chow et al., 2010). As in the case of the *SLCO1A2* gene no direct homologue to the human gene can be pinpointed in rodents, the species conservation is not a relevant question.

Based on our findings in Caco-2 cell culture, we propose a role for VDR in regulation of *SLCO1A2* gene expression in intestinal epithelial cells, and that via this mechanism vitamin D_3 enhances the intestinal absorption of OATP1A2 substrates. OATP1A2 was originally cloned from human liver cDNA by homology to the rat Oatp1a1 (Kullak-Ublick et al., 1995). However, its expression in human hepatocytes has proved to be low. Similarly, human hepatocytes only express very low levels of VDR (Gascon-Barre et al., 2003; own data not shown), implying that VDR-mediated regulation of the *SLCO1A2* gene expression may not occur within hepatocytes. We note that both OATP1A2 (Lee et al., 2005) and VDR (Gascon-Barre et al., 2003) are more notably expressed in cholangiocytes, which form the bile duct epithelium, and it will be of interest to study whether the VDR-dependent regulation occurs in these cells.

Our current results further emphasize the emerging role for VDR in regulation of intestinal membrane transport, drug disposition, and enteric compound absorption, in parallel with PXR, CAR, and the bile acid receptor FXR (*NR1H4*), which have well-established roles in these processes (Eloranta et al., 2005). The second generation H_1-receptor antagonist antihistamine fexofenadine has been shown to be a substrate of OATP1A2, OATP2B1, and OATP1B3 (Shimizu et al., 2005). Based on 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. In order to gain *in vivo* evidence to support our finding on regulation of *SLCO1A2* gene expression by vitamin D_3, we are currently designing a follow-up study in human volunteers, where vitamin D_3 administration will be followed by measurements of fexofenadine pharmacokinetics and by astrointestinal endoscopy for duodenal biopsies to

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measure OATP1A2 expression levels. Our current study should also warrant an investigation into possible interactions between pharmacologically administered OATP1A2 transport substrates and vitamin D_3.

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Authorship contributions

Participated in research design: Eloranta and Kullak-Ublick

Conducted experiments: Eloranta, Hiller, and Jüttner

Contributed new reagents: Eloranta and Hiller

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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Footnotes:

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Legends for figures

Fig. 1. OATP1A2 mRNA and protein expression are induced by agonistic ligands for VDR in Caco-2 cells. Treatment of cells with 500 nM vitamin D_3 (D_3) for 24 hours increases the mRNA expression of OATP1A2 (A) and PCFT (B). C, Another VDR agonist LCA (50 μM) similarly increases OATP1A2 mRNA expression. D, OATP1A2 protein expression is enhanced by treatment of cells with vitamin D_3 for 8 and 24 hours, after which (48 hours) the induction is attenuated. ***, $P<0.001$.

Fig. 2. Transfection of short interfering RNAs (siRNAs) specifically targeting VDR (siVDR) attenuate the OATP1A2 mRNA induction by vitamin D_3 in Caco-2 cells, when compared to the control siRNA (siCon)-transfected cells. A, VDR-specific siRNAs significantly suppress endogenous VDR mRNA expression in both vehicle (ethanol)- and vitamin D_3-treated cells. B, VDR-specific siRNAs significantly suppress OATP1A2 mRNA induction by vitamin D_3. VDR and OATP1A2 mRNA expression levels were normalized to those of the housekeeping gene β-actin. ***, $P<0.001$.

Fig. 3. The proximal promoter sequence of the *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 "*Homo sapiens* chromosome 12 genomic contig *NT_009714.17* (*www.ncbi.nih.gov*). A, The *SLCO1A2* promoter variant 1. The location of the (+202/+216) *DR-3*-like element are indicated with arrows and by framing. The locations of the ChIP oligonucleotide primers are underlined B, The *SLCO1A2* promoter variant 2.

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Fig. 4. VDR:RXRα heterodimers specifically bind to the +202/+216 *DR-3*-like motif of the *SLCO1A2* promoter variant 1 in electrophoretic mobility shift assays (EMSAs). A, Recombinant VDR and RXRα bind 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 radiolabelled EMSA probes are indicated below the lanes. D, EMSA competition studies confirm the specific DNA-binding of endogenous VDR-RXRα heterodimers in Caco-2 cells to the (+202/+216) *DR-3*-like element of the *SLCO1A2* promoter variant 1. The consensus *VDRE* was used as the radiolabelled EMSA probe in all tests and the identities and molar excesses of the unlabelled competitor oligonucleotides are shown above the lanes. The VDR-specific antibody added to the binding reaction produces a supershift (indicated with an arrow), confirming the identity of the endogenous complexes forming on the radiolabelled probe. C, Two critical point mutations within the *SLCO1A2*(+202/+216) *DR-3*-like element strongly reduce the binding of endogenous VDR:RXRα in Caco-2 nuclear extracts. Double-stranded oligonucleotides employed as radiolabelled EMSA probes are indicated below the lanes. In all EMSA tests, 500 nM vitamin D_3 was included in the binding reactions. WT, wild-type; MT, mutant; con, consensus; ab, antibody.

Fig. 5. VDR interacts with the vitamin D_3-responsive region of the *SLCO1A2* promoter within Caco-2 cells. Cells were treated with 500 nM vitamin D_3 for 24 hours, after which the ChIP assays were perfomed. Two different VDR-specific antibodies were efficient in precipitating the promoter variant 1 region *SLCO1A2*(+83/+334) containing the (+202/+216) motif in cells treated with the VDR ligand, whereas no signal was obtained for a region *SLCO1A2*(-3431/-3182) located in the *SLCO1A2* promoter variant 2.

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Fig. 6. The *SLCO1A2* promoter variant 1 is activated by VDR and vitamin D_3 (D_3) in Caco-2 cells via the (+202/+216) *DR-3*-like motif. A, In heterologous promoter-reporter assays, the (+202/+216) mediates a strong response to VDR. B, Only the *SLCO1A2* promoter variant 1, but not the promoter variant 2, is responsive to VDR and its ligand. C, Site-directed mutagenesis of the (+202/+216) element abolishes the VDR-mediated promoter activation of the *SLCO1A2* variant 1. D, The *SLCO1A2*(-227/+347) promoter is responsive to the bile acid ligand of VDR, LCA, in addition to vitamin D_3. ***, $P<0.001$.

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Table 1. Sequences of oligonucleotides used for cloning, site-directed mutagenesis, and as EMSA probes. 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
SLCO1A2 (-227)fwd	ACGCGTGCTGGGAGACCCACTGCTCTCTTC (MluI)	Cloning *SLCO1A2* (-227/+347)luc
SLCO1A2 (+347)rev	GATATCTGCATTTCCATCTGAGGTACCGGGTTCATCTC (EcoRV)	Cloning *SLCO1A2* (-227/+347)luc
SLCO1A2 (+202/+216)sdm_top	CTTTGACTCAGAAAG*GGAACTCCCTG**G**CA**C**CTTGAGCTTCCCAAG	Mutagenesis of *SLCO1A2* (+202/+216)
*VDRE*con EMSA_top	AGCTGCAGGGGG*AGGTCAAAGAGGTCA*CACTAGTA	*VDRE* consensus EMSA probe; cloning HSV-*tk*-luc constructs
SLCO1A2 (+202/+216)wt EMSA_top	AGCTTCAGAAAG*GGAACTCCCTGACCC*CTTGAGCT	*SLCO1A2* (+202/+216) EMSA probe/ competitor; cloning HSV-*tk*-luc constructs
SLCO1A2 (+202/+216)mut EMSA_top	AGCTTCAGAAAG*GGAACTCCCTG**G**CA**C**CTTGAGC	*SLCO1A2* (+202/+216) EMSA probe/ competitor
SLCO1A2 (-101/-87) EMSA_top	AGCTAGGCAGGC*AGGCCTTCTTGAGCT*GTGGTGGG	*SLCO1A2* (-101/-87) EMSA probe; cloning HSV-*tk*-luc constructs
SLCO1A2 (+83/+334) CHIP_fwd	CTGAGGTACCGGGTTCATCTCAC	CHIP assay
SLCO1A2 (+83/+334) CHIP_rev	GTGCGGGATATAATCTTGTGGCGCG	CHIP assay
*SLCO1A2*var2 (-3431/-3182) CHIP_fwd	CTGTGTTTAGGGGAATTGGTTG	CHIP assay
SLCO1A2 (-3431/-3182) CHIP_rev	CAAGAGTGAGGGATTCTCTCC	CHIP assay

Fig. 1

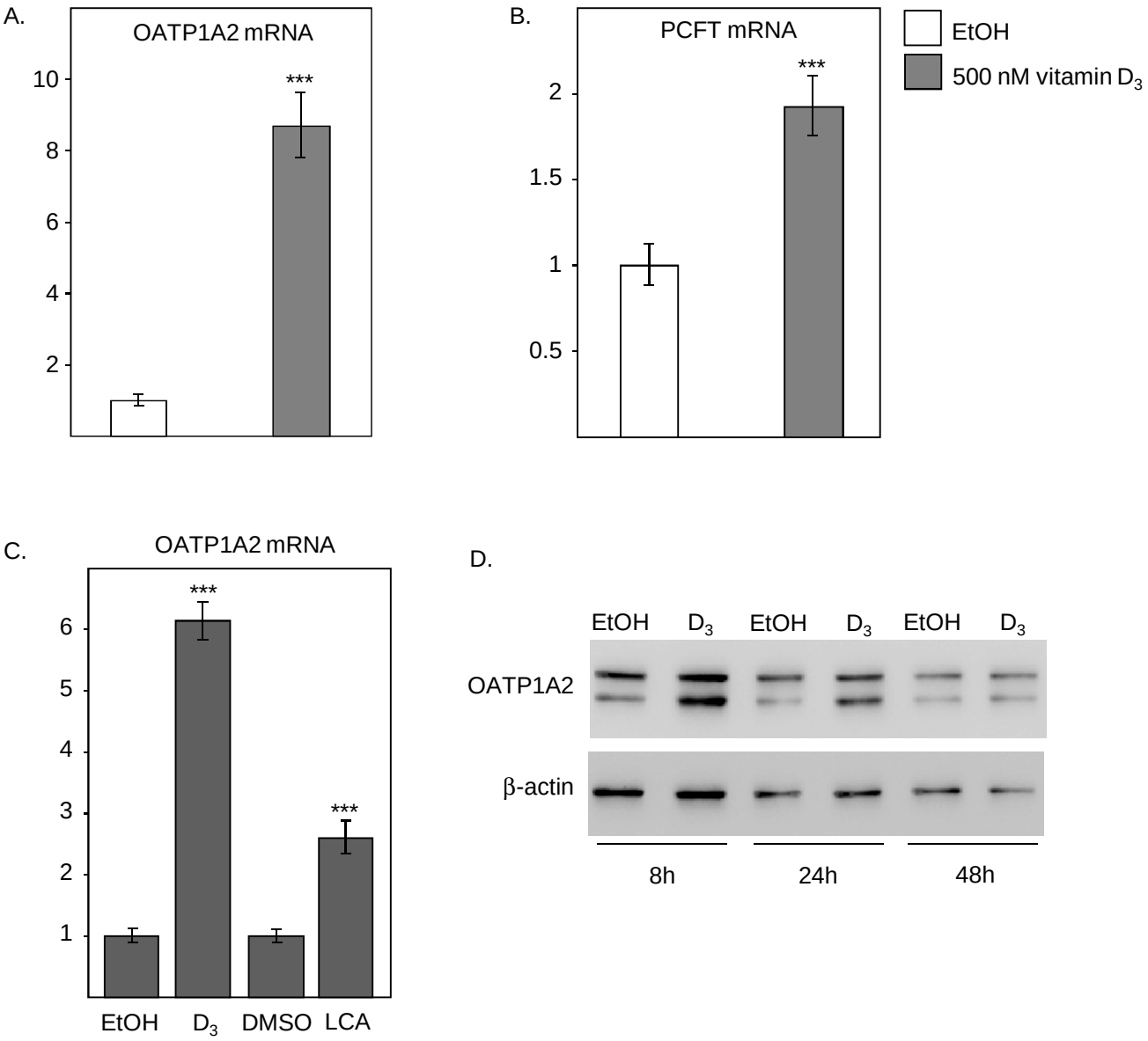


Fig. 2

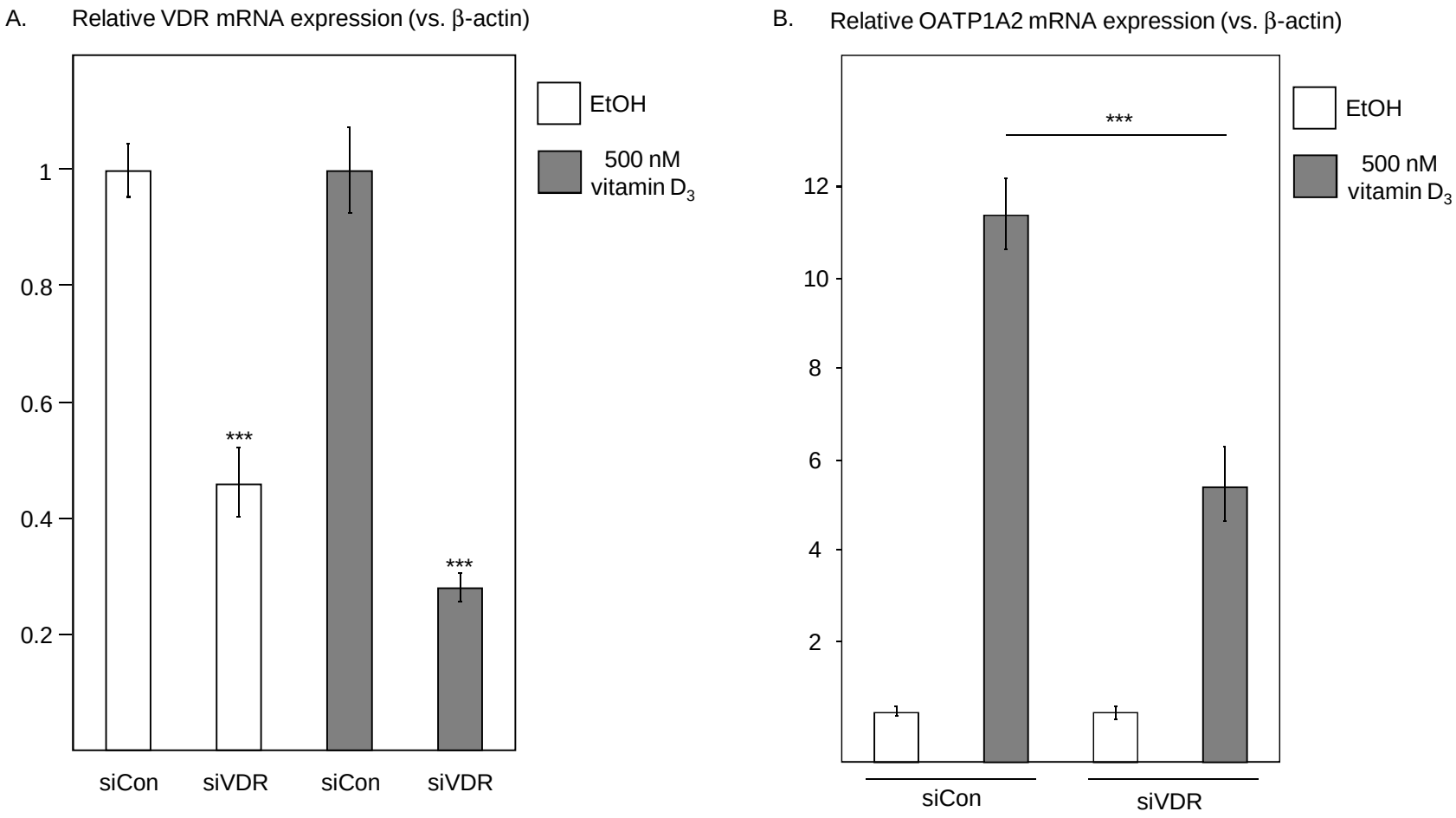
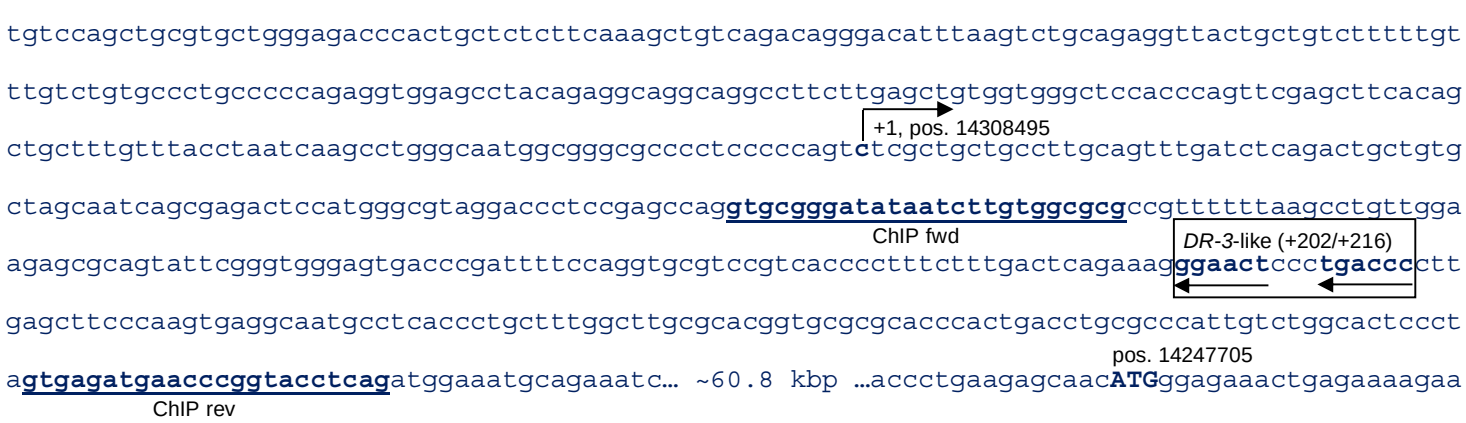


Fig. 3

A. *NT_009714.17: SLCO1A2* promoter variant 1 (transcript variant 1; NM_134431.3; 7695 bp)



B. *NT_009714.17: SLCO1A2* promoter variant 2 (transcript variant 2; NM_021094.3; 7225 bp)

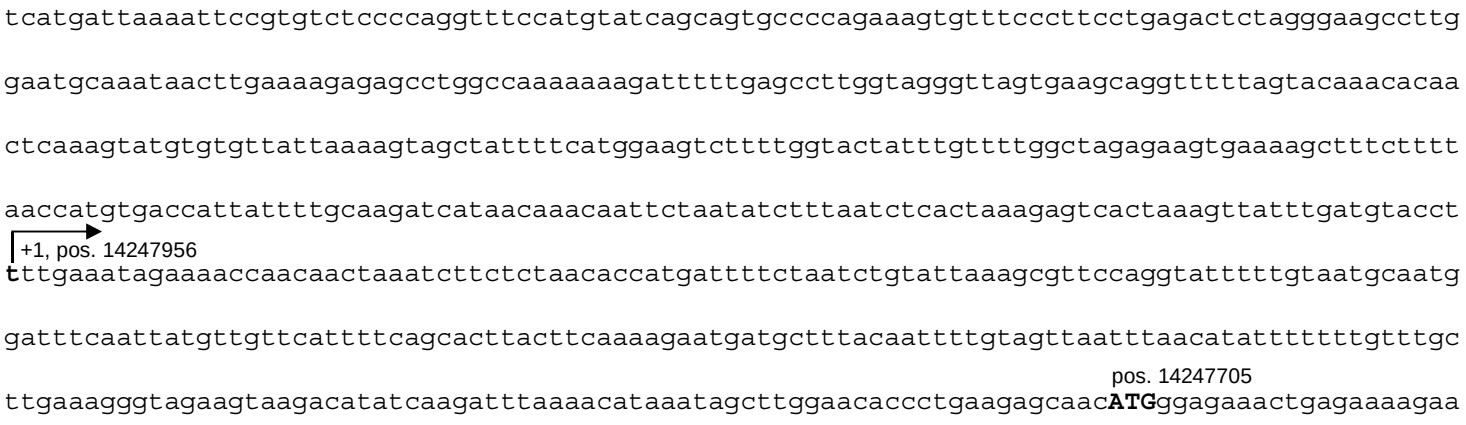


Fig. 4

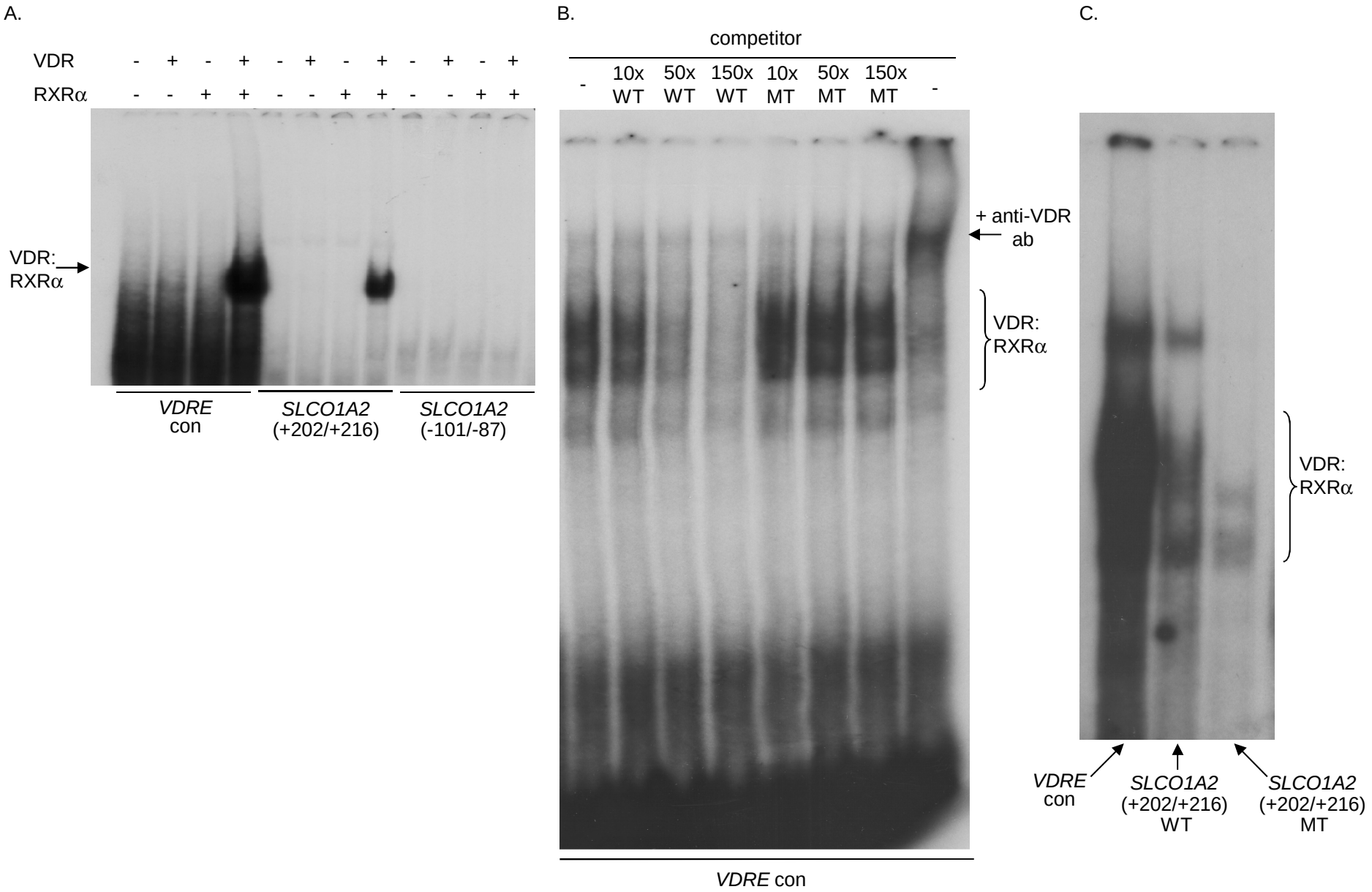


Fig. 5

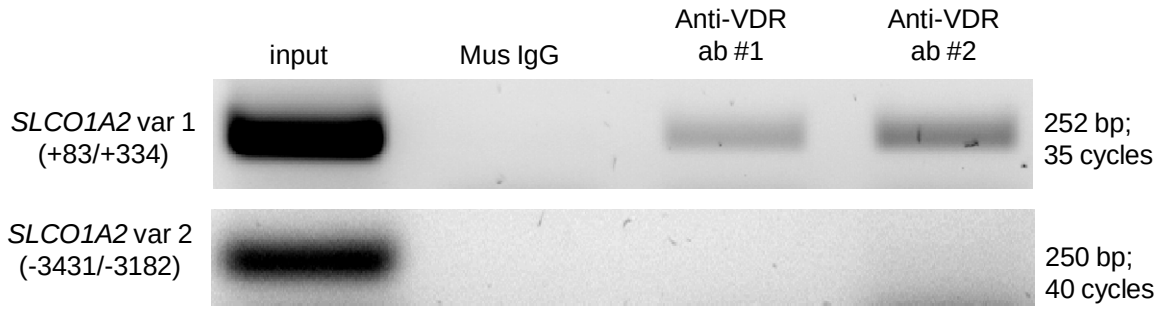


Fig. 6