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Vitamin D_3 and its Nuclear Receptor Increase the Expression and Activity of the Human Proton-Coupled Folate Transporter

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Running title: VDR regulates *PCFT* gene expression

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ABBREVIATIONS: 9-cis RA, 9-cis retinoic acid; ChIP, chromatin immunoprecipitation; *DR-3*, direct repeat-3; EMSA, electrophoretic mobility shift assay; IBD, inflammatory bowel disease; MTX, methotrexate; PCFT/Pcft, proton-coupled folate transporter; RFC, reduced folate carrier; RXRα, retinoid X receptor-α; vitamin D_3, 1,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; *VDRE*, vitamin D response element.

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ABSTRACT

Folates are essential for nucleic acid synthesis and required particularly in rapidly proliferating tissues, such as intestinal epithelium and hemopoietic cells. Availability of dietary folates is determined by their absorption across the intestinal epithelium, mediated by the proton-coupled folate transporter (PCFT) at the apical enterocyte membranes. Whereas transport properties of PCFT are well characterized, regulation of *PCFT* gene expression remains less elucidated. We have studied the mechanisms that regulate *PCFT* promoter activity and expression in intestine-derived cells. PCFT mRNA levels are increased in Caco-2 cells treated with 1,25-dihydroxyvitamin D_3 (vitamin D_3) in a dose-dependent fashion, and the duodenal rat Pcft mRNA expression is induced by vitamin D_3 *ex vivo*. The *PCFT* promoter region is transactivated by the vitamin D receptor (VDR) and its heterodimeric partner retinoid X receptor-α (RXRα) in the presence of vitamin D_3. *In silico* analyses predicted a VDR response element (*VDRE*) in the *PCFT* promoter region -1694/-1680. DNA-binding assays showed direct and specific binding of the VDR:RXRα heterodimer to the *PCFT*(-1694/-1680), and chromatin immunoprecipitations verified that this interaction occurs within living cells. Mutational promoter analyses confirmed that the *PCFT*(-1694/-1680) motif mediates a transcriptional response to vitamin D_3. In functional support of this regulatory mechanism, treatment with vitamin D_3 significantly increased the uptake of [^{3}H]-folic acid into Caco-2 cells at pH5.5. In conclusion, vitamin D_3 and VDR increase intestinal PCFT expression, resulting in enhanced cellular folate uptake. Pharmacological treatment of patients with vitamin D_3 may have the added therapeutic benefit of enhancing the intestinal absorption of folates.

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Folates are water-soluble B vitamins that act as one-carbon donors required for purine biosynthesis and for cellular methylation reactions. They are essential for *de novo* synthesis of nucleic acids, and thus for production and maintenance of new cells, particularly in rapidly dividing tissues, such as bone marrow and intestinal epithelium (Kamen, 1997). Adequate dietary folate availability is especially important during periods of rapid cell division, such as during pregnancy and infancy. Folate deficiency has been associated with reduced erythropoiesis, which can lead to megaloblastic anemia in both children and adults (Ifergan and Assaraf, 2008). Deficiency of folate availability in pregnant women has been linked to neural tube defects, such as spina bifida, in children (Pitkin, 2007). This has prompted the application of folate supplementation schemes either as pills or via fortification of grain products with folates (Eichholzer et al., 2006). Folates have also been proposed to act as protective agents against colorectal neoplasia, although contradictory results have also been reported (Sanderson et al., 2007).

The availability of diet-derived folates is primarily determined by the rate of their uptake into the epithelial cells of the intestine, mediated by the proton-coupled folate transporter (PCFT, gene symbol *SLC46A1*), localized at the apical brush-border membranes of enterocytes (Subramanian et al., 2008a). PCFT is an electrogenic transporter, which functions optimally at a low pH (Qiu et al., 2006; Umapathy et al., 2007). Despite being abundantly expressed in enterocytes, the second folate transporter, termed reduced folate carrier (RFC, gene symbol *SLC19A1*), has recently been shown not to play an important role in intestinal folate absorption (Zhao et al., 2004; Wang et al., 2005).

The human *PCFT* gene resides on chromosome 17, contains five exons, and is expressed as two prominent mRNA isoforms of 2.1 kb and 2.7 kb (Qiu et al., 2006). Mutations in the *PCFT* gene have been associated with hereditary folate malabsorption, a rare autosomal recessive disorder (Qiu et al., 2006; Zhao et al., 2007). The PCFT protein is predicted to have a

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structure harbouring twelve transmembrane domains (Qiu et al., 2007; Subramanian et al., 2008a). While the transport function of PCFT has been studied extensively, relatively little is known about the regulation of *PCFT* gene expression. Recently, it was shown that *PCFT* promoter activity may be epigenetically regulated by its methylation status in human tumor cell lines (Gonen et al., 2008). Furthermore, both the PCFT mRNA expression levels and *PCFT* promoter activity positively correlate with the level of differentiation of colon-derived Caco-2 cells (Subramanian et al., 2008b).

In addition to its well-known roles in regulating calcium homeostasis and bone mineralization, the biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 (vitamin D_3), executes many other important functions, particularly in the intestine. For example, vitamin D_3 promotes the integrity of mucosal tight junctions (Kong et al., 2008). Many effects of vitamin D_3 are mediated via its action as a ligand for the vitamin D receptor (VDR; gene symbol *NR1I1*), a member of the nuclear receptor family of transcription factors (Dusso et al., 2005). VDR typically regulates gene expression by directly interacting with so-called direct repeat-3 (*DR-3*; a direct repeat of AGGTCA-like hexamers separated by three nucleotides) motifs within the target promoters, as a heterodimer with another nuclear receptor, retinoid X receptor-α (RXRα; gene symbol *NR2B1*) (Haussler et al., 1997). Genetic variants of VDR have been associated with inflammatory bowel disease (IBD) (Simmons et al., 2000; Naderi et al., 2008). Similarly to folates, both VDR and its ligand vitamin D_3 have been proposed to be protective against intestinal neoplasia (Ali and Vaidya, 2007). It has been suggested that dietary folate intake regulates gene expression of the components of the vitamin D system, possibly via epigenetic control through the function of folates as methyl donors (Cross et al., 2006). Several intestinally expressed transporter genes, such as those encoding the multidrug resistance protein 1 (MDR1) and multidrug resistance-associated protein 2 (MRP2), have recently been shown to be induced by vitamin D_3 (Fan et al., 2009). We

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investigated, whether in a reverse manner vitamin D_3 regulates the expression of the *PCFT* gene, encoding a transporter crucial for intestinal folate absorption. The human well-polarized enterocyte-derived Caco-2 cells exhibit many of the characteristics associated with mature enterocytes and were used here to investigate the effects of vitamin D_3 on *PCFT* gene expression and folate transport activity.

Materials and Methods

Chemicals. Deoxyadenosine 5'-[α-^{32}P]-triphosphate (6000 Ci/mmol) was purchased from Perkin Elmer (Schwerzenbach, Switzerland) and [^{3}H]-labelled folic acid diammonium salt (0.2 μCi/ml) was from Moravek Biochemicals (Campro Scientific, Berlin, Germany). Restriction enzymes were from Roche Diagnostics (Rotkreuz, Switzerland), and the T4 DNA Ligase from Promega (Dübendorf, Switzerland). The oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). All other chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland), unless stated otherwise.

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). 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 70-80% confluent Caco-2 cells grown on 6-wells were isolated with TRIzol reagent (Invitrogen). RNAs were quantified spectrophotometrically at 260 nm (NanoDrop ND-1000, Thermo Fisher Scientific, Wilmington, Delaware), and 4 μg of total RNAs were reverse transcribed using random primers and Reverse Transcription Kit (Promega). Complementary DNAs (cDNAs) were diluted to a final volume of 200 μl with nuclease-free water. For

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quantitative real-time PCR reactions, 5 μl of diluted cDNAs were used per reaction. TaqMan Gene Expression Assays Hs00611081_m1 and Hs00953344_m1 (Applied Biosystems, Rotkreuz, Switzerland) were used to measure PCFT and RFC cDNA, respectively, using the ABI Prism 7900HT Fast Real-Time PCR system (Applied Biosystems). The intestinal epithelial housekeeping gene human villin cDNA (TaqMan Gene Expression Assay Hs00200229_m1; Applied Biosystems) was measured to normalize the relative PCFT expression levels, which were calculated using the comparative threshold cycle method ($\Delta\Delta C_T$). All PCR tests were performed in triplicate.

Animal *Ex Vivo* Experiments. Six male Wistar rats were obtained from Charles River (Sulzfeld, Germany) and housed in a 12:12-h light/dark cycle and permitted free consumption of water and a standard diet. The study was covered by the licence (#68/2007) from the Local Committee for Care and Use of Laboratory Animals. After sacrificing the rats, their duodena were excised, rinsed in saline, transferred into phosphate-buffered saline, and cut into 3-5 mm tissue pieces. Six duodenal pieces were transferred to each indicated culture condition in DMEM in a randomized manner. After 8 hour incubation at 37°C in a humidified atmosphere containing 5% CO_2, the tissue pieces were individually transferred to 1 ml TRIzol, disintegrated by repeated syringing, and the RNAs isolated as above. Following RNA isolation, 1 μg of six RNAs from each condition were pooled, and 3 ug of the RNA pools reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Complementary DNAs were diluted to a final volume of 130 μl with nuclease-free water. For quantitative real-time PCR reactions, 5 μl of diluted cDNAs were used per reaction. TaqMan Gene Expression Assay Rn01471183_m1 (Applied Biosystems) was used to measure rat Pcft cDNA, as described above. The intestinal epithelial housekeeping gene rat villin cDNA (TaqMan Gene Expression Assay Rn01400772_g1; Applied Biosystems) was measured to normalize the relative Pcft expression levels, which were calculated as above. All PCR tests were performed in triplicates.

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DNA Constructs. Using a pool of human genomic DNAs (Clontech, Saint-Germain-en-Laye, France) as the template and Platinum Pfx DNA Polymerase (Invitrogen, Basel, Switzerland), the human *PCFT* promoter region -2800/+96 (according to the GenBank entry EU185738) was obtained by PCR using oligonucleotide primers listed in Table 1. The PCR product *PCFT*(-2800/+96) was subcloned into the pGL3basic luciferase reporter vector (Promega, Dübendorf, Switzerland) using the engineered recognition sites for the restriction enzymes XhoI and NcoI. The resulting *PCFT*(-2800/+96)luc construct was employed as the template for PCR cloning of the promoter variants *PCFT*(-1674/+96), *PCFT*(-1098/+96), and *PCFT*(-843/+96), using the oligonucleotide primers in Table 1. The *PCFT*(-1674/+96) and *PCFT*(-843/+96) fragments were subcloned to SmaI-NcoI digested pGL3basic vector, and the *PCFT*(-1098/+96) fragment to XhoI-NcoI-digested pGL3basic vector to create plasmids *PCFT*(-1674/+96)luc, *PCFT*(-843/+96)luc, and *PCFT*(-1098/+96)luc, respectively. The plasmid *PCFT*(-2231/+96)luc was constructed by digesting the *PCFT*(-2800/+96)luc plasmid with EcoRV and NcoI and subcloning the *PCFT* promoter fragment to SmaI-NcoI-digested pGL3basic. Point mutations within the *PCFT*(-1694/-1680) were created in the *PCFT*(-2231/+96)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 wild-type *PCFT*(-1694/-1680) or a mutated version of it, single-stranded oligonucleotides (Table 1) containing HindIII and BamHI overhangs were annealed and 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).

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Transfections and 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 or RXRα expression plasmids, using the FuGENE HD reagent (Roche Diagnostics, Rotkreuz, 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 transfection cells were treated with the ligands 100 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 1x Passive Lysis Buffer (Promega) and luciferase activities measured using a Luminoskan Ascent luminometer (Thermo Electron, Allschwil, Switzerland). 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 condition.

Electromobility Shift Assays (EMSAs). EMSA binding reactions and gel runs were performed as described previously (Saborowski et al., 2006), except that 100 nM vitamin D_3 and 1 μM 9-cis retinoic acid were included in all binding reactions. 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 SuperScript II (Invitrogen) reverse transcriptase. Recombinant proteins VDR and RXRα were synthesized with the TNT T7 Coupled Reticulocyte Lysate System and using the plasmids pCMX-VDR and pCMX-RXRα as templates (Promega). Caco-2 nuclear protein extracts were prepared

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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 Biotechnology, LabForce, Nunningen, Switzerland) and/or 1 μg of the RXRα antibody (D-20X; Santa Cruz Biotechnology) were 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 100 nM vitamin D_3 and 1 μM 9-cis retinoic acid or the respective vehicles ethanol and DMSO. 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 test, using PuRe *Taq* Ready-To-Go PCR beads (GE Healthcare, Glattbrugg, Switzerland) and the oligonucleotide primers listed in Table 1: *PCFT*(-1789/-1543) containing the *PCFT*(-1694/-1680) element and *PCFT*(intron1) located within the first intron of the *PCFT* gene. After the

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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 30 cycles, ten microliters of each PCR product were resolved on 1.5% agarose gels and detected with SYBR Safe DNA gel stain (Invitrogen).

Cellular Uptake Measurements. For transport experiments, Caco-2 cells were grown on 3 cm dishes and used 3-5 days after reaching confluence. Cells were treated with the indicated concentrations of vitamin D_3 and/or the vehicle ethanol for three days, after which the uptake experiments were performed in Krebs buffer (123 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 0.85 mM $CaCl_2$, 5 mM D-glucose, 10 mM Hepes, 10 mM MES, adjusted to pH 5.5 or 7.4 with NaOH. In brief, cells were rinsed three times with Krebs buffer at 37°C, after which 6.7 nM [^{3}H]-labelled folic acid diammonium salt were added. After incubation at 37 °C for the indicated times, the folic acid-containing buffer was removed and the cells immediately rinsed four times with ice-cold Krebs buffer to terminate the reactions. Cells were solubilised with 1% (w/vol) Triton X-100 for at least 1 h and the radioactivity measured using a TRI-CARB 2200CA scintillation counter (Canberra Packard, Rüsselsheim, Germany). Protein concentrations of the lysates were determined using the BCA kit, and the level of uptake expressed as pmol [^{3}H]-folate/mg protein.

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 and the uptake experiment shown in Fig. 7B, one-way ANOVAs, followed by post-hoc Tukey's tests, were performed to determine statistical significance. Two-way unpaired t-tests were used to analyze the transport data in Fig. 7A and 7C.

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Results

Vitamin D_3 Increases PCFT mRNA Expression in a Dose-Dependent Manner. We treated near-confluent Caco-2 cells with increasing concentrations (0-500 nM) of vitamin D_3, and measured endogenous PCFT mRNA expression by quantitative real-time PCR. As shown in Fig. 1A, already the lowest vitamin D_3 concentration (50 nM) significantly increased PCFT mRNA expression. This effect was further enhanced in a step-wise manner upon gradually elevating the vitamin D_3 concentration to 500 nM. The vitamin D_3-dependent increase in PCFT levels was similar, whether villin (Fig. 1A) or β-actin (data not shown) was employed as the normalization gene, confirming that vitamin D_3 specifically affects PCFT expression. In contrast to PCFT, the mRNA levels of the other folate carrier expressed in Caco-2 cells, RFC, were unaffected by vitamin D_3 treatment (Fig. 1B). Exogenous overexpression of VDR and/or RXRα did not influence the effect of vitamin D_3 on PCFT mRNA expression (data not shown), probably because Caco-2 cells abundantly express these nuclear receptors endogenously, as indicated by the EMSA experiments below (Fig. 4). Co-treatment of Caco-2 cells with both VDR and RXRα ligands, vitamin D_3 and 9-cis retinoic acid, respectively, did not enhance the vitamin D_3 effect on PCFT mRNA expression (data not shown). To obtain further support for the physiological relevance of the vitamin D_3-mediated induction of PCFT expression, we studied, whether this phenomenon is conserved in rodents. Duodenal explants taken from six Wistar rats were incubated for 8 hours in the presence of 0-500 nM vitamin D_3. As shown in Figure 1C, a significant increase in rat Pcft mRNA levels was already observed with 100 nM vitamin D_3, and this elevation was further enhanced in a dose-dependent manner at higher vitamin D_3 concentrations.

VDR:RXRα Heterodimers Induce *PCFT* Promoter Activity in a Ligand-Dependent Fashion. To investigate, whether the nuclear receptors VDR and RXRα directly regulate the

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expression of the human *PCFT* gene, the *PCFT* promoter region between the nucleotides -2231 and +96 (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 RXRα and their respective ligands, vitamin D_3 and or 9-cis-retinoic acid. The highest levels of *PCFT*(-2231/+96) promoter activity were observed in Caco-2 cells transfected with both the VDR and RXRα expression plasmids, and in the presence of their ligands (Fig. 2). Cells exogenously expressing VDR only and treated with vitamin D_3 alone exhibited approximately 50% lower *PCFT* promoter activity. In the absence of their ligands, exogenous expression of either VDR alone or VDR and RXRα together did not result in a notable increase in *PCFT* promoter activity, demonstrating that VDR transactivates the human *PCFT* promoter in a ligand-dependent fashion.

Deletion Mapping of VDR Response on the *PCFT* Promoter. To analyze the location of the DNA element(s) mediating the vitamin D_3 induction of the *PCFT* promoter, we created a series of 5'-deletion fragments upstream of the luciferase reporter gene. When these promoter reporter constructs were used in transient transfections of Caco-2 cells, the variant *PCFT*(-2231/+96) clearly exhibited the highest response to cotransfected VDR and RXRα in the presence of vitamin D_3 and 9-cis retinoic acid (Fig. 3A). The promoter deletion variants *PCFT*(-1674/+96), *PCFT*(-1098/+96), and *PCFT*(-843/+96) showed significantly reduced VDR-dependent luciferase activities, but there were no major differences between the VDR-responses of these three shorter promoter variants.

A VDR-Responsive Element is Located between Nucleotides -1694 and -1680 of the Human *PCFT* Promoter. VDR:RXRα heterodimers preferentially bind to *DR*-3-like motifs on their target promoters (Colnot et al., 1995). Using the NUBIscan *in silico* algorithm (Podvinec et al., 2002), specifically designed to predict DNA-binding sites for members of the

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nuclear receptor family of transcription factors, we identified one *DR-3*-like motif with a threshold score higher than 0.75 within the human *PCFT* promoter. This putative VDR-responsive element is located between the nucleotides -1694 and -1680, ie. within the region -2231/-1674 mediating the strongest response to VDR and vitamin D_3 in our deletional analysis above. The *PCFT*(-1694/-1680) differs from the most preferred DNA-binding site (Colnot et al., 1995) of the VDR:RXRα heterodimer, 5'-AGGTCA(N_3)AGGTCA-3', by only two bases (Fig. 3B).

VDR:RXRα Heterodimers Interact Directly and Specifically with the *PCFT*(-1694/-1680) DR-3-Like Motif. We next investigated, whether the VDR:RXRα heterodimers may directly interact with the above identified -1694/-1680 *DR-3*-like motif on the *PCFT* promoter, using electrophoretic mobility shift assays (EMSAs). When both *in vitro* translated recombinant VDR and RXRα were mixed with the double-stranded radioactively labelled *PCFT*(-1694/-1680) probe in the presence of vitamin D_3 and retinoic acid, a distinct protein-DNA complex with an identical mobility to the complex forming on the *VDRE* consensus probe could be observed, albeit to a lesser degree than on the consensus probe (Fig. 4A). This complex only formed when both VDR and RXRα were added to the binding reaction, indicating that VDR binds to the *PCFT*(-1694/-1680) element as a heterodimer with RXRα, not as a monomer or homodimer. A similar distinct endogenous complex was formed when nuclear protein extracts prepared from Caco-2 cells were incubated with the radiolabelled *PCFT*(-1694/-1680) probe in the presence of VDR and RXRα ligands (Fig. 4B). In order to verify the identity of proteins present in Caco-2 nuclear extracts that are binding to the *PCFT*(-1694/-1680) sequence, we performed antibody supershift tests. When either anti-VDR antibodies or anti-RXRα-antibodies, or both together, were added to the binding reactions, the formation of the specific complex was efficiently abolished, and in the case of the anti-VDR antibody, supershifted complexes could be observed. In search of further confirmation of the

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specificity of the formation of protein-DNA complexes, we radioactively labelled annealed oligonucleotides representing a mutated version of the *PCFT*(-1694/-1680) *DR-3* motif ("*PCFT* mut"), where two bases have been changed, predicted to disrupt the binding by VDR:RXRα heterodimers (Table 1) (Colnot et al., 1995). When examined in parallel with either the *VDRE* consensus or the wild-type *PCFT*(-1694/-1680) EMSA probes, the mutated version was incapable of binding either the recombinant VDR:RXRα complexes, or VDR:RXRα complexes endogenously present in Caco-2 nuclear extracts (Fig. 4C). As a final confirmation of the specific nature of the DNA-binding by VDR:RXRα to the *PCFT*(-1694/-1680) element, we performed EMSA competition experiments: unlabelled double-stranded oligonucleotides containing the wild-type *PCFT*(-1694/-1680) region were efficient at competing off both the recombinant and endogenous VDR-RXRα complexes forming on the radiolabelled *VDRE* consensus probe when in molar excess, while the mutant version of the same element could not notably compete for the binding by VDR-RXRα, even when present in 100-fold molar excess (Fig. 4D).

VDR interacts with the vitamin D_3-responsive region of the *PCFT* promoter within living cells. To study, whether there is a direct interaction between the *PCFT*(-1694/-1680) *DR-3* element and VDR in the context of living cells, we performed chromatin immunoprecipitation analyses. Caco-2 cells were treated with 100 nM vitamin D_3 and 1 μM 9-cis retinoic acid or the vehicles 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 antibodies raised against VDR. As shown in Figure 5A, both VDR antibodies efficiently precipitated the promoter region -1789/-1543 of the endogenous *PCFT* gene in ligand-treated but not in vehicle-treated cells, while the non-specific mouse IgG antibodies failed to precipitate the vitamin D_3-responsive promoter region in either sample. As an additional specificity control, we amplified a 193 bp region from the first intron

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of the *PCFT* gene using the same ChIP samples as templates. Neither VDR antibody was able to precipitate this intronic region of the *PCFT* gene (Fig. 5B).

The *PCFT*(-1694/-1680) Element Is a Functional Mediator of the VDR:RXRα-Induced Transactivation of the *PCFT* Promoter. Having established that the *DR-3* element present in the -1694/-1680 region of the human *PCFT* promoter can specifically bind both recombinant and endogenous VDR:RXRα complexes, we aimed to confirm that this element can also functionally mediate *PCFT* promoter activation in response to vitamin D_3. To test this hypothesis in a heterologous promoter context, we annealed oligonucleotides containing either the wild-type (wt) or mutated (mut) *PCFT*(-1694/-1680) and cloned the double-stranded oligonucleotides upstream of the herpes simplex virus thymidine kinase (HSV-*TK*) 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. Whereas the wild-type *PCFT*(-1694/-1680)-containing construct was highly responsive to exogenous VDR:RXRα expression in the presence of their ligands, the mutated version of the same *DR-3* element lacked any responsiveness (Fig. 6A). This demonstrates that the *PCFT*(-1694/-1680) *DR-3*-like motif can function as an independent VDR-responsive element. In the next step, we introduced the same two point mutations to the *PCFT*(-1694/-1680) sequence in the context of the native *PCFT*(-2231/+96) promoter. The mutagenized reporter construct was transfected into Caco-2 cells in parallel with the wild-type variant, with or without the expression plasmids for VDR and RXRα and the ligands for these nuclear receptors. We observed a significant ~50% decrease in the VDR:RXRα-dependent activity of the mutagenized *PCFT* promoter (Fig. 6B). This degree of reduction in *PCFT* promoter activity was to the same level as observed with promoter deletion variants lacking the *PCFT*(-1694/-1680) motif (Fig. 3A).

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Cellular Uptake of Folic Acid is Enhanced in the Presence of Vitamin D_3. We next examined, whether the induction of PCFT mRNA expression by vitamin D_3 results in a functional effect on cellular folate uptake. Caco-2 cells were treated with 500 nM vitamin D_3 for three days, followed by uptake measurements of the prototypic PCFT transport substrate folate. As shown in Fig. 7A, we observed an increase in [^{3}H]-labelled folic acid uptake into Caco-2 cells with increasing incubation time, both in vehicle- and vitamin D_3-treated cells at pH5.5. Treatment of cells with vitamin D_3 moderately, but significantly, increased the uptake of folates at all timepoints. The uptake of folate at pH 7.4 was negligible both in control cells and in vitamin D_3-treated cells, demonstrating that Caco-2 cells display the typical characteristic of PCFT-mediated transport, which is clearly more active in acidic extracellular pH (Qiu et al., 2006; Nakai et al., 2007; Unal et al. 2009). In addition, the results show that vitamin D_3 treatment does not alter the barrier function of the apical membranes of Caco-2 cells. Next, we investigated the dependence of the induction of PCFT-mediated folate transport on vitamin D_3 concentration. We observed an increased folate uptake upon treating the cells with vitamin D_3 concentrations ranging from 0-500 nM for three days (Fig. 7B). Finally, we verified in parallel that PCFT mRNA is also increased in Caco-2 cells under the same conditions that lead to enhanced folate uptake: in cells treated with 250 nM vitamin D_3 for three days, both folate uptake and PCFT mRNA expression were significantly increased (Fig. 7C).

Discussion

Vitamin D_3 regulates the expression of its target genes primarily by acting as an agonistic ligand for its DNA-binding nuclear receptor VDR, although non-genomic actions by vitamin D_3 have also been described (Christakos et al., 2003; Dusso et al., 2005). VDR, an important regulator of differentiation and proliferation of enterocytes, typically activates gene expression

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by heterodimerizing with its nuclear receptor partner RXRα. VDR:RXRα heterodimers then directly bind to *DR-3* like elements on the target genes. It should be noted that other modes of VDR-mediated regulation either via direct interaction with other DNA-binding factors or through non-genomic actions have also been reported (Dusso et al., 2005).

Here we demonstrate that VDR is a ligand-dependent transactivator of the human *PCFT* gene, coding for a vital transporter for intestinal absorption of dietary folates. PCFT mRNA is also abundantly expressed in the liver (Qiu et al., 2006). However, VDR is expressed at very low levels in primary human hepatocytes or hepatocyte-derived cell lines (Gascon-Barre et al., 2003; data not shown), suggesting that VDR-mediated regulation of the *PCFT* gene may not occur in hepatocytes.

Endogenous PCFT mRNA levels were induced by vitamin D_3 in a dose-dependent manner in Caco-2 cells (Fig. 1A). This increase was not further enhanced by co-treatment of cells with the RXRα ligand 9-cis retinoic acid (data not shown), consistent with a previous report that VDR:RXRα heterodimers, at least in some promoter contexts, may not respond to RXRα ligands (Forman et al., 1995). Alternatively, saturating levels of RXRα ligands may already be endogenously present in cells in these experimental conditions. In transient transfection assays, the *PCFT* promoter fragment -2231/+96 exhibited significant response to exogenous expression of VDR alone in the presence of its ligand (Fig. 2), most probably supported by endogenously expressed RXRα in Caco-2 cells.

Supporting the importance of the VDR:RXRα heterodimer formation for *PCFT* promoter regulation, the luciferase values were further significantly elevated upon exogenous expression of RXRα. Exogenous expression of VDR in the absence of vitamin D_3 did not notably influence the activity of the *PCFT*(-2231/+96) promoter, indicating ligand-dependence of VDR action. In deletional transfection analysis, the strongest induction in response to VDR and RXRα in the presence of their ligands was achieved with the *PCFT*(-2231/+96) promoter

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fragment (Fig. 3A). Induction of the shortest deletion variant tested (*PCFT*(-843/+96)luc) was approximately 50% of that achieved for the *PCFT*(-2231/+96), indicating that this more proximal region is likely to contain further DNA elements mediating a response to vitamin D_3. However, in our current study, we focussed on the distal region between the nucleotides -2231 and -1674 upstream of the transcriptional start site of the human *PCFT* gene, which confers maximal response to vitamin D_3. In our computational analysis we identified a putative *VDRE* within the *PCFT* promoter region between nucleotides -1694 and -1680. We have not so far been successful in identifying further binding sites for the VDR:RXRα heterodimer in the more proximal region of the *PCFT* promoter. It may be that, in addition to direct DNA-binding to the *PCFT*(-1694/-1680) element identified here, VDR may also affect *PCFT* promoter activity indirectly, via interactions with other DNA-binding factors. For example, it has been proposed that the $p27^{Kip1}$ gene is regulated by VDR via response elements for unrelated DNA-binding transcription factors Sp1 and NF-Y (Huang et al., 2004).

Both endogenously expressed and recombinant VDR and RXRα bound to the *PCFT*(-1694/-1680) element specifically, and as obligate heterodimers (Fig. 4). The interaction between VDR and this region of the *PCFT* promoter within living cells treated with VDR and RXRα ligands was confirmed by chromatin immunoprecipitation tests (Fig. 5). Heterologous promoter assays proved that the *PCFT*(-1694/-1680) element can function as an independent VDR response element. The significant decrease in VDR:RXRα-mediated induction upon mutagenesis of the *PCFT*(-1694/-1680) element confirmed that it is an important functional mediator of the effect (Fig 6A and 6B).

While we observed vitamin D_3-mediated increase of rat Pcft mRNA expression *ex vivo* (Fig. 1C), the rat *Pcft* promoter (chromosome 10, GenBank accession number NW_047336) exhibits no significant overall homology with the human *PCFT* promoter over the proximal

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3000 bp regions. This suggests that despite the divergence of the promoter sequences between human and rodent *PCFT*/*Pcft* genes, the functional response to vitamin D_3 is conserved.

The activation of *PCFT* gene transcription by VDR also translates into an increase in PCFT protein function. Vitamin D_3 treatment of Caco-2 cells led to significantly increased uptake of folate across the apical membrane, in a dose-dependent manner (Fig. 7). In keeping with the fact that PCFT strongly prefers an acidic milieu for its transport function (Qiu et al., 2006; Nakai et al., 2007; Unal et al., 2009), we only observed vitamin D_3-stimulated transport activity at pH5.5, but not at neutral pH. These data strongly suggest that vitamin D_3-mediated transcriptional activation of *PCFT* gene expression leads to an increase of PCFT transport function. Consistent with our model, mRNA expression of the other known folate carrier expressed in Caco-2 cells, RFC, which functions efficiently at neutral pH (Ganapathy et al., 2004; Wang et al., 2004), was not affected by vitamin D_3 treatment (Fig. 1B). Recently, it has been reported that vitamin D_3-induced gene expression increases as Caco-2 cells differentiate (Cui et al., 2009). Thus, our current findings on VDR-mediated regulation of PCFT expression provide a possible molecular mechanism for a prior observation that folate uptake into Caco-2 cells is enhanced upon confluence-associated differentiation (Subramanian et al., 2008b).

Our results suggest that intestinal folate absorption may be enhanced by an increase in dietary vitamin D_3 intake. Food products are often supplemented with folates, because of their proposed beneficial health effects. Based on our current study, supplementation of vitamin D_3 may enhance the intestinal absorption of folates. PCFT also transports the antifolate drug methotrexate (MTX) (Inoue et al., 2008; Yuasa et al., 2008) widely used in the treatment of autoimmune diseases and cancer. MTX interferes with folate metabolism by competitively inhibiting the enzyme dihydrofolate reductase. Our results may further suggest a potential mechanism to increase intestinal absorption of MTX via simultaneous treatment with vitamin D_3, thereby affecting the bioavailability of MTX. Patients suffering from IBD are frequently on

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long-term treatment with calcium and vitamin D_3 as a prophylaxis against osteopenia and osteoporosis (Lichtenstein et al., 2006). This patient group is frequently treated with folates (in the case of folate deficiency) or MTX (as a second-line immunosuppressant) (Rizzello et al., 2002). MTX therapy *per se* requires prophylactic administration of folates, and these patients often receive additional calcium/vitamin D_3. Our current results may warrant a closer investigation into potential drug-drug interactions between pharmacologically administered vitamin D_3, MTX, and folates.

Taking into account the previous report that folates regulate the expression of genes involved in vitamin D_3 metabolism, it may be that folate and vitamin D_3 homeostasis are closely interlinked through such mutual regulatory interactions.

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Footnotes:

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Legends for figures

Fig. 1. Analysis of effects of vitamin D_3 treatment on PCFT/Pcft and RFC mRNA expression. A, Treatment of Caco-2 with vitamin D_3 cells increases PCFT mRNA expression in a dose-dependent manner. B, RFC mRNA expression levels are not affected by vitamin D_3. Cells were treated with increasing concentrations (0-500 nM) of vitamin D_3 for 24 hours, before RNA isolation, reverse transcription, and quantitative real-time PCR. C, *Ex vivo* treatment of rat duodenum biopsies with vitamin D_3 for 8 hours induces Pcft mRNA expression in a dose-dependent manner. PCFT/Pcft and RFC mRNA expression levels were normalized to those of the housekeeping gene villin. **, P<0.01; ***, P<0.001; ns, not significant.

Fig. 2. VDR:RXRα heterodimers activate the human *PCFT* promoter in a ligand-dependent manner. A, Caco-2 cells were transiently transfected with the plasmid containing the *PCFT* promoter region -2231/+96 cloned upstream of a luciferase reporter gene, together with expression constructs for human VDR and its heterodimerization partner RXRα. Highest *PCFT* promoter activity was observed in the presence of both VDR and RXRα and their respective ligands vitamin D_3 and 9-cis RA. **, P<0.01; ***, P<0.001.

Fig. 3. Deletion analysis of the *PCFT* promoter. A, Mapping of VDR-responsive regions within the *PCFT* promoter via deletional promoter analysis. Luciferase linked 5'-deletion variants of the *PCFT* promoter were transiently transfected into Caco-2 cells, either in the absence or presence of the VDR and RXRα expression plasmids and the respective ligands for these nuclear receptors. Highest induction by VDR:RXRα and their ligands was observed for the promoter construct *PCFT*(-2231/+96)luc, while the promoter variants *PCFT*(-1674/+96), *PCFT*(-1098/+96), and *PCFT*(-843/+96) exhibited significantly reduced VDR:RXRα-

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dependent stimulation, at the level of approximately 50% of that observed for the promoter region *PCFT*(-2231/+96). ***, P<0.001. B, Alignment of the identified *DR-3*-like element with the NUBIscan treshold score of above 0.75 in the human *PCFT* promoter with the *DR-3* consensus motif for a VDR-response element. The numbering of the nucleotides is relative to the transcriptional start site of the human *PCFT* gene.

Fig. 4. VDR:RXRα heterodimers bind specifically to the -1694/-1680 *DR-3*-like motif of the human *PCFT* promoter in electrophoretic mobility shift assays (EMSAs). A, Recombinant VDR and RXRα bind to the *PCFT*(-1694/-1680) 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. The two panels are derived from the same exposure of the same EMSA gel. B, VDR:RXRα heterodimers endogenously present in Caco-2 nuclear extracts bind to the *PCFT*(-1694/-1680) element. Oligonucleotides derived from the *PCFT*(-1694/-1680) region were used as radiolabelled EMSA probes, and anti-VDR and/or anti-RXRα antibodies were added to the binding reactions as indicated above the lanes. C, Point mutations in the *PCFT*(-1694/-1680) *DR-3*-like element abolish the binding of both recombinant and endogenous VDR:RXRα heterodimers. Double-stranded oligonucleotides employed as radiolabelled EMSA probes are indicated above the lanes. D, EMSA competition studies confirm the specific DNA-binding of both recombinant and endogenous VDR-RXRα heterodimers to the -1694/-1680 *DR-3*-like element of the *PCFT* promoter. 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. In all EMSA tests, 100 nM vitamin D_3 and 1 μM 9-cis retinoic acid were included in the binding reactions. For all sections: ivt, *in vitro* translated; NE, nuclear extract; wt, wild-type; mut, mutant.

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Fig. 5. VDR interacts with the vitamin D_3-responsive region of the *PCFT* promoter within Caco-2 cells. Cells were treated with 100 nM vitamin D_3 and 1 μM 9-cis retinoic acid or the vehicles (control) for 24 hours, after which the ChIP assays were perfomed. Two different VDR-specific antibodies were efficient in precipitating the promoter region *PCFT*(-1789/-1543) in cells treated with VDR and RXRα ligands (A), whereas no signal was obtained for the intronic region of the endogenous *PCFT* gene (B).

Fig. 6. Mutation of the *PCFT*(-1694/-1680) *DR-3*-like motif leads to decreased VDR:RXRα-mediated induction of *PCFT* promoter activity in Caco-2 cells. A, Heterologous promoter assays show that the *PCFT*(-1694/-1680) motif can function as an independent VDR response element. When placed upstream of the HSV-*TK* core promoter, the wild-type *PCFT*(-1694/-1680) element, but not the mutated version of it, renders the hybrid promoter responsive to transactivation by VDR:RXRα heterodimers, in the presence of VDR and RXRα ligands. B, Point mutations in the *PCFT*(-1694/-1680) *DR-3*-like element significantly reduce the VDR:RXRα-mediated transactivation of the native *PCFT*(-2231/+96) promoter. *, $P<0.05$; ***, $P<0.001$; ns, not significant.

Fig. 7. Analysis of vitamin D_3-dependent effects on folate transport activity. A, Vitamin D_3 stimulates folate uptake into Caco-2 cells. Cellular uptake of [^{3}H]-labelled folic acid was measured at timepoints 0, 1, 3, and 5 minutes after treatment of cells with 500 nM vitamin D_3 for three days. B, Folate transport into Caco-2 cells is enhanced upon increasing vitamin D_3 concentrations. Cellular uptake of [^{3}H]-labelled folic acid was measured at timepoints 0 and 1 minutes after treatment of cells with 0-500 nM vitamin D_3 for three days. C, Vitamin D_3-dependent stimulation of cellular folate uptake correlates with increased PCFT mRNA levels. Cellular uptake of [^{3}H]-labelled folic acid was measured at timepoints 0 and 1 minutes after

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treatment of cells with 250 nM vitamin D_3 for three days, and PCFT mRNA levels were quantified from Caco-2 cells treated in identical conditions. For (A), statistical analysis shown refers to the difference between vitamin D_3- and vehicle-treated cells at pH 5.5. **, $P<0.01$; ***, $P<0.001$.

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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 EMSA assays. Where applicable, restriction sites introduced are underlined and the corresponding enzymes used are given in parentheses. The *DR-3* motifs are shaded grey, and mutated nucleotides within the *PCFT*(-1694/-1680) *DR-3* element are indicated in bold.

Oligonucleotide	Sequence (5'-3')	Purpose
PCFT(-2800)fwd	CTCGAGAGATCTTTTCCTTTGCAATAGC (XhoI)	Cloning *PCFT*(-2800/+96)luc
PCFT(-1674)fwd	ATCTAGCCAGCACCTACCCATCC	Cloning *PCFT*(-1674/+96)luc
PCFT(-1098)fwd	CTCGAGAGCATAGGTTCACACCCAAGGACC (XhoI)	Cloning *PCFT*(-1098/+96)luc
PCFT(-843)fwd	CTCAATTGATCCTCCCACCTCAGCCACCCG	Cloning *PCFT*(-843/+96)luc
PCFT(+96)rev	CTTTCTTTATGTTTTTGGCGTCTTCCATGGTGCGTGC GCGGCGGAGCTGTCGCCAGG (NcoI)	Cloning *PCFT*-luc constructs
PCFT(-1694/-1680) sdm_top	GCTTGCAGGGGGA**T**G**C**TATTCAGTTCACTAGTATCTAGCC	Mutagenesis of *PCFT*(-1694/-1680)
*VDRE*con_EMSA_top	AGCTGCAGGGGGAGGTCAAAGAGGTCACACTAGTA	*VDRE* consensus EMSA probe
PCFT(-1694/-1680) wt_EMSA_top	AGCTGCAGGGGGAGGTTATTCAGTTCACTAGTA	Wild-type *PCFT*(-1694/-1680) EMSA probe/competitor; Cloning *TK*-luc constructs
PCFT(-1694/-1680) mut_EMSA_top	AGCTGCAGGGGGA**T**G**C**TATTCAGTTCACTAGTA	Mutagenized *PCFT*(-1694/-1680) EMSA probe/competitor: Cloning *TK*-luc constructs
PCFT(-1789/-1543) ChIP_fwd	GAGGGAGGAGAGTGGAGCCAATC	ChIP assay
PCFT(-1789/-1543) ChIP_rev	CTGATTCTTGGGAGGATGGATGG	ChIP assay
PCFT(intron1) ChIP_fwd	CTTGGTCCATGTCCTTCCCCCTC	ChIP assay
PCFT(intron1) ChIP_rev	GGTAAGGGTCTCCACTTCCTGTAG	ChIP assay

Figure 1

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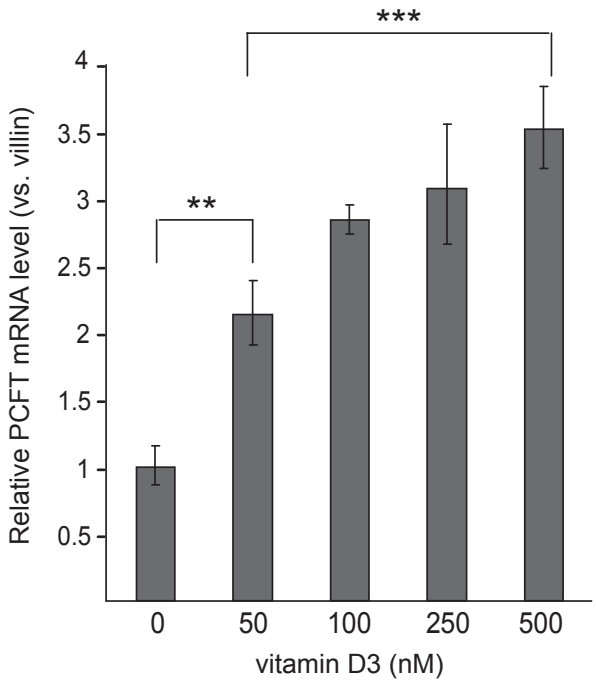

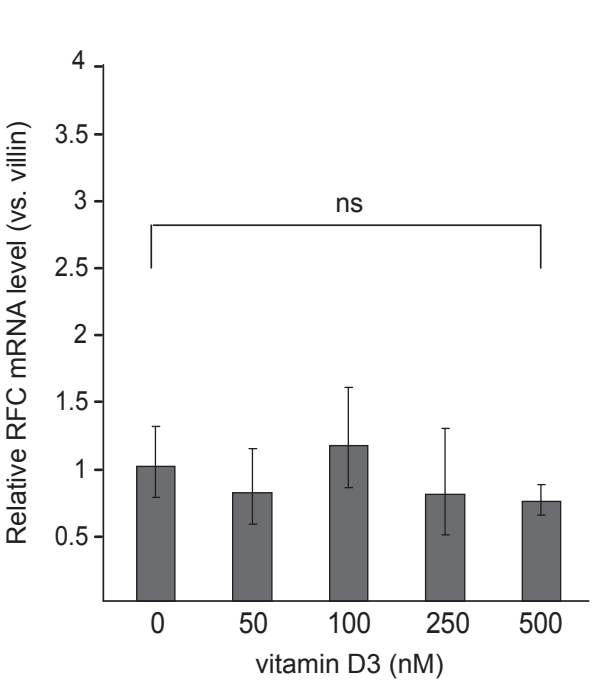

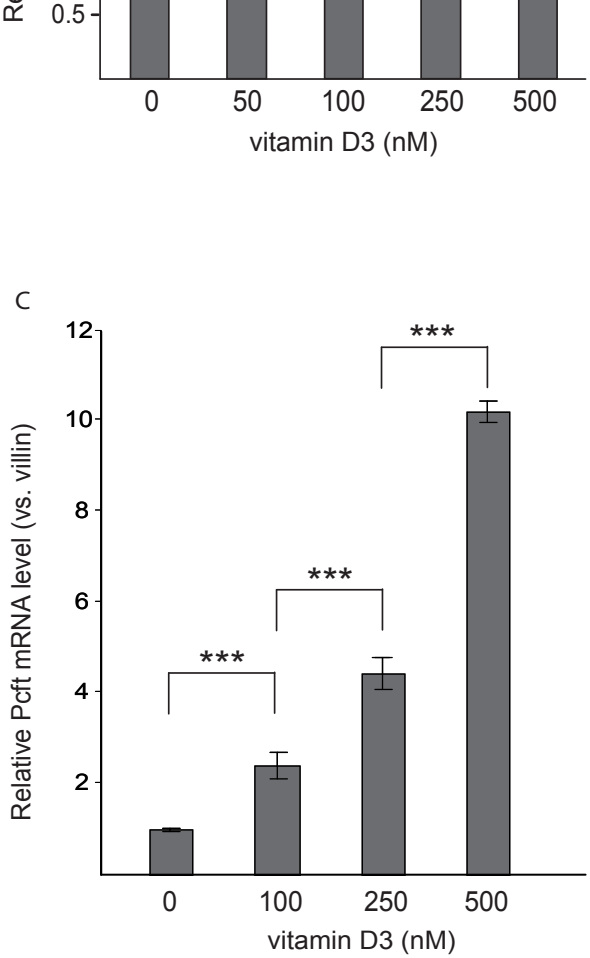

Figure 2

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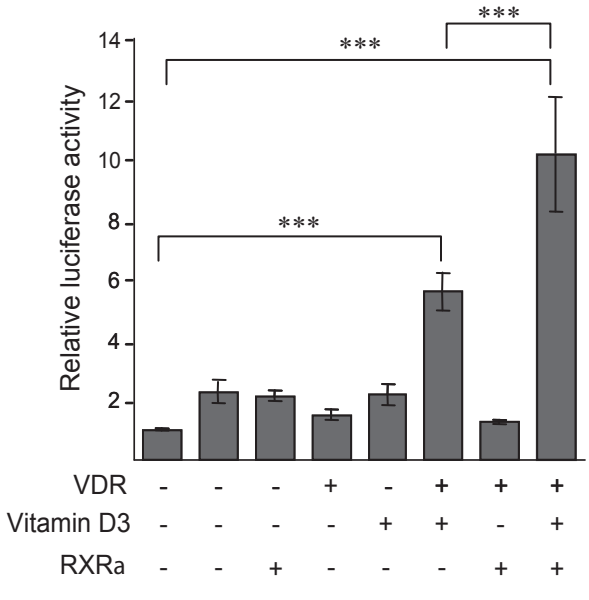

Figure 3

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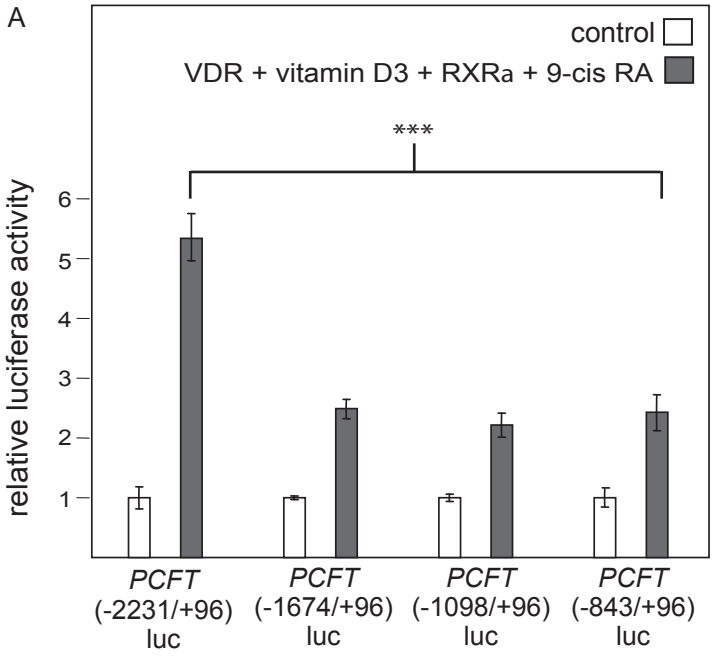

B

VDRE consensus	AGGTCAnnnAGGTCA
	|||| |||||| |||
Human *PCFT*(-1694/-1680)	AGGTTATTCAGTTCA

Figure 4

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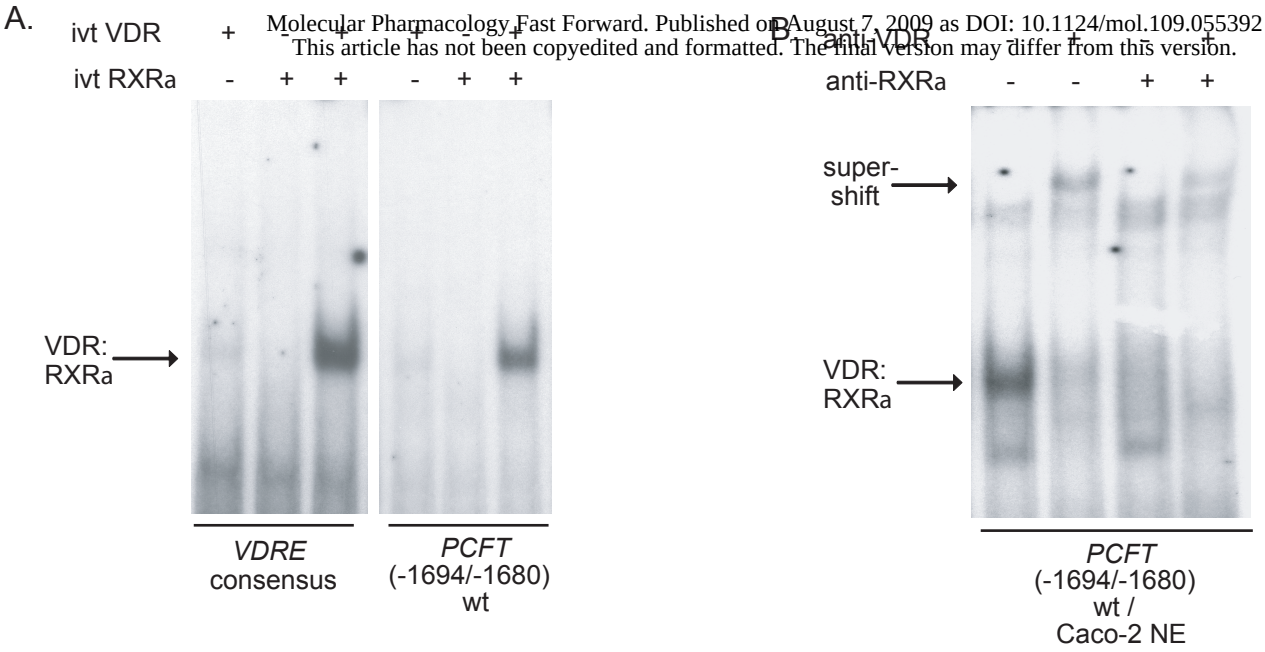

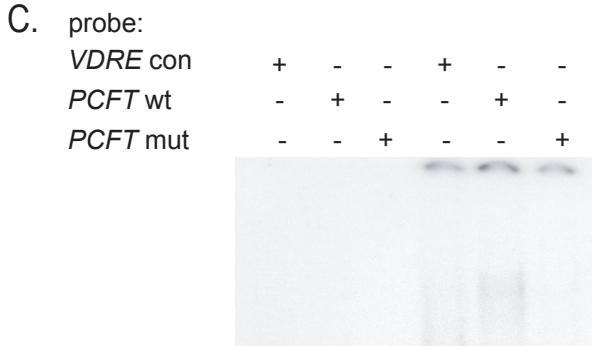

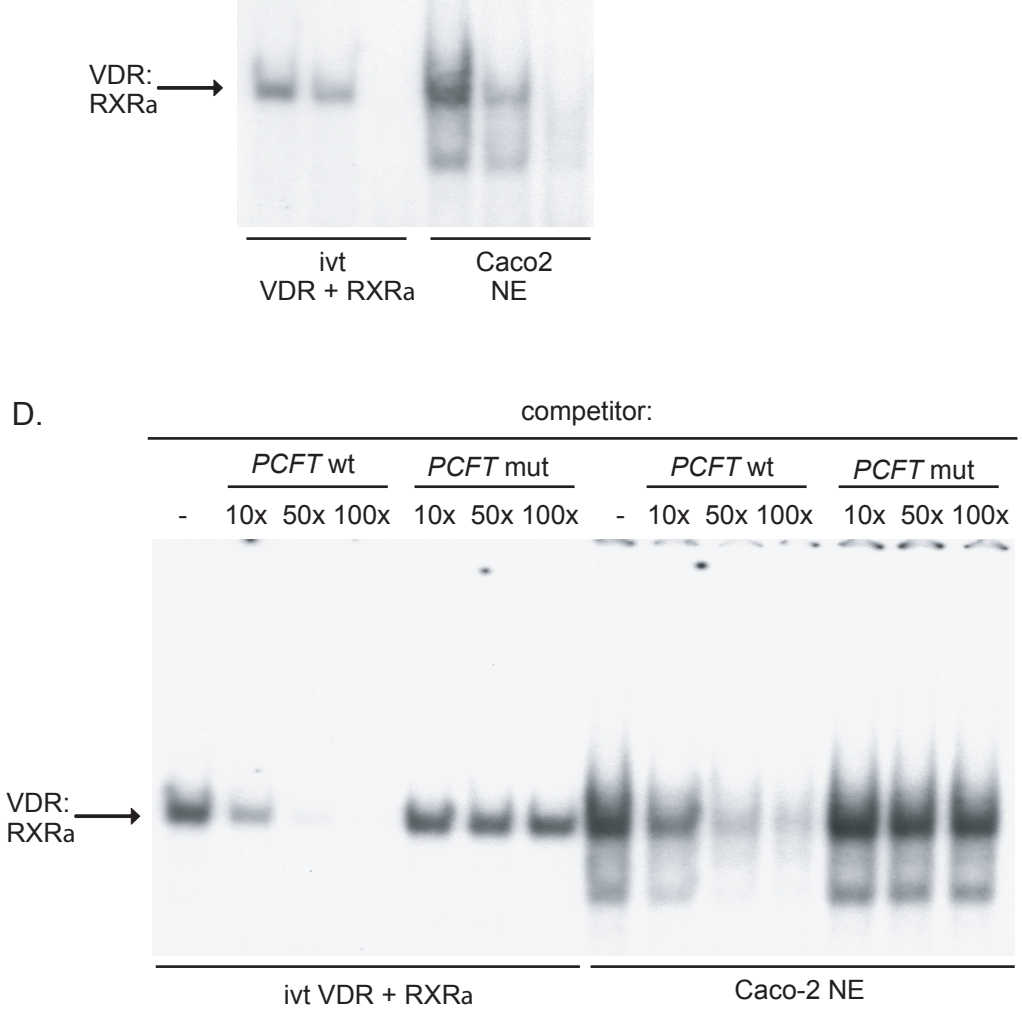

Figure 5

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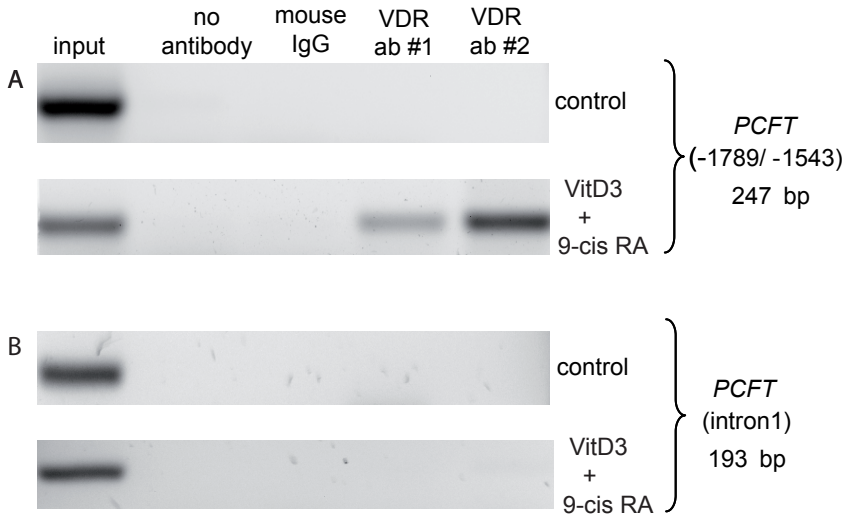

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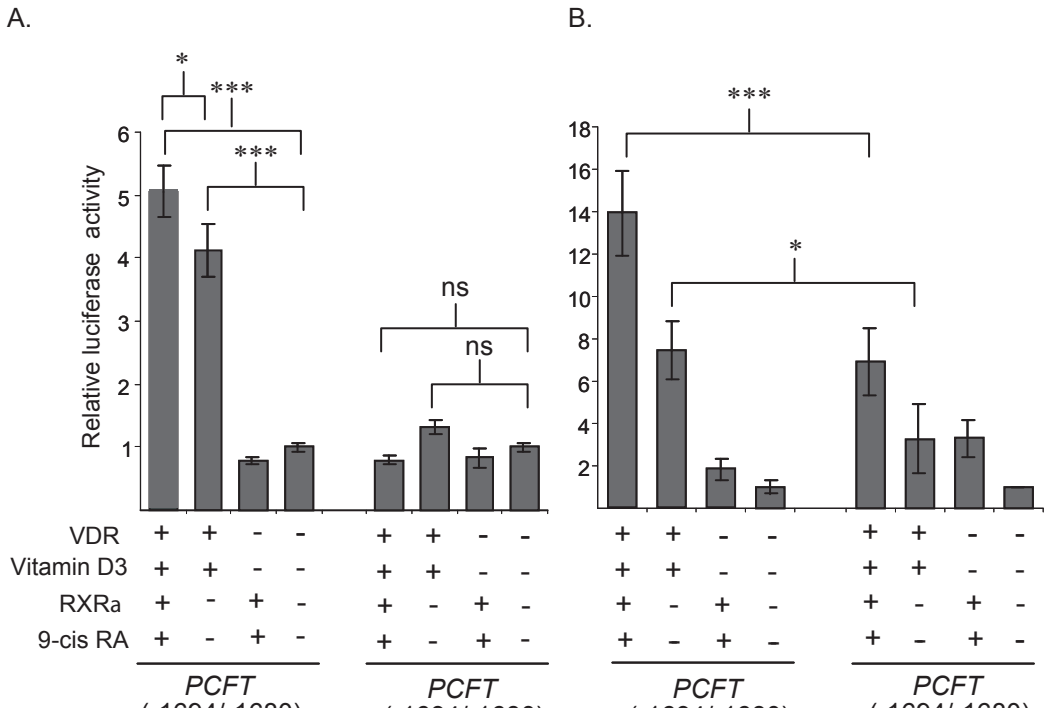

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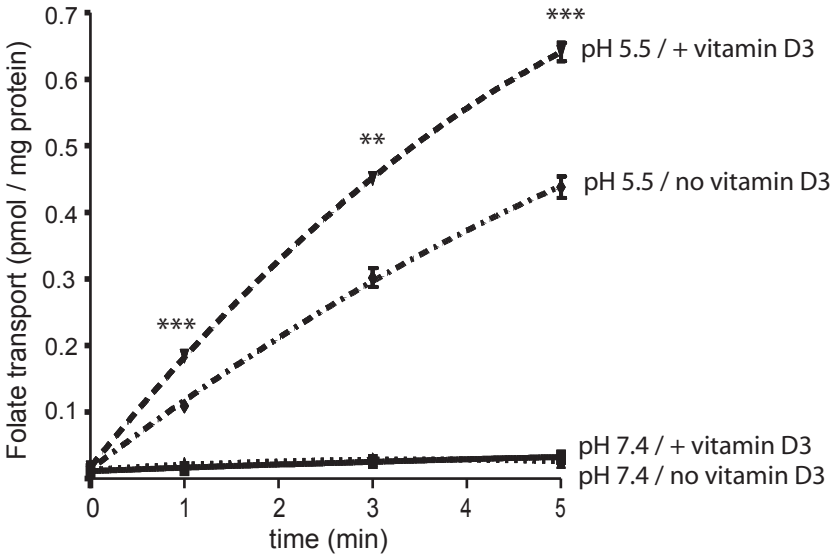
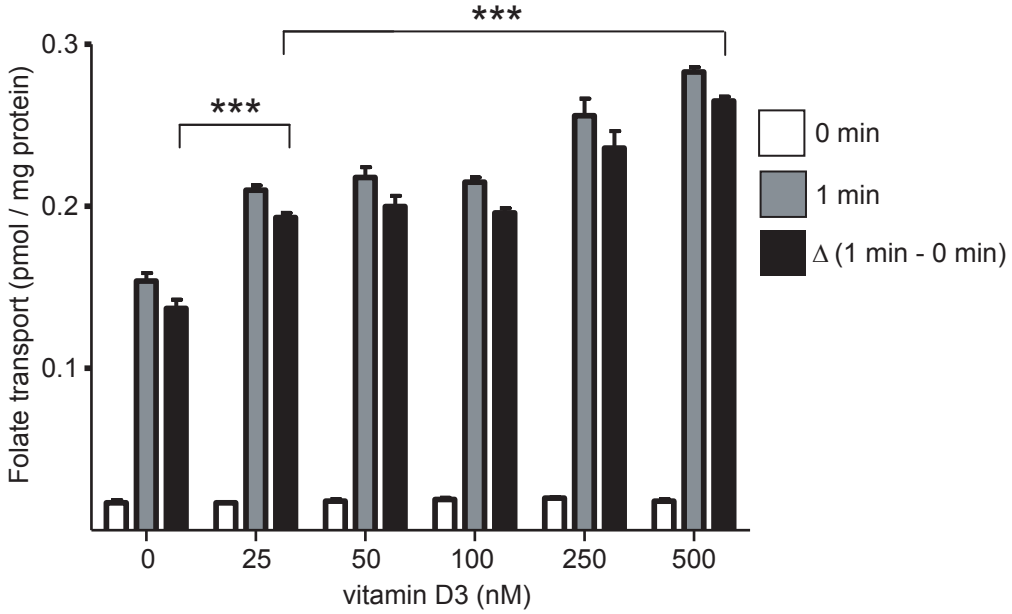
A.



B.



C.