

Vitamin D₃ and Its Nuclear Receptor Increase the Expression and Activity of the Human Proton-Coupled Folate Transporter

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Received February 9, 2009; accepted August 7, 2009

ABSTRACT

Folates are essential for nucleic acid synthesis and are particularly required 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 D₃ (vitamin D₃) in a dose-dependent fashion, and the duodenal rat Pcf mRNA expression is induced by vitamin D₃ ex vivo. The *PCFT* promoter region is transactivated by the vitamin D receptor (VDR) and its heterodimeric partner retinoid X receptor- α

(RXR α) in the presence of vitamin D₃. 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:RXR α 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 D₃. In functional support of this regulatory mechanism, treatment with vitamin D₃ significantly increased the uptake of [³H]folic acid into Caco-2 cells at pH 5.5. In conclusion, vitamin D₃ and VDR increase intestinal PCFT expression, resulting in enhanced cellular folate uptake. Pharmacological treatment of patients with vitamin D₃ may have the added therapeutic benefit of enhancing the intestinal absorption of folates.

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 re-

duced erythropoiesis, which can lead to megaloblastic anemia in both children and adults (Ifeghan 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 that 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

This study was supported by the Swiss National Science Foundation [Grant 32-120463/1]; the Swiss Inflammatory Bowel Disease Cohort Study [SNF Grant 33CSC0-108792]; the Zurich Center of Integrative Human Physiology; the Center of Clinical Research at the University Hospital Zurich; and the Novartis Foundation for Biomedical Research.

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Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.109.055392.

ABBREVIATIONS: PCFT, proton-coupled folate transporter; RFC, reduced folate carrier; vitamin D₃, 1,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; VDRE, vitamin D response element; DR-3, direct repeat-3; RXR α , retinoid X receptor- α ; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; MES, 2-(N-morpholino)ethanesulfonic acid; HSV-TK, herpes simplex virus thymidine kinase; bp, base pair(s); MTX, methotrexate.

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 5 exons, and is expressed as two prominent mRNA isoforms of 2.1 and 2.7 kilobase pairs (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 structure harboring 12 transmembrane domains (Qiu et al., 2007; Subramanian et al., 2008a). Although the transport function of PCFT has been studied extensively, relatively little is known about the regulation of *PCFT* gene expression. *PCFT* promoter activity has been shown possibly to 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, 1,25-dihydroxyvitamin D₃ (vitamin D₃), the biologically active metabolite of vitamin D, executes many other important functions, particularly in the intestine. For example, vitamin D₃ promotes the integrity of mucosal tight junctions (Kong et al., 2008). Many effects of vitamin D₃ are mediated via its action as a ligand for the vitamin D receptor (VDR; gene symbol *NR1H1*), 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 (RXR α ; gene symbol *NR2B1*) (Haussler et al., 1997). Genetic variants of VDR have been associated with inflammatory bowel disease (Simmons et al., 2000; Naderi et al., 2008). Similarly to folates, both VDR and its ligand vitamin D₃ have been proposed to be protective against intestinal neoplasia (Ali and Vaidya, 2007). Dietary folate intake has been suggested to regulate 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 and multidrug resistance-associated protein 2, have recently been shown to be induced by vitamin D₃ (Fan et al., 2009). We investigated whether vitamin D₃ 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 D₃ on *PCFT* gene expression and folate transport activity.

Materials and Methods

Chemicals. [α -³²P]deoxy-ATP (6000 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Schwerzenbach, Switzerland) and ³H-labeled folic acid diammonium salt (0.2 μ Ci/ml) was from Moravsek Biochemicals (Berlin, Germany). Restriction enzymes were from Roche Diagnostics (Rotkreuz, Switzerland), and the T4 DNA ligase was 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, Switzerland), derived from human colon carcinoma, were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Buchs, Switzerland), supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen, Basel, Switzerland). Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂.

Isolation of RNA, Reverse Transcription, and Quantitative Real-Time PCR. Total RNAs from 70 to 80% confluent Caco-2 cells grown on six-well plates were isolated with TRIzol reagent (Invitrogen). RNAs were quantified spectrophotometrically at 260 nm (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, MA), and 4 μ g of total RNA was reverse-transcribed using random primers and Reverse Transcription Kit (Promega). cDNAs were diluted to a final volume of 200 μ l with nuclease-free water. For quantitative real-time PCR reactions, 5 μ l 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-h light/dark cycle and permitted free consumption of water and a standard diet. The study was covered by the license (no. 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- to 5-mm tissue pieces. Six duodenal pieces were transferred to each indicated culture condition in Dulbecco's modified Eagle's medium in a randomized manner. After 8-h incubation at 37°C in a humidified atmosphere containing 5% CO₂, the tissue pieces were individually transferred to 1 ml of TRIzol and disintegrated by repeated syringing; the RNAs were isolated as above. After RNA isolation, 1 μ g of six RNAs from each condition were pooled, and 3 μ g of the RNA pools were reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNAs were diluted to a final volume of 130 μ l with nuclease-free water. For quantitative real-time PCR reactions, 5 μ l 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 triplicate.

DNA Constructs. Using a pool of human genomic DNAs (Clontech, Saint-Germain-en-Laye, France) as the template and Platinum Pfx DNA Polymerase, 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) 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 con-

the *PCFT* gene. After the initial denaturation stage at 94°C for 3 min, the PCR cycling conditions were 94°C for 20 s, 60°C for 30 s, and 72°C for 1 min. After 30 cycles, 10 μ l 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 to 5 days after reaching confluence. Cells were treated with the indicated concentrations of vitamin D₃ and/or the vehicle ethanol for 3 days, after which the uptake experiments were performed in Krebs buffer (123 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 0.85 mM CaCl₂, 5 mM D-glucose, 10 mM HEPES, and 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 [³H]-labeled folic acid diammonium salt were added. After incubation at 37°C for the times indicated in Fig. 7, the folic acid-containing buffer was removed and the cells were immediately rinsed four times with ice-cold Krebs buffer to terminate the reactions. Cells were solubilized with 1% (w/v) Triton X-100 for at least 1 h, and the radioactivity was measured using a TRI-CARB 2200CA scintillation counter (Canberra Packard, Rüsselsheim, Germany). Protein concentrations of the lysates were determined using the bicinchoninic acid kit, and the level of uptake expressed as picomoles of [³H]folate per milligram of protein.

Statistical Analysis. All experiments shown were repeated from two to three times. Statistical analyses were performed with Prism (GraphPad Software, San Diego, CA). Error bars represent S.D. of the mean values. For luciferase and real-time PCR experiments and

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

Results

Vitamin D₃ Increases PCFT mRNA Expression in a Dose-Dependent Manner. We treated near-confluent Caco-2 cells with increasing concentrations (0–500 nM) of vitamin D₃ and measured endogenous PCFT mRNA expression by quantitative real-time PCR. As shown in Fig. 1A, the lowest vitamin D₃ concentration (50 nM) significantly increased PCFT mRNA expression. This effect was further enhanced in a step-wise manner upon gradually elevating the vitamin D₃ concentration to 500 nM. The vitamin D₃-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 D₃ specifically affects PCFT expression. In contrast to PCFT, the mRNA levels of RFC, the other folate carrier expressed in Caco-2 cells, were unaffected by vitamin D₃ treatment (Fig. 1B). Exogenous overexpression of VDR and/or RXR α did not influence the effect of vitamin D₃ on PCFT mRNA expression (data not shown), probably because Caco-2 cells abundantly

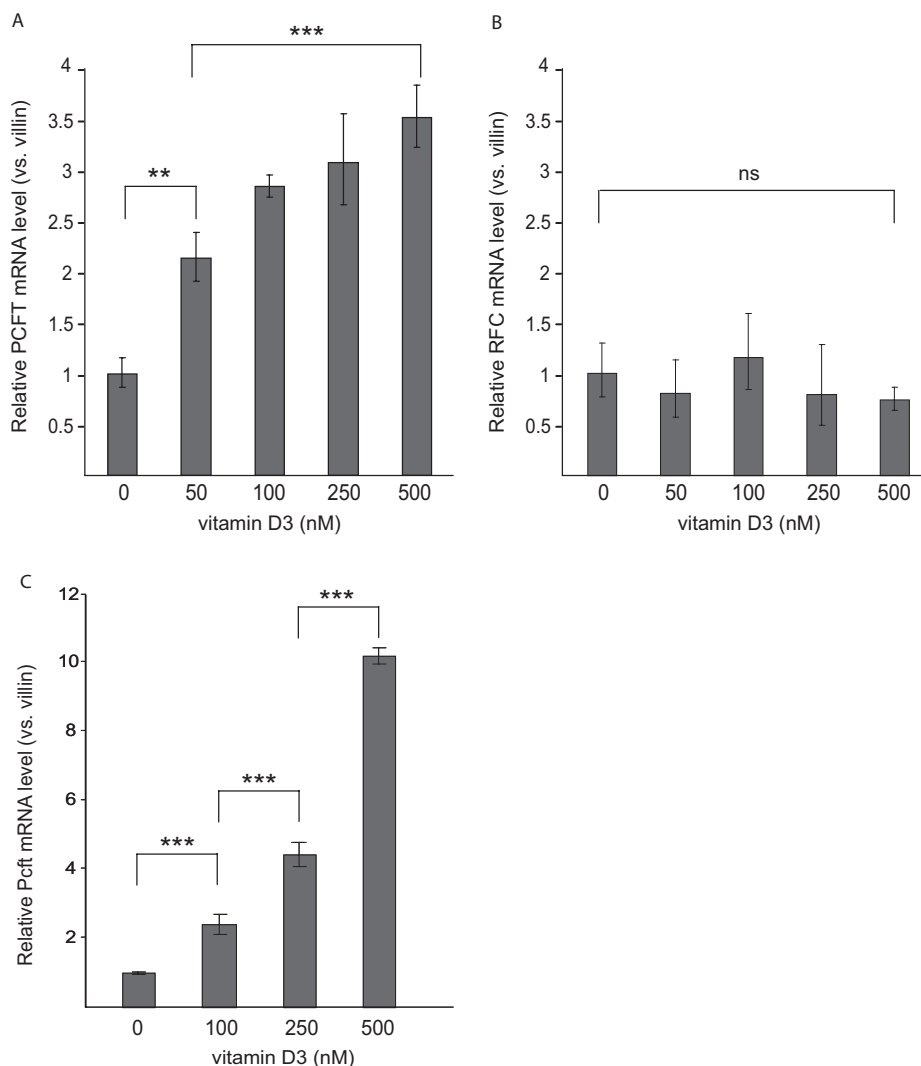


Fig. 1. Analysis of effects of vitamin D₃ treatment on PCFT/Pcft and RFC mRNA expression. A, treatment of Caco-2 with vitamin D₃ cells increases PCFT mRNA expression in a dose-dependent manner. B, RFC mRNA expression levels are not affected by vitamin D₃. Cells were treated with increasing concentrations (0–500 nM) of vitamin D₃ for 24 h, before RNA isolation, reverse transcription, and quantitative real-time PCR. C, ex vivo treatment of rat duodenum biopsies with vitamin D₃ for 8 h 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; n.s., not significant.

express these nuclear receptors endogenously, as indicated by the EMSA experiments below (Fig. 4). Cotreatment of Caco-2 cells with both VDR and RXR α ligands, vitamin D₃, and 9-*cis* retinoic acid, respectively, did not enhance the vitamin D₃ effect on PCFT mRNA expression (data not shown). To obtain further support for the physiological relevance of the vitamin D₃-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 h in the presence of 0 to 500 nM vitamin D₃. As shown in Fig. 1C, a significant increase in rat Pcf mRNA levels was already observed with 100 nM vitamin D₃, and this elevation was further enhanced in a dose-dependent manner at higher vitamin D₃ concentrations.

VDR:RXR α Heterodimers Induce PCFT Promoter Activity in a Ligand-Dependent Fashion. To investigate whether the nuclear receptors VDR and RXR α directly regulate the 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 RXR α and their respective ligands, vitamin D₃ 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 RXR α expression plasmids, and in the presence of their ligands (Fig. 2). Cells exogenously expressing VDR only and treated with vitamin D₃ alone exhibited approximately 50% lower PCFT promoter activity. In the absence of their ligands, exogenous expression of either VDR alone or VDR and RXR α 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.

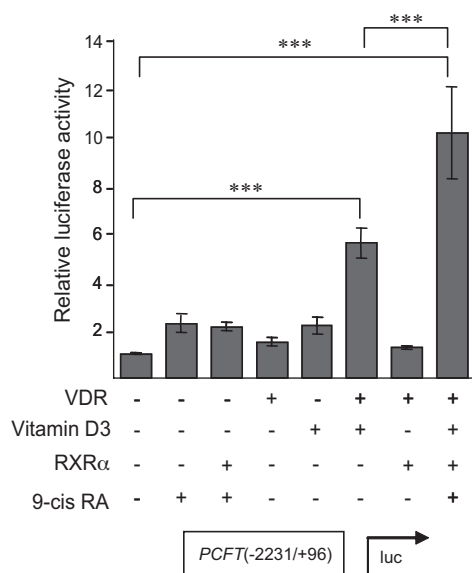


Fig. 2. VDR:RXR α heterodimers activate the human PCFT promoter in a ligand-dependent manner. A, Caco-2 cells were transiently transfected with the plasmid containing the PCFT promoter region -2231/+96 cloned upstream of a luciferase reporter gene, together with expression constructs for human VDR and its heterodimerization partner RXR α . Highest PCFT promoter activity was observed in the presence of both VDR and RXR α and their respective ligands vitamin D₃ and 9-*cis* retinoic acid (9-*cis* RA). **, $P < 0.01$; ***, $P < 0.001$.

Deletion Mapping of VDR Response on the PCFT Promoter. To analyze the location of the DNA element(s) mediating the vitamin D₃ 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 RXR α in the presence of vitamin D₃ 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:RXR α heterodimers preferentially bind to DR-3-like motifs on their target promoters (Colnot et al., 1995). Using the NUBIsScan in silico algorithm (Podvinec et al., 2002), specifically designed to predict DNA-binding sites for members of the 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 (i.e., within the region -2231/-1674 mediating the strongest response to VDR and vitamin D₃ in our deletional analysis above). The PCFT(-1694/

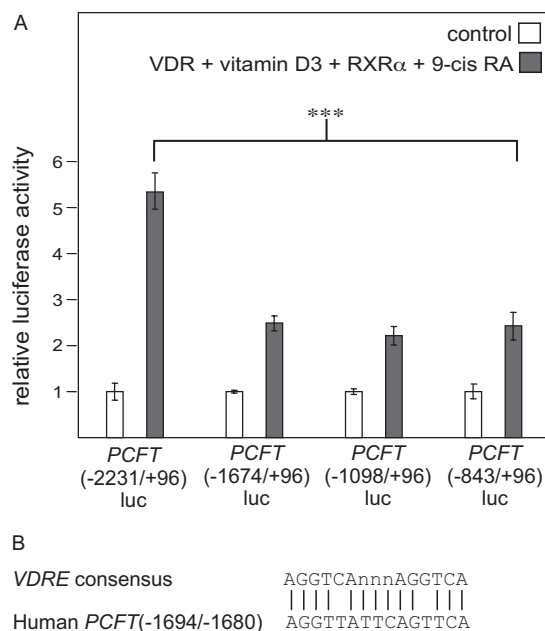


Fig. 3. Deletion analysis of the PCFT promoter. A, mapping of VDR-responsive regions within the PCFT promoter via deletional promoter analysis. Luciferase linked 5'-deletion variants of the PCFT promoter were transiently transfected into Caco-2 cells, either in the absence or presence of the VDR and RXR α expression plasmids and the respective ligands for these nuclear receptors. Highest induction by VDR:RXR α and their ligands was observed for the promoter construct PCFT(-2231/+96)luc, whereas the promoter variants PCFT(-1674/+96), PCFT(-1098/+96), and PCFT(-843/+96) exhibited significantly reduced VDR:RXR α -dependent stimulation, at the level of approximately 50% of that observed for the promoter region PCFT(-2231/+96). ***, $P < 0.001$. 9-*cis* RA, 9-*cis* retinoic acid. B, alignment of the identified DR-3-like element with the NUBIsScan threshold 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.

–1680) differs from the most preferred DNA-binding site (Colnot et al., 1995) of the VDR:RXR α heterodimer, 5'-AGGTCA(N₃)AGGTCA-3', by only two bases (Fig. 3B).

VDR:RXR α Heterodimers Interact Directly and Specifically with the *PCFT*(–1694/–1680) DR-3-Like Motif. We next investigated whether the VDR:RXR α heterodimers might directly interact with the above-identified –1694/–1680 DR-3-like motif on the *PCFT* promoter by using EMSAs. When both in vitro-translated recombinant VDR and RXR α were mixed with the double-stranded radioactively

labeled *PCFT*(–1694/–1680) probe in the presence of vitamin D₃ and retinoic acid, a distinct protein-DNA complex with a mobility identical to that of 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 RXR α were added to the binding reaction, indicating that VDR binds to the *PCFT*(–1694/–1680) element as a heterodimer with RXR α , not as a monomer or homodimer. A similar distinct endogenous complex was formed when nuclear protein extracts pre-

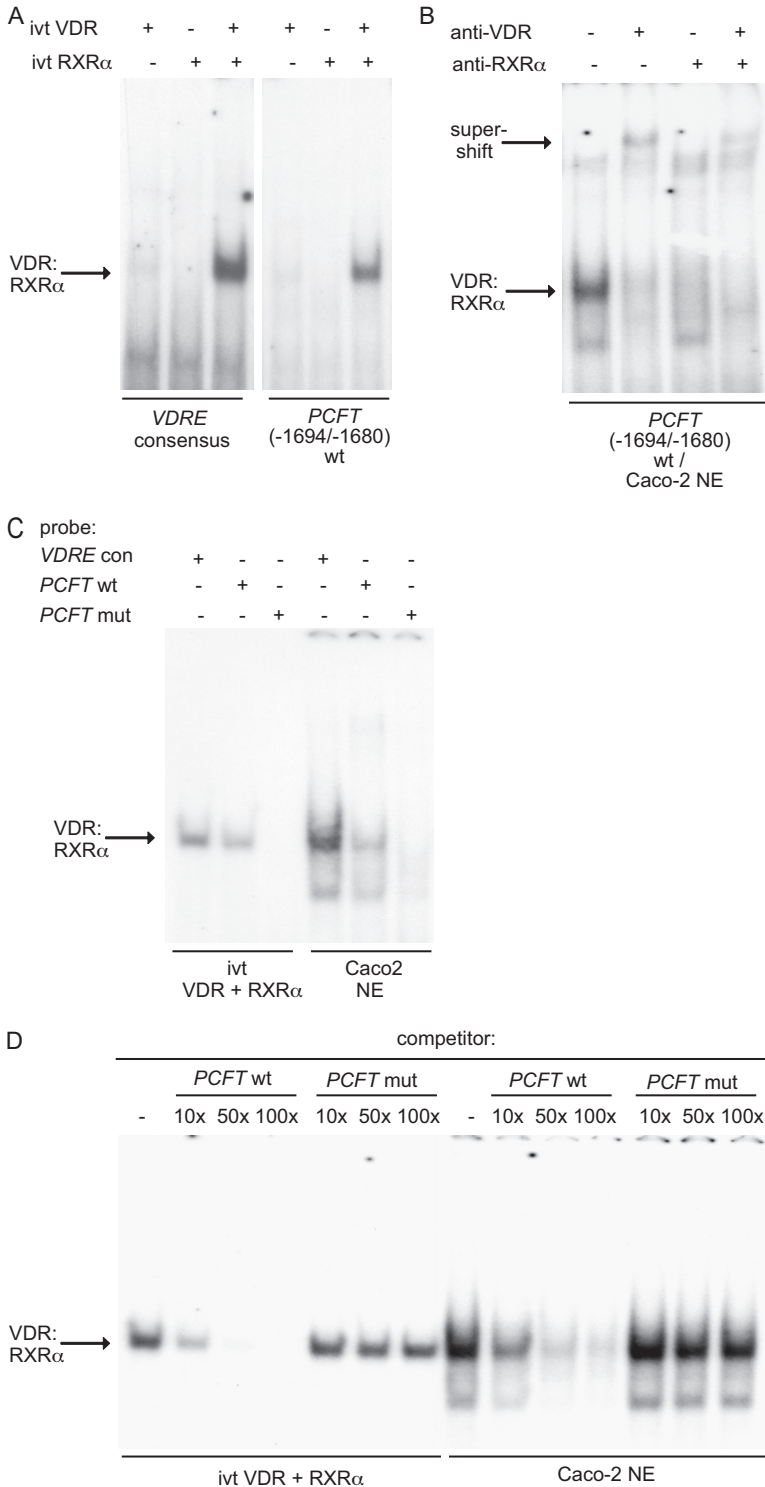


Fig. 4. VDR:RXR α heterodimers bind specifically to the –1694/–1680 DR-3-like motif of the human *PCFT* promoter in electrophoretic mobility shift assays (EMSAs). A, recombinant VDR and RXR α bind to the *PCFT*(–1694/–1680) sequence as obligate heterodimers. In vitro-translated proteins added to the binding reactions are indicated above the lanes, and the double-stranded oligonucleotides used as radiolabeled EMSA probes are indicated below the lanes. The two panels are derived from the same exposure of the same EMSA gel. B, VDR:RXR α 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 radiolabeled EMSA probes, and anti-VDR and/or anti-RXR α 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:RXR α heterodimers. Double-stranded oligonucleotides employed as radiolabeled EMSA probes are indicated above the lanes. D, EMSA competition studies confirm the specific DNA-binding of both recombinant and endogenous VDR:RXR α heterodimers to the –1694/–1680 DR-3-like element of the *PCFT* promoter. The consensus *VDRE* was used as the radiolabeled EMSA probe in all tests and the identities and molar excesses of the unlabeled competitor oligonucleotides are shown above the lanes. In all EMSA tests, 100 nM vitamin D₃ and 1 μ M 9-*cis* retinoic acid were included in the binding reactions. ivt, in vitro-translated; NE, nuclear extract; wt, wild type; mut, mutant.

pared from Caco-2 cells were incubated with the radiolabeled *PCFT*(-1694/-1680) probe in the presence of VDR and RXR α ligands (Fig. 4B). 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-RXR α -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 specificity of the formation of protein-DNA complexes, we radioactively labeled annealed oligonucleotides representing a mutated version of the *PCFT*(-1694/-1680) DR-3 motif ("PCFT mut"), where two bases have been changed, which was predicted to disrupt the binding by VDR:RXR α 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:RXR α complexes or VDR:RXR α complexes endogenously present in Caco-2 nuclear extracts (Fig. 4C). As a final confirmation of the specific nature of the DNA-binding by VDR:RXR α to the *PCFT*(-1694/-1680) element, we performed EMSA competition experiments: unlabeled double-stranded oligonucleotides containing the wild-type *PCFT*(-1694/-1680) region were efficient at competing off both the recombinant and endogenous VDR:RXR α complexes forming on the radiolabeled VDRE consensus probe when in molar excess, whereas the mutant version of the same element could not notably compete for the binding by VDR:RXR α , even when present in 100-fold molar excess (Fig. 4D).

VDR Interacts with the Vitamin D₃-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 D₃ and 1 μ M 9-cis retinoic acid or the vehicles for 24 h, after which proteins were cross-linked to DNA in vivo using formaldehyde, and the cells were lysed. After shearing the genomic DNA into fragments of 300 to 600 bp, we performed immunoprecipitations using two antibodies raised against VDR. As shown in Fig. 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, whereas the nonspecific mouse IgG antibodies failed to precipitate the vitamin D₃-responsive promoter region in either sample. As an additional specificity control, we amplified a 193-bp region from the first intron 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:RXR α -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:RXR α complexes, we aimed to confirm that this element can also functionally mediate *PCFT* promoter activation in response to vitamin D₃. To test this hypothesis in a heterologous promoter context, we annealed oligonucle-

otides containing either the wild-type or mutated *PCFT*(-1694/-1680) and cloned the double-stranded oligonucleotides upstream of the 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 RXR α . Whereas the wild-type *PCFT*(-1694/-1680)-containing construct was highly responsive to exogenous VDR:RXR α 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 RXR α and the ligands for these nuclear receptors. We observed a significant ~50% decrease in the VDR:RXR α -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).

Cellular Uptake of Folic Acid Is Enhanced in the Presence of Vitamin D₃. We next examined whether the induction of *PCFT* mRNA expression by vitamin D₃ results in a functional effect on cellular folate uptake. Caco-2 cells were treated with 500 nM vitamin D₃ for 3 days, followed by uptake measurements of the prototypic *PCFT* transport substrate folate. As shown in Fig. 7A, we observed an increase in ³H-labeled folic acid uptake into Caco-2 cells with increasing incubation time, both in vehicle- and vitamin D₃-treated cells at pH 5.5. Treatment of cells with vitamin D₃ moderately, but significantly, increased the uptake of folates at all time points. The uptake of folate at pH 7.4 was negligible both in control cells and in vitamin D₃-treated cells, demonstrating that Caco-2 cells display the typical characteristic of *PCFT*-mediated transport, which is clearly more active in acidic

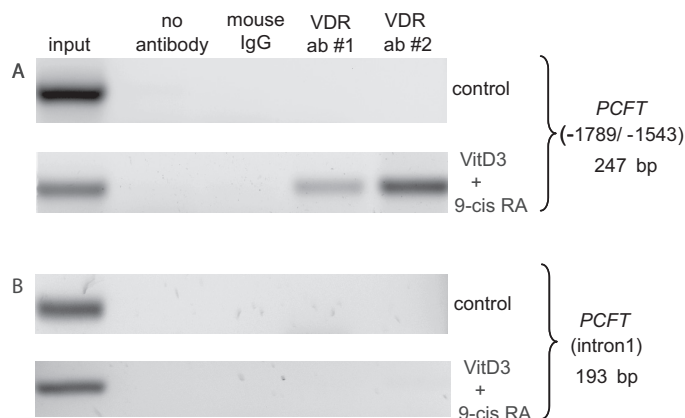


Fig. 5. VDR interacts with the vitamin D₃-responsive region of the *PCFT* promoter within Caco-2 cells. Cells were treated with 100 nM vitamin D₃ and 1 μ M 9-cis retinoic acid (9-cis RA) or the vehicles (control) for 24 h, after which the ChIP assays were performed. Two different VDR-specific antibodies were efficient in precipitating the promoter region *PCFT*(-1789/-1543) in cells treated with VDR and RXR α ligands (A), whereas no signal was obtained for the intronic region of the endogenous *PCFT* gene (B).

extracellular pH (Qiu et al., 2006; Nakai et al., 2007; Unal et al., 2009). In addition, the results show that vitamin D₃ 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 D₃ concentration. We observed an increased folate uptake upon treating the cells with vitamin D₃ concentrations ranging from 0 to 500 nM for 3 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 D₃ for 3 days, both folate uptake and PCFT mRNA expression were significantly increased (Fig. 7C).

Discussion

Vitamin D₃ regulates the expression of its target genes primarily by acting as an agonistic ligand for its DNA-binding nuclear receptor VDR, although nongenomic actions by vitamin D₃ have also been described previously (Christakos et al., 2003; Dusso et al., 2005). VDR, an important regulator of differentiation and proliferation of enterocytes, typically activates gene expression by heterodimerizing with its nuclear receptor partner RXR α . VDR:RXR α 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 nongenomic 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 D₃ in a dose-dependent manner in Caco-2 cells (Fig. 1A). This increase was not further enhanced by cotreatment of cells with the RXR α ligand 9-*cis* retinoic acid (data not shown), consistent with a previous report that VDR:RXR α heterodimers, at least in some promoter contexts, may not respond to RXR α ligands (Forman et al., 1995). Alternatively, saturating levels of RXR α 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 RXR α in Caco-2 cells.

Supporting the importance of the VDR:RXR α heterodimer formation for *PCFT* promoter regulation, the luciferase values were further significantly elevated upon exogenous expression of RXR α . Exogenous expression of VDR in the absence of vitamin D₃ did not notably influence the activity of the *PCFT*(–2231/+96) promoter, indicating ligand-dependence of VDR action. In deletion transfection analysis, the strongest induction in response to VDR and RXR α in the presence of their ligands was achieved with the *PCFT*(–2231/+96) promoter 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 D₃. However, in our current study, we focused 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 D₃. In our computational analysis, we identified a puta-

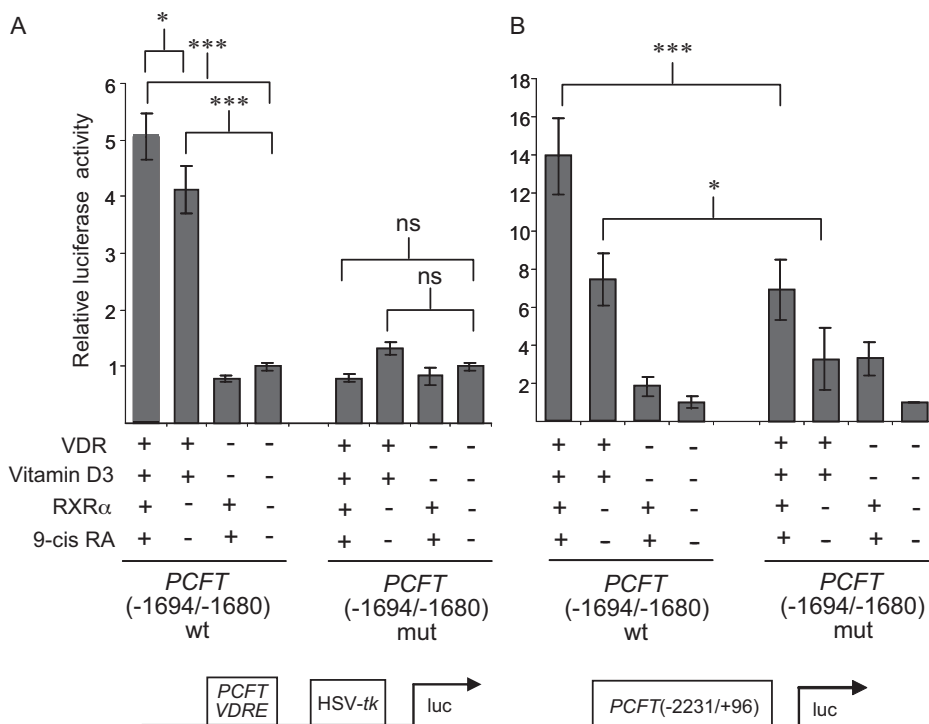


Fig. 6. Mutation of the *PCFT*(–1694/–1680) DR-3-like motif leads to decreased VDR:RXR α -mediated induction of *PCFT* promoter activity in Caco-2 cells. A, heterologous promoter assays show that the *PCFT*(–1694/–1680) motif can function as an independent VDR response element. When placed upstream of 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:RXR α heterodimers, in the presence of VDR and RXR α ligands. B, point mutations in the *PCFT*(–1694/–1680) DR-3-like element significantly reduce the VDR:RXR α -mediated transactivation of the native *PCFT*(–2231/+96) promoter. *, $P < 0.05$; ***, $P < 0.001$; ns, not significant; 9-*cis* RA, 9-*cis* retinoic acid.

tive 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:RXR α 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 RXR α 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 RXR α 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:RXR α -mediated induction upon mutagenesis of the *PCFT*(-1694/-1680) element confirmed that it is an important functional mediator of the effect (Fig. 6, A and B).

Although we observed vitamin D₃-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 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 D₃ is conserved.

The activation of *PCFT* gene transcription by VDR also translates into an increase in PCFT protein function. Vitamin D₃ 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 D₃-stimulated transport activity at pH 5.5, but not at neutral pH. These data strongly suggest that vitamin D₃-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 D₃ treatment (Fig. 1B). It has been reported that vitamin D₃-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 D₃ intake. Food products are often supplemented with folates, because of their proposed beneficial health effects. Based on our current study, supplementation of vitamin D₃ may enhance the intestinal absorption of folates. PCFT also transports the antifolate drug methotrexate (MTX) (Inoue et al., 2008; Yuasa et al., 2009) 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 D₃, thereby affecting the bioavailability of MTX. Patients suffering from inflammatory bowel disease are frequently on long-term treatment with calcium and vitamin D₃ as a prophylaxis against osteopenia

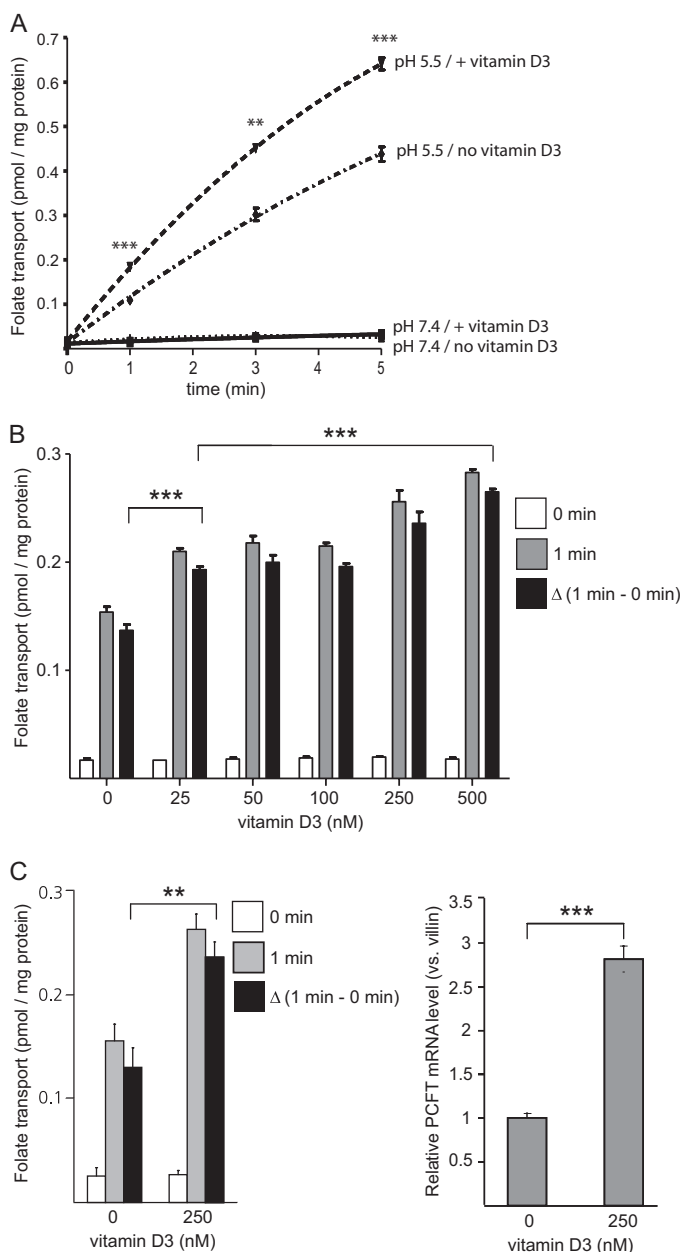


Fig. 7. Analysis of vitamin D₃-dependent effects on folate transport activity. **A**, vitamin D₃ stimulates folate uptake into Caco-2 cells. Cellular uptake of ³H-labeled folate was measured at time points 0, 1, 3, and 5 min after treatment of cells with 500 nM vitamin D₃ for 3 days. **B**, folate transport into Caco-2 cells is enhanced upon increasing vitamin D₃ concentrations. Cellular uptake of ³H-labeled folate was measured at time points 0 and 1 min after treatment of cells with 0–500 nM vitamin D₃ for 3 days. **C**, vitamin D₃-dependent stimulation of cellular folate uptake correlates with increased PCFT mRNA levels. Cellular uptake of ³H-labeled folate was measured at time points 0 and 1 min after treatment of cells with 250 nM vitamin D₃ 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 D₃- and vehicle-treated cells at pH 5.5. **, *P* < 0.01; ***, *P* < 0.001.

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 D₃. Our current results may warrant a closer investigation into potential drug-drug interactions between pharmacologically administered vitamin D₃, MTX, and folates. Taking into account the previous report that folates regulate the expression of genes involved in vitamin D₃ metabolism, it may be that folate and vitamin D₃ homeostasis are closely interlinked through such mutual regulatory interactions.

Acknowledgments

We thank Lia Hofstetter for technical assistance, Dr. David Mangelsdorf for donating the pCMX-VDR and pCMX-RXR α expression plasmids, and members of our team for helpful comments on the manuscript.

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Correction to “Vitamin D₃ and Its Nuclear Receptor Increase the Expression and Activity of the Human Proton-Coupled Folate Transporter”

In the above article [Eloranta JJ, Hiller C, Häusler S, Stieger B, and Kullak-Ublick GA (2009) *Mol Pharmacol* **76**:1062–1071], an author name was inadvertently omitted. The correct authors of the article are Jyrki J. Eloranta, Zoulikha M. Zaïr, Christian Hiller, Stephanie Häusler, Bruno Stieger, and Gerd A. Kullak-Ublick. In addition, the contributions of the added author, Zoulikha M. Zaïr, are equal to those of Jyrki J. Eloranta. Finally, the current affiliation of Zoulikha M. Zaïr is as follows: Cancer Research, Swansea University School of Medicine, Swansea, Wales, United Kingdom.

The online version of this article has been corrected in departure from the print version.

The authors regret this error and apologize for any confusion or inconvenience it may have caused.