

Eigene Arbeiten

Calcitriol controls the epithelial calcium channel in kidney

Hoenderop JGJ, **Müller D**, van der Kemp AWCM, Hartog A, Suzuki M, Ishibashi K, Imai M, Sweep F, Willems PGHM, van Os CH, Bindels RJM

J Am Soc Nephrol 2001; 12:1342-1349

Calcitriol Controls the Epithelial Calcium Channel in Kidney

JOOST G. J. HOENDEROP,* DOMINIK MÜLLER,*
ANNEMIETE W. C. M. VAN DER KEMP,* ANITA HARTOG,* MAKOTO SUZUKI,[§]
KENICHI ISHIBASHI,[§] MASASHI IMAI,[§] FRED SWEEP,[‡]
PETER H. G. M. WILLEMS,[†] CAREL H. VAN OS,* and RENÉ J. M. BINDELS*
*Departments of *Cell Physiology, [†]Biochemistry, and [‡]Endocrinology, University Medical Center Nijmegen, Nijmegen, The Netherlands; and [§]Department of Pharmacology, Jichi Medical School, Tochigi, Japan.*

Abstract. The recently cloned epithelial Ca^{2+} channel (ECaC), which is expressed primarily in 1,25-dihydroxyvitamin D_3 ($1,25(\text{OH})_2\text{D}_3$)-responsive Ca^{2+} -transporting epithelia, is postulated to constitute the rate-limiting step in active Ca^{2+} reabsorption. In the present study, the effect of $1,25(\text{OH})_2\text{D}_3$ was investigated on ECaC mRNA and protein levels in kidneys of rats that were raised on a vitamin D-depleting diet. This diet decreased the serum $1,25(\text{OH})_2\text{D}_3$ concentration significantly, which was accompanied by a marked drop in serum Ca^{2+} level. Both $1,25(\text{OH})_2\text{D}_3$ and Ca^{2+} levels were normalized within 48 h after $1,25(\text{OH})_2\text{D}_3$ administration. In $1,25(\text{OH})_2\text{D}_3$ -deficient rats, ECaC mRNA and protein levels of the kidney cortex were significantly decreased compared with

the repleted animals, suggesting that $1,25(\text{OH})_2\text{D}_3$ exerts its stimulatory effect on Ca^{2+} reabsorption via increased ECaC expression. In agreement with this observation, the elucidated human ECaC promoter contains several consensus vitamin D-responsive elements. ECaC was restricted to the apical membrane of the distal part of the distal convoluted and the connecting tubule. This conclusion was based on only minor overlap with the localization of the thiazide-sensitive NaCl co-transporter and complete co-localization with the $1,25(\text{OH})_2\text{D}_3$ -dependent Ca^{2+} binding protein, calbindin- $\text{D}_{28\text{K}}$. In conclusion, ECaC, present in the distal part of the nephron, is an important target for $1,25(\text{OH})_2\text{D}_3$ -mediated Ca^{2+} reabsorption.

Vitamin D is one of the most important regulators of Ca^{2+} homeostasis of the body and is required for proper development and maintenance of bone mineralization. This is reflected by severe disorders resulting from mutations in the genes coding for 1α -hydroxylase, a renal enzyme that controls the synthesis of the biologic active form of vitamin D, calcitriol, or 1,25-dihydroxyvitamin D_3 ($1,25(\text{OH})_2\text{D}_3$), and for the vitamin D receptor (1,2). $1,25(\text{OH})_2\text{D}_3$ is of primary importance because it enhances the active Ca^{2+} absorption in small intestine and stimulates Ca^{2+} reabsorption in kidney (3–5). The distal nephron determines the final excretion of Ca^{2+} into the urine and is a target for regulation of the extracellular Ca^{2+} concentration. Ca^{2+} reabsorption in these latter segments is active and generally envisioned as a three-step process consisting of passive entry of Ca^{2+} across the apical membrane, cytosolic diffusion of Ca^{2+} bound to calbindin- $\text{D}_{28\text{K}}$, and active extrusion of Ca^{2+} across the opposite basolateral membrane by the Na^+ - Ca^{2+} exchanger and the plasma Ca^{2+} ATPase (4,6). A major breakthrough was the recent discovery of the Ca^{2+} influx protein, named epithelial Ca^{2+} channel (ECaC) (7). ECaC is present primarily in $1,25(\text{OH})_2\text{D}_3$ -responsive epithelia

and exhibits the distinctive properties for being the gatekeeper of active Ca^{2+} reabsorption in the distal part of the nephron (6,8).

In the present study, we postulated that $1,25(\text{OH})_2\text{D}_3$ acts on the distal part of the renal tubule by increasing the expression of ECaC, which will result in increased Ca^{2+} reabsorption. In general, the genomic mechanism of $1,25(\text{OH})_2\text{D}_3$ action involves direct interaction of the vitamin D receptor with regulatory domains on the promoter region of the gene known as vitamin D-responsive elements (VDRE). The VDRE has been reported to consist of two imperfect repeats separated by three nucleotide pairs (9). On the basis of promoter sequence analysis in vitamin D-responsive genes such as calbindin- $\text{D}_{28\text{K}}$ (10), calbindin- $\text{D}_{9\text{K}}$ (11), osteocalcin (12), and the 24-hydroxylase gene (13,14), several VDRE were identified.

To provide evidence for this hypothesis, we raised vitamin D-deficient rats to study the effect of $1,25(\text{OH})_2\text{D}_3$ on the expression of ECaC. In addition, the human ECaC promoter was elucidated and analyzed for VDRE. Using ECaC-specific ribonuclease protection and immunofluorescence assays, we could demonstrate that decreases in circulating levels of $1,25(\text{OH})_2\text{D}_3$ resulted in a marked decline in ECaC mRNA and protein expression. In agreement with this finding, the human ECaC promoter sequence was shown to contain several putative VDRE. Taken together, this study indicates that the recently elucidated ECaC, which is present exclusively in the distal part of distal convoluted tubule (DCT) and connecting tubule (CNT), is an important target for $1,25(\text{OH})_2\text{D}_3$ to regulate renal Ca^{2+} excretion.

Received August 24, 2000. Accepted December 6, 2000.

Correspondence to Dr. René Bindels, Department of Cell Physiology, University Medical Center Nijmegen, Institute of Cellular Signalling, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Phone: +31-24-3614211; Fax: +31-20-8675224; E-mail: reneb@sci.kun.nl

1046-6673/1207-1342

Journal of the American Society of Nephrology

Copyright © 2001 by the American Society of Nephrology

Materials and Methods

Vitamin D–Deficient Rats

Male Wistar rats were raised for 7 to 8 wk on a vitamin D₃–deficient diet as described previously (15). Briefly, rats were fed with a normal vitamin D–containing standard diet during the first 2 wk after birth. Subsequently, the rats (pups and mothers) were placed in the dark on a nonrachitogenic vitamin D–deficient diet lacking manganese and zinc but having normal levels of calcium (1.1% wt/wt) and phosphorus (0.8% wt/wt). The animals had free access to deionized water. After 2 wk, the rats were weaned and the mothers were removed. After the rats were fed with this diet for the following 3 wk, they then were maintained on an essentially calcium-free diet for 2 wk. This later step was included to deplete the endogenous vitamin D₃ stores. After the 2-wk challenge, the vitamin D–deficient rats were fed with the nonrachitogenic calcium-containing diet for 2 wk; at 11 wk of age, blood samples were obtained to determine serum Ca²⁺ levels. Subsequently, one group of vitamin D–deficient rats received two intraperitoneal injections of 160 ng 1,25(OH)₂D₃ at 48 and 24 h before analysis (referred to as vitamin D–repleted animals) and another group received an injection of an equal volume of solvent (ethanol; referred to as vitamin D–deficient animals). They were killed for analysis of serum Ca²⁺ and vitamin D₃ levels as well as ECaC mRNA and protein expression.

Serum Parameters

Total Ca²⁺ concentrations in serum were measured with the use of a colorimetric assay kit as described previously (16). The concentration of 1,25(OH)₂D₃ in serum was assessed with the use of a radio-receptor assay after extraction of the samples followed by paper chromatography, as described previously (17).

Ribonuclease Protection Assay

Riboprobes for rat ECaC (nucleotides 2130 to 2330) (7), calbindin-D_{28K} (nucleotides 1420 to 1570) (18), and β-actin (nucleotides 2220 to 2420) (19) were prepared by transcription of subcloned cDNA fragments into pGEM-Teasy (Promega, Madison, WI) with the use of either SP6 or T7 RNA polymerases and [γ -³²P]UTP (3000 Ci/mmol; Amersham, Buckinghamshire, UK) as described previously. The ribonuclease protection assay (RPA III kit; Ambion, Austin, TX) was performed with the use of total RNA (5 to 20 μg), isolated from kidney cortex with Trizol reagent (Life Technologies BRL, Breda, The Netherlands). This RNA was hybridized with labeled riboprobe (10⁵ cpm) for 16 h at 42°C and treated with RNase A/T₁ (25/100 U/ml) mix for 1 h at 37°C. The protected fragments were precipitated, heat denatured, and separated on 6% (wt/vol) denaturing polyacrylamide gels. The gels were exposed to a film (Kodak, Rochester, NY) for quantification of radioactive signals under conditions in which linearity was achieved. The mRNA expression levels were quantified by computer-assisted densitometry with the use of a flatbed scanner in combination with Molecular Analyst software (Imaging Densitometer GS-690; Biorad, Richmond, CA).

Immunoblotting

Kidney cortex tissue was removed, immediately frozen in liquid nitrogen, and homogenized in phosphate-buffered saline. All samples (20 μg of protein) were separated on 12% (wt/vol) sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and blotted to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Bedford, MA). Blots were incubated for 16 h with calbindin-D_{28K} antibody (1:10000), and immunoreactive protein was detected with the use of the enhanced chemiluminescence method as described

previously (5). The protein expression levels were quantified by computer-assisted densitometry with the use of a flatbed scanner as described above.

Immunohistochemistry

Kidney tissue was cut into pieces, placed in 1% (wt/vol) periodate-lysine-paraformaldehyde fixative for 2 h at room temperature, and incubated overnight at 4°C in phosphate-buffered saline containing 15% (wt/vol) sucrose as described previously (20). Subsequently, kidney samples were frozen in liquid nitrogen and 7-μm frozen sections were cut for different staining procedures. The kidney sections were stained with guinea pig anti-ECaC antiserum (1:200) as described previously. Sections that were double stained for ECaC and thiazide-sensitive NaCl co-transporter (NCCT) were incubated simultaneously for 16 h at 4°C with antiserum against ECaC and NCCT (1:3200) (20). Double staining of ECaC with calbindin-D_{28K} was performed simultaneously with anti-ECaC antiserum (1:200) and rabbit antiserum against calbindin-D_{28K} (1:200) as described previously. To visualize ECaC, calbindin-D_{28K} and NCCT, we stained sections with goat anti-guinea pig-Alexa 594-conjugated anti-IgG (1:300), goat anti-rabbit-Alexa 488-conjugated anti IgG (1:300), or goat anti-mouse Alexa 488-conjugated anti-IgG (1:300), respectively (Molecular Probes, Eugene, OR). All negative controls, including sections incubated with preimmune serum, antiserum preabsorbed for 1 h with 10 μg/ml ECaC–glutathione-S-transferase fusion protein, or conjugated antibodies alone, were devoid of any staining. Photographs were taken with a Zeiss Axioskop microscope (Thornwood, NY) equipped for epifluorescence illumination with the use of Kodak EPH P1600X film.

5' Rapid Amplification of cDNA Ends and Isolation of ECaC Genomic Fragments

The 5' terminus of human ECaC (hECaC) cDNA was identified with the use of the 5' rapid amplification of cDNA ends (RACE) technique on adapter-ligated human cDNA (Clontech, Palo Alto, CA) as described by Müller *et al.* (21). An ECaC-specific antisense primer (5'-GGCAAGCAGTGC GCGCACCAGGTTCA-3') at nucleotide position 506 to 480 on cDNA and an adapter-1 sense primer (5'-CCATCCTAATACGACTCACTATAGGGC-3') were used for 5' RACE. Subsequently, a 5' radiolabeled hECaC-specific genomic DNA probe, containing the 5'UTR and exon 1, was used to screen a genomic library (Stratagene, La Jolla, CA) to obtain the promoter region of hECaC. After several rounds of screening, one specific clone that contained a part of the 5' ECaC gene including the promoter region could be identified. Dye termination sequencing was performed with the use of the BigDye Termination kit (Perkin Elmer, Foster City, CA) following the manufacturer's protocol, and separation was carried out at the Research Sequence Facility, University Medical Center Nijmegen.

Results

Effect of Vitamin D–Deficient Diet on Ca²⁺ Homeostasis and 1,25(OH)₂D₃ Levels

When 2-wk-old Wistar rats were fed a nonrachitogenic vitamin D–deficient diet containing adequate Ca²⁺ (1.1% wt/vol), no significant decrease in serum Ca²⁺ concentration was observed compared with rats that were fed a normal vitamin D–containing standard diet (15). However, when subsequently a Ca²⁺-free period of 2 wk was included in the vitamin D–deficient regimen as described in the Materials and Methods section, these animals developed severe hypocalcemia with

plasma Ca^{2+} levels of 1.8 ± 0.1 mM ($P < 0.05$, $n = 4$) compared with 2.6 ± 0.2 mM ($n = 4$) for rats that were fed the standard diet. Repleting these deficient rats by two injections of 160 ng $1,25(\text{OH})_2\text{D}_3$ (intraperitoneally) at 48 and 24 h before serum was analyzed completely normalized their plasma Ca^{2+} concentration (2.5 ± 0.2 mM; $n = 4$). Measurements of $1,25(\text{OH})_2\text{D}_3$ levels in these blood samples confirmed the $1,25(\text{OH})_2\text{D}_3$ -deficient status of these animals (149 ± 22 pM; $P < 0.05$, $n = 4$) and confirmed the $1,25(\text{OH})_2\text{D}_3$ levels in the repleted animals (486 ± 60 pM; $n = 4$).

Effect of $1,25(\text{OH})_2\text{D}_3$ on ECaC Expression in Kidney

To investigate the effect of $1,25(\text{OH})_2\text{D}_3$ on ECaC expression in the kidney, we performed RNase protection assays. Total RNA from kidney cortex was isolated from vitamin D-deficient and -repleted rats and control rats fed a normal standard diet and hybridized with an ECaC-specific radioactive labeled RNA probe (Figure 1). These data show that 48 h of treatment of vitamin D-deficient rats with $1,25(\text{OH})_2\text{D}_3$ resulted in a marked increase in ECaC mRNA abundance ($183 \pm 9\%$), comparable with the expression level observed in control rats. To confirm the correct size of the protected ECaC fragment, we used 1 μg of rabbit ECaC cRNA transcribed from pSPORT-ECaC (Promega, Madison, WI) (7). As a control, β -actin-specific hybridizations were performed with an equal quantity of total RNA (Figure 1). Scanning of the β -actin corresponding bands revealed little or no variation in the densities, thus excluding the possibility that the difference in ECaC expression between vitamin D-deficient and -repleted rats was due to unequal loading. In the next experiment, the abundance of ECaC protein was examined. Figure 2 shows representative immunofluorescence labeling of distal tubules in sections of kidney cortex from control rats and vitamin D-deficient and -repleted rats. Reduction of the $1,25(\text{OH})_2\text{D}_3$ levels had a major effect on ECaC protein expression as indicated by the virtually complete absence of staining in the kidney cortex, whereas normalization of the $1,25(\text{OH})_2\text{D}_3$ levels in the repleted animals restored ECaC expression to levels comparable to those observed in control rats (Figure 2, A through C). ECaC is localized predominantly to the apical membrane of distal tubule segments in vitamin D-repleted and control animals.

Thus, the increased expression in the renal cortex observed in the RNase protection assays is not associated with a major redistribution of ECaC within the distal tubule segments. The ECaC antibody used in this study has been characterized extensively but is, unfortunately, not working on immunoblots (20). To semiquantify ECaC protein expression, we counted immunopositive tubules in 10 random microscopic fields for each animal. Figure 2D depicts the average values obtained from four vitamin D-deficient rats and demonstrates that ECaC expression is virtually abolished. This is in contrast to the vitamin D-repleted rats in which immunopositive tubules were observed easily for each animal and in number identical to those in normal-fed rats (Figure 2, E and F).

Effect of $1,25(\text{OH})_2\text{D}_3$ on Calbindin- $\text{D}_{28\text{K}}$ Expression in Kidney

The expression level of the vitamin D-dependent calcium binding protein, calbindin- $\text{D}_{28\text{K}}$, was determined at the mRNA and protein levels. RNase protection assays clearly demonstrated an upregulation of calbindin- $\text{D}_{28\text{K}}$ mRNA in vitamin D-repleted ($286 \pm 21\%$; $P < 0.005$, $n = 4$) compared with vitamin D-deficient animals (Figure 3A). The immunoblot shown in Figure 3B clearly demonstrates an increase in calbindin- $\text{D}_{28\text{K}}$ after repletion of $1,25(\text{OH})_2\text{D}_3$ ($312 \pm 47\%$; $P < 0.005$, $n = 4$). Immunohistochemistry confirmed this upregulation of calbindin- $\text{D}_{28\text{K}}$ protein by $1,25(\text{OH})_2\text{D}_3$ treatment of the deficient rats (Figure 3, C and D). The observed protein expression levels for calbindin- $\text{D}_{28\text{K}}$ in the repleted rats were not significantly different from control rats that were fed a normal standard diet (data not shown).

Distribution of ECaC in Rat Kidney Cortex

Co-localization studies of ECaC, calbindin- $\text{D}_{28\text{K}}$, and the thiazide-sensitive NCCT were performed to investigate the distribution of these proteins in rat kidney. Figure 4A shows distal tubule segments expressing ECaC in a control rat. As shown before, ECaC was located predominantly to the apical membrane. Co-localization studies revealed that ECaC and calbindin- $\text{D}_{28\text{K}}$ were bracketed exclusively to the same tubule segments (Figure 4B). Within the ECaC-immunopositive tubules, some negative cells (indicated by white arrowheads)

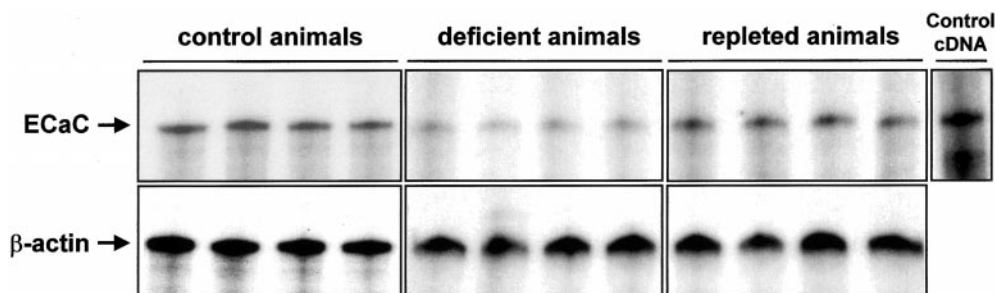


Figure 1. Effect of 1,25-dihydroxyvitamin D_3 ($1,25(\text{OH})_2\text{D}_3$) on the epithelial Ca^{2+} channel (ECaC) mRNA expression level in vitamin D-deficient and -repleted rats and control rats. Ribonuclease protection assays were performed to semiquantify the ECaC and β -actin mRNA levels. Each lane was loaded with equal amounts of total RNA from a different rat ($n = 4$). As a control, ECaC cRNA was transcribed from pSPORT containing the full-length rabbit ECaC sequence (7).

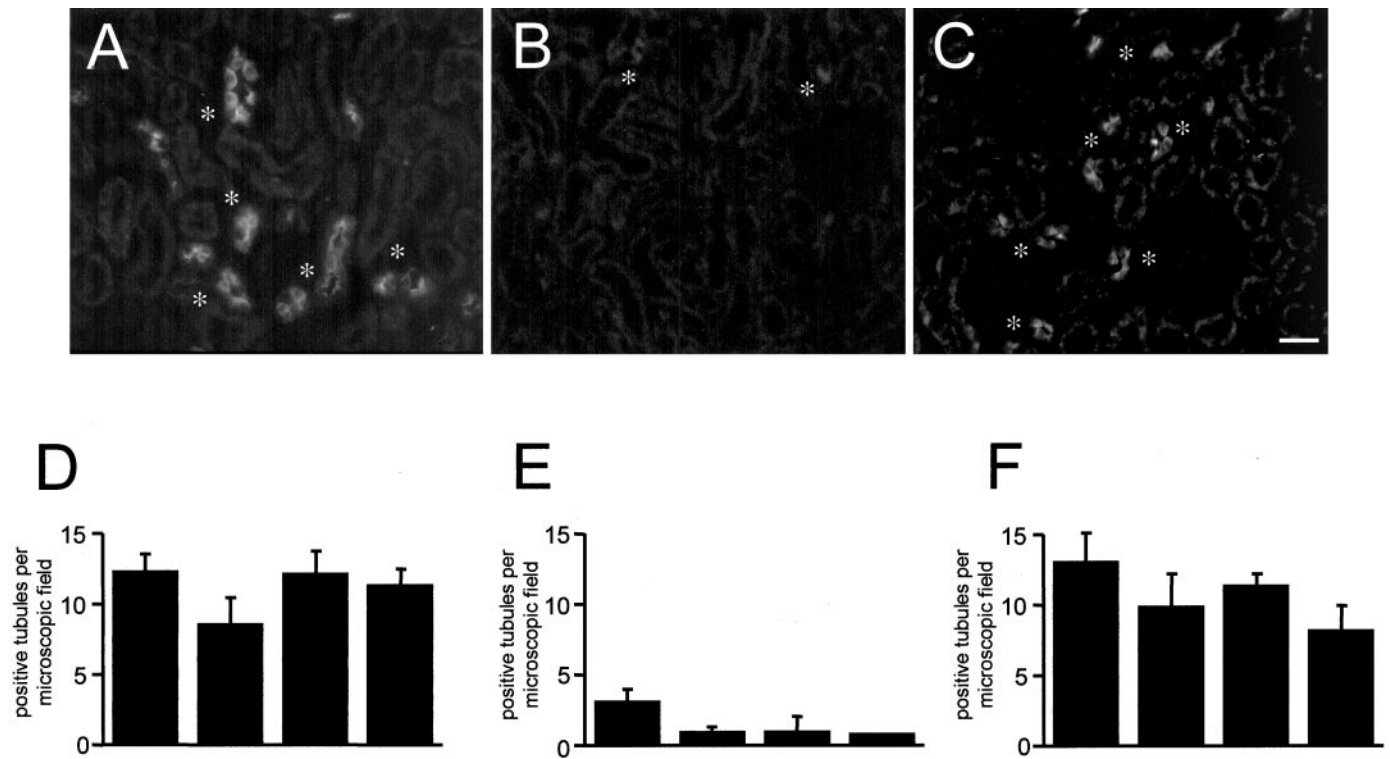


Figure 2. Immunofluorescence localization of ECaC, indicated by asterisks in control rats (A), vitamin D–deficient rats (B), and vitamin D–repleted rats (C). Semiquantitative results were obtained by counting randomly 10 microscopic fields for ECaC-immunopositive distal tubule segments in control rats (D), vitamin D–deficient rats (E), and vitamin D–repleted rats (F). Bar = 25 μ m.

were observed, which were identified as intercalated cells. Figure 4, C and D, shows double labeling of rat kidney cortex for the presence of ECaC and NCCT. Some distal tubule segments (indicated by an asterisk) stained intensively for NCCT but were negative for ECaC. Importantly, a transition (arrowheads) was found from NCCT immunopositive to negative within one tubule, whereas the whole segment stained for ECaC. Because the NCCT-positive segments were identified previously as the first part of DCT (DCT1) (22), this finding suggests that ECaC and, therefore, calbindin- D_{28K} are present exclusively in the more distal part of DCT (DCT2) and the CNT (Figure 4E). There was no indication that ECaC expression in the deficient rats was decreased selectively in DCT2 or CNT.

Identification of the Human ECaC Promoter

To identify the transcription initiation site, we performed 5'-RACE experiments on hECaC cDNA. Figure 5 shows this transcription site, designated +1, at 90 bp upstream from the translation initiation ATG codon. Subsequently, a human genomic library was screened with the use of a 5' genomic hECaC probe containing the 5'UTR, exon 1, and the 5' part of the adjacent intron, resulting in the isolation of a single clone containing a part of the 5' ECaC gene including the transcription initiation site and 2.4-kb upstream information (Figure 5) (GenBank AJ278604). Neither a classical TATA box nor a CCAAT box was identified close to the 5' region of the transcription initiation site. However,

at position –25, the sequence ATAAA was located. Although TATA and CCAAT boxes were positioned farther upstream, their distance to the transcription site is considered too far to have a promoter function. The hECaC promoter contains a number of potential *cis*-acting elements recognized by well-characterized transcription factors that may play a role in the regulation of the ECaC gene (Figure 5). Importantly, several putative VDRE were identified in the ECaC promoter region (indicated by black boxes). In addition, many other regulatory sites were identified, including sites for activator protein 1, 2, and 3 (AP-1, AP-2, and AP-3), a stimulatory protein (SP-1) box, and a NF κ B recognition domain. Evaluation of the physiologic meaning of these sites in the gene awaits further studies.

Discussion

ECaC constitutes the rate-limiting step in the process of active Ca^{2+} reabsorption by facilitating apical Ca^{2+} influx into cells of the mammalian distal renal tubule. Investigating this Ca^{2+} influx pathway was hampered severely because the molecular identity of this Ca^{2+} influx protein was elusive until now. The recent elucidation of ECaC and its predominant localization in vitamin D–responsive epithelia offers for the first time the possibility to study the vitamin D dependency of Ca^{2+} influx into renal distal tubule cells. The conclusion from the present study is that $1,25(OH)_2D_3$ upregulates the expression of ECaC in the distal part of DCT and in CNT, an effect

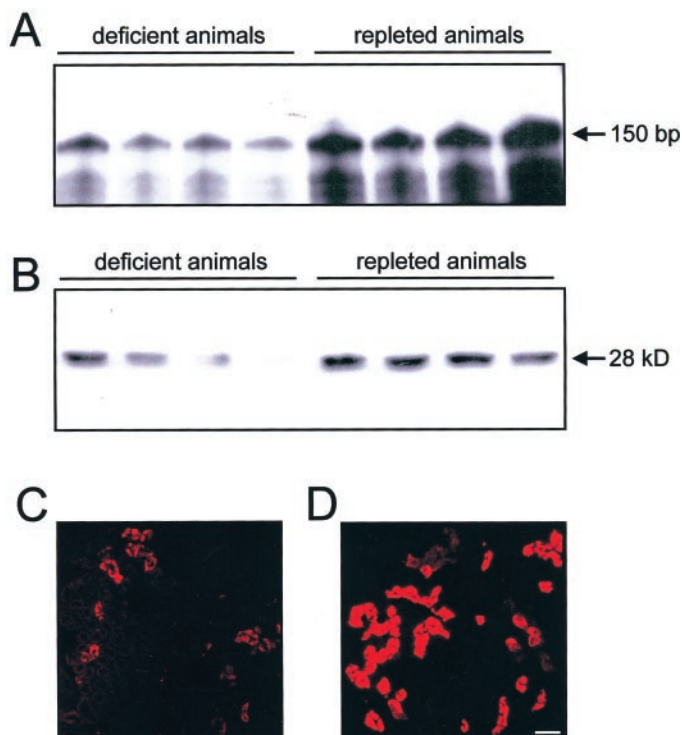


Figure 3. Effect of $1,25(\text{OH})_2\text{D}_3$ on the calbindin- $\text{D}_{28\text{K}}$ expression in vitamin D–deficient and –repleted rats. Comparison of vitamin D–deficient and –repleted rats for calbindin- $\text{D}_{28\text{K}}$ mRNA (A) and protein abundance (B). Immunofluorescence localization of calbindin- $\text{D}_{28\text{K}}$ in vitamin D–deficient (C) and –repleted (D) rats. Bar = 40 μm .

that is likely to have a major impact on active Ca^{2+} reabsorption.

The vitamin D–deficient status of the rats used in our study was illustrated by severe hypocalcemia. Administration of $1,25(\text{OH})_2\text{D}_3$ completely normalized this situation within 48 h and was accompanied by a modest upregulation of ECaC mRNA and a marked increase in ECaC protein expression. These observations suggest that in addition to an increase in mRNA abundance, translational regulation of ECaC may take place, which results in increased channel activity at the apical cell surface. In theory, the enhanced ECaC mRNA expression could be due to increased transcriptional activity and/or mRNA stabilization. The steroid character of $1,25(\text{OH})_2\text{D}_3$ and the presence of VDRE in the ECaC promoter, however, are in line with a regulation at the transcriptional level, but extensive promoter analysis is necessary to demonstrate the functional role of these elements.

In addition, $1,25(\text{OH})_2\text{D}_3$ enhances the renal expression of calbindin- $\text{D}_{28\text{K}}$ as demonstrated in previous studies (reviewed in reference 23) and by our group in primary cultures of CNT cells (5). Because the activity of ECaC is tightly controlled by the cytosolic Ca^{2+} concentration, it is essential that the number of ECaC channels at the plasma membrane be matched by the cytosolic Ca^{2+} buffering capacity of these cells (24). The present observation that both ECaC and calbindin- $\text{D}_{28\text{K}}$ are synchronically upregulated demonstrates that this requirement is fulfilled. The necessity of sufficient Ca^{2+} buffering for

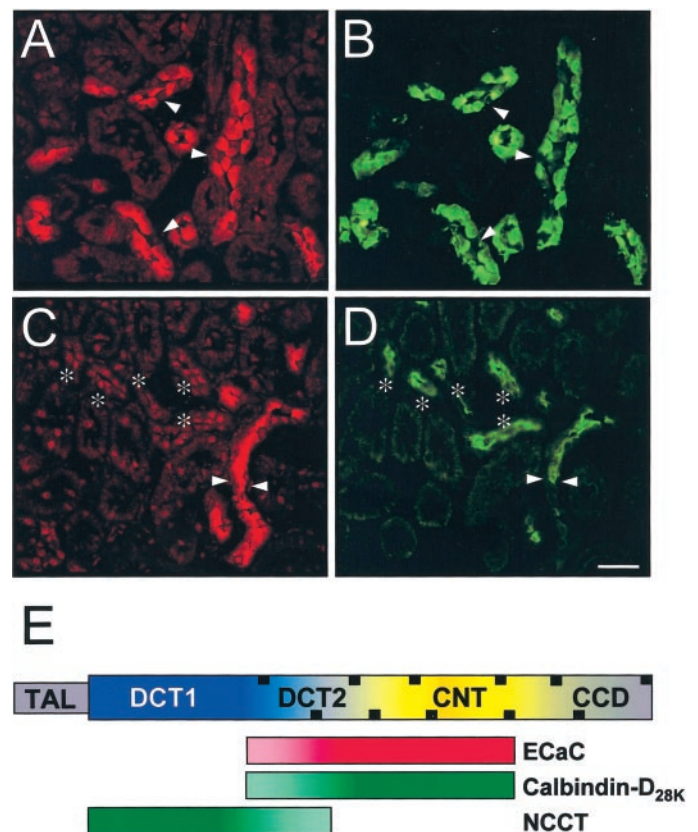


Figure 4. Immunolocalization of ECaC along the distal nephron. Double immunofluorescence staining of rat kidney cortex sections showing staining for ECaC (A, C), calbindin- $\text{D}_{28\text{K}}$ (B), and the thiazide-sensitive NaCl co-transporter (NCCT) (D). The asterisks indicate distal convoluted tubule 1 (DCT1) cells that stained intensively for NCCT but negative for ECaC. The arrowheads depict a transition between DCT2 and CNT in which DCT2 is positive for NCCT, whereas the whole segment is stained for ECaC. (E) Schematic distribution of the ion transporters in the distal part of the rat nephron. TAL, thick ascending limb of Henle's loop; CCD, cortical collecting duct. Bars = 25 μm in A through D.

optimal ECaC functioning is underlined by the conspicuous co-localization of ECaC and calbindins in Ca^{2+} transporting tissues (21).

Importantly, the hECaC promoter contains several putative VDRE within an area ranging between 20 and 2000 bases upstream, which might be involved in the observed $1,25(\text{OH})_2\text{D}_3$ -dependent ECaC mRNA expression. The core binding motif sequences of the VDRE known to date are rather divergent, but the hexameric sequence RRKNSA ($R = \text{A or G}$, $K = \text{G or T}$, $S = \text{C or G}$) seems to be a distinct consensus sequence (25). In human and rat osteocalcin promoters, an AP-1 consensus sequence closely juxtaposed to the VDRE has been identified. It has been demonstrated that AP-1 sites synergistically enhance the activation by $1,25(\text{OH})_2\text{D}_3$ (13). In the ECaC promoter, putative VDRE are surrounded by an AP-1 site. However, further promoter analysis will be required to identify the key regulatory sites involved in vitamin D–mediated transcription. The proximal promoter region does not

```

-2367 TTCTTGCTCTCTCTAAAGGTTATTTTGTCCATCCCTCAATCTTGGGAGCAGATCTCTCTTTCTTACAACAAAAGAAGGGGGCAGTCCCCAC
-2269 TCCTCAAGACCCAGAGGGAGAGTCCCAGCGTATCCTCACGCTGGTCTCTCACACCTTTACAGCCAAAAGATTCCCAAGAAGCAACAAGGTG
-2171 TGGAGAGTGGTTTGGAGCTGAGATCCCAAGTTCATGGCCAGTTACAGTCTCGCCACTAGCCATGGGACTTGGGGCCAGCACACCCAAGAATT
-2073 CTCACAGACCGAATCTTCTCTTCTTCTCATTACCTGATAGGTCTATTGAGTGAATGCAATTCATAATATTGATGTACCTCTTTGAAAACA
-1975 ATGAGAACAGAAATGAAACAATGAGAACAACCTGGACACAGGAAGGGGAACATCACACACCAGGACCTGCTCGTGGGGTGGGGGAGGGGGAGG
-1877 GATACATAGGAGATATACCTAATGTAATGACAGTAAATGGGTGCAGCACACCAACATGGCACATGTATACATATGTAACAAACCTGCATA
-1779 TTGTGCACATGACCCCTAGAAGCTAAAGTATAATAAAAAAATGAGAACAATAACAAGCTATCATATACAGTAGTACCTTTTATCTC
-1681 TCAGGGGATACATTCCAAGACGCCCGAGTGGATGCCAGGAACCTTAGATAGCACCAAACCTGTATATACTATCTTTTTCTACACGTACA
-1093 TACATACATACATACATACATACATACATACATACATATGATGAAGTTAATTTATACATTAGGCACAGTGAAGAGTTTAGGCC
-1583 GGGCATAGTGACTCACGCCGTGAATCCAGCACATTCAGGAGGCCGAGGCAAGCAGATCACCTGAGGTGAGGAGTTCGAGACTAGCCTGGCCA
-1485 ACATGGTAAAACCTGTCTCTACTAAAAATACAAAAATTAGCCAGGCGTGGTGGTGGGCACCTGTAATCCAGCTACTAGGGAGGCTGCGGC
-1387 AGGAGAATAACTTGAACCTGGAAGGTGGATGTTGCAGTGAGCCAAGATCACACCATTGCTCTCCAGCCTGGGCAACGAAAGTGAACCTCTGT
-1289 CTCAAAAAATAAATAAAGAGTAAACAACAATAAAATGAAATATAATAATAAAGTTATGTGAATGTTCTCTTTCTCTCTGTCTCTC
-1191 AAAATATGTTATAGTACTGTACTCACCTATTTTCCAACCTGGCTGACCTTAGGTAACGAACCATCAGAGAGTGAACCTGGGATAAGGGA
-1093 AACTACTTAATATAAAAAATAATAAAAAATAATAAAATAGTGAACATTACTTATCTCTCTGTTTTCATTCTGGAAAGAGTCCCA
-995 AACATGGAATCACGATTTATTTGCTGCTACCTTTGCTCAGTTTCTGCTGTTCAACCTCCATAATAACTCGAGGCCCTCTGGGGCTCTCTG
-897 ACCTGCGTCCCCAGGTACCAGAGCCTGCTCGGCAGCCTCCAGGAAGCTGGCCGGTCAAGGTCATGAGGACTTCTCACAGAGAGGGATTGGC
-799 CTGGGAAGTGGGGCTCAAAAACCTCCTTTGAGGTACAGGGTGGAGGACAGAGATGACCACAAGACACATAAAATGGGAAGCTCTGAGAAGCA
-701 GCCCGGGGCTCTCTTGGCCCTCTTGTGAAGGAGGATCGGCAGTGATTGAGTACTTGTGACACATGCTTTACAGATGCTCCCGTGAGACGG
-603 GCATAGCCGTTACCCCAATTTACAGGTTAGGAACTGAGGCTCAGAAAGCTCACCTAAGTGAAGGATAAGAGTCAAGATTGAACCAAGGC
-505 TCTGCCCTGACTCTTGAAGCTGGAAGTCTTTATTTCTCTCTCCCTCATGCAACTAAGTGTGACCTGTTTGTGGACCCACCTTCCATCCCT
-407 TTTCTTTTCCAAATCCTCTGCCCCACCTCTTAGCTTCTCGGGTCTCAGCCATCCCAGCCACAGTTAGGAACCTTCTCTCTCTCTCTCT
-309 TTTCTTCCCCAGGGTGGTTCTGTGGCTGTGGGGCTCAGCTCTGCAAAAGAGCTCTCCCCCTCCAGGAAAAGCCTTCTCCCCAGGCAGGGCG
-211 GTCAGTCCCCCAGCGCACCTGCATGCACACACACCCTCACACACACACTGCATGCACACACATACACCACAGCCACACACTGTGCAT
-113 ACACGCACACCCCAACACCACTACTGCATGCACACACACACCTACAAGCTGCATGCTGCATACACAAAGTCATACAGGAGATAAACTCA
-15 GAGTCCCAGCCCCAATAGACCCCATCTCTTGCTCAGTTGCTGTCATCCTAGACCTGTTCTTTGCCCACATTTCTATAATCTGCCAGTGTG
83 TGCAAGGAGAAGACATGGGGGTTTCTACCTAAGCCAGAAGGGCCGGGAGCCAACTCCAGAACTTCTGCCTCTTTCTGGTCAGAGAA
M G G F L P K A E G P G S Q L O K L L P S V R E
181 CAAGACTGGGACCAGCCTGGACAAGCTTCATATGCTGCAGCAGAAGAGGTAGGCTTCTTAGAACGATCCGTGCCCTACCBCGTGATCC
Q D W D Q H L D K L H M L Q Q K R L -> intron1
279 TCAGACCTCTGACCCACCTGGGCTGCTTTCCGAATACAGCACCTAGAAGTATTGTGTCAGTGCAGTCACTCAGACATACCTTTCTCTT
377 CCAGCCACCTAATTACAGAATCCCAGCTGGCCCTTTTGTGGCGTGGAGGATGTGGCTTGGGCCACACAGGAAAATCTTGAAGGTAGA
    
```

Figure 5. Nucleotide sequence of the 5' region of the human ECaC gene. Nucleotide +1 denotes the transcription start site and is marked with a dashed arrow. The amino acid sequence that corresponds to exon 1 starting with the translation initiation codon at position 98 is in capital letters and indicated below the coding strand. The TATA and CCAAT boxes are underlined, whereas other putative transcription factor binding motifs (AP-1, AP-2, AP-3, SP-1, and NFκB) are boxed. Regions containing potential vitamin D-responsive elements are indicated by black boxes.

contain typical TATA or CCAAT boxes; however, a similar site is present at -20 (ATAAA). Although these elements have been demonstrated to play an important role in the transcription machinery, many genes have been described without these sequences (26). Alternatively, SP-1 regions (GGGCGG) present at position -209 may drive transcription as indicated for other genes. For the chicken VDR promoter, which contains no TATA box but possesses GC boxes or SP-1 sites, a series of deletion promoter constructs established that the proximal GC boxes are the major drivers of gene transcription (27).
 Micropuncture studies of superficial nephron segments of the rat have demonstrated that the distal part of the nephron is responsible for reabsorption of 15% of filtered Ca²⁺. The portion of the distal tubule normally assessed by micropuncture

is a combination of the DCT, CNT, and initial collecting tubule. However, the exact contribution of the individual segments to active Ca²⁺ transport was still unresolved (4,6,8,22). Immunolocalization studies in rabbit kidney cortex demonstrated that the expression of ECaC is restricted to the connecting tubule that is the predominant Ca²⁺ transporting segment in this species (4,6,8). In other species, including rat, Ca²⁺ reabsorption seems to be located primarily in DCT (4,22). However, the present localization study demonstrates that like in rabbit, there is very little overlap in the localization of NCCT and ECaC in rat kidney. The expression of NCCT is limited to DCT cells. In human, rat, and mouse, expression of this co-transporter extends from the proximal end of the DCT into a transitional segment, referred to as DCT2, that shares

properties of both DCT and CNT (22). Conversely, ECaC and calbindin-D_{28K} are restricted to DCT2 and CNT. Thus, thiazide-sensitive NaCl cotransport and 1,25(OH)₂D₃-regulated Ca²⁺ reabsorption are spatially dissociated in that NCCT is restricted to DCT1 and ECaC is restricted to CNT, whereas DCT2 is a transitional segment that expresses both transport systems. This functional separation has important implications for understanding the hypocalcemia observed in patients with Gitelman's syndrome and during administration of thiazide diuretics (28,29). So far, the effects of thiazides on Ca²⁺ reabsorption were explained by a hyperpolarization that increased the driving force for transcellular Ca²⁺ transport (4,29). Because the localization of NCCT and ECaC are dissociated, other mechanisms must be at work.

The regulation of ECaC by 1,25(OH)₂D₃ may shed new light on Ca²⁺ metabolism under pathophysiologic circumstances. The treatment with vitamin D that has been proved to be beneficial in various clinical situations (*e.g.*, the prevention of rickets during infancy) now can be explained at the molecular level. Primary or secondary involvement of ECaC can be expected in several pathologic situations. Among these, conditions associated with hypercalciuria certainly are of interest because of the dominant localization of ECaC in the kidney. Closely correlated with hypercalciuria is nephrolithiasis, which, because of its high prevalence, has a considerable socioeconomic impact. Intensive investigations, therefore, have been performed in the past to clarify the underlying mechanisms (30,31). Recent studies suggest that the pathogenesis is heterogeneous, because different molecular pathways have been causally related to this disease (32). Interest of research centers around defective regulation of Ca²⁺ homeostasis and the related vitamin D metabolism. Potential candidate genes have been screened for their involvement but several genes (*e.g.*, 1 α -hydroxylase) have been excluded (33). Alternatively, dysregulation of 1,25(OH)₂D₃-sensitive Ca²⁺ reabsorption through ECaC that controls partly the Ca²⁺ flux into the blood compartment could be one factor in the pathogenesis of Ca²⁺-related kidney stone disease.

After the recent molecular identification and subsequent electrophysiologic characterization of ECaC, the present study demonstrates unequivocally that the expression of ECaC in DCT2 and CNT is controlled by the calciotropic hormone 1,25(OH)₂D₃. Future studies may substantiate the role of 1,25(OH)₂D₃-responsive ECaC expression in the pathogenesis of Ca²⁺ homeostasis-related disorders.

Acknowledgments

D.M. was supported by a grant from the Stifterverband für Deutsche Wissenschaft (TS114/02.074/98). This work was supported by the Dutch Kidney Foundation (grant C00.1881). We thank Dr. S. C. Hebert for kindly providing the antibody against NCCT.

References

1. Kitanaka S, Takeyama K, Murayama A, Sato T, Okumura K, Nogami M, Hasegawa Y, Niimi H, Yanagisawa J, Tanaka T, Kato S: Inactivating mutations in the 25-hydroxyvitamin D₃ 1 α -hydroxylase gene in patients with pseudovitamin D-deficiency rickets. *N Engl J Med* 338: 653–661, 1998
2. Hughes MR, Malloy PJ, Kieback DG, Kesterson RA, Pike JW, Feldman D, O'Malley BW: Point mutations in the human vitamin D receptor gene associated with hypocalcemic rickets. *Science* 242: 1702–1705, 1988
3. Brown AJ, Dusso A, Slatopolsky E: Vitamin D. *Am J Physiol* 277: 157–175, 1999
4. Friedman PA, Gesek FA: Cellular calcium transport in renal epithelia: Measurement, mechanisms, and regulation. *Physiol Rev* 75: 429–471, 1995
5. Van Baal J, Yu A, Hartog A, Franssen JAM, Willems PHGM, Bindels RJM: Localization and regulation by vitamin D of calcium transport proteins in rabbit cortical collecting system. *Am J Physiol* 271: F985–F993, 1993
6. Hoenderop JGJ, Willems PHGM, Bindels RJM: Toward a comprehensive molecular model of active calcium reabsorption. *Am J Physiol* 278: F352–F360, 2000
7. Hoenderop JGJ, van der Kemp AWCM, Hartog A, van de Graaf SFJ, Van Os CH, Willems PHGM, Bindels RJM: Molecular identification of the apical Ca²⁺ channel in 1,25-dihydroxyvitamin D₃-responsive epithelia. *J Biol Chem* 274: 8375–8378, 1999
8. Hoenderop JGJ, Müller D, Suzuki M, van Os CH, Bindels RJM: The epithelial calcium channel: Gatekeeper of active calcium reabsorption. *Curr Opin Nephrol Hypertens* 9: 335–340, 2000
9. Christakos S, Raval-Pandya M, Wernyl RP, Yang W: Genomic mechanisms involved in the pleiotropic actions of 1,25-dihydroxyvitamin D₃. *Biochem J* 316: 361–371, 1996
10. Gill R, Christakos S: Identification of sequence elements in mouse calbindin-D_{28k} gene that confer 1,25-dihydroxyvitamin D₃- and butyrate-inducible responses. *Proc Natl Acad Sci USA* 90: 2984–2988, 1993
11. Darwish HM, DeLuca HF: Identification of a 1,25-dihydroxyvitamin D₃-response element in the 5'-flanking region of the rat calbindin D-9k gene. *Proc Natl Acad Sci USA* 89: 603–607, 1992
12. Demay MB, Gerardi JM, DeLuca HF, Kronenberg HM: DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin D₃ receptor and confer responsiveness to 1,25-dihydroxyvitamin D₃. *Proc Natl Acad Sci USA* 87: 369–373, 1990
13. Ozono K, Liao J, Kerner SA, Scott RA, Pike JW: The vitamin D-responsive element in the human osteocalcin gene. Association with a nuclear proto-oncogene enhancer. *J Biol Chem* 265: 21881–21888, 1990
14. Chen KS, DeLuca HF: Cloning of the human 1 α ,25-dihydroxyvitamin D₃ 24-hydroxylase gene promoter and identification of two vitamin D-responsive elements. *Biochim Biophys Acta* 1263: 1–9, 1995
15. Ghijssen WE, Van Os CH: 1 α ,25-Dihydroxyvitamin D₃ regulates ATP-dependent calcium transport in basolateral plasma membranes of rat enterocytes. *Biochim Biophys Acta* 689: 170–172, 1992
16. Bindels RJM, Hartog A, Abrahamse SL, Van Os CH: Effects of pH on apical calcium entry and active calcium transport in rabbit cortical collecting system. *Am J Physiol* 266: F620–F627, 1994
17. Van Hoof HJC, Swinkels LMJW, Van Stevenhagen JJ, Van den Berg H, Ross HA, Benraad TJ: Advantages of paper chromatography as a preparative step in the assay of 1,25-dihydroxyvitamin D. *J Chromatogr* 17: 33–39, 1993
18. Hunziker W, Schrickel S: Rat brain calbindin D₂₈: Six domain structure and extensive amino acid homology with chicken calbindin D₂₈. *Mol Endocrinol* 2: 465–473, 1988

19. Nudel U, Zakut R, Shani M, Neuman S, Levy Z, Yaffe D: The nucleotide sequence of the rat cytoplasmic beta-actin gene. *Nucleic Acids Res* 11: 1759–1771, 1983
20. Hoenderop JGJ, Hartog A, Stuiver M, Doucet A, Willems PHGM, Bindels RJM: Localization of the epithelial Ca²⁺ channel in rabbit kidney and intestine. *J Am Soc Nephrol* 11: 1171–1178, 2000
21. Müller D, Hoenderop JGJ, Meij IC, van den Heuvel LPJ, Knoers NVAM, den Hollander AI, Eggert P, Garcia-Nieto V, Claverie-Martin F, Bindels RJM: Molecular cloning, tissue distribution, and chromosomal mapping of the human epithelial calcium channel. *Genomics* 67: 48–53, 2000
22. Reilly RF, Ellison DH: Mammalian distal tubule: Physiology, pathophysiology, and molecular anatomy. *Physiol Rev* 80: 277–313, 2000
23. Gross M, Kumar R: Physiology and biochemistry of vitamin D-dependent calcium binding proteins. *Am J Physiol* 28: F195–F209, 1990
24. Vennekens R, Hoenderop JGJ, Prenen J, Stuiver M, Willems PHGM, Droogmans G, Nilius B, Bindels RJM: Permeation and gating properties of the novel epithelial calcium channel, ECaC. *J Biol Chem* 275: 3963–3969, 2000.
25. Carlberg C: Mechanisms of nuclear signalling by vitamin D₃. Interplay with retinoid and thyroid hormone signalling. *Eur J Biochem* 231: 517–527, 1995
26. Martinez E, Chiang CM, Ge H, Roeder RG: TATA-binding protein-associated factor(s) in TFIID function through the initiator to direct basal transcription from a TATA-less class II promoter. *EMBO J* 13: 3115–3126, 1994
27. Lu Z, Jehan F, Zierold C, DeLuca HF: Isolation and characterization of the chicken vitamin D receptor gene and its promoter. *J Cell Biochem* 77: 92–102, 2000
28. Scheinman SJ, Guay-Woodford LM, Thakker RV, Warnock DG: Genetic disorders of the renal electrolyte transport. *N Engl J Med* 15: 1177–1187, 1999
29. Simon DB, Lifton RP: Ion transporter mutations in Gitelman's and Bartter's syndromes. *Curr Opin Nephrol Hypertens* 7: 43–47, 1998
30. Asplin JR, Favus MJ, Coe FL: *Nephrolithiasis in the Kidney*, 5th Ed., edited by Brenner BM, Philadelphia, WB Saunders, 1996, pp. 1893–1935
31. Scheinman SJ: Nephrolithiasis. *Semin Nephrol* 19: 381–388, 1999
32. Lloyd SE, Pearce SH, Fisher SE, Steinmeyer K, Schwappach B, Scheinman SJ, Harding B, Bolino A, Devoto M, Goodyer P, Rigden SP, Wrong O, Jentsch TJ, Craig IW, Thakker RV: A common molecular basis for three inherited kidney stone diseases. *Nature* 379: 445–449, 1996
33. Scott P, Ouimet D, Proulx Y, Trouvé ML, Guay G, Gagnon B, Valiquette L, Bonnardeaux A: The 1 α -hydroxylase locus is not linked to calcium stone formation or calciuric phenotypes in French-Canadian families. *J Am Soc Nephrol* 9: 425–432, 1998