Biochemical and Biophysical Research Communications **275,** 47–52 (2000)
doi:10.1006/bbrc.2000.3227, available online at http://www.idealibrary.com on IDEAL®

Gene Structure and Chromosomal Mapping of Human Epithelial Calcium Channel

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Received July 10, 2000

The epithelial Ca^{2+} channel, ECaC, represents the rate-limiting step of vitamin D_3-regulated Ca^{2+} (re)absorption in kidney and intestine, and provides, therefore, a new candidate gene for Ca^{2+}-related disorders. To supply the basis for direct mutation analysis, we report here the structure of the human ECaC gene (ECAC1[2]). It consists of 16 exons spanning 25 kb with introns ranging from 98 to 8500 bp. The 5′-flanking region of ECAC1 contains four putative vitamin D_3-responsive elements. At positions −92 and −13 transcription initiation sites were identified, but the former lacks the canonical TATA or CAAT boxes. ECAC1 was mapped to chromosome 7q35 by fluorescent *in situ* hybridization, reassigning a previous radiation hybrid mapping to 7q31.1-2. The gene of a recently identified rat intestine homologue of ECaC, named Ca^{2+} transporter 1, was found juxtaposed to the ECaC gene, indicating that both genes are the products of evolutionary local gene duplication. © 2000 Academic Press

Key Words: **calcium absorption; calcium reabsorption; calcitriol; EcaC.**

The recently identified epithelial Ca^{2+} channel, ECaC, constitutes the apical Ca^{2+} influx pathway in 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)-responsive epithelia present in kidney and small intestine [1–6]. ECaC exhibits distinctive properties including a constitutively activated Ca^{2+} permeability at physiological membrane potentials, a high selectivity for Ca^{2+}, an anomalous mole-fraction behavior and hyperpolarization-stimulated and Ca^{2+}-dependent feedback regulation of channel activity [3]. To date, ECaC has been cloned from three different species including rabbit (Accession No. AJ133128), rat (Accession No. AB032019), and human (Accession No. AJ271207). The obtained sequences exhibit an overall homology on amino acid level of about 85% [5]. Recently, the ECaC family has been extended by Peng *et al.* who identified a Ca^{2+} transporter (CaT1) from rat small intestine, which is highly homologues to ECaC [7].

These channels represent the first members of a new family of Ca^{2+}-selective channels involved in active Ca^{2+} (re)absorption. This transcellular Ca^{2+} pathway confers the only mechanism by which the organism can influence the Ca^{2+} uptake in kidney and intestine specifically. ECaC could, therefore, play a crucial role in Ca^{2+} homeostasis and is a serious candidate gene in disorders associated with alterations of absorption or excretion of Ca^{2+} [4]. Dysfunctioning of the channel, either as a result of mutations in the gene itself or its regulatory pathways, could ultimately impair the Ca^{2+} conserving capacity of the body contributing to Ca^{2+}-associated kidney stone disease and age-related bone disorders.

The aim of the present study was to elucidate the gene structure and chromosomal localization of human ECaC to allow mutation analysis in patients suffering from Ca^{2+} homeostasis-related disorders.

MATERIALS AND METHODS

Library screening and long range PCR. To determine the genomic organization of ECaC, a PAC clone 209-H-9 was isolated from the RPCI-6 human PAC library by PCR-screening. The approximate insert length of this clone was estimated by digestion with different restriction enzymes (*Bam*HI, *Eco*RI, and *Hind*III). Clone 209-H-9 was then digested with *Bam*HI and *Eco*RI and the fragments were randomly subcloned into a pBluescript II KS (+/−) vector (Stratagene). These subclones were sequenced by means of standard procedures using Sp6 and T3 promoter primers on a semi-

The sequence of the human ECaC gene has been deposited with the EMBL/GenBank Data Libraries under Accession No. AJ401155.

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[2] The HGMW-approved symbol of the human ECaC gene.

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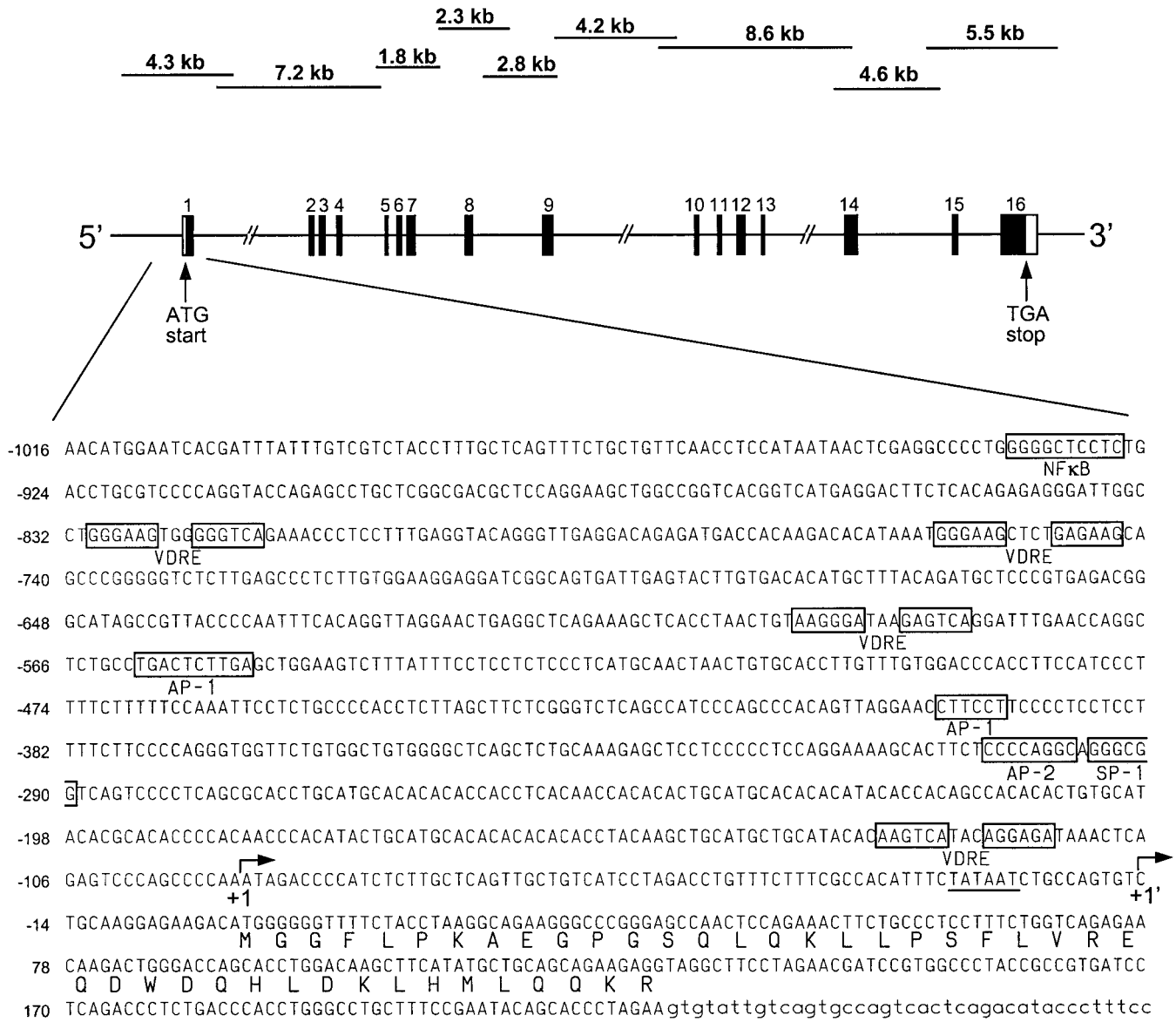

FIG. 1. The gene structure of human ECaC. Filled boxes represent coding sequences and open boxes represent untranslated regions. The exons are numbered 1–16. The start codon (ATG) and stop codon (TAA) are indicated. As an insert the sequence of the transition of the proximal promoter, exon 1 and the first intron is shown. The two different transcription initiation sites are marked by +1 and +1′. The corresponding amino acid sequence is depicted. The putative vitamin D responsive elements are boxed and marked "VDRE." The TATA box is underlined. Additional sites (NFκB, AP-1, AP-2, and SP-1) are indicated. The length of the subclones and individual PCR fragments covering the ECaC gene are indicated at the top of the figure.

automated sequencer (Perkin-Elmer). Based on the cDNA sequence of human ECaC (Accession No. AJ271207), primers were designed to amplify missing parts of the gene, using purified DNA from PAC clone 209-H-9 as a template. For amplification, a LA-Taq was used (TaKaRA), according to the manufacturer's instructions. The products obtained were subjected to direct sequencing analysis (Perkin-Elmer).

5′- and 3′- RACE. 5′-rapid amplification of cDNA ends (RACE) procedures were carried out according to the manufacturer's protocol (Clontech). 5′-RACE was performed by using an ECaC specific primer (5′-CAGGAGCGGGATGAGCTGTGGAGGGCCA-3′) that was combined with the AP1 primer supplied by the manufacturer. 3′-RACE experiments for ECaC and CaT1 were performed by using a primer located in an area of high homology (>95%) between ECaC and CaT1 (forward 5′-CCATCATCGCCAACTGCTCATGCTCAA-3′). As a reverse primer, Clontech's AP1 was used. PCR products were isolated from the agarose gel (Qiagen), cloned into a pGEM-T Easy Vector (Promega) and sequenced as described above. The sequences obtained were used to design specific primers for human CaT1 (forward 5′-ACAGGCAGCAAAGGACAAAGGGTGCTC-3′ and reverse 5′-TCTCGACCCTTATAGTCTAGA-3′). By using long range PCR (see above) a genomic 701 bp fragment was amplified, isolated, and sequenced. There was no homology when compared with the ECaC gene.

TABLE 1

Sequence of the Exon-Intron Junctions of the Human ECaC Gene

Exon number	Exon length (bp)	Boundaries (5′-intron EXON....EXON intron-3′)	Intron length (bp)
1	219	cccaaATAGA....AAGAGgtagg	~3500
2	98	cccagGATTC....AAGAGgtggg	168
3	123	tccagGAGCC....TGCAGgtaag	489
4	106	tgggcCAGGT....CACAGcact-	~2500
5	42	ctacaGCAGG....CTTTGctgag	306
6	99	cacagGGGAG....CCAGGgtcaa	164
7	176	cccagGAAAC....CTGTGgtaag	456
8	147	ctcagATGTT....GAGAGgtatg	2346
9	214	gccagGCTCG....ACAGGtgatt	~8500
10	86	cacatAGGAG....TAGAGgtgac	98
11	77	cccagATTCC....TTCATgtgag	260
12	166	cccagCATCA....AGAAGgtcag	172
13	57	tgcagATGAT....CTCCGgtaag	1893
14	269	acgagCGTTC....GGGAGgtgag	2885
15	107	tccagGTCGT....CTGCGgtgag	681
16	785	atcagGGTTG....CTCCTaatac	

Note. Depicted are exon length, boundaries, and intron length (Caps: Exon; small caps: intron). The sequence of the human ECaC gene has been deposited with the EMBL/GenBank data Libraries under Accession No. AJ401155.

Fluorescent in situ hybridization (FISH). A 5 kb genomic probe of ECaC was prepared by long range PCR and subsequently used for FISH analysis. This probe spanned the transmembrane domains 4, 5, and 6 as well as the pore forming region. The probe DNA was biotine-14-dATP labeled and precipitated (Gibco). After denaturation, slides with normal human metaphase lymphocytes (>20) were incubated. The probe was visualized by incubation of antibodies conjugated with avidin fluorescein-isothiocyanate (Vector laboratories) [8].

RESULTS AND DISCUSSION

Genomic structure of human ECaC. In this study, we first characterized the genomic organization of the human epithelial calcium channel, ECaC. To this end, a human genomic PAC library was screened with an ECaC-specific probe of 721 bp and a single clone, numbered 209-H-9, was isolated. Digestion of this PAC clone yielded an insert of approximately 100 kb. Characterization of this clone revealed that the ECaC gene consists of 16 exons spanning about 25 kb with introns ranging from 98 to 8500 bp (Fig. 1 and Table 1). The 5′- and 3′-UTRs are located within the first and the last exon (1 and 16), respectively. 3′-RACE revealed an untranslated region of 472 bp including a terminal poly-A signal (AATAA). No alternative polyadenylation signals were found after this site within the next 2.5 kb. Two different transcription start points of ECaC were identified at −92 and −13 bp. As far as the longer transcript is considered, the proximal promoter of ECaC does not display classical CAAT or TATA boxes. The shorter transcript, however, does have a canonical TATA box at −27 bp. It has been shown that a short 5′-UTR, if in combination with a strong KOZAK sequence (CATGG), is sufficient to avoid leaky ribosomal scanning [9]. The first ATG is indeed located within a KOZAK consensus sequence (ACATGGG) [10]. Besides NFκB, SP-1, AP-1, and AP-2 sites, we also found four sites of imperfect hexameric repeats which were spaced by three or four nucleotides. These sites were located within a range of −150 to −600 bp from the ATG and could serve as vitamin D-responsive elements (VDRE's) [11, 12].

The demonstration of VDRE's substantiates the manifest tissue distribution of ECaC in $1,25(OH)_2D_3$-responsive epithelia and provides further evidence that ECaC could be a molecular target of $1,25(OH)_2D_3$. Diseases like vitamin D resistant rickets with end-organ unresponsiveness to $1,25(OH)_2D_3$ caused by mutations in the gene of the vitamin D receptor demonstrate how crucial this system is for the overall Ca^{2+} homeostasis [13]. However, many disturbances in Ca^{2+} metabolism remain still unexplained. Among those, idiopathic hypercalciuria (OMIM 143870) is one of the most important. Due to its high prevalence in the population and its close relation to Ca^{2+}-related kidney stone disease, it confers, beyond its medical aspects, a major socio-economic problem. Although many efforts have been taken in the past, the molecular basis of this disease still awaits its elucidation [14, 15]. Considering the fact that hypercalciuria can be caused in principle by increased intestinal Ca^{2+} absorption as well as by increased renal excretion, ECaC represents a timely candidate gene for different subtypes of this disorder.

Chromosomal assignment of human ECaC. Subsequently, the chromosomal assignment of ECaC was carried out by FISH analysis. Using a genomic 5 kb

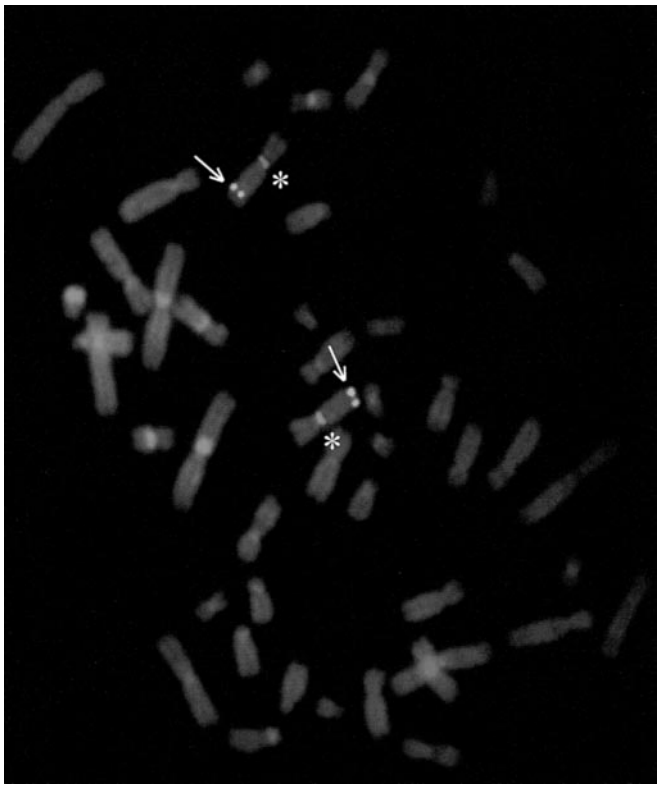

FIG. 2. The chromosomal assignment of human ECaC was carried out by FISH analysis. The specific signal detected on human chromosome 7q35 is marked by an arrow. The centromere marker is indicated by an asterisk.

ECaC probe, one specific signal was detected on human chromosome 7q35 and no signal was detectable on any other chromosome (Fig. 2). This localization contrasts with the previously reported mapping of ECaC on chromosome 7q31.1-2 [6]. In this latter study, radiation hybrid mapping was used and the identified sequence tagged sites (STS) were assigned to this particular chromosomal area using the Stanford G3 Radiation Hybrid Panel. This is a widely accepted procedure for chromosomal mapping of human genes. However, we now screened three additional databases containing the previously obtained STS (Fig. 3). According to this new analysis, ECaC was mapped to different chromosomal areas that span several bands from 7q21.1 (GeneMap99 G3 and NCBI integrated RH Map) to 7q22.1 (Stanford G3 Map). None of the four available databases did assign ECaC to the localization on 7q35 as obtained by FISH. Additionally, it was found that subclones of the isolated human PAC clone 209-H-9 fully match with a previously published BAC clone that has been mapped by FISH to chromosome 7q36 (Accession No. AF107256), confirming that ECaC is located close to the telomere. Taken together, these findings unequivocally demonstrate that the human ECaC gene maps to chromosome 7q35 and that the set of STS as obtained from the radiation hybrid panel is, despite a LOD score of >10 [6], not suitable for this particular area.

The divergent data of the chromosomal assignment of ECaC, demonstrate the necessity to confirm findings

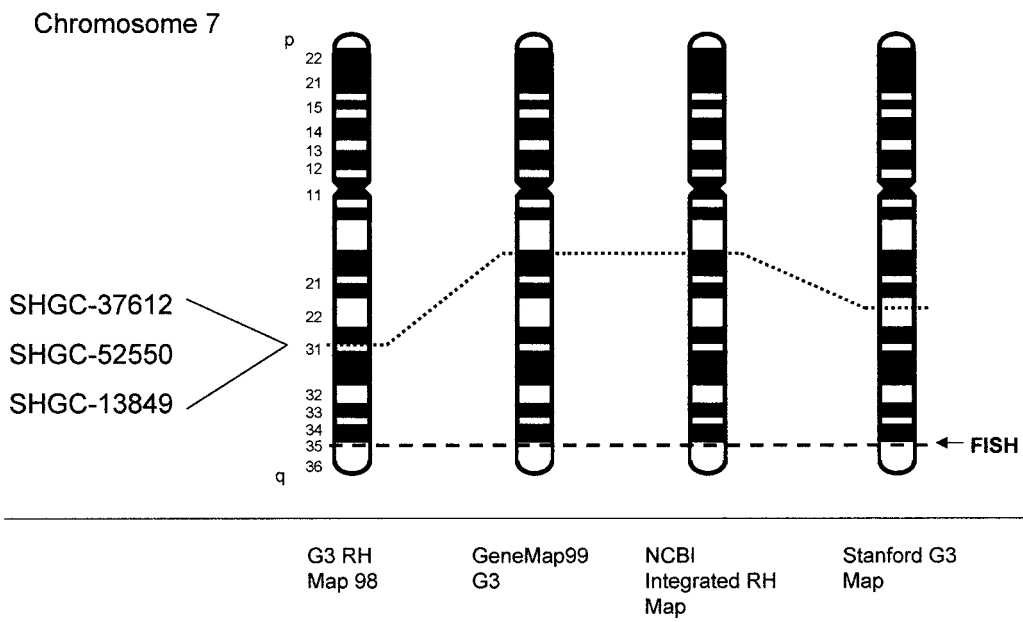

FIG. 3. Localization of three sequence-tagged sites (STS) on chromosome 7 obtained by radiation hybrid mapping using different maps. The localization of ECaC as revealed by FISH is depicted.

of radiation hybrid mapping by conventional FISH analysis. Even though this might become a minor issue as the human genome project progresses, it is still of vital importance if linkage studies are performed when the area of interest is mapped by radiation hybrid screening only. This is also true for other organisms for which a whole genome project is not yet settled.

ECaC gene duplication. During the 3′-RACE experiments, we also obtained the C-terminal part of the ECaC homologue named CaT1. Based on this sequence, primers were designed and a corresponding genomic fragment of CaT1 was amplified from the PAC clone. This 701 bp fragment was juxtaposed to the ECaC gene, demonstrating that ECaC and CaT1 are transcripts of two neighboring genes rather than being splice variants of the same gene. This data gives evidence for evolutionary gene duplication that in general provides one of the major driving forces of evolution [16]. The acquisition of different functions for the corresponding proteins prevents one of the copies from being silenced and remaining as a pseudogene and can usually be illustrated by a different tissue distribution [17]. Conversely, human ECaC is expressed primarily in kidney, pancreas, placenta and intestine, while rat CaT1 is mainly restricted to duodenum [6, 7]. Both channels are highly homologues in the areas presumed to be essential for channel function such as transmembrane segment 5 and 6 including the pore forming stretch, but the N- and C-terminus are divergent. However, the precise functional discrimination between ECaC and CaT1 awaits further detailed electrophysiological characterization of both epithelial channels.

It is interesting to note that the nearest relatives of the ECaC family, the vanilloid receptor (VRL, Accession No. AF029310) and the olfactory receptor (OSM-9, Accession No. AF031408) arise from a cluster of genes located on chromosome 17p13.3 [18, 19]. These proteins share with ECaC and CaT1 the structure of six transmembrane domains (TM) with a short hydrophobic stretch between TM5 and TM6. However, even though OSM-9, VRL, CaT1, and ECaC display the properties of a cation channel, they exhibit different functions in the organism. Whereas ECaC and CaT1 facilitate transcellular Ca^{2+} transport, VRL, and OSM-9 are involved in pain sensation and smelling, respectively [20, 21].

ACKNOWLEDGMENT

D.M. was supported by a grant from the Stifterverband für die Deutsche Wissenschaft (D.M. TS114/02.074/98).

REFERENCES

1. Hoenderop, J. G. J., van der Kemp, A. W. C. M., Hartog, A., van de Graaf, S. F. J., van Os, C. H., Willems, P. H. G. M., and Bindels, R. J. M. (1999) Molecular identification of the apical Ca^{2+} channel in 1,25-dihydroxyvitamin D_3-responsive epithelia. *J. Biol. Chem.* **274,** 8375–8378.
2. Hoenderop, J. G. J., Hartog, A., Stuiver, M., Doucet, A., Willems, P. H. G. M., and Bindels, R. J. M. (2000) Localization of the epithelial Ca^{2+} channel in rabbit kidney and intestine *J. Am. Soc. Nephrol.* **11,** 1171–1178.
3. Vennekens, R., Hoenderop, J. G. J., Prenen, J., Stuiver, M., Willems, P. H. G. M., Droogmans, G., Nilius, B., and Bindels, R. J. M. (2000) Permeation and gating properties of the novel epithelial calcium channel, ECaC. *J. Biol. Chem.* **275,** 3963–3969.
4. Hoenderop, J. G. J., Willems, P. H. G. M., and Bindels, R. J. M. (2000) Toward a comprehensive molecular model of active calcium reabsorption. *Am. J. Physiol.* **274,** F736–F743.
5. Hoenderop, J. G. J., Müller, D., Suzuki, M., van Os, C. H., and Bindels, R. J. M. (2000) Epithelial calcium channel: Gatekeeper of active calcium reabsorption. *Curr. Opin. Nephrol. Hypertens.* **9,** 335–340.
6. Müller, D., Hoenderop, J. G. J., Meij, I. C., van den Heuvel, L. P. J., Knoers, N. V. A. M., den Hollander, A. I., Eggert, P., Garcia-Nieto, V., Claverie-Martin, F., and Bindels R. J. M. (2000) Molecular cloning, tissue distribution, and chromosomal mapping of the human epithelial calcium channel. *Genomics* **67,** 48–53.
7. Peng, J. B., Chen, X. Z., Berger, U., Vassilev, P. M., Tsukaguchi, H., Brown, E. M., and Hediger, M. (1999) Molecular cloning and characterization of a channel-like transporter mediating intestinal calcium absorption. *J. Biol. Chem.* **274,** 22739–22746.
8. Suijkerbuijk, R. F., Sinke, R. J., Olde Weghuis, D., Roque, L., Forus, A., Stellink, F., Siepman, A., van de Kaa, C., Soares, J., and Geurts van Kessel, A. (1994) Amplification of chromosome subregion 12p11.2-p12.1 in a metastasis of an i(12p)-negative seminoma: Relationship to tumor progression? *Cancer Genet. Cytogenet.* **78,** 145–152.
9. Werten, P. J. L., Stege, G. J. J., and de Jong, W. W. (1999) The short 5′ untranslated region of the betaA3/A1-crystallin mRNA is responsible for leaky ribosomal scanning. *Mol. Biol. Rep.* **26,** 201–205.
10. Kozak, M. (1996) Interpreting cDNA sequences: Some insights from studies on translation. *Mamm. Genome* **7,** 563–574.
11. Christakos, S., Raval-Pandya, M., Wernyj, R. P., and Yang, W. (1996) Genomic mechanisms involved in the pleotropic actions of 1,25-dihydroxyvitamin D_3. *Biochem. J.* **316,** 361–371.
12. Carlberg, C. (1995) Mechanisms of nuclear signaling by vitamin D_3. Interplay with retinoid and thyroid hormone signaling. *Eur. J. Biochem.* **231,** 517–527.
13. Hughes, M. R., Malloy, P. J., Kiebach, D. G., Kesterson, R. A., Pike, J. W., Feldmann, D., and O'Malley, B. W. (1988) Point mutations in the human vitamin D receptor gene associated with hypocalcemic rickets. *Science* **242,** 1702–1705.
14. Petrucci, M., Scott, P., Ouimet, D., Trouvé, L., Proulx, Y., Valiquette, L., Guay, G., and Bonnardeaux, A. (2000) Evaluation of the calcium-sensing receptor gene in idiopathic hypercalciuria and calcium nephrolithiasis. *Kidney Int.* **58,** 38–42.
15. Scott, P., Ouimet, D., Proulx, Y., Trouvé, M. L., Guay, G., Gagnaon, B., Valiquette, L., and Bonnardeaux A. (1998) The 1α-hydroxylase locus is not linked to calcium stone formation or calciuric phenotypes in French-Canadian families. *J. Am. Soc. Nephrol.* **9,** 425–432.
16. Lynch, M., and Force, A. (2000) The probability of duplicate gene preservation by subfunctionalization *Genetics* **154,** 459–473.
17. Ohta, T. (1988) Evolution by gene duplication and compensatory advantageous mutations. *Genetics* **120,** 841–847.

18. Touchman, J. W., Anikster, Y., Dietrich, N. L., Maduro, V. V., McDowell, G., Shotelersuk, V., Bouffard, G. G., Bechstrom-Sternberg, S. M., Gahl, W. A., and Green, E. D. (2000) The genomic region encompassing the nephropathic cystinosis gene (CTNS): Complete sequencing of a 200-kb segment and discovery of a novel gene within the common cystinosis-causing deletion. *Genome Res.* **10,** 165–173.
19. Glusman, G., Sosinsy, A., Ben-Asher, E., Avidan, N., Sonkin, D., Bahar, A., Rosenthal, A., Clifton, S., Roe, B., Ferraz, C., Demaille, J., and Lancet, D. (2000) Sequence, structure, and evolution of a complete human olfactory receptor gene cluster. *Genomics* **63,** 227–245.
20. Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., and Julius, D. (1997) The capsaicin receptor: A heat-activated ion channel in the pain pathway. *Nature* **389,** 816–824.
21. Colbert, H. A., Smith, T. L., and Bargmann, C. I. (1997) OSM-9, a novel protein with structural similarity to channels, is required for olfaction, mechanosensation, and olfactory adaptation in *Caenorhabditis elegans. J. Neurosci.* **17,** 8259–8269.