Factors involved in the duodenal expression of the human calbindin-D9k gene

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Calbindin-D9k is expressed in the cytoplasm of intestinal cells, where it is critical for dietary calcium absorption. Two striking aspects of the expression of this gene are its vitamin-D dependency and regional differences in expression, with high levels only in duodenum. We report studies of the human calbindin-D9k promoter. Differences between the reported sequences of the human calbindin-D9k promoter were first clarified before undertaking a functional analysis of this sequence. Studies of the rat gene have indicated that several transcription factors, including the caudal-related homeobox factor (CDX-2), hepatic nuclear factor-4 and CCAAT-enhancer-binding protein α (C/EBPα), could interact with elements in the promoter. Although these elements are conserved in the human gene, we show here that their intestinal distribution makes them unlikely to be critical positive factors. The calbindin-D9k gene contains multiple potential binding sites for homeobox transcription factors; one of these, known as IPF-1 or PDX-1, co-localizes in the intestine with calbindin-D9k. We show in gel-shift assays that the sequence within a putative vitamin-D-response element in the human calbindin-D9k promoter can bind expressed IPF-1/PDX-1 protein, although we cannot confirm binding of the vitamin-D-receptor protein. CDX-2 binds to the region around the TATA box, as in the rat gene, and may act as a negative factor in the distal intestine. Transfection studies in Caco-2 and MCF-7 cells with heterologous reporter vectors containing up to 1303 bp of the gene showed that this functioned as a weak promoter and indicated the presence of suppressor sequences, but did not show vitamin-D responsiveness. This indicates that other elements are also needed for the control of human calbindin-D9k expression.

Key words: calcium absorption, homeobox transcription factors, intestine, vitamin D.

INTRODUCTION

Calbindin-D9k, the mammalian intestinal vitamin-D-dependent Ca^{2+}-binding protein, is a key factor in the absorption of dietary calcium. The protein is expressed at high concentrations in the regions of the small intestine that transport Ca^{2+} by the active transcellular pathway. Calbindin-D9k is thought to act by buffering cytoplasmic Ca^{2+}, increasing transcellular Ca^{2+} diffusion and stimulating rates of Ca^{2+} extrusion by the basolateral membrane Ca^{2+}-pumping ATPase. It has been the subject of many animal studies and, in some species, may also be found in non-intestinal tissues such as uterus, placenta and kidney [1–4]. In mice that do not express the vitamin-D receptor (VDR), intestinal calbindin-D9k mRNA expression is reduced to low levels by weaning, when hypocalcaemia and other phenotypic changes develop [5].

Human calbindin-D9k has been less thoroughly studied. We reported the sequence of calbindin-D9k cDNA in humans [6], as did another group independently [7], and we showed high levels of expression only in the duodenum. The human protein has been studied by Staun and colleagues [8,9]. They have shown that 1,25-dihydroxycholecalciferol [1,25$(OH)_2D_3$] affects levels of calbindin-D9k protein [9] and we have demonstrated further that this hormone correlates with mRNA expression [10].

In order to investigate the transcriptional control of calbindin-D9k expression in humans, the 5′-flanking region of the human calbindin-D9k gene has been sequenced. The sequence that we determined [11] (GenBank/EMBL accession number X76451) differs from that determined by Jeung et al. [12] (L13042) and our first aim was to resolve these differences to see if they could be polymorphic sites. One of these differences affects a region where Schräder et al. have reported a vitamin-D-response element (VDRE) [13]. This VDRE is unusual, as it is an inverted palindrome spaced by nine bases (IP9) rather than the more accepted type, a direct repeat spaced by three bases (DR3) [14,15]. These bind the retinoid-X receptor as well as the VDR in a ligand- and salt-dependent manner [16,17].

Studies of the promoter of the rat calbindin-D9k gene have described functional elements using DNase I footprinting, gel-shift, transfection and transgenic studies [18–21]. Several transcription factors, including CDX-2, CCAAT-enhancer-binding protein α (C/EBPα), HNF (hepatic nuclear factor)-1 and HNF-4 have been suggested to bind to elements in the rat promoter and we wished to determine whether any of these could define the proximo-distal gradients of intestinal expression in humans. Following our recent study of regional differences in human intestinal homeobox factors [22], it was apparent that one of these, known as IPF-1 (insulin-promoter-factor-1) or PDX-1 (pancreatic-duodenal-homeobox factor-1), had a regional distribution within the small intestine similar to that of calbindin-D9k. (PDX-1 is the name given by the International Committee on Standardized Genetic Nomenclature for Mice; IPF-1 is the name assigned by the Human Genome Database Committee to the homologous gene, which has also been called IDX-1, STF-1 and

Abbreviations used: 1,25$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; AEBSF, (4-aminoethyl)benzenesulphonyl fluoride; GST, glutathione S-transferase; VDR, vitamin-D receptor; VDRE, vitamin-D-response element; TBP, TATA-binding protein; C/EBPα, CCAAT-enhancer-binding protein α; DR3, direct repeat spaced by three bases; HNF, hepatic nuclear factor; DTT, dithiothreitol; IPF-1, insulin-promoter-factor-1; PDX-1, pancreatic-duodenal-homeobox factor-1; SV40, simian virus 40; SIF1, sucrase-isomaltase footprint 1.

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IUF-1. We will refer to it as IPF-1/PDX-1). We have therefore investigated whether IPF-1/PDX-1 can interact with the human calbindin-D9k promoter.

EXPERIMENTAL

Sequence of calbindin-D9k promoter

A human genomic library was screened with a probe derived from our published cDNA sequence [6]. Regions of the calbindin-D9k gene were amplified by PCR using specific primers derived from this and the other published human genomic sequence [12]. At least three independent sequences were read; comparisons with other sequences were made using the GCG software package.

Clarification of sequence differences

Genomic DNA was prepared from pelleted blood cells of 37 individuals (23 Caucasians, 6 Afro-Caribbeans and 8 Asians) by SDS/urea lysis and successive phenol/chloroform extractions [10]. PCR was used to study two regions: near the start site of transcription (region 1) and in the proposed VDRE (region 2). For region 1, PCR amplification was performed with the following primers: 5′-TCATAATCAGGGTGGC-3′ (forward) and 5′-CGCGAATTCAAGGAAGCAGAGTATTAATGC-3′ (reverse). Amplification proceeded over 45 cycles of 45 s at 95 °C, 2 min at 55 °C and 2 min at 70 °C. Products were cut with the restriction enzyme *Alu*I, stained with ethidium bromide and separated on 2.5% agarose gels. For region 2, two PCR amplifications were attempted with specific 5′ primers: 5′-CTCGGTACCTGGATCTTGCCCTTA-3′ (forward primer A) and 5′-CTCGGTACCTGGATCTTGCCCTTC-3′ (forward primer C; where underlining indicates the difference between the primers). The reverse primer used was 5′-CGCGAATTCAAGGAAGCAGAGTATTAATGC-3′ and amplification was performed with 45 cycles of 45 s at 95 °C, 30 s at 62 °C and 2 min at 70 °C. Products were detected with ethidium bromide on 1.4% agarose gels.

Northern blotting

Small-intestinal mucosal samples obtained from human proximal (duodenal) and distal (ileal) regions [22] and from six regions of rat intestine [23] were studied. RNA was extracted by acid guanidinium phenol/chloroform extraction, run on agarose gels, blotted and hybridized using standard techniques described previously [24]. cDNA probes for calbindin-D9k were synthesized as before [6]. A 333-bp probe for HNF-4 was amplified from rat intestinal cDNA using primers comprising bases 435–451 and 751–767 of the published sequence (accession number X57133). A C/EBPα probe was amplified using primers made from positions 872–891 and 1040–1052 of the rat cDNA (X12752). Rat CDX-2 was amplified as described before [22]. Glyceraldehyde phosphate dehydrogenase and 18-S rRNA probes were used to confirm equal loading of blots for human and rat cDNAs respectively. PCR products were cloned in M13 and sequenced. Probe sequences were excised from the vector and labelled with [α-^{32}P]dCTP using random primers.

Preparation of nuclear extracts

Caco-2 or MCF-7 cells ($\approx 1.5 \times 10^7$) were harvested, washed and lysed in buffer containing 0.5% Nonidet P40, 0.32 M sucrose, 10 mM Tris/HCl, pH 8.0, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.5 mM AEBSF [(4-aminoethyl)benzenesulphonyl fluoride] on ice for 5 min [25]. The lysate was centrifuged to pellet the nuclei, resuspended in the above buffer without Nonidet P40 and re-centrifuged. The nuclei were resuspended in a low-salt buffer (20 mM Hepes, pH 7.9, 25% glycerol, 0.02 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM AEBSF); the salt was increased to 0.8 M KCl and the mixture incubated on ice for 20 min. After dilution, the supernatant was separated by centrifugation at 13000 ***g*** for 15 min at 4 °C, and frozen immediately in aliquots, which were stored at −70 °C. The extracts were not refrozen after thawing. Their protein concentrations were determined using the Bio-Rad protein assay, with BSA as a standard.

Purified proteins

IPF-1/PDX-1 was expressed as a glutathione S-transferase-fusion protein (GST–PDX-1). The coding sequence of human IPF-1/PDX-1 (bases 21–872) [26] was amplified from duodenal cDNA using 30 cycles of 45 s at 95 °C, 1 min at 60 °C and 2 min at 70 °C and standard conditions, with the addition of 'Q solution' (Qiagen, Crawley, West Sussex, U.K.). The PCR product was cloned into the prokaryotic expression vector pGEX-2T (Pharmacia, St. Albans, Herts, U.K.) at the *Bam*HI and *Eco*RI sites and sequenced. *Escherichia coli* XL1B cells were transformed with the vector containing the insert with the coding sequence and grown overnight. Protein production was induced by adding IPTG to the culture medium. The GST–PDX-1 fusion protein was isolated using a glutathione–agarose column (Sigma, Poole, Dorset, U.K.). The eluted protein was checked by SDS/PAGE and its identity confirmed by Western blotting with an antibody characterized previously [27].

TATA-binding protein (TBP) and antibody were purchased from Promega (Southampton, Hants, U.K.). VDR protein, expressed in a baculovirus expression system, and the VDR antibody 9A7, were purchased from Alexis Corporation (Nottingham, U.K.).

Gel-shift assays

Single-stranded oligonucleotides were usually purchased from Life Technologies (Paisley, Scotland, U.K.) and are shown in Table 1. They were annealed to give double-stranded oligonucleotides and end-labelled using polynucleotide kinase and [γ-^{32}P]ATP. Double-stranded oligonucleotides were separated from single-stranded DNA on 5% non-denaturing polyacrylamide gels, eluted and stored at 4 °C.

Reactions were carried out at room temperature in 20 μl of buffer containing either 50 or 150 mM KCl, 50 mM NaCl, 50 mM $MgCl_2$, 10 mM Hepes, pH 8.0, 0.1 mM EDTA, 4 mM spermidine, 2 mM DTT, 10 μg/ml BSA, 4% Ficoll and 1 μg poly(dI-dC). Nuclear extracts (≈ 5 μg of protein) were incubated with unlabelled competitor oligonucleotides for 5 min before addition of the ^{32}P-labelled probe (≈ 1 ng). After a further 30-min incubation, the reactions were run on polyacrylamide gels, usually 5% with 0.25 × TBE (where 1 × TBE = 45 mM Tris/borate/1 mM EDTA) and 2.5% glycerol. $MgCl_2$ (5 mM) was added to the gel and to the running buffer when the purified TBP was used. The gels were dried and exposed to film. For supershift assays, the reactions were incubated with the appropriate antibody for 20 min at room temperature or overnight at 4 °C.

Transfection studies

Constructs of the human calbindin-D9k gene were used in transient-transfection studies. The pGL2- and pGL3-series lu-

Table 1 Definition and sequences of the oligonucleotides used in gel shift assays in this study

The oligonucleotides are shown as single-stranded 5′–3′. In the site-A oligonucleotides, underlined bases are those that have been changed from the consensus CDX-2 elements (YTTTAYNR or YNRTAAAR [47], shown in bold). Also underlined are the differences in calbindin-D9k sequences between VDRE-A and VDRE-C.

Name	Nucleotide sequence: sense strand	Description
Calbindin-D9k site A	GTGGCGTGC**CCGTAAAG**A**CTATAAAA**GTGTCAT	Human equivalent of rat site A
M3	TGCCCGTAAAGAGTATATATGTG	Mutated 3′ CDX-2 element
M5	TGCGCGTAGACACTATAAAAGTG	Mutated 5′ CDX-2 element
M35	TGCGCGTAGACAGTATATATGTG	Mutated 5′ and 3′ elements
Rat site A	GTGGTGTGT**CTGTAAAG**A**CTATAAAA**GAGCTCCT	From [19]
SIF1	GGGTG**CAATAAAACTTTATGA**GTA	Control CDX-2 site from sucrase isomaltase gene [33]
VDRE-C	TGCCCTTCCTTATGGGGTTCA	Proposed VDRE (IP9) from human calbindin-D9k gene reported in [12]
VDRE-A	TGCCCTTAATTATGGGGTTCA	Corresponding region in calbindin-D9k gene in [6]
DR3	TCAGGTCAAGGAGGTCAGAG	Prototypic version of a DR3 VDRE [14]
SMS-UEB	TTTTGCGAGGCTAATGGTGCGTAA	Control PDX-1 site from the rat somatostatin gene [41]

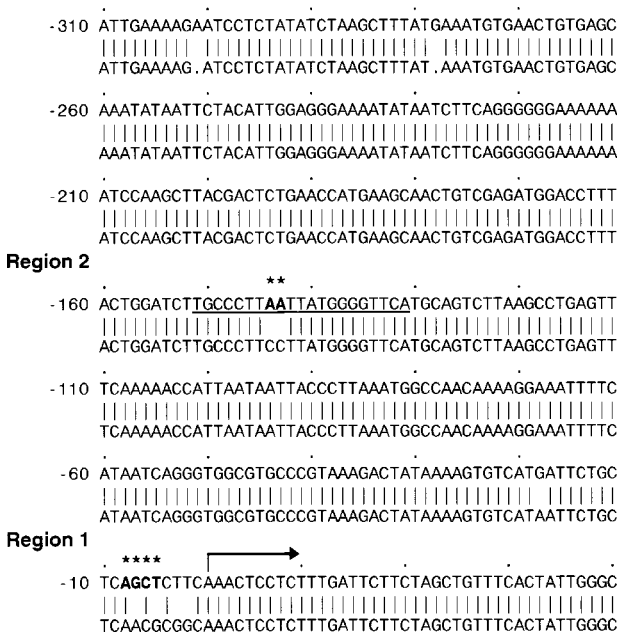

Figure 1 Comparison of sequences of the human calbindin-D9k gene promoter determined by Howard et al. [11] (top line) and Jeung et al. [12] (bottom line)

Numbering is derived from the 5′ end of the cDNA determined previously [6]. The differences studied are marked by asterisks: region 1 is near the transcription start site (arrow) with the *Alu*I site (in bold) indicated. Region 2 is within the reported VDRE (underlined) with the two bases that differ marked.

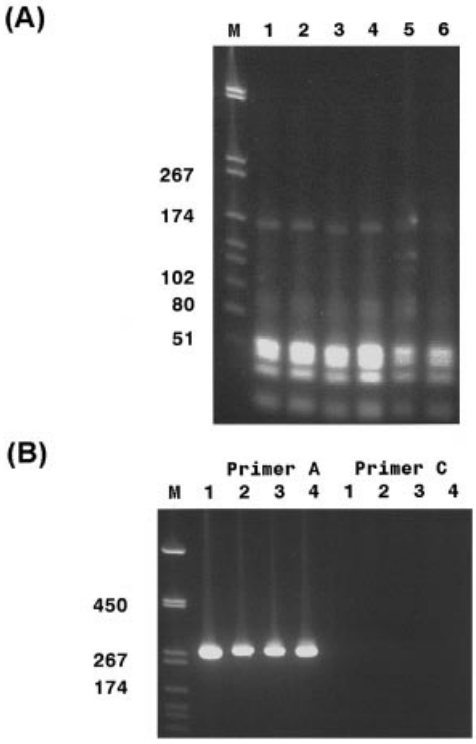

Figure 2 Results of PCR amplification of genomic DNA in two regions of the human calbindin-D9k promoter with sequence differences

Ethidium bromide-visualized products are shown. (**A**) Region 1, amplified from six individuals and digested with *Alu*I. (**B**) Region 2, amplified for four subjects, with either primer A or primer C. DNA size markers in lane M are an *Hae*III digest of pBluescript.

ciferase reporter vectors (Promega) were used, which include the pGL2- and pGL3-basic vectors, and the pGL2- and pGL3-promoter vectors containing the simian virus 40 (SV40) promoter. Constructs of the calbindin-D9k gene included bases −1303 to +4 in the pGL2- and pGL3-basic vectors, bases −1303 to −648 in the pGL2-promoter vector and bases −1303 to −1174 in the pGL2-promoter vector. Other constructs were bases −829 to −340 of the human osteocalcin enhancer [28] in the pGL2- and pGL3-promoter vectors, and the pSV-β-galactosidase control vector (Promega) to correct for transfection differences. The constructs were purified using the plasmid-purification kits from Qiagen.

Caco-2 cells or MCF-7 cells were transfected with luciferase reporter vector constructs using the MBS® mammalian transfection kit (Stratagene, Cambridge, U.K.) as described previously [29]. Cells were seeded at $4 \times 10^4/cm^2$. Caco-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10%

fetal calf serum, 50 μg/ml penicillin and streptomycin, 25 mM Hepes and 1% non-essential amino acids. MCF-7 cells were grown in the same medium containing 5% fetal calf serum and no added Hepes. 1,25(OH)$_2$D$_3$ (Leo Pharmaceuticals, Ballerup, Denmark) was added where indicated. The cells were usually harvested 16 h after transfection and assayed for cytoplasmic luciferase and β-galactosidase activities. Luciferase activities were normalized to those found with the SV40 promoter vector control. Each set of experiments was performed at least in triplicate, and the data are shown as means ± S.E.M.

RESULTS AND DISCUSSION

The sequence of the human calbindin-D9k promoter

The 1272 bp of 5′-flanking sequence that we reported (GenBank/EMBL accession number X76451) [11] had several differences from that published independently [12]. Two of these were in potentially critical areas: one was near the transcription start site (region 1, Figure 1) and the other (region 2) was included in the sequence suggested to contain a VDRE [13]. We looked to see whether these differences might represent common polymorphisms.

Genomic DNA was prepared from 37 individuals. At region 1, near the transcription start site, our sequence predicts the presence of an *Alu*I restriction site (AGCT) at positions −8 to −5, which is not present in the other sequence. PCR amplification produced the expected product of 176 bp which, when digested with *Alu*I, produced four bands of 56, 49, 42 and 29 bp, as predicted from our sequence, and no band at 85 bp (Figure 2A). The presence of the *Alu*I restriction site was demonstrated in all 37 individuals. At region 2, the VDRE region, amplification with the forward primer A, based on our sequence, gave the expected 281-bp product (Figure 2B). No amplification occurred in any individual with the forward primer C. Sequencing was performed on the products from several individuals and, in all cases, we found the sequence we reported previously, which contains TAATT. This is the core element recognized by many homeobox factors (see below).

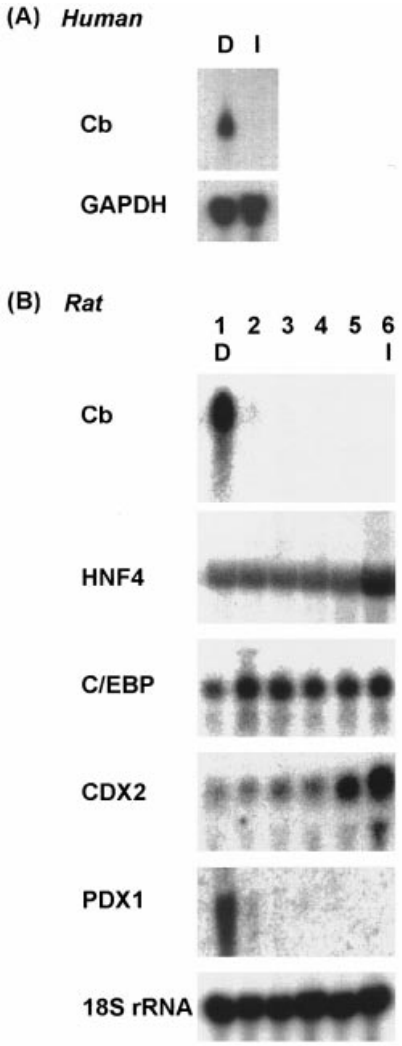

Figure 3 Northern blots of RNA in proximal and distal regions of intestine

(**A**) Calbindin-D9k (Cb) hybridization with RNA prepared from mucosa of human duodenum (D) and ileum (I). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used to show equivalent RNA loading. (**B**) Calbindin-D9k, HNF-4, C/EBPα, CDX-2 and PDX-1 hybridization with RNA from mucosa of rat small intestine divided into six equal segments (1–6) from duodenum to ileum. 18S rRNA was used as a control.

Table 2 Comparison of proposed elements in the calbindin-D9k promoters of rats and humans

Sequences in the human gene, H, (top) and rat gene, R, (bottom) are shown for areas where transcription factor binding in the rat has been demonstrated previously [13,19]. Consensus sequences for the proposed elements are underlined.

Species	Position	Sequence
Site A	(CDX-2/TATA box)	
H	-42/-25	CCGTAAAGACTATAAAAG
R	-40/-23	CTGTAAAGACTATAAAAG
Site B	(C/EBP)	
H	-70/-50	GGAAATTTTCATAATCAGGGT
R	-67/-48	GGAAA.TTTCATAATCAGGGT
Site C	(HNF-1)	
H	-113/-77	GTTTCAAAAACCATTAATAATTACCCTTAAATGGCCA
R	-110/-74	GTCTCAGAAACCATTAATCATTACCCTTAAATAGTAA
Proposed	Human VDRE	
H	-151/-123	TGCCCTTAATTATGGGGTTCATGCAGTCT
R	-137/-119	TGACCTTAATTGAGAG.....TCT
Site D	(?C/EBP)	
H	-196/-173	CTCTGAACCATGAAGCAACTGTCG
R	-182/-159	CTCTGAACCATTAAGCAATTGTTG
Site E	(?HNF-4)	
H	-271/-248	GAACTGTGAGCAA..ATATAATTCTA
R	-261/-236	GGACTGTGCGCAAGGTCAAAATTCTA

Interpretation of the sequence

The sequence of the human calbindin-D9k promoter is very similar to that of the rat. When repetitive elements were excluded, such as the Alu element in the human sequence between −664 and −365, there was 76% identity. However, some differences were apparent. The VDRE described in the rat gene between −489 and −445 [30] appeared to be in a rat B1 repetitive sequence and was not found in a corresponding location in the human gene. A possible VDRE, comprised of a typical DR3, was suggested in the human gene at −1247 to −1233 [11,12]. However, this sequence did not confer 1,25(OH)$_2$D$_3$-responsiveness, whereas another VDRE, at −151 to −130, was reported to be functional

Table 3 Proposed IPF-1/PDX-1 elements

Sequences of some elements reported to bind IPF-1/PDX-1. Many of these elements are known by alternative names.

Gene	Species	Element	Position	Sequence	Reference
Possible consensus				**TAATK**	[42]
				CCY**TAATG**GG	[39]
Insulin I	Rat	TAAT1	−87/−71	GAGCCCT**TAATG**GGCCA	
Insulin II	Rat	TAAT1	−87/−71	CCACCCT**TAATG**GGACA	
		TAAT2	−204/−192	CTC**TAATT**ACCCT	[27]
		TAAT3r	−205/−216	TCT**TAAT**AGGGG	[43]
Somatostatin	Rat	TSEI/UEB	−101/−86	CGAGGC**TAATG**GTGCG	[40]
		TAAT1	−438/−462	CTGATTGCATAT**TAATT**CTCAGAT	[42]
		TSEII/TAAT2	−303/−280	GGATCTCAG**TAATT**AATCATGCACC	[42]
Islet amyloid polypeptide	Human	AT-1r	−199/−210	CAGT**TAATG**GTG	[44]
		AT-2	−153/−137	GAGT**TAATG**TAATAATGACC	
		AT-3	−91/−80	AAAT**TAATG**ACA	
GLUT2	Mouse		−649/−632	GACT**TAAT**AATAACAGTA	
	Human		−465/−448	GCAA**TAAT**AATAACAACC	[45]
Calbindin-D9k	Human		−151/−131	TGCCCT**TAATT**ATGGGGTTCA	[6]

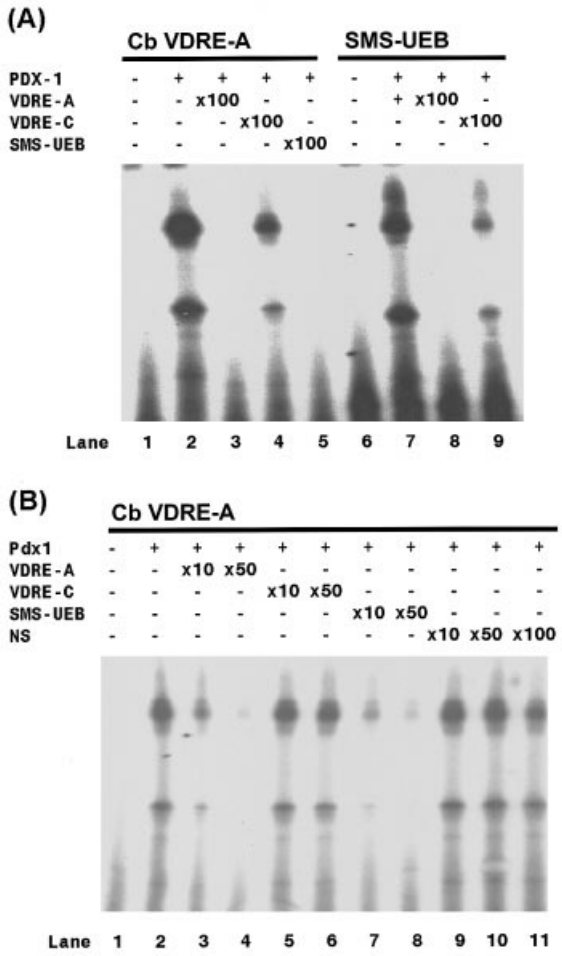

Figure 4 Gel-shift studies showing purified PDX-1-fusion protein binding to elements in the human calbindin-D9k (Cb) and somatostatin promoters

(**A**) Binding patterns and competition are similar with labelled calbindin-D9k VDRE-A (lanes 1–5) and SMS-UEB oligonucleotide probes (lanes 6–9). (**B**) Competition of PDX-1 binding to calbindin-D9k VDRE-A (lane 2) occurs with 10- and 50-fold excesses of VDRE-A (lanes 3 and 4) and SMS-UEB (lanes 7 and 8) but not with VDRE-C (lanes 5 and 6) or a non-specific (NS) oligonucleotide (lanes 9–11).

[13]. This element is in a region where an extra 10 bp are present in the human promoter compared with the rat.

Table 2 compares the human and rat sequences in the regions where Lambert et al. [19] described interactions of transcription factors with the rat promoter following DNaseI-footprinting studies. These regions were described as site A (binding CDX-2 and including the TATA box), site B (C/EBPα), site C (HNF-1), site D (C/EBPα) and site E (HNF-4). The conservation seen in most of these elements suggests that similar interactions might be expected to occur and to be functional in the human promoter, although the effects of individual base changes cannot be predicted. Indeed, it has already been shown that the oestrogen-responsive element in the first intron of the rat gene has two critical bases mutated in the human gene and is probably not functional [12].

Proximo-distal intestinal gradients of calbindin-D9k expression

Intestinal expression of calbindin-D9k occurs proximally in the duodenum and not more distally. Previously this has been shown in adult human intestine for the protein [9] and for RNA and protein in fetal gut [31]. We confirmed this distribution of mRNA in adult human intestine (Figure 3A), and in rat small intestine, where expression is abundant only in the most proximal segment (Figure 3B). We postulated that the transcription factors which determined such a distribution would show similar differences in expression and investigated the distribution of expression of candidate factors.

HNF-4, CDX-2 and C/EBPα were expressed throughout the rat small intestine (Figure 3B) and none of these factors had the same duodenal distribution as calbindin-D9k. CDX-2 was more abundant distally in ileum, as shown previously in mouse [32,33]. We found no evidence to support the previously reported proximal pattern of expression of C/EBPα [34]. Furthermore, both HNF-1 and VDR have been shown to be widespread in the intestine [35,36].

The lack of a clear positive association between these factors and calbindin-D9k expression led us to ask whether other factors, such as homeobox factors involved in developmental axial patterning, could be involved. We looked for differences in homeobox-factor expression in human proximal and distal intestinal regions, finding that only IPF-1/PDX-1 was present at high levels in duodenum but not ileum [22]. Similarly, in rat small

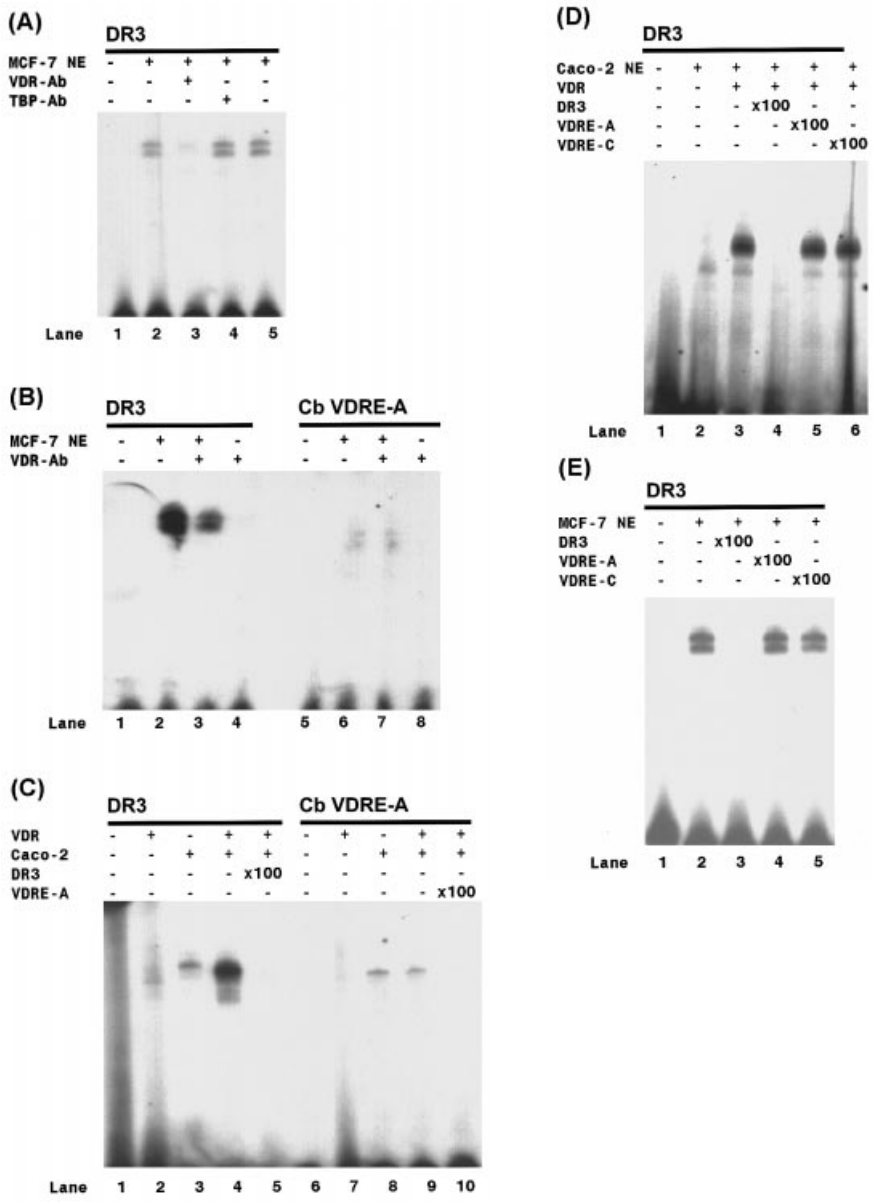

Figure 5 Gel-shift studies with cell extracts and purified VDR

(**A**) MCF-7 cell nuclear extracts (NE) produce a specific band pattern with the DR3 oligonucleotide, which is reduced when pre-incubated with the VDR antibody (VDR-Ab, lane 3) but not with the TBP antibody (TBP-Ab, lane 4). (**B**) The band pattern with the calbindin-D9k (Cb) VDRE-A oligonucleotide is different and unaffected by the VDR antibody. (**C**) With Caco-2 cell nuclear extracts, additional VDR increased binding only with the DR3 oligonucleotide (lane 4 > lane 3) and not with the calbindin-D9k VDRE-A probe (lanes 8 and 9 are similar). (**D** and **E**) Competition experiments show that 100-fold excesses of calbindin-D9k oligonucleotides (VDRE-A and VDRE-C) do not compete with the labelled DR3 oligonucleotide for binding with Caco-2 (**D**) and MCF-7 (**E**) cell nuclear extracts.

intestine, IPF-1/PDX-1 was expressed in the same proximal segment as calbindin-D9k (Figure 3B).

IPF-1/PDX-1 is involved in pancreatic and duodenal development [37,38]. It is also expressed in adult pancreatic islet β and δ cells. It has previously been shown to activate several genes, including insulin, somatostatin, islet amyloid polypeptide and GLUT2 [27,39–46]. We looked for potential binding sites for IPF-1/PDX-1 in the calbindin-D9k genes (Table 3). The simplest consensus sequence, TAATK [42], recognized by many homeobox factors, was present at 11 possible sites in 1303 bases of the human calbindin-D9k gene. Several of these sites approximate a proposed longer consensus sequence [39], one of these being at -151 to -130, the region previously suggested to contain the VDRE [13].

IPF-1/PDX-1 binding to the putative VDRE in the human calbindin-D9k promoter

To determine whether IPF-1/PDX-1 was able to interact with this element in the calbindin-D9k promoter, we expressed and purified a GST–PDX-1-fusion protein. Two bands were detected on gel electrophoresis, the smaller possibly representing a truncated form of the protein, and both interacted with a previously well-characterized PDX-1 antibody [27] on Western blots (results

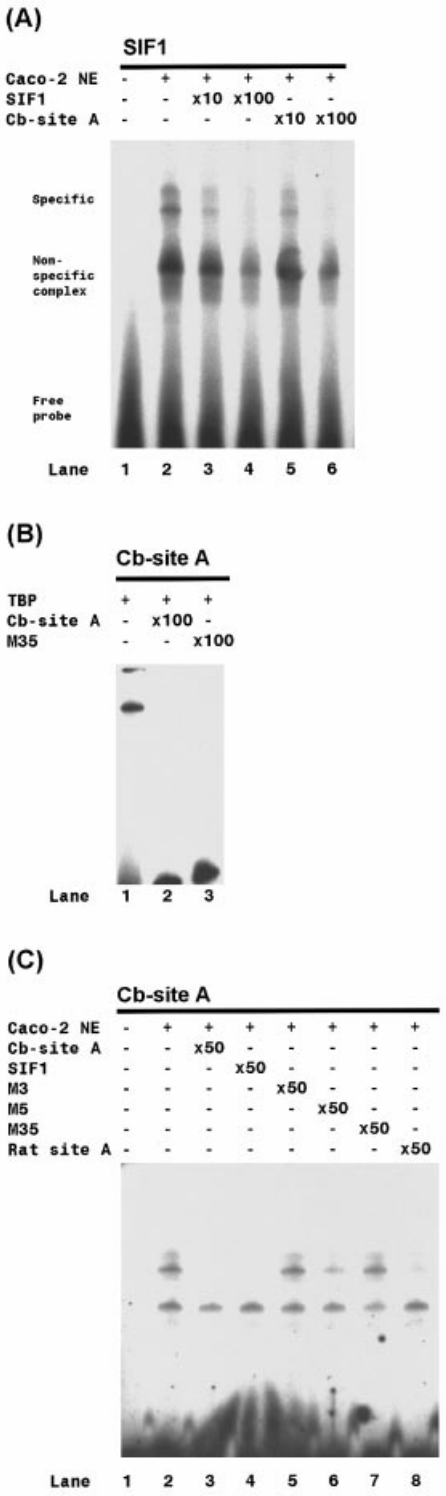

Figure 6 Gel-shift experiments studying the calbindin-D9k site A

(**A**) Complexes of Caco-2 cell nuclear extracts (NE) and labelled SIF1 oligonucleotide were competed similarly with 10- and 100-fold excesses of unlabelled SIF1 (lanes 3 and 4) and the calbindin-D9k (Cb) site-A oligonucleotide (lanes 5 and 6). (**B**) Binding to calbindin-D9k site-A oligonucleotide of purified TBP was competed by 100-fold excess of unlabelled site A (lane 2) and the M35 mutated oligonucleotide (lane 3). (**C**) Competition of specific binding of Caco-2 nuclear-extract proteins to calbindin-D9k site A by 50-fold excesses of unlabelled human site-A, SIF1, M3, M5, M35 and rat site-A oligonucleotides.

not shown). Electrophorectic-mobility-shift assays were performed with this expressed PDX-1-fusion protein.

The mobility of the labelled calbindin-D9k oligonucleotide VDRE-A, containing the sequence TAATT, was retarded by the PDX-1-fusion protein to a similar extent as a control oligonucleotide, SMS-UEB (see Table 1), which includes the IPF-1/PDX-1 site from the somatostatin promoter [42] (Figure 4A). Addition of unlabelled oligonucleotides from VDRE-A or SMS-UEB abolished the band shift, whereas competition was not found with the mutated oligonucleotide (VDRE-C) based on the sequence [12] containing TCCTT (Figure 4B). Addition of the PDX-1 antibody changed the pattern of the shifted bands (results not shown).

Experiments were performed to see whether this oligonucleotide, VDRE-A, was able to bind the VDR. As a positive control, band-shift experiments were performed with a prototypic DR3-type VDRE [14] using nuclear extracts from MCF-7 and Caco-2 cells, which contain varying amounts of VDR and retinoid-X receptor. These band shifts were reduced by pre-incubation with the VDR antibody 9A7 (Figure 5A), but similar results were not found with the calbindin-D9k VDRE-A oligonucleotide (Figure 5B). With Caco-2 nuclear extracts, the intensity of the DR3 band shift increased after the addition of purified VDR; similar results were not observed with VDRE-A (Figure 5C). Using an excess of unlabelled VDRE-A had no effect on the binding of nuclear extracts to the labelled DR3 probe (Figures 5D and 5E). Varying the ionic strength by changing the KCl concentration between 50 and 130 mM, or the addition of $1,25(OH)_2D_3$ at concentrations up to 10^{-6} M, did not affect these negative results.

CDX-2 binding to the human calbindin-D9k promoter

In studies of the rat calbindin-D9k promoter, it was found that the intestinal homeobox factor CDX-2 was capable of binding to the same region as the TATA box [19]. In the human calbindin-D9k gene, the proposed CDX-2 consensus [47] was conserved in this region (site A, Table 2), but as the orientation and spacing of the two half elements differed from that found in the prototypic CDX-2 site in the sucrase-isomaltase footprint 1 (SIF1), we considered it important to confirm this unusual interaction. Gel-shift experiments using Caco-2-cell nuclear extracts as a source of CDX-2 showed that the human calbindin-D9k site-A oligonucleotide could compete for binding to SIF1 (Figure 6A), and the band shift could be inhibited by pre-incubation with a specific CDX-2 antibody (result not shown).

The calbindin-D9k site A comprises a repeat of two consensus CDX-2 sites with the 3′ site overlying the TATA box. To investigate whether both bound CDX-2, mutant oligonucleotides were synthesized (see Table 1) where the TATA consensus was conserved but the CDX-2 site had been altered at the 5′-half site (M5), the 3′-half site (M3) or both (M35). We confirmed (Figure 6B) that this wild-type site can bind pure TBP and that the M35 oligonucleotide was able to compete for this. This M35 oligonucleotide was unable to compete for the CDX-2 binding in Caco-2 nuclear extracts (Figure 6C). The oligonucleotide with only the 3′-site mutation (M3) was also unable to compete, whereas the 5′-site mutation (M5) was partially effective. This suggests that the 3′ CDX-2 site, overlying the TATA box, had the stronger interaction with CDX-2.

These results confirm the suggestion that CDX-2 could act as a negative factor in transcriptional regulation, inhibiting TBP binding to the calbindin-D9k TATA box in cells with high CDX-2 concentrations. As CDX-2 increases distally in the intestine (Figure 3 and [22]), this could be a factor explaining the regional

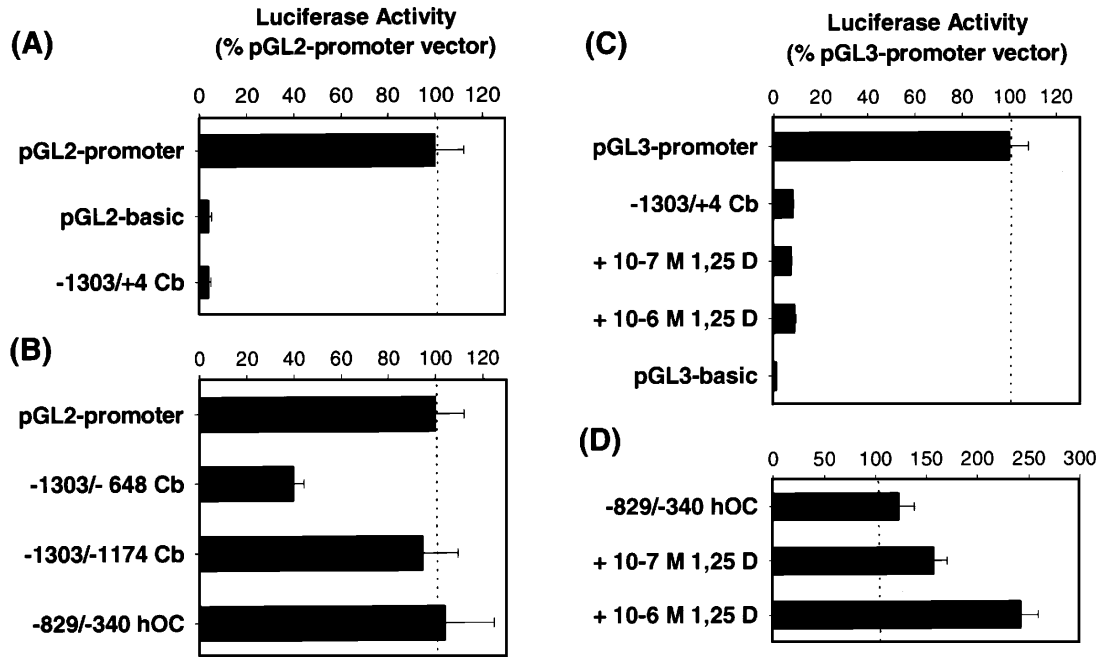

Figure 7 Effects of constructs of the calbindin-D9k (Cb) 5′-flanking sequence on heterologous luciferase reporter-gene activity

Results are the means ± S.E. of normalized luciferase activities. (**A**) Caco-2 cells were transfected with the pGL2-basic and pGL2-promoter vectors and the construct of bases −1303/+4 of the human calbindin-D9k gene in the pGL2-basic vector. (**B**) Caco-2 cells transfected with constructs in the pGL2-promoter vector of bases −1303/−648, bases −1303/−1174 of the calbindin-D9k promoter or −829/−340 of the human osteocalcin (hOC) promoter. (**C**) MCF-7 cells were transfected with the pGL3-promoter vector, the pGL3-basic vector, or with the construct of calbindin-D9k bases −1303/+4 in the pGL3-basic vector (−1303/+4 Cb). $1,25(OH)_2D_3$ (1,25 D) was added, where shown, to cells transfected with the −1303/+4 Cb vector to give final concentrations of 10^{-7} or 10^{-6} M. (**D**) Results in the same experiments using the human osteocalcin −829/−340 in pGL3-promoter construct and additions of $1,25(OH)_2D_3$ to give 10^{-7} or 10^{-6} M.

Table 4 Sequence of a suppressor element found in the intestinal fatty acid-binding protein having similarity with an element in human and rat calbindin-D9k

Bases identical to those in the rat fatty acid-binding protein sequence are shown in bold [50].

Gene	Species	Position	Sequence
Intestinal fatty acid-binding protein	Rat	−263/−244	**AGGTGGAAGCCATCACACTT**
	Mouse	−265/−246	**A**AG**T**C**G**C**AGCCATCA**TTACC
	Human	−291/−272	TTAGAAA**A**AC**A****ATCA**TTAGA
Calbindin-D9k	Rat	−999/−980	**A**T**G**CAC**AA**AG**CATCA**GTA**T**A
	Human	−743/−725	**ATGT.AAAG**A**CATCA**G**A**AGG

difference in calbindin-D9k expression. CDX-2 has also previously been shown to repress transcription of the apolipoprotein B promoter where an interaction with members of the C/EBP family of proteins has been suggested [48].

Reporter-gene assays with human calbindin-D9k promoter constructs

The Caco-2 cell line, derived from a colon carcinoma, has been used to study the expression of small-intestinal genes such as sucrase-isomaltase and fatty acid-binding proteins. Constitutive calbindin-D9k expression had been reported in these cells using reverse transcriptase PCR [49], which led us initially to use them in transfection experiments to study the calbindin-D9k promoter with a heterologous luciferase reporter.

Constructs of the human calbindin-D9k gene (bases −1303/+4) in the pGL2 basic vector, without any other promoter or enhancer, produced no measurable reporter activity (Figure 7A). Shorter constructs (bases −998/+4 and −650/+4) also gave no evidence of endogenous promoter activity. A construct containing bases −1303/−648 of the calbindin-D9k gene in the pGL2-promoter vector suppressed luciferase expression driven by the SV40 promoter by more than 50% (Figure 7B). As controls, constructs with bases −1303/−1174 of the calbindin-D9k gene or with bases −829/−340 of the human osteocalcin gene produced no suppression of the same expression vector. These experiments suggest the presence of powerful negative elements, both in the proximal promoter and also more distally between bases −1174 and −648. As described above, CDX-2 binding to the TATA-box could be one such negative factor. Furthermore, bases −743 to −725 in the human calbindin-D9k gene are similar to a 20-bp sequence described in the intestinal fatty acid-binding protein gene and thought to act as a suppressor in distal small intestine and colon [50] (Table 4).

As an alternative approach, we transfected MCF-7 cells, which had been used with the human calbindin-D9k promoter in the report suggesting a VDRE at −151 to −130 [13]. Our previous findings indicated that the pGL2 series of vectors was unreliable for showing effects of $1,25(OH)_2D_3$ [29], so constructs were made in the pGL3 series of vectors which, among other changes, produce higher levels of reporter activity. Expression of luciferase activity with the construct containing bases −1303/+4 of calbindin-D9k in the pGL3-basic vector was about 10% of that found with the pGL3-promoter vector, which uses the SV40 promoter (Figure 7C).

Although we could show weak promoter activity, we have been unable to demonstrate any effect of $1,25(OH)_2D_3$ with this construct of −1303/+4 of calbindin-D9k in the pGL3-basic vector (Figure 7C). In parallel transfections, we confirmed vitamin-D responsiveness of a control construct of bases −829/−340 of human osteocalcin in the pGL3-promoter vector (Figure 7D). Another experiment using −1303/+4 of calbindin-D9k in an enhancer setting with a heterologous thymidine kinase promoter in the pGL3-basic vector also found no effects of $1,25(OH)_2D_3$ (results not shown).

Interpretation of these results

The lack of a suitable duodenal cell line has impaired the study of the transcriptional control of intestinal regional gene expression of the human calbindin-D9k gene. Positive factors, such as the interaction of the homeobox factor IPF-1/PDX-1, and negative factors, including the interaction of CDX-2 with the TATA-box region, seem likely. These require further study, as does the basis for the vitamin-D responsiveness.

It is of interest to compare these findings with those from mice expressing transgenes containing constructs of the rat calbindin-D9k promoter [20,21]. Intestinal expression was not found with constructs containing 1011 and 117 bp of the 5′-flanking sequence. Small-intestinal expression of the transgene was found with a construct that had 4580 bp of the 5′-flanking sequence; this had the appropriate proximally restricted expression, but ectopic expression was also found in the colon. A distal site (−3735/−2894) linked to the 117-bp construct was variably expressed in different lines at lower levels. Vitamin-D responsiveness was found with both these constructs even though one lacked the putative VDRE in the rat calbindin-D9k gene between −489 and −445 [30]. The transgenic approach has also led to the unexpected development of a model of uterine leiomyomas where T-antigen expression in uterus was driven by the construct comprising −117/+365 of the calbindin-D9k gene.

Other genes, which resemble calbindin-D9k in being expressed in duodenum but not more distally in gut, include enteropeptidase, adenine deaminase, the calcium-ATPase PMCA1 and alkaline phosphatase. For none of these has the basis for their restricted intestinal distribution been established clearly. It remains to be determined whether there are common factors, or whether a variety of mechanisms are responsible for the restricted expression of these genes in the proximal duodenal intestinal mucosa.

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