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Control of differentiation-induced calbindin-D_{9k} gene expression in Caco-2 cells by cdx-2 and HNF-1 α

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Abstract

Calbindin D_{9k} (CaBP) is critical for intestinal calcium absorption; its in vivo expression is restricted to differentiated enterocytes of the small intestine. Our goal was to identify factors controlling the transcriptional regulation of this gene in the human intestine. Both the natural gene and a 4600-bp promoter construct were strongly regulated by differentiation (>100-fold) but not by treatment with 1,25(OH)₂ vitamin D (<2-fold) in the Caco-2 clone TC7. Deletion-mutation studies revealed that conserved promoter sequences for cdx-2 (at –3158 bp) and hepatocyte nuclear factor (HNF)-1 (at –3131 and at –98 bp) combined to control CaBP expression during differentiation. Other putative response elements were not important for CaBP regulation in TC7 cells (CCAAT enhancer binding protein, pancreatic duodenal homeobox-1 (pdx-1), a proximal cdx-2 element). Mutation of the distal HNF-1 site had the greatest impact on CaBP gene expression through disruption of HNF-1 α binding; both basal and differentiation-mediated CaBP expression was reduced by 80%. In contrast, mutation of the distal cdx-2 element reduced only basal CaBP expression. Whereas a 60% reduction of CaBP mRNA in the duodenum of HNF-1 α null mice confirmed the physiological importance of HNF-1 α for CaBP gene regulation, additional studies showed that maximal CaBP expression requires the presence of both HNF-1 α and cdx-2. Our data suggest that cdx-2 is a permissive factor that influences basal CaBP expression in enterocytes and that HNF-1 α modulates CaBP gene expression during cellular differentiation.

Keywords

intestine; enterocyte; 1,25 dihydroxyvitamin D

The Normal Function of Intestinal mucosa relies on the appropriate renewal and differentiation of cells along the cryptvillus axis (25,29). Developing therapies that promote recovery of a damaged epithelium or prevent malignancy (50) require understanding of the molecular

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pathways programming enterocyte differentiation. A classical approach to studying the program dictating this process has been to examine the regulation of intestine-specific genes, such as sucrase-isomaltase and lactase-phlorizin hydrolase. Extensive studies (3,4,17,19,33, 40) on their promoters have identified several transcription factors important for intestinal expression of these genes [cdx-2, hepatocyte nuclear factor (HNF)-1, and GATA family members]. However, intestinal expression of villin and adenosine deaminase depends on enhancer elements in introns 1 and 2, respectively (16,37). Thus the study of other differentiation-associated genes in intestine will provide new insight into the programming essential for development of a functional absorptive surface.

One such gene encodes calbindin D_{9k}, an EF hand calcium-binding protein critical for calcium absorption that is expressed in the duodenum and cecum of rodents (5,7). The expression of calbindin D_{9k} is regulated by the hormonally active form of vitamin D, 1,25 dihydroxyvitamin D [1,25(OH)₂ D] (7,22,47). Whereas previous reports have identified putative vitamin D response elements in the calbindin D_{9k} promoter from various species (12,28,45), other data suggest that posttranscriptional regulation may be a more important mode of calbindin D_{9k} regulation by 1,25(OH)₂ D (14,15,23,46). In addition to vitamin D regulation, calbindin D_{9k} expression is restricted to the differentiated villus compartment (24,38,49,53), indicating that it is under the control of factors programming intestinal cell differentiation; mutation of a cdx-2 response element at -3.5 kb decreased the intestinal expression of a calbindin D_{9k} reporter gene construct by 99% in transgenic mice (9). Several other potential regulatory sites in the proximal rat calbindin D_{9k} promoter have been reported on the basis of footprinting and gel-shift assays (1,34), i.e., a proximal cdx-2 element and sites for HNF-1, HNF-4, and CCAAT enhancer binding protein (C/EBP). None of those sites has been assigned a functional role in the regulation of calbindin D_{9k} promoter activity.

Here we characterized 1,25(OH)₂ D- and differentiation-mediated regulation of the human calbindin D_{9k} gene promoter in Caco-2 cells, an in vitro model for small intestinal differentiation (2,13,18,51). Our findings demonstrate that the calbindin D_{9k} gene is poorly regulated at the transcriptional level by 1,25(OH)₂ D but strongly upregulated during spontaneous differentiation to an enterocyte-like phenotype. Two HNF-1 binding sites, one at -98 and a novel, distal HNF-1 binding site at -3131 are essential for the differentiation-dependent expression of calbindin D_{9k}. In addition, our findings suggest that cdx-2 is a permissive factor that influences the basal expression level and that HNF-1-mediated transcriptional regulation of the calbindin D_{9k} promoter requires a complex interaction between the proximal and the distal HNF-1 response element.

MATERIALS AND METHODS

Cell Culture

Unless otherwise noted, all chemicals were obtained from Fisher Scientific (Fair Lawn, NJ), the media and supplements were purchased from Sigma (St. Louis, MO), and tissue culture plates were purchased from Corning Costar (Cambridge, MA).

The parental strain of Caco-2 cell (HTB-37) and the BBe clone (CRL-2102) were obtained from American Type Culture Collection (Rockville, MD). TC7 cells were first described by Chantret et al. (6). These were provided to us at *passage 69* by Dr. Mark Failla (Ohio State University). Cells were cultured as previously described (20). The BBe and TC7 clones were used because they have previously been demonstrated to develop a more differentiated phenotype (e.g., higher sucrase activity) compared with the parental line (6,42). To ensure that the cell lines would reach confluence concurrently (4 days in culture), different seeding densities were used for each cell line, i.e., parental Caco-2, BBe, and TC7 cells were seeded onto six-well dishes at a density of 128,000, 256,000, and 64,000 cells/well, respectively. For

transfection experiments, TC7 cells were seeded onto 24-well dishes at a density of 15,000 cells/well.

Regulation of Calbindin D_{9k} Expression by 1,25(OH)₂ D and Spontaneous Differentiation

Spontaneous differentiation—Parental Caco-2, BBe, and TC7 cells were grown in culture until 50% confluent (2 days in culture), confluent (4 days in culture), 4 days postconfluent (8 days in culture), and 11 days postconfluent (15 days in culture). At each time point, cells ($n = 3$ wells per cell line) were harvested into 1 ml TriReagent (Molecular Research Center, Cincinnati, Ohio), total RNA was isolated following the manufacturer's directions, and specific messages were assessed by RT-PCR. The experiment was repeated twice ($n = 6$ per time point per cell line).

To evaluate whether the expression of calbindin D_{9k} mRNA was transcriptionally activated during spontaneous differentiation, expression of a full-length calbindin D_{9k} promoter reporter gene construct (CaBP4600) was examined under two protocols. In one study, proliferating cultures of TC7 cells were transfected and then harvested at 50% confluent, 5 days in culture (1 days postconfluent), 9 days in culture (5 days postconfluent), and 13 days in culture (9 days postconfluent). In the second study, 2- or 9-day cultures of BBe and TC7 cells were transiently transfected and studied 24 h later. This second protocol was utilized for all subsequent experiments. Cells were either seeded onto six-well dishes (for the study of proliferating cells) or 24-well dishes (for the study of postproliferative cultures) and transfected with the Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA). Briefly, 2 μ g of the specific experimental vector was cotransfected along with a *Renilla*-expressing control vector, pRL-CMV (Promega, Madison, WI; molar ratio between the experimental and control vector = 250:1). In proliferating cells, transfection medium was replaced with 10% FBS DMEM 2 h after transfection. For differentiated cells, an equal volume of 20% FBS DMEM was added to each well 8 h after transfection, and new medium was added at 24 h. The prolonged exposure of differentiated cells to plasmid was necessary to overcome the low transfection efficiency we observed in these tight monolayers of nonproliferating cells. Cells were harvested, and luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) in a luminometer (model TD-20/20; Twin Designs, Sunnyvale, CA). The firefly luciferase activity (experimental plasmid) was normalized to *Renilla* luciferase activity (control plasmid) to control for variability in transfection efficiency. All treatments were examined in triplicate, and each experiment was conducted at least twice.

1,25(OH)₂ D treatment—TC7 cells were seeded into six-well dishes and cultured for 2, 8, or 15 days. Cells were treated with either 100 nM 1,25(OH)₂ D or ethanol vehicle (0.1% final concentration) for 48 h and then harvested for the examination of gene expression by RT-PCR.

Transcriptional regulation of the CaBP4600 construct was examined in 13-day cultures of TC7 cells. Cells were transiently transfected with either the CaBP4600 construct or a rat 24-hydroxylase promoter reporter construct provided to us by Dr. John Omdahl (30). Twenty-four hours after the transfection, cells were treated with 100 nM 1,25(OH)₂ D for 24 h, and promoter activity was assessed after an additional 24 h as described above. For both natural and reporter-gene studies, the treatments were examined in triplicate and each experiment was conducted twice.

RT-PCR analysis—Levels of specific messages were assessed by RT-PCR as described previously (21,22). PCR primers used were sucrase (GenBank accession no. X63597): forward, 5'-GGTGGTCACATCCTACCATGTCAAG-3', reverse, 5'-CTGGGATATCTTTTACTAA-3' (annealing temperature = 55°C); calbindin D_{9k} (GenBank accession no. X65869): forward, 5'-ATGAGTACTAAAAAGTCTCCT-3', reverse, 5'-

CTGGGATATCTTTTTTACTAA-3' (annealing temperature = 55°C); cdx-2 (GenBank accession no. U51096): forward, 5'-AGCCAAGTGAACACCAGGAC-3', reverse, 5'-CAGGGACAGAGCCAGACAC-3' (annealing temperature = 55°C); GAPDH (GenBank accession no. X02231): forward, 5'-CCATACAGGCAGCTTCGG-3', reverse, 5'-AGTCATCCACGAGCGATTTG-3' (annealing temperature = 55°C); Pdx-1 (GenBank accession no. U35632): forward, 5'-GCCGCATGAAGTGGAAAAAGG-3', reverse, 5'-TGTGGCGACGCGCTTAAGG-3' (annealing temperature = 55°C); HNF-1 α (GenBank accession no. NM_000545): forward, 5'-TAGTGGAGGAGTGCAACAGGGC-3', reverse, 5'-TGGAAGAACTGGACGGGCTG-3' (annealing temperature = 60°C); HNF-1 β (GenBank accession no. NM_000458): forward, 5'-GATGCCCACACACCACTTAC-3', reverse, 5'-TACGGCTTTCTTGCTTCCTC-3' (annealing temperature = 57°C); C/EBP α (GenBank accession no. XM009180): forward, 5'-CCCGAGTCACACCAGAAAG-3', reverse, 5'-CCGAGCAAAACCAAAACAA-3' (annealing temperature = 57°C). All PCR reactions used the cycling conditions of 94°C for 15 s and primer annealing temperature for 1 min 30 s, and 72°C for 45 s. PCR cycle numbers used were sucrase-isomaltase (27 cycles), cdx-2 (27 cycles), GAPDH (17 cycles), calbindin D_{9k} (2 and 4 days = 35 cycles; 8 days = 27 cycles for BBe and P, 23 cycles for TC7, 15 days = 27 cycles for BBe and P; 21 cycles for TC7), HNF-1 α (28 cycles), HNF-1 β (24 cycles), C/EBP α (27 cycles), and Pdx-1 (35 cycles). These cycles were chosen so that the amplification was conducted in the linear range of amplification efficiency for each of the primer sets (data not shown). The resulting PCR products were subjected to electrophoresis on 2.5% agarose gels containing ethidium bromide, and bands were visualized under UV light. Gel data were recorded by using the Bio-Rad FluorS Imaging System, and relative densities of the bands were determined by using Quantity One software (Bio-Rad laboratories, Hercules, CA). Data were normalized for the expression of GAPDH within the sample. For calbindin D_{9k}, we adjusted the data to account for differences in cycle numbers using the equation $A_n = A_o(1 + R)^n$ to determine a correction factor [where A_n = the amount of product produced at n cycles, A_o = the starting amount of cDNA, R = the amplification efficiency (assumed to be 0.8), and n = the cycle number]. Use of this correction factor permitted us to compare the transcript levels for calbindin D_{9k} across time and between cell lines.

In Silico Analysis of the Calbindin D_{9k} Gene Promoter

A computer-based comparison of the calbindin D_{9k} gene promoter from mouse, rat, and human was conducted by using sequences available in GenBank (rat = GenBank accession no. X16635; mouse = GenBank accession no. AY034822 submitted from our laboratory; human = GenBank accession nos. X13042 and AL445467). The Compare (16 bp/21 bp window homology match) and DotPlot programs (GCG, Madison, WI) were used to identify promoter regions (clusters) with high cross-species sequence homology. These conserved clusters were examined for potential response elements using the computer program SIGSCAN (Advanced Bio-science Computer Center of The National Institutes of Health, <http://bimas.dcrf.nih.gov/molbio/signal/>) and the mammalian response element database TRANSFAC (54). When a putative response element was identified in the human calbindin D_{9k} promoter, we examined the rat and mouse promoters to determine whether the element was conserved in these species.

Deletion-Mutation and Transcription Factor Activation Studies of the Human Calbindin D_{9k} Gene Promoter

TC7 cells were either seeded onto six-well dishes (for the study of proliferating cells) or 24-well dishes (for the study of postproliferative cultures) and transfected with various constructs containing deletions of large promoter regions or mutations of specific response elements. Analysis of promoter activity was conducted as described earlier in *Spontaneous differentiation*. All treatments were examined in triplicate, and each experiment was conducted

at least twice. In other experiments, proliferating cultures of TC7 or HeLa cells were transiently transfected with the CaBP4600 construct in the presence or absence of expression vectors for mouse *cdx-2*, HNF-1 α , or HNF-1 β either alone or in combination. Promoter activity was examined 24 h after transfection. Expression vectors for HNF-1 α (pBJ5-HNF-1 α) and HNF-1 β (pBJ5-HNF-1 β) were gifts from Dr. G. R. Crabtree, Stanford University. The expression vector for mouse *cdx-2* (pCB-mcdx-2) and its control empty vector (pCB6) were previously described (36). The sucrase-isomaltase promoter-luciferase reporter gene vector (pGL3-SI256) was provided by Dr. J. T. Troelsen (University of Copenhagen, Copenhagen, Denmark).

Reporter gene plasmid construction—A 5.4-kb fragment of the human calbindin D_{9k} promoter (−4478 to +889 bp) was obtained by PCR using human genomic DNA as template and the PCR primers: forward, 5′-GCTGCTATGGTTTGAATGCATTCC-3′; reverse, 5′-TCCTCTTCAGTTCCTCAGGAGAC-3′. A gel-purified PCR fragment was subcloned into the pCR4-Blunt vector (Invitrogen, Carlsbad, CA; pCR4-CaBP5400) and resequenced (Iowa State University DNA Sequencing Facility, Ames, IA). A full-length calbindin D_{9k} promoter-luciferase reporter gene construct was assembled in three steps. First, a 3400-bp *SacI* digestion fragment of pCR4-CaBP5400 (−3342 to +74) was subcloned into the *SacI* site of pGL3-basic (Promega, Madison, WI; pGL3-CaBP3400). Second, a 3.1-kb *EcoRI* fragment of pCR4-CaBP5400 (−4580 to −1500) was subcloned into pCR2.1 (Invitrogen, Carlsbad, CA; pCR2.1-CaBP3100). Finally, the pGL3-CaBP3400 and pCR2.1-CaBP3100 vectors were digested with *KpnI* and *ApaI*, and the fragment from pCR2.1-CaBP3100 (−4478 to −2898) was ligated into the backbone from pGL3-CaBP3400 to yield pGL3-CaBP4600 (−4478 to +74 bp).

A construct lacking conserved *cluster I* was prepared by digesting pGL3-CaBP3400 with *KpnI* and *NsiI*. The sticky ends were blunted by using Klenow Large Fragment (New England Biolabs, Beverly, MA) and then religated to create pGL3-CaBP1700 (−1558 to +74 bp). A construct lacking conserved *clusters I* and *II* was prepared by digesting pGL3-CaBP3400 with *KpnI* and *BstXI* followed by bluntend ligation of the backbone to form pGL3-CaBP500 (−408 to +74 bp). A construct lacking conserved *cluster II* (pGL3-CaBP1&3) was prepared by deleting the 1603-bp *BbvCI* fragment (−2153 to −550) from pGL3-CaBP4600, whereas a construct lacking three-fourths of *cluster III* (pGL3-CaBP1 and 2) was prepared by deleting the 631-bp *MscI* fragment (−710 to −79) from pGL3-CaBP4600.

Point mutations in specific response elements were introduced onto the wild-type calbindin D_{9k} promoter-luciferase reporter gene plasmid (pGL3-CaBP4600) using the QuikChanger XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's directions. The oligonucleotides used to introduce mutations were: distal *cdx-2*, 5′-CTCTTGTTGAACACACCCTGTAA(TT/GG)ATCACTTGTCTCC-3′; distal HNF-1, 5′-CACTTGTCTCCATATATTTTAGT(TA/GG)ATGTTTCACAAGAGTATCTATTAG-3′; proximal C/EBP, 5′-CGACTCTGAACCATGAAGC(AA/GG)CTGTGCGAGATGGACC-3′; proximal *cdx-2*, 5′-GGTGGCGTGCCCGT(AA/GG)AGACTATAAAAGTGTGCATG-3′; and proximal HNF-1, 5′-GCCTGAGTTTCAAAAACCAT(TA/GG)TAATTACCCTTAAATGGCCAAC-3′, where the underlined section is the response element and the bracketed bases represent the wild-type sequence followed by bolded nucleotides that replaced them in the mutated version. Plasmids with mutated response elements were isolated by using the Plasmid Mini Kit (Qiagen, Valencia, CA). All plasmids were sequenced to confirm the presence of the mutation (Iowa State University DNA Sequencing Facility, Ames, IA).

EMSA Analysis of Novel HNF-1 Response Element

Single-stranded oligonucleotides were synthesized and purified by Integrated DNA Technologies (Coralville, IA). The sequences used were lactase promoter HNF-1 site, 5′-

AACCCAGTTAAATATTAAGTC-3', -125 to -105 (33); calbindin D_{9k} distal HNF-1 site, 5'-TTTAGTTAATGTTTCACAAGAGTAT-3', -3151 to -3127; and mutated calbindin D_{9k} distal HNF-1 site, 5'-TTTAGTGGATGGTTTCACAAGAGTAT-3', -3151 to -3127 (underlined portions refer to the HNF-1 site; the bold GG in the mutated version is the site of mutation). Double-stranded probes were made by annealing equal molar amounts of two complementary oligonucleotides in annealing buffer (10 mM Tris-HCl, pH = 7.5, 1 mM EDTA, 100 mM NaCl). The annealing reaction was conducted by heating samples at 90°C for 10 min with gentle agitation and then slowly cooling to room temperature for 1 h. The double-stranded probe was labeled with ³²P by end-labeling. Briefly, 3.5 pmol of the probe was incubated with 1 µl of T₄ polynucleotide kinase 10× buffer (700 mM Tris-HCl, pH 7.6, 100 mM MgCl₂, 50 mM DTT), 1 µl of [γ -³²P]ATP (3,000 Ci/mmol at 10 mCi/ml, Amersham Biosciences, Piscataway, NJ), and 1 µl of T₄ polynucleotide kinase (5–10 U/µl) in a 10 µl reaction at 37°C for 10 min. The reaction was stopped by adding 1 µl of 0.5 M EDTA. The labeled probe was diluted 10-fold with Tris-EDTA buffer (pH = 7.4) and stored at -20°C for ≤2 wk. Only probes with >40% labeling efficiency were used.

Nuclear extracts were isolated from 50% confluent and 5-day postconfluent TC7 cell cultures using the Nuclear Extract Kit (Active Motif, Carlsbad, CA). Protein yield was determined by the Bio-Rad protein assay. Ten micrograms of nuclear extract were preincubated for 10 min at room temperature in binding buffer (in mM): 10 Tris-HCl, pH 7.5, 5 MgCl₂, 50 NaCl, 0.5 DTT, 0.5 EDTA, and 4% glycerol plus 0.025 µg/ml poly(dIdC) with or without unlabeled competitor probes or antibodies (anti-HNF-1α antibody or goat IgG, 2 mg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) in 9-µl total volume. ³²P-labeled probe (0.035 pmol) was then added, and the reaction was incubated at room temperature for another 20 min, at which time 1 µl of 10× gel loading buffer was added (250 mM Tris-HCl, pH 7.5, 0.2% bromophenol blue, 40% glycerol). The whole reaction was loaded onto a 6% DNA retardation gel (Invitrogen, Carlsbad, CA), and electrophoresis was conducted by using 0.5× TBE running buffer (44.5 mM Tris base, pH 8.3, 44.5 mM boric acid, 22.1 mM EDTA) at 250 V for 15 min. The gel was dried by using a GelAir gel drying system (Bio-Rad) for 1 h, and specific bands were detected by autoradiography using Kodak XOMAT film.

Analysis of Calbindin D_{9k} Expression in HNF-1α Null Mice

HNF-1α null mice were generated by using Cre-loxP-mediated deletion of exon 1 of the HNF-1α gene. The description and characterization of these mice has been presented elsewhere (35). Eight to twelve-wk-old HNF-1α heterozygous (+/-) and age-matched HNF-1α homozygous null (-/-) mice were used in this study (*n* = 6 per genotype). Mice were anesthetized with avertin (240 mg/kg), and the proximal 1 cm of small intestine adjacent to the pylorus was removed and immediately frozen on dry ice. All dissections were conducted between 1:00 and 4:00 PM to minimize variability due to circadian expression of HNF-1α (43).

The abundance of calbindin D_{9k} mRNA in HNF-1α +/- and HNF-1α -/- mice was determined by RNase protection assays (RPA) using previously established methods (31). Total RNA was isolated from mouse tissues using the RNeasy kit (Qiagen), quantified by optical density at A260 nm, and checked on an agarose gel. Antisense RNA probes for mouse calbindin D_{9k} (a cDNA for the coding region of the mouse gene in pCR2.1) and mouse β-actin (32) were linearized, transcribed with T7 RNA polymerase, labeled by using [³²P]ATP (Perkin-Elmer Life Sciences) and hybridized to RNA at 68°C in 50% formamide overnight, and digested with RNase T1 (1 µg/µl), and the protected fragments were separated on 6% denaturing polyacrylamide gels and then detected by autoradiography. The bands were quantified by using a Gel Doc system for image acquisition and Quantity One (Bio-Rad) software for analysis of band density. Calbindin D_{9k} mRNA levels were corrected for the expression of β-actin mRNA levels before analysis.

These studies were conducted under protocols approved by the Animal Research at Children's Hospital Animal Care and Use Committee.

Statistical Analysis

Data are reported as the means $\pm$ SE. Studies were examined by ANOVA or *t*-test (HNF-1 α null mouse only) using the SYSTAT statistical software package (SAS Institute, Cary, NC). Comparisons of multiple group means were performed by using Fishers protected least significant difference. Comparisons with $P < 0.05$ were considered significantly different.

RESULTS

Regulation of Calbindin D_{9k} Gene Expression by Differentiation

In the parental Caco-2 line and the two well-differentiating clones, BBe and TC7, sucrase-isomaltase and calbindin D_{9k} mRNA levels significantly increased as the cells differentiated (Fig. 1), and the two clonal lines expressed sucrase-isomaltase mRNA at a significantly higher level at every point after confluence (Fig. 1B). None of the three cell lines expressed high levels of calbindin D_{9k} mRNA at 2 or 4 days in culture. However, by 8 days in culture, the calbindin D_{9k} message was higher in TC7 (1036.40 ± 43.42 arbitrary units) than either parental or BBe cells (66.43 ± 3.97 and 86.85 ± 18.60 arbitrary units, $P < 0.001$, Fig. 1A). This pattern was also seen at 15 days in culture (TC7 60-fold $>$ BBe = parental). Figure 1C shows that the level of cdx-2 mRNA was similar in proliferating cells (parental = 0.29 ± 0.04 , BBe = 0.49 ± 0.01 , TC7 = 0.27 ± 0.02) and increased significantly in all three Caco-2 lines from 2 to 15 days in culture (parental = 0.89 ± 0.12 , BBe = 1.68 ± 0.18 , TC7 = 2.23 ± 0.40).

We confirmed that the full-length calbindin D_{9k} promoter-reporter gene construct recapitulated the natural calbindin D_{9k}. In TC7 cells transfected with the CaBP4600 promoter construct while proliferating and then studied at 1, 5, and 9 days postconfluence (5, 9, and 13 days in culture), calbindin D_{9k} promoter activity increased dramatically relative to the activity measured in proliferating TC7 cells (Fig. 2A). A 30-fold increase in calbindin D_{9k} promoter activity from 50% confluence (2 days in culture) to 5 days postconfluence (9 days in culture) was also observed by using the protocol in which TC7 cells were transiently transfected during proliferation or at 4 days postconfluence and studied 24 h later (data not shown). With the use of the later protocol, we found that the increase in promoter activity between 2 and 9 days in BBe cells was less than that observed in TC7 cells (BBe ratio = 10% TC7 response, data not shown).

Several transcription factors have been implicated in the regulation of calbindin D_{9k} in the enterocyte. A brief survey of their expression revealed that Pdx-1 mRNA was not expressed in either proliferating or differentiated TC7 or BBe cells. In contrast, cdx-2, HNF-1 α , HNF-1 β , and C/EBP α mRNA were all expressed in proliferating cells, and their mRNA levels were higher in differentiated cells (data not shown).

Regulation of Calbindin D_{9k} Expression in TC7 Cells by 1,25(OH)₂ D

1,25(OH)₂ D treatment caused a small (2-fold) increase in the expression of calbindin D_{9k} mRNA in proliferating, postproliferative, and differentiated TC7 cells (data not shown). In contrast, calbindin D_{9k} promoter activity was not influenced by 100 nM 1,25(OH)₂ D treatment, although CYP24 promoter activity was increased > 100 -fold under the same conditions (Fig. 2B).

Identification of Conserved Sequence Clusters and Response Elements in the Human Calbindin D_{9k} Gene Promoter

Conserved promoter regions are more likely to contain regulatory elements controlling the expression of a gene (26). In silico analysis of the human, rat, and mouse calbindin D_{9k} promoter sequences revealed three clusters with a high degree of homology across species. In the human promoter, these were located at -3512 to -2932 (*cluster I*), -1452 to -682 (*cluster II*) and -272 to -1 (*cluster III*, Fig. 3A). DNase I hypersensitive sites previously identified in the rat promoter (41) occur within *cluster I* (HS1), between *clusters II* and *III* (HS2 and 3), and in *cluster III* (HS4 and 5). Table 1 lists all of the previously reported transcription factor binding sites and relates their location to the conserved clusters we identified. Only the proximal cdx-2 response element (including the TATA box), a putative Pdx-1 response element, and the C/EBP response element at -61 bp were 100% conserved across all three species. HS2, HS3 (neither of which are intestine specific) are within a segment that was deleted in mice and humans. Of the previously identified vitamin D response elements (VDREs), none were well conserved between species (e.g., VDRE3 had missing segments in mouse and rat; VDRE1 is in a nonconserved portion of the promoter and is absent in human). SIGSCAN analysis of the three clusters identified 13 putative sites that were completely conserved among all three species, including a HNF-1 site [-3131 to -3120 bp, (-) strand, 5'-GGTTAATNATTATCA-3', other data not shown]. We also identified two 100% conserved stretches among the species in *cluster II*: a 19-bp sequence [on the (+) strand starting at -807 bp 5'-TTAAACCTGCTTCTGAAGC-3'] and a 16-bp sequence [on the (-) strand starting at -932 bp 5'-TTTTATTTCATTAT-3']. The 16-bp stretch (5'-TTTTATTTC-3') is similar to a cdx-2 consensus sequence (5'-TTTTTATA/GGC-3'), but no known response element consensus sequence was found in the 19-bp stretch.

Role of Conserved Promoter Sequence Clusters and Potential cis Elements in Calbindin D_{9k} Gene Expression

To investigate the contribution of each conserved promoter cluster to the control of calbindin D_{9k} gene expression, we made a series of deletion constructs as illustrated in Fig. 3A. The result of studies using these deletion constructs are shown in Fig. 3B. Removal of *cluster I* reduced basal promoter activity (expression in 2-day cultures) by 80% and the fold change associated with differentiation (9:2 days expression ratio) by 90% (CaBP1700). Removal of *cluster II* significantly increased promoter activity in both 2- and 9-day cultures by 60% (CaBP1 and 3), whereas removal of three-fourths of *cluster III* significantly blocked basal promoter activities as well as the fold change associated with differentiation by 50% (CaBP1 and 2).

Five *cis* elements were examined in this study. Within *cluster I*, mutation of the cdx-2 site (-3158 to -3152 bp, cdx-2 mut1), the HNF-1 site (-3131 to -3120 bp, HNF-1 mut1), or both of the sites (Cdx-2 mut + HNF-1 mut) reduced the basal promoter activity by 75, 80, and 75%, respectively ($P < 0.01$), and the fold induction due to differentiation by 12, 75, and 90% ($P < 0.01$), respectively (Fig. 4A). In *cluster III*, mutation of either the proximal cdx-2 site (-40 to -25 bp, Cdx-2 mut3) or the C/EBP site (-183 to -174 bp) had no detectable effect on calbindin D_{9k} promoter activity at either stage (Fig. 4B). Similar to the removal of three-fourths of *cluster III* (CaBP1 and 2), mutation of the proximal HNF-1 site in *cluster III* (-98 to -86 bp, HNF-1 mut3) decreased basal promoter activity by 36% ($P < 0.01$), and suppressed the differentiation-associated increase by 40% ($P < 0.01$, Fig. 4B).

Characterization of the Distal HNF-1 Site by Competitive EMSA

To further characterize the novel distal HNF-1 site in *cluster I*, a competitive EMSA was conducted. A major complex was formed by incubating the [³²P]calbindin D_{9k}-HNF-1 probe with Caco-2 nuclear extract from either preconfluent or postconfluent cultures. Binding in 9-day extracts was greater than that seen in 2-day extracts (Fig. 5). This complex disappeared in

the presence of a 30-fold molar excess of unlabeled calbindin D_{9k}-HNF-1 probe as well as a probe for the HNF-1 site previously characterized in the lactase gene promoter (33). A 30-fold molar excess of unlabeled mutated calbindin D_{9k}-HNF-1 probe or a nonspecific AP2 probe did not compete with the Caco-2 nuclear extract binding to the labeled calbindin D_{9k}-HNF-1 probe (Fig. 5). The major complexes formed with the calbindin D_{9k}-HNF-1 probe or the lactase-HNF-1 probe could be supershifted by the addition of an anti-HNF-1 α antibody, but not IgG, to the binding reaction (Fig. 5).

Effect of HNF-1 α Overexpression on Human Calbindin D_{9k} Promoter Activity

In proliferating TC7 cells, which naturally express both cdx-2 and HNF-1 α , overexpression of cdx-2 stimulated calbindin D_{9k} promoter activity by only 2.5-fold, whereas overexpression of HNF-1 α increased calbindin D_{9k} promoter activity by 10-fold ($P < 0.001$, Fig. 6B). The overexpression of both cdx-2 and HNF-1 α had an additive effect on promoter activity (12-fold, $P < 0.001$). In HeLa cells, which lack cdx-2 and HNF-1 α , overexpression of either cdx-2 or HNF-1 α stimulated calbindin D_{9k} promoter activity by 5.3-fold and 3.2-fold, respectively, and the combined overexpression of cdx-2 and HNF-1 α in HeLa cells had a synergetic effect on calbindin D_{9k} promoter activity (Fig. 6A, 14.5-fold, $P < 0.001$ vs. control; $P = 0.012$ for cdx-2 and HNF-1 α interaction).

HNF-1 β significantly suppressed the induction of calbindin D_{9k} promoter activity by HNF-1 α in proliferating TC7 cells (Fig. 6C). HNF-1 α -induced calbindin D_{9k} promoter activity was suppressed by 24, 30, and 43% in the presence of 0.05, 0.1, and 0.2 μ g of the HNF-1 β -expressing vector, respectively.

Calbindin D_{9k} Expression in HNF-1 α Null Mice

To further define the importance of our findings regarding HNF-1 α -mediated calbindin D_{9k} gene expression during enterocyte differentiation, we sought to determine whether these results could be extended to an in vivo setting. Duodenal RNA from HNF-1 α knockout mice were examined for calbindin D_{9k} expression by RPA, and this was compared with the expression seen in HNF-1 α $\pm$ mice. HNF-1 α $\pm$ mice are phenotypically normal and comparable to their wild-type littermates (35). As shown for the representative samples in Fig. 7, actin-corrected calbindin D_{9k} mRNA levels in HNF-1 α $-/-$ mice (0.16 ± 0.03 arbitrary units) were 60% lower than those of the age-matched HNF-1 α $\pm$ mice (0.41 ± 0.07 arbitrary units, $P < 0.05$).

DISCUSSION

Due to its proposed role in the control of intestinal calcium absorption (5), most of the research on calbindin D_{9k} gene expression has focused on its regulation by 1,25(OH)₂ D. Our findings indicate that in TC7 cells, calbindin D_{9k} mRNA levels are not transcriptionally regulated by 1,25(OH)₂ D. This is consistent with our previous work in the parental Caco-2 cell line and in mice, suggesting a posttranscriptional regulation mechanism, i.e., in Caco-2 cells. Induction of calbindin D_{9k} mRNA requires high doses, occurs gradually (over 48 h), and is inhibited by cycloheximide (22,23), whereas in mice, calbindin D_{9k} mRNA slowly rises in response to a large dose of 1,25(OH)₂ D (47) and significant calbindin D_{9k} expression is seen in the duodenum of vitamin D receptor null mice (46). Dupret et al. (14,15) previously showed that induction of calbindin D_{9k} mRNA was not inhibited by inhibitors of transcription and that posttranscriptional events account for the accumulation of calbindin D_{9k} mRNA in rat duodenum. The lack of a transcriptional response is also consistent with the observation that the calbindin D_{9k} VDREs do not have a high degree of similarity to the consensus VDRE (5'-GGGTCA NNR RGTTC A-3', where N = any base and R = G or A) and that these VDREs are not well conserved across species. For example, the direct repeat with 3-bp spacing (DR3)-

type VDRE identified in the rat promoter by Darwish et al. (10) and studied by Colnot et al. (8) is not present in the human promoter. In addition, the inverted palindromes spaced by nine nucleotides VDRE identified in the human promoter by Schrader et al. (45) is not intact in the rat or mouse promoter and has not been demonstrated to be active in a natural promoter context.

In contrast to the lack of vitamin D-mediated gene regulation, the calbindin D_{9k} promoter was strongly activated during TC7 cell differentiation to an enterocyte-like phenotype. This is consistent with earlier in vivo data (24,38,49,53). Previous research implicated two cdx-2 response elements as the important determinants for intestinal expression of the calbindin D_{9k} gene (9,34). Both elements bind to cdx-2 by ESMA (1,9,34), and mutation of the distal element at -3500 bp almost completely eliminated expression of a 4.6-kb rat calbindin D_{9k} promoter-CAT reporter gene construct in transgenic mice (9). In our studies, mutation of the distal cdx-2 response element reduced basal promoter activity in TC7 cells but did not have a strong effect on differentiation-associated promoter activity. This indicates that other factors are important for increased calbindin D_{9k} gene expression during enterocyte differentiation. We took several steps to identify these factors.

First, we showed that whereas GATA 4-5 and Pdx-1 have been implicated in the control of other intestinal genes, they may not be critical for calbindin D_{9k} gene regulation during enterocyte differentiation. For example, whereas a previously identified Pdx-1 site (1) was reasonably conserved across species, Pdx-1 mRNA was not detectable in either proliferating or differentiated TC7 cells (data not shown) and therefore cannot account for the dramatic upregulation of calbindin D_{9k} mRNA levels observed during TC7 cell differentiation. However, the participation of Pdx-1 in the regulation of the calbindin D_{9k} gene in vivo, specifically in the duodenum in which Pdx-1 is expressed, cannot be ruled out based on our experiments. We were also unable to find phylogenically conserved GATA sites in the conserved clusters of the calbindin D_{9k} promoter using the variants of the GATA 4/5/6 consensus sequence reported by Krasinski et al. (33). Finally, we identified a HNF-1 site that was within 25 bp of the cdx-2 response element in *cluster I*. Our subsequent analysis focused on the distal HNF-1 and cdx-2 sites as well as a number of phylogenically conserved sites that had been previously identified within *cluster III*, i.e., cdx-2 (the 5' half site), C/EBP (-53 to -61 bp), and HNF-1 (-86 to -98 bp) response elements (34).

The major finding of our analysis is that the newly identified distal HNF-1 site is critical for the control of both basal and differentiation-associated calbindin D_{9k} promoter activity. Whereas mutation of the distal cdx-2 site lowered only the basal promoter activity (by 80%), mutation of the distal HNF-1 site reduced differentiation-associated calbindin D_{9k} promoter activity by 75% and accounted for >90% of the influence of *cluster I* on calbindin D_{9k} gene expression. We subsequently showed that the putative HNF-1 response element binds avidly to a protein from 9-day TC7 cell nuclear extracts, that the binding is reduced in the presence of a the HNF-1 response element from the lactase gene (33), and that the specific binding to this sequence can be supershifted by an antibody against HNF-1 α . In addition, a proximal HNF-1 site in *cluster III* is also required for basal and differentiation-associated calbindin D_{9k} gene expression in enterocytes; mutation of this site reduced promoter activity by 40% in 9-day cultures. The impact of HNF-1 site mutations we observed in TC7 cells was similar to the reduction in calbindin D_{9k} mRNA level we observed in HNF-1 α null mice (60% reduction). This confirms that HNF-1 α is important for intestinal expression of calbindin D_{9k} and demonstrates that regulation of promoter activity through the two HNF-1 α sites is physiologically relevant.

Our data identifies at least two important determinants that modulate HNF-1 α -mediated regulation of calbindin D_{9k} gene expression. First is the presence of cdx-2. We found that in HeLa cells with low natural expression of cdx-2 and HNF-1 α , coexpression of the two

transcription factors was required for maximal induction of calbindin D_{9k} promoter activity. When our data are considered in combination with the earlier studies by Colnot et al. (9), our studies support a model whereby cdx-2 is a permissive factor necessary for basal expression of the calbindin D_{9k} gene in enterocytes and in which HNF-1 α accounts for the increased expression of this gene along the crypt-villus axis of the small intestine. The close proximity of the distal HNF-1 and cdx-2 sites (within 25 bases) suggests that the two transcription factors may directly interact to modulate calbindin D_{9k} gene expression; [i.e., as for the lactase gene (40)]. Additional studies will be necessary to confirm these interactions as well as to assess the potential for direct communication between the proximal and distal HNF-1 response elements.

In addition to the role that cdx-2 plays, we found that HNF-1 β antagonizes HNF-1 α action similar to what Boudreau et al. (4) reported for sucrase-isomaltase gene regulation. Although Boudreau et al. (4) showed that the β - to α -protein ratio falls with Caco-2 cell differentiation (from ~0.8 to 0.3 due to a decline in HNF-1 β levels), we found that β - to α -transfection ratios >4:1 did not completely suppress calbindin D_{9k} promoter activity (43% reduction). This suggests that the release of HNF-1 β -mediated suppression alone does not account for the dramatic increase in calbindin D_{9k} gene expression we see during TC7 cell differentiation. Because HNF-1 and cdx-2 are both abundant in proliferating Caco-2 cells (4), a more likely mechanism for their role in calbindin D_{9k} gene regulation is that these transcription factors are activated during enterocyte differentiation. It has been well documented that posttranscriptional modification (e.g., phosphorylation) can alter the DNA binding activity of transcription factors including cdx-2 (27,44). Hence, it is possible that differentiation-associated changes in the phosphorylation status of HNF-1 α or cdx-2 induces direct and indirect interactions between the transcription factors at critical DNA response elements or with essential coactivators (e.g., dimerization co-factor of HNF-1, cAMP response element binding protein, and CREB-binding protein-associated factors) (3,39,40,48,52). Future studies are necessary to define whether posttranslational modifications of HNF-1 α or cdx-2 are necessary for differentiation-induced calbindin D_{9k} gene activation.

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Fig. 1.

Comparison of calbindin D_{9k}, sucrase-isomaltase, and cdx-2 mRNA level among three Caco-2 cell lines. Parental Caco-2 (P) and two well differentiating clones of Caco-2 (BBe and TC7) were harvested at 2 days (50% confluent, proliferating cultures), 4 days (confluence), 8 days (4 days postconfluent, postproliferative), and 15 days (11 days postconfluent, well-differentiated) after seeding. Calbindin D_{9k}, sucrase-isomaltase, and cdx-2 mRNA levels were analyzed by semiquantitative RT-PCR. Each value represents the means $\pm$ SE ($n = 6$) for calbindin D_{9k} (A), sucrase-isomaltase (B), and cdx-2 (C). a, Significantly different from 2-day expression; b, significantly different from parental expression (Fishers least significant difference test, $P < 0.05$).

Fig. 2.

Regulation of calbindin D_{9k} mRNA levels and promoter activity by differentiation. *A*: regulation of calbindin D_{9k} promoter activity during TC7 cell differentiation using a full-length calbindin D_{9k} promoter-reporter gene construct (CaBP4600). TC7 cells were transfected with a CaBP4600 at 1 day in culture, and reporter activity was examined at 2, 5, 9, and 13 days in culture by measuring luciferase activity. *B*: vitamin D (VD) regulated promoter activity in 15-day cultures of TC7 cells. Cells were transfected with either CaBP4600 or a 24-hydroxylase promoter construct (CYP24), treated for 24 h with 100 nM 1,25(OH)₂ vitamin D, and promoter activity was assessed by measuring luciferase activity. Bars represent the means $\pm$ SE. Bars with different letters (in *A*) are significantly different ($P < 0.05$). In *B*, the bar with an asterisk (*) indicates significant difference from vehicle treatment ($P < 0.05$).

Fig. 3.

Deletion analysis of conserved clusters in the human calbindin D_{9k} gene reveals important regulatory regions. **A**: schematic representation of the promoter constructs [CaBP4600 or truncated calbindin D_{9k} promoters (CaBP1700, CaBP1&2, CaBP1&3, and CaBP500)] used in experiments. The sequence containing all 3 of the conserved sequence clusters is CaBP4600. Versions of the promoter lacking one or more conserved clusters are shown under the full-length promoter construct. In CaBP4600, arrows point to the sites of putative response elements that were functionally evaluated by mutation analysis. **B**: reporter constructs containing the sucrase-isomaltase promoter (SI256), CaBP4600, or CaBP1700, CaBP1&2, CaBP1&3, and CaBP500 were transiently transfected into 50% confluent (*day 2*) and 5-day postconfluent

(day 9) TC7 cells. After 24 h, promoter activity was assessed by measuring luciferase activity. All data are expressed relative to the expression observed for CaBP4600 at 2 days (relative value = 1). Values in parentheses are the ratio of promoter activity at 9 days to that measured at 2 days. Each experiment was repeated at least 3 times with triplicate observations within each experiment. Data from 1 representative experiment is shown as means $\pm$ SE.

Fig. 4.

The role of *cis* elements in conserved *clusters I* and *III* in differentiation-induced regulation of calbindin D_{9k} promoter activity. Luciferase reporter constructs containing CaBP4600 or various truncated or mutated promoter constructs were transiently transfected into 50% confluent (*day 2*) and 5-day postconfluent TC7 cells (*day 9*). After 24 h, promoter activity was assessed by measuring luciferase activity. All data are expressed relative to the expression observed for CaBP4600 at 2 days (relative value = 1). Values in parentheses are the ratio of promoter activity at 9 days to that measured at 2 days. Each experiment was repeated at least 3 times with triplicate observations within each experiment. Data from 1 representative experiment are shown as means $\pm$ SE. *A*: *cluster I* mutants. A truncated calbindin D_{9k} promoter

without *cluster I* (CaBP1700), or promoters with mutations within the distal cdx-2 site (Cdx-2 mut1), the distal HNF-1 site (HNF-1 mut1), or both sites (Cdx-2 mut + HNF-1 mut) were examined. *B: cluster III* mutants. A truncated calbindin D_{9k} promoter without $\frac{3}{4}$ of *cluster III* (CaBP1&2) or promoters with mutations at the proximal cdx-2 site (Cdx-2 mut3), the C/EBP site (C/EBP), or the proximal HNF-1 site (HNF-1 mut3) were examined.

Fig. 5.

EMSA analysis of HNF-1 binding to the distal HNF-1 response element in conserved *cluster I* of the calbindin D_{9k} promoter. Oligonucleotides containing the putative HNF-1 site from *cluster I* in the human calbindin D_{9k} promoter (CaBP D_{9k}) or a well-characterized HNF-1 site from the lactase promoter (Lactase) were labeled with [γ -³²P]ATP by T4 polynucleotide kinase. *Left*: competitive gel-shift assay. Nuclear extracts (Nuc Extract; 10 μ g) from preconfluent (2 days) or 5-day postconfluent (9 days) TC7 cells were used for each binding reaction. The specific HNF-1 α -containing complex is marked with an arrow. Specificity of complex formation was confirmed by competition with a 6-fold (6 $\times$) or 30-fold (30 $\times$) molar excess of unlabeled probe (9k, CaBP-HNF-1; M, mutated CaBP-HNF-1; L, lactase HNF-1; A, AP2). Specificity of complex formation on the labeled lactase HNF-1 probe was confirmed by competition with a 50-fold molar excess of unlabeled calbindin D_{9k} HNF-1 probe or lactase HNF-1 probe. *Right*, HNF-1 α supershift assay. Nuclear extracts (10 μ g) from 9-day cultures of TC7 cells were preincubated with HNF-1 α antibody (H) or a goat IgG (Ig) before incubation with ³²P-labeled calbindin D_{9k} HNF-1 probe or lactase HNF-1 probe.

Fig. 6.

Effect of HNF-1 α , HNF-1 β , and cdx-2 expression on calbindin D_{9k} promoter activity in intestinal (TC7) and nonintestinal (HeLa) cells. Luciferase reporter constructs containing CaBP4600 were cotransfected into 50% confluent HeLa (A) or TC7 (B) cells with control vector (250 ng of pCB6), mouse cdx-2 expression vector (250 ng of pCB6-mcdx-2), mouse HNF-1 α expression vector (250 ng of pBJ5-HNF-1 α), or both cdx-2 and HNF-1 α expression vector (250 ng of each). C: 50% confluent cultures of TC7 cells were transfected with CaBP4600 along with control vector, HNF-1 α expression vector (0.05 μ g), HNF-1 β expression vector (0.1 μ g), or HNF-1 α expression vector mixed with increasing amounts of HNF-1 β expression vector (0.05, 0.1, or 0.2 μ g). Promoter activities were assessed 24 h after transfection

and are expressed relative to the induction seen in the control vector transfected cells (relative value = 1). In *A* and *B*, each bar represents the means $\pm$ SE ($n = 6$). In *C*, each bar represents the means $\pm$ SE ($n = 3$). Bars with different letters are significantly different from one another (Fishers least significant difference test, $P < 0.05$).

Fig. 7.

HNF-1 α is an activator of calbindin D_{9k} gene expression in vivo. RNase protection assays were carried out on RNA isolated from the duodenum of mice heterozygous (+/-) and homozygous (-/-) for the HNF-1 α null allele using labeled antisense RNA probes for mouse calbindin D_{9k} and β -actin. Two micrograms of RNA were used for calbindin D_{9k} analysis, and 1 μ g of RNA was used for β -actin analysis. The analysis from 3 representative mice is shown.

Table 1Response elements in the calbindin D_{9k} promoter

Name	Species	Position	Sequence*	Ref. No.
Cdx-2 (P)	H	-40/-25	GTAAAG ACT ATAAAAG	34
	R	-38/-23	<u>GTAAAG</u> ACT <u>ATAAAAG</u>	
	M	-43/-28	GTAAAG ACT ATAAAAG	
Cdx-2 (D)	H	-3158/-3152	TGtAaTTATcaCt	9
	R	-3439/-3433	<u>TGCATTTATGGCA</u>	
	M	-3044/-3038	TGCcTTTATGGCA	
ERE	H	+51/+63	GGTtA GTG TGATt	11
	R	+50/+62	<u>GGTCA</u> GGG <u>TGATC</u>	
	M	+50/+62	GGTCA GTG TGgTC	
PDX-1	H	-145/-141	<u>CCTTAATTATG</u>	1
	R	-131/-127	CCTTAATTgaG	
	M	-136/-132	CCTTAATTgaG	
C/EBP	H	-61/-53	TTTCATAAT	34
	R	-62/-54	<u>TTTCATAAT</u>	
	M	-67/-59	TTTCATAAT	
HNF-1	H	-98/-86	ATTAATaATTACC	34
	R	-98/-86	<u>ATTAATCATTACC</u>	
	M	-103/-91	ATTAATCATTACC	
C/EBP	H	-183/-174	TgAAGCAAcT	34
	R	-172/-164	<u>TTAAGCAAT</u>	
	M	-177/-169	TTAAGCAAT	
HNF-4	H	-260/-251	TGaGCAaA-T-A	34
	R	-256/-245	<u>TGCGCAAGGTCA</u>	
	M	-261/-250	TGCGCAAGGTCA	
VDRE1	H	n/a	Not present	11
	R	-489/-475	<u>GGGTGT</u> CGG <u>AAGCCC</u>	
	M	-508/-493	GGGTGT CAG AAaCCC	
VDRE2	H	-1247/-1233	<u>AGGTGA</u> TAT <u>AAGGCA</u>	28
	R	-1648/-1634	AGGgaA TAT ActGtA	
	M	-1260/-1246	AGGTaA TAT ActGtA	
VDRE3	H	-151/-130	<u>TGCCCT</u> TAATTATGG <u>GGTTCATGCAGT</u>	45
	R	-137/-122	TGaCCT TAATTGAG - - - - - AGT	
	M	-142/-127	TGCCCT TAATTGAG - - - - - AGT	

* Underlined is the consensus sequence reported in the reference; bases different from the reported sequence are written in lower case. All sequences are from the (+) strand and are written 5'- to -3'. P, proximal; D, distal; ERE, estrogen response element; PDX-1, pancreatic duodenal homebox-1; C/EBP, CCAAT enhancer binding protein; HNF-1, hepatocyte nuclear factor 1; VDRE, vitamin D response element; H, human; R, rat; M, mouse.