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Identification of sequence elements in mouse calbindin-D_{28k} gene that confer 1,25-dihydroxyvitamin D_3- and butyrate-inducible responses

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ABSTRACT **We have examined the 5′ flanking region of the mouse calbindin-D_{28k} gene and identified a 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]-responsive element by deletion mutant analysis of the native promoter as well as by studies with a heterologous thymidine kinase (TK) promoter. The segment between residues −200 and −169 was found to confer a dose-dependent 1,25-$(OH)_2D_3$ responsiveness through the TK promoter in Ros 17/2.8 cells as well as in CV-1 cells cotransfected with pAV-hVDR (human vitamin D receptor expression vector). This region contains sequences homologous to the rat osteocalcin vitamin D response element (VDRE). Incubation of this element with nuclear extracts from 1,25-$(OH)_2D_3$-treated Ros 17/2.8 cells or from 1,25-$(OH)_2D_3$-treated COS cells that had been transfected with pAV-hVDR resulted in a specific protein–DNA interaction. In addition to 1,25-$(OH)_2D_3$, sodium butyrate, a differentiating agent, has also been found to modulate expression of calbindin-D_{28k}. Deletion analysis of the mouse calbindin-D_{28k} promoter as well as studies with a heterologous TK promoter resulted in identification of a butyrate-responsive element between −180 and −150 that was found to bind specifically to nuclear factors from butyrate-treated Ros 17/2.8 cells. This butyrate-responsive element may represent a genetic element acted upon by enhancer binding proteins. In summary, the 5′ flanking region of the mouse calbindin-D_{28k} gene contains responsive elements that interact with nuclear factors and may mediate, at least in part, the enhanced expression of this gene by 1,25-$(OH)_2D_3$ and butyrate.**

The major proteins in intestine and kidney whose synthesis is regulated by vitamin D are calcium binding proteins, known as calbindins. Calbindin-D_{9k} is a M_r 9000 protein that is present in mammalian intestine and calbindin-D_{28k} is a M_r 28,000 protein that is present in avian intestine and avian and mammalian kidney (1). It has been suggested that calbindin in these tissues is involved in intracellular calcium translocation (2, 3). Besides intestine and kidney, the calbindins have been reported in many other tissues, including bone and tissues that are not regulators of serum calcium such as pancreas, brain, uterus, and placenta (1). The presence of calbindin in tissues that are not regulators of serum calcium suggests that calbindin may indeed have functions other than facilitating calcium diffusion. We recently found that calbindin increases with differentiation. Sodium butyrate induces rat insulinoma cells {RIN-1046; a β-cell line, which contains both calbindin and receptors for 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]} to differentiate to a more islet cell phenotype. Butyrate treatment of these cells was found to increase calbindin protein and mRNA 4- to 8-fold and resulted in a parallel increase in insulin secretion (4). Thus, calbindin increased in accord with cell differentiation. These findings suggest the possibility that calbindin-D_{28k}, similar to other calcium binding proteins, may play a regulatory role in cellular differentiation, a process that involves changes in intracellular calcium. Although the exact role of the calbindins remains to be determined, biosynthesis of calbindin has provided a model for studies that have resulted in an important basic understanding of the mechanism of action of 1,25-$(OH)_2D_3$ in major target tissues such as intestine and kidney. *In vivo* experiments have suggested that 1,25-$(OH)_2D_3$ regulates calbindin gene expression at the transcriptional and posttranscriptional level (4–7). To study the regulatory elements involved in transcriptional activation, we have recently cloned the genomic DNA encoding mouse calbindin-D_{28k} and have linked the 5′ flanking region of this gene to a reporter gene coding for bacterial chloramphenicol acetyltransferase (CAT). We have used this chimeric gene construct to investigate the basal and inducible expression of calbindin-D_{28k}.†

MATERIALS AND METHODS

Cloning of Genomic DNA Encoding Mouse Calbindin D_{28k}. Clones were obtained by screening a mouse (BALB/c) kidney genomic library in pJB8 cosmid [from Wood and Tonegawa (8)] using ^{32}P-labeled mouse calbindin-D_{28k} cDNA (9) as a probe. Mouse calbindin-D_{28k} cDNA was labeled by the random oligonucleotide priming method of Feinberg and Vogelstein (10). Hybridization was carried out as described (11). Positive clones were selected and purified.

Southern Blot Analysis and Isolation of the 5′ Flanking Region. Mouse genomic DNA was analyzed by Southern blot hybridization (12). Fragments containing the 5′ flanking region were identified by probing to a 33-mer oligonucleotide probe complementary to the first 11 amino acids of rat calbindin-D_{28k} (13, 14) [there is 95% sequence homology between mouse and rat calbindin-D_{28k} (9)]. This oligonucleotide was 5′-end-labeled with [γ-^{32}P]ATP and polynucleotide kinase (15). A 1.3-kb *Pst* fragment that hybridized with the oligomer was subcloned into $M_{13}um_{31}$ and sequenced by the dideoxynucleotide chain-termination method (16). DNA sequence analysis of the region of the gene between +1 and +154 indicated that this region is identical to the rat calbindin-D_{28k} sequence +1 to +154 (13, 14).

Recombinant Plasmid Construction. A 1.1-kb *Pst* I/*Sac* II DNA fragment extending from −1075 to +34 (−1075/+34) was blunt end ligated at the *Sma* I site of pHCAT (which is derived from pSV2CAT by deleting the simian virus 40

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Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CAT, chloramphenicol acetyltransferase; VDR, vitamin D receptor; VDRE, vitamin D response element; TK, thymidine kinase.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L11891).

promoter; pHCAT was a gift from M. Tocci, Merck Sharpe & Dohme). For experiments involving deletion analysis, the −1075/+34 fragment was cleaved with *Pvu* II, *Bsm* I, *Ava* III, and *Hin*fI to create fragments −702/+34, −488/+34, −390/+34, and −175/+34, respectively, which were cloned into pHCAT. In addition, for experiments with a heterologous viral thymidine kinase (TK) promoter, a 31-bp sequence corresponding to the region −200 to −169 or a 30-bp sequence corresponding to the region −180 to −150 was synthesized (by Oligos Etc., Guilford, CT) and blunt end ligated at the *Bam*HI site of the pBL2CAT vector upstream from the TK promoter (the pBL2CAT construct was a gift from J. W. Pike, Ligand Pharmaceuticals, San Diego). pAV-hVDR, which directs expression of the full-length human vitamin D receptor (VDR), was also a gift of J. W. Pike (17). Plasmid DNA from all clones was isolated through two cesium chloride gradients by standard protocols (15).

Cell Transfection and CAT Assay. Ros 17/2.8 cells (18) were cultured at 37°C and 5% CO_2/95% air in Ham's F-12 medium with glutamine (GIBCO) supplemented with 10% Nu-Serum (Collaborative Research). CV-1 monkey kidney fibroblasts (American Type Culture Collection) were grown at 37°C and 5% CO_2/95% air in minimal essential medium (MEM) (GIBCO) supplemented with 10% fetal calf serum (GIBCO). Transfection of Ros 17/2.8 cells was carried out by DEAE-dextran (15) using 4 μg of plasmid DNA per 10^6 cells. Transfection of CV-1 cells was carried out with $CaPO_4$ (15) using 1 μg of heterologous DNA chimera, 3 μg of human VDR and 4 μg of β-galactosidase reporter gene (pCH110 from Pharmacia). After transfection of CV-1 cells, serum was removed for 5–6 h. CV-1 cells were then cultured in MEM supplemented with 5% Nu-Serum and treated with 1,25-$(OH)_2D_3$. At 48 or 65 h (48 h for the heterologous promoter construct and 65 h for all other studies) posttransfection cells were harvested in 250 mM Tris·HCl (pH 8.0). The cells were lysed by repeated freeze–thawing and the protein concentration of the lysate was quantitated by the method of Bradford (19). CAT activity was assayed with 50 μg of lysate protein as described (20). CAT activity was quantitated by densitometric scanning of autoradiograms using a Shimadzu CS-9000 U dual-wavelength flying spot scanner (Shimadzu, Columbia, MD). CAT activity was also quantitated by scanning TLC plates with the AMBIS radioanalytic imaging system (San Diego). Cotransfection with the β-galactosidase reporter gene as a control resulted in no difference in β-galactosidase activity (21) under conditions in which changes in CAT activity were observed.

Gel-Retardation Assay. DNA fragments of the mouse calbindin-D_{28k} promoter corresponding to −208/−158 and −180/−150 were labeled with ^{32}P. Nuclear extracts were prepared from Ros 17/2.8 cells treated with 10 nM 1,25-$(OH)_2D_3$ or 2 mM sodium butyrate for 48 h and COS cells that had been transfected with pAV-hVDR or mock transfected (nuclear extracts from transfected and nontransfected COS cells were a generous gift of J. W. Pike). The gel-retardation assay was performed as described (20) with 1 ng of labeled probe and 7.5 μg of Ros 17/2.8 or 5 μg of COS nuclear extracts. Nuclear extracts were prepared by the method of Dignam *et al.* (22) as modified by Holthuis *et al.* (23).

RESULTS

The mouse (BALB/c) kidney genomic library was screened with a ^{32}P-labeled mouse calbindin-D_{28k} cDNA, which contains most of the coding region and the 3′ noncoding region (this cDNA is 400 bp short on the 5′ end and starts at exon 4; ref. 9). Analysis of 8×10^6 clones by colony hybridization resulted in 10 positive clones. Restriction analysis indicated that all positive clones contained an ≈35-kb insert. The 5′ end of the gene was identified by probing Southern blots with a 33-mer oligonucleotide probe starting from the initiation site of rat calbindin-D_{28k}. A 1.3-kb *Pst* I fragment that hybridized with the probe but not with the mouse calbindin-D_{28k} cDNA was subcloned and sequenced using the 33-mer oligonucleotide as a primer. Sequence analysis indicated that a TATA box is present 30 bp upstream from the cap site. There are two GC boxes at −37 and −88. The consensus for the CAT box is present at −255 (Fig. 1).

Transient transfection of the mouse calbindin-D_{28k} promoter CAT construct (−1075/+34) in various VDR-containing cells including rat insulinoma cells (RIN_r), which also contain endogenous calbindin-D_{28k} (4), pig kidney cells ($LLC\text{-}PK_1$), and rat osteosarcoma cells (Ros 17/2.8), resulted in basal CAT expression only when the promoter was in the sense orientation. 1,25-$(OH)_2D_3$ (1–100 nM)-dependent CAT activity was not detected with the −1075/+34 construct after transfection in RIN cells, $LLC\text{-}PK_1$ cells, or Ros 17/2.8 cells (data not shown). In RIN and $LLC\text{-}PK_1$ cells, basal CAT expression was low. The highest level of basal expression was observed in Ros 17/2.8 cells, which were used for all further studies. Using the Ros 17/2.8 cells and the −1075/+34 CAT construct, we found that treatment with estradiol (100 nM; using phenol red-free medium) or dexamethasone (100 nM) also did not result in increased CAT activity (data not shown). However, the 5′ flanking region was found to mediate a dose-dependent stimulation by sodium butyrate, a differentiating agent that has been reported to modulate expression of a number of genes (24) including calbindin (4) [an 8-fold induction is observed at 2 mM sodium butyrate (Fig. 2)].

We next used deletion mutant analysis (Fig. 3) to identify regions involved in basal and inducible expression of calbindin-D_{28k}. Based on our findings with butyrate (Fig. 2), we also treated cells with 1,25-$(OH)_2D_3$ in the presence of butyrate to test the possibility that butyrate may activate factors that may be involved in a 1,25-$(OH)_2D_3$-dependent response. Deletion mutant analysis indicated that basal CAT expression increased 5- to 6-fold with the deletion of 587 bp of upstream sequence (data not shown), suggesting the presence of an upstream repressor sequence. In addition, deletion analysis indicated that the sequences involved in butyrate-mediated induction of CAT activity were located in the −175/+34 fragment (Fig. 3). Besides this direct effect, a segment located between −702 and −488 was consistently found to confer a 1,25-$(OH)_2D_3$-dependent increase in CAT activity in the presence of butyrate (Fig. 3*B*). Incubation of cells in the

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-1075      GACTT GTCTGGAGAA GACAATTGGT TGCCTCACAA TCCCACTATT GGAGGAAACA
-1020 CAAATGTGGT GAATTCCATA TTCTATGACA CACGTGAAAC CTGTGGCGCT TCATGAGCTC
 - 960 GTTTTGAAAG TTTATACCAC ACTGGTTTGC TTTTGTGTTA CTGTAACCAG ACACATAGGG
 - 900 TTCATTTTCA GCAGACCATA AGGTCTCAAA AGATGGACAA TTAGATAACT TAGAAATACT
 - 840 TACGTAAAAG AAAACCTAAA TACAGTATTT GTCATATTAA AATCCAATTG TAACATGTAG
 - 780 CCGGATATTT TTCCCACCTC TAATGATTTC CAGTTTCTGG AAAAAAATCC CTCACCTAGA
 - 720 GATAGAAGCA GCGCAGCTGT AAAATCAGTG AGTGGGGCTG CTACGGAGTC ACTGGTTAGC
 - 660 CTGGTGACAT TTCTTTCAGT TTCACTTTGT AAGATGCAAG TAACTAATGG CATCGATAAA
 - 600 ATCACTTCCT TCCTAACATC TTAAATTCTT ATAAGTTAAT TCTACTACAT TCCAATAATT
 - 540 CTGCTTCAAG CTCAAAAAGT AACAACAGCA AGAGCAGCAG ACTCCTGCAT TCTGCTGTCT
 - 480 CTAAGCATAG CTCACATCTT AAACAGCCAC GTGATGGTCT CCATTAGCGA ATATGAAGCA
 - 420 TTGTTACAAT TAACCACAGC AACGTATGCA TTAATCAAAT TAAACTAACA CTTGACATCT
 - 360 GATTTTGTTC AAATACTCAA CTGCCTCGAT AAATACTAAG TAGACAAAAT CTCCACTGAC
 - 300 GTGGTTTATC AGTCAGCTTT CCCTTCCATC TGAAAAAAAA TCAAACAATT CTAGGTATGT
 - 240 TGCTTTACTC TAACATTCAG GAGTGAAAGC CTCCCTGAAC CTGGGGGATG TGAGGAGAAA
 - 180 TGAGTCTGAG CAAGCTCCAA AACTCCAGCT CCAGCTATTT CCTGGGAAGA GAGAAATCGG
 - 120 AGGGGAGGGG AAGAAGGTTG GTGAGAGCAA GAGGCGGGAG CTAGGAAAAG GAGGCAGGAG
 - 60 GAGGCGTGGC CCGGCCTGGG GCGGCGGGA TAAATACAGA GAACTGGGTG CGGGGTGCGG
+  1 AGAACTCCGG AGGACGCCCG AACGGAGCAG CACCGCGGAC AGCGCCCCGC CGCGCCGCGC
  61 CCAGCTCAGC CTGCGCAGCC CTCTCGCCCG AGGTTCGCGC TCCGCGCACT CTCAAACTAG
 121 CCGCTGCACC ACGATGGCAG AATCCCACCT GCAG
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FIG. 1. Nucleotide sequence of the 5′ flanking region of the mouse calbindin-D_{28k} gene. Transcription initiation site (single underlining), TATA box (underlining and overlining), GC box (dashed underlining), CAT box (double dashed underlining), and translation initiation site (double underlining) are indicated.

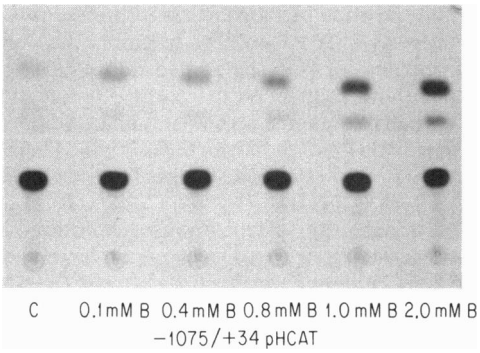

FIG. 2. Transcriptional activity of the −1075/+34 pHCAT construct in Ros 17/2.8 cells. After transfection, cells were treated with various concentrations of sodium butyrate (B). CAT activities were assayed 65 h after transfection. Data are representative of four to six separate assays. C, control.

presence of 1,25-$(OH)_2D_3$ and butyrate (0.4 and 0.8 mM) resulted in a 3- to 4-fold stimulation in CAT activity above the stimulation observed with butyrate alone. Further analysis indicated that butyrate could confer responsiveness in this upstream region to dexamethasone (100 nM) (data not shown) as well as to 1,25-$(OH)_2D_3$, suggesting that the potentiating effect of butyrate was not specific for 1,25-$(OH)_2D_3$. Since the effect was not specific, the mechanism of the enhancement of the butyrate effect by the steroids was not further investigated at this time. However, another segment located between −390 and −175 consistently contributed a significant 2- to 3-fold induction of CAT activity in the presence of 1,25-$(OH)_2D_3$ alone (Fig. 3*D*). In this region, the sequences between −198 and −176 have homology to the rat (25, 26) and human (27) osteocalcin vitamin D response elements (VDREs):

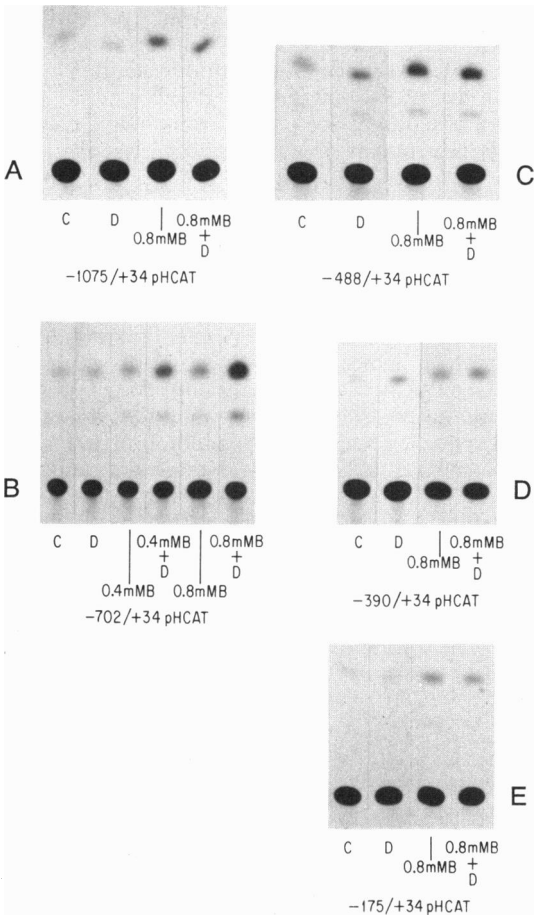

FIG. 3. Transcriptional activity of deletion mutants. Cells were transfected with various CAT constructs containing fragments of the calbindin-D_{28k} promoter. (*A*) −1075/+34 pHCAT. (*B*) −702/+34 pHCAT. (*C*) −488/+34 pHCAT. (*D*) −390/+34 pHCAT. (*E*) −175/+34 pHCAT. After transfection, Ros 17/2.8 cells were treated with either vehicle (C), 100 nM 1,25-$(OH)_2D_3$ (D), 0.4 mM butyrate (0.4 mM B), 0.8 mM butyrate (0.8 mM B), 0.4 mM butyrate and 100 nM 1,25-$(OH)_2D_3$ (0.4 mM B+D), or 0.8 mM butyrate and 100 nM 1,25-$(OH)_2D_3$ (0.8 mM B+D). CAT activities were assayed 65 h after transfection. Controls in *A–E* are not directly comparable since the data shown in each are from separate experiments representing different autoradiographic exposure times. Data shown are representative of six to eight similar experiments.

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                    -457                              -432
rat osteocalcin:    CTGGGTGAATGAGGACATTACTGACC

                    -200                              -170
mouse calbindin-D28k: CTGGGGGATGTGAGGAGAAATGAGTCTGAGC
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To further analyze the 1,25-$(OH)_2D_3$ responsive region between −390 and −175, a 31-bp sequence corresponding to the region −200 to −169 was synthesized. We found when multiple copies of the −200/−169 fragment were blunt end ligated at the *Bam*HI site of the pBL2CAT vector upstream from the TK promoter, there was a dose-dependent induction of CAT activity by 1,25-$(OH)_2D_3$ [2.0-fold at 1 nM, 4-fold at 10 nM, and 5-fold at 100 nM ($P < 0.05$); Fig. 4*A*), indicating that 1,25-$(OH)_2D_3$ responsiveness can be observed with this specific homologous segment cloned upstream from a heterologous promoter.

To examine whether the region containing the homologous calbindin promoter sequences binds to nuclear factors, gel-retardation assays were performed. Incubation of a ^{32}P-labeled probe (calbindin promoter sequences −208/−158) with the Ros cell nuclear extracts resulted in a specific DNA–protein interaction, as indicated by a band of retarded mobility (Fig. 4*Ba*, lane 2). Addition of unlabeled oligonucleotide representing the calbindin VDRE (−200/−169) or unlabeled nucleotide representing the rat osteocalcin VDRE (−469/−433) specifically competed with the probe, and this competition was demonstrated by abolishing the band of retarded mobility (lanes 4 and 5, respectively). However, addition of a nonspecific fragment from the promoter region did not compete with the probe (lane 3). In addition, when the calbindin-D $_{28k}$ fragment, which included the sequences homologous to the rat osteocalcin VDRE, was incubated with nuclear extracts from 1,25-$(OH)_2D_3$-treated COS cells that had been transfected with pAV-hVDR (Fig. 4*Bb*, lane 1) or mock transfected (lane 2), a band unique to pAV-hVDR-transfected cells was consistently observed (lane 1).

When the human VDR expression vector was cotransfected with the calbindin VDRE (−200/−169) TK CAT construct into receptor-negative CV-1 cells, we found that treatment with 1,25-$(OH)_2D_3$ led to a receptor-dependent induction in CAT activity (Fig. 4*C*), thus suggesting a direct role for VDR in 1,25-$(OH)_2D_3$-dependent transcriptional regulation of calbindin-D_{28k}.

After identifying the vitamin D responsive region in the calbindin promoter, we analyzed the butyrate responsive region, which we had found to be located in the −175/+34 fragment. After transfection of a −78/+34 CAT construct (obtained by using *Mae* I restriction enzyme), we found that CAT activity was not enhanced by butyrate (Fig. 5*A*), suggesting that the butyrate responsive region is located between −175 and −78. In this region, sequences located between −172 and −160 were found to contain sequences homologous to a 14-bp fragment in human immunodeficiency virus type 1 long terminal repeat promoter, which has been shown to enhance transcription in response to sodium bu-

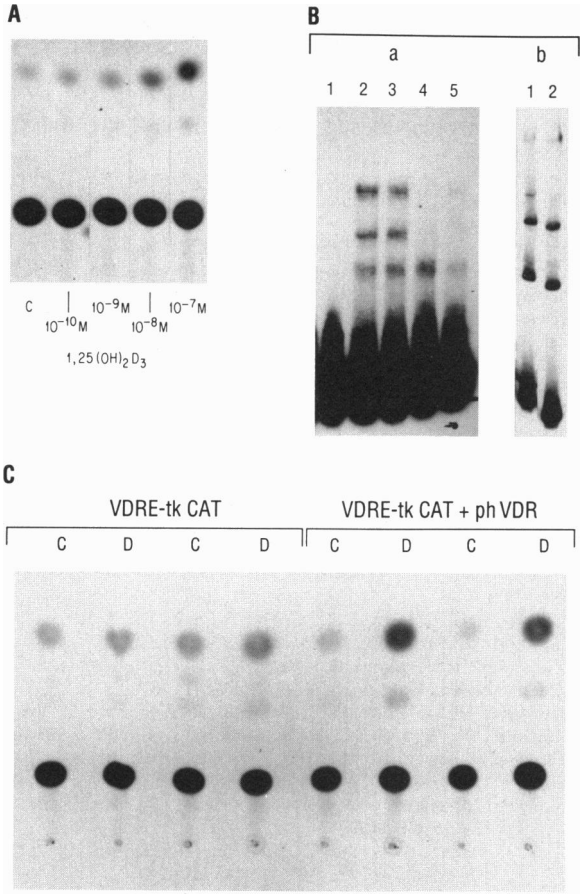

FIG. 4. Enhancement of transcription of a heterologous promoter using a synthetic oligonucleotide representing the mouse calbindin-D $_{28k}$ VDRE and gel-retardation assay using the putative calbindin-D $_{28k}$ VDRE. (*A*) Multiple copies of synthetic VDRE (−200/−169) were ligated at the *Bam*HI site of pBL2CAT to generate a heterologous (VDRE-TKCAT) promoter construct. After transfection, Ros 17/2.8 cells were treated with either vehicle (C) or various concentrations of 1,25-$(OH)_2D_3$. CAT activities were assayed 48 h after transfection. Similar results were observed in three additional experiments. (*B*) Gel-retardation assay. Labeled −208/−158 was used as a probe. (*a*) Nuclear extracts were prepared from 1,25-$(OH)_2D_3$-treated Ros 17/2.8 cells. Lanes: 1, probe alone; 2, crude nuclear extracts incubated with labeled probe; 3, competition experiment done in the presence of an unlabeled nonspecific DNA fragment from the calbindin promoter region −390/−249 (100-fold excess); 4, competition experiment done with 100-fold excess unlabeled oligonucleotide −200/−169; 5, competition experiment done in the presence of 100-fold excess unlabeled oligonucleotide representing rat osteocalcin VDRE (−469/−433). (*b*) Nuclear extracts were prepared from COS cells. ^{32}P-labeled probe was incubated in the presence of nuclear extracts prepared from COS cells transfected with human VDR expression vector (lane 1) or in the presence of nuclear extracts prepared from mock-transfected COS cells (lane 2). Similar results were observed in two additional experiments. (*C*) Cotransfection of human VDR expression vector and the calbindin VDRE (−200/−169) tkCAT construct into CV-1 cells. CV-1 cells were transfected with VDRE tkCAT alone (VDRE-tkCAT) or VDRE tkCAT together with the human VDR expression vector (VDRE-tkCAT + phVDR). Cells were treated with vehicle (C) or 10 nM 1,25-$(OH)_2D_3$ (D) and harvested 48 h after transfection. CAT assay was performed at constant β-galactosidase activity. Data are representative of four separate assays.

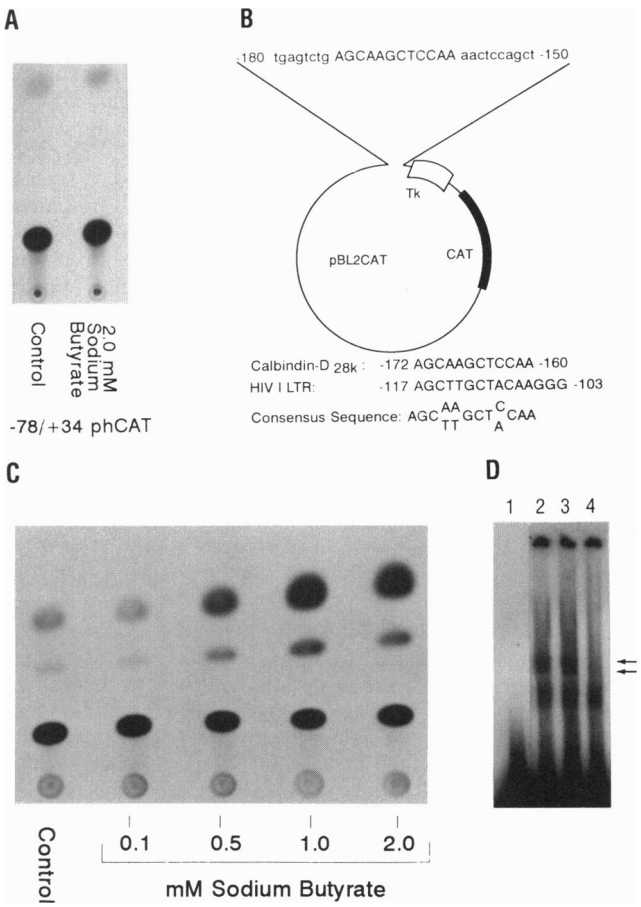

FIG. 5. Characterization of the butyrate responsive region in the calbindin-D_{28k} promoter. (*A*) Deletion analysis indicating that after transfection of a −78/+34 CAT construct, CAT activity is not enhanced by butyrate. (*B*) Schematic representation of a chimeric gene construct containing the putative butyrate responsive region (−180/−150) blunt end ligated at the *Bam*HI site of the pBL2CAT vector upstream from the TK promoter. Sequences showing homology to the butyrate-inducible elements in the human immunodeficiency virus type 1 long terminal repeat (HIV-1 LTR) are indicated. (*C*) Enhancement of transcription by butyrate of a heterologous promoter using a synthetic oligonucleotide representing sequences −180/−150 of the calbindin promoter. After transfection, Ros 17/2.8 cells were treated either with vehicle (C) or with various concentrations of butyrate. (*D*) Gel-retardation assay. ^{32}P-labeled −180/−150 was used as a probe. Nuclear extracts were prepared from 2 mM sodium butyrate-treated Ros 17/2.8 cells. Lanes: 1, labeled probe alone; 2, crude nuclear extracts incubated with labeled probe; 3, competition experiment done in the presence of an unlabeled nonspecific fragment from the calbindin promoter region −390/−249 (100-fold excess); 4, competition experiment done in the presence of 100-fold excess unlabeled oligonucleotide −180/−150.

tyrate by deletion mutant analysis (ref. 28; Fig. 5*B*). When the −180/−150 fragment was blunt end ligated at the *Bam*HI site of the pBL2CAT vector upstream from the TK promoter, a dose-dependent induction of CAT activity by butyrate was observed (Fig. 5*C*). Incubation of the butyrate responsive element with nuclear extracts from butyrate-treated Ros cells resulted in formation of protein–DNA complexes (Fig. 5*D*, lane 2). Addition of unlabeled nucleotide (−180/−150) resulted in loss of the upper bands (lane 4). A nonspecific fragment from the promoter was unable to affect this complex (lane 3).

DISCUSSION

In this report, we have identified sequence elements in the mouse calbindin-D_{28k} gene promoter that are involved in basal activation and respond to 1,25-$(OH)_2D_3$ and butyrate

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-200                                                    -160
  ct GGGGGA tgtg AGGAGA aa TGAGT ctg AGCAAGCTCCAA
     VDRE                                  butyrate
                                           response
                                           element
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Our findings represent functional analysis of the mammalian calbindin-D_{28k} promoter. At this time, little information is available concerning sequences important in regulation of the calbindin-D_{28k} gene. Functional analysis of the chicken calbindin-D_{28k} promoter indicated that a 1176-bp fragment was capable of inducing CAT activity in response to 1,25-$(OH)_2D_3$ 2-fold (29). However, the 1,25-$(OH)_2D_3$ responsive region was not further defined. We have found by dot matrix analysis no sequence homology between the chicken and mouse calbindin-D_{28k} promoters except in the region of the TATA box (unpublished data). More clearly defined are the sequences involved in regulating the 1,25-$(OH)_2D_3$-modulated bone proteins osteocalcin and osteopontin (25–27, 30–32). When comparing calbindin and osteocalcin 1,25-$(OH)_2D_3$ responsive sequences, it should be noted that the vitamin D response observed using calbindin-D_{9k} or calbindin-D_{28k} responsive sequences is modest (refs. 29 and 33; Fig. 3) compared to the induction of CAT activity by 1,25-$(OH)_2D_3$ observed using the osteocalcin VDRE (25–27, 30, 31). This may be due to lack of a suitable cell line that contains both high levels of endogenous VDR and calbindin (Ros 17/2.8 cells contain endogenous osteocalcin). Alternatively, the modest induction by 1,25-$(OH)_2D_3$ of calbindin gene transcription may be reflecting previous *in vivo* findings. We have previously shown that 1,25-$(OH)_2D_3$ can induce renal calbindin gene expression by a rapid transcriptional stimulation (peak at 1–3 h; 3.5-fold induction), which precedes the peak of accumulation of calbindin mRNA (12 h; >100-fold induction) and calbindin protein (48 h) (7). These results suggest that the small, early increases in calbindin mRNA and protein are due to stimulation of transcription. The large accumulation of mRNA long after 1,25-$(OH)_2D_3$ treatment suggests an effect on stabilization of calbindin-D_{28k} by 1,25-$(OH)_2D_3$. Similar *in vivo* findings were observed for regulation of chicken intestinal calbindin-D_{28k} (5) and rat intestinal calbindin-D_{9k} (6) by 1,25-$(OH)_2D_3$. Thus, there is only a modest 1,25-$(OH)_2D_3$-dependent transcriptional induction of calbindin (as indicated by both CAT activity and *in vivo* studies) and the large induction of calbindin mRNA by 1,25-$(OH)_2D_3$ (>100-fold) is primarily due to posttranscriptional mechanisms.

Although the exact mechanism by which butyrate induces calbindin-D_{28k} gene transcription is unknown, it is of interest that the region between −172 and −160 contains sequences homologous to butyrate responsive sequences in the promoter of the human immunodeficiency virus type 1 long terminal repeat (28). Besides calbindin-D_{28k} and human immunodeficiency virus type 1 long terminal repeat, sodium butyrate specifically stimulates transcription of a number of other genes, including metallothionein (34), chorionic gonadotropin (35), insulin (36), and glucagon (36). Comparison of the promoter regulatory regions of the calbindin-D_{28k} gene with the promoter regulatory regions of other genes induced by sodium butyrate may demonstrate common mechanisms involved in activation of cellular factors that are important in induction of these genes. It is possible that the butyrate responsive sequences may represent genetic elements acted upon by enhancer binding proteins and/or by nuclear factors that may be involved in differentiation.

In conclusion, these studies result in an increased understanding of the molecular mechanisms involved in regulation of the calbindin-D_{28k} gene. Our studies suggest that further definition of not only transcriptional but also posttranscriptional mechanisms involved in 1,25-$(OH)_2D_3$ action is needed to further our understanding of the regulatory mechanisms involved in the multiple actions of the vitamin D endocrine system. In addition, it will be of interest in future studies to further define the VDRE and the butyrate response element and to examine the responsiveness of the calbindin promoter to estrogen (37) and retinoic acid (38) in order to obtain increased insight into multiple steroid interactions as well as interactions of various transcription factors that may be involved in regulation of calbindin gene expression.

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