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Identification of a 1,25-dihydroxyvitamin D_3-response element in the 5′-flanking region of the rat calbindin D-9k gene

(hormone-responsive elements/calcium-binding protein gene)

HISHAM M. DARWISH AND HECTOR F. DELUCA*

Department of Biochemistry, University of Wisconsin–Madison, 420 Henry Mall, Madison, WI 53706

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ABSTRACT **The rat calbindin D-9k gene is transcriptionally regulated by 1,25-dihydroxyvitamin D_3 in the intestine. We have examined the 5′-flanking region of this gene and identified a 1,25-dihydroxyvitamin D_3-responsive element (DRE) between nucleotides −489 and −445. This element confers 1,25-dihydroxyvitamin D_3 responsiveness through its native promoter and the heterologous thymidine kinase promoter, and it contains the sequence GGGTGTCGGAAGCCC, which is homologous to the other previously identified DREs. Incubation of this element with the 1,25-dihydroxyvitamin D_3 receptor produced a specific protein–DNA complex, which shifted to a higher molecular weight form upon the addition of a monoclonal antibody specific to the 1,25-dihydroxyvitamin D_3 receptor. Therefore, the 5′-flanking region of the rat calbindin D-9k gene contains a DRE that mediates the enhanced expression of this gene by 1,25-dihydroxyvitamin D_3 in the intestine.**

The calbindin D-9k calcium-binding protein is a member of a large family of intracellular calcium-binding proteins that act to control the transport, availability, and storage of calcium (1, 2). This protein has been detected in various tissues, including the intestine (3–5), placenta (6), uterus (7, 8), yolk sac (9, 10), and fallopian tube of the rat (11). It has also been detected in small quantities in the kidney (12) and the growth cartilage (13). The primary sequences of the mouse (14), rat (15, 16), bovine (17), and porcine (18) proteins have been determined. The rat calbindin D-9k cDNA has been isolated and characterized (19). The rat gene for calbindin D-9k has also been isolated and sequenced (20, 21). It is a relatively small gene, spanning about three kilobases (kb) of genomic DNA, and consists of three exons flanked by two introns (20, 21).

In the intestine, vitamin D appears to be the primary regulator for the calbindin D-9k gene at the transcriptional level (22–25). However, no regulation by vitamin D is exerted on the gene in the uterus, where it seems to be primarily under estrogen regulation (7, 26). An active estrogen-responsive element (ERE) has been detected in the first intron of the gene at the border with the first exon (27), which is presumed to mediate the responsiveness of the gene to estrogen in the uterus. However, no vitamin D-responsive element has yet been identified in or around the calbindin D-9k gene. A number of vitamin D-responsive elements (DREs) have been identified and characterized in the 5′-flanking region of various other vitamin D-dependent genes, including the rat osteocalcin gene (28), the human osteocalcin gene (29, 30), and the mouse osteopontin gene (31). All of these DREs were shown to bind the vitamin D receptor and are able to mediate vitamin D-dependent transcriptional activation.

Since the isolation of this gene, we have tried to localize and identify a potential DRE with limited success. We describe here the identification and characterization of an active DRE in the 5′ upstream region of the calbindin D-9k gene.† This element, which is homologous to the other identified DREs, lies between −445 and −489 nucleotide from the transcription initiation start, binds vitamin D receptor, and confers vitamin D-dependent transcriptional activity.

MATERIALS AND METHODS

Recombinant Plasmid Construction. An ≈2.1-kb DNA fragment extending from the *Bam*HI site at ≈−2.0 kb to the *Ava* II site at nucleotide +88 was subcloned in the unique *Hin*dIII site of the pSVOCAT vector (32) to generate the pSVAV24CAT construct. This construct contains the promoter, the entire first exon, and 36 nucleotides of the first intron of the calbindin D-9k gene. The pSVAV24CAT construct was generated by cloning an *Ava* II fragment of the calbindin D-9k gene extending from nucleotides −431 to +88 into the *Hin*dIII site of the same pSVOCAT. Another set of deletion mutations in the 5′ upstream region of the calbindin D-9k gene was constructed by inserting the indicated fragments (see *Results* for details) into the *Bam*HI site of the pBLCAT2 vector upstream from the viral thymidine kinase (TK) promoter (33). The rat vitamin D receptor expression vector pVDR was constructed and characterized (unpublished results). All plasmids were purified twice after centrifugation on a cesium chloride gradient by using standard protocols (34).

Cell Transfection and Chloramphenicol Acetyltransferase (CAT) Assay. CV-1 African green monkey kidney cells were cultured to 65–70% confluency 24 hr prior to transfection. The cells were cotransfected with 6 μg of the vitamin D receptor expression plasmid and 10 μg of the test plasmid by using the standard calcium phosphate coprecipitation method (35). The transfected cells were dosed with either ethanol (vehicle) or 10 nM 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] and incubated for 72 hr at 37°C. The cells were harvested by scraping the cells into 0.25 M Tris·HCl buffer, pH 8.0. The cells were lysed by three cycles of freezing and thawing and the CAT enzyme was assayed as previously described (32), using 100 μg of lysate protein as measured by the Lowry protein assay (36). The acetylated chloramphenicol was quantitated by using a Betascope 603 blot analyzer (Betagen, Waltham, MA).

Gel Retardation Assay. A double-stranded 45-nucleotide DNA fragment corresponding to the sequence between nucleotides −489 and −445 was synthesized with *Bam*HI ends and used as a probe in the binding assay with the vitamin D

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Abbreviations: ERE, estrogen-responsive element; DRE, vitamin D-responsive element; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; PNE, porcine intestinal nuclear extract.

*No reprints will be available.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M83775).

receptor. The DNA was labeled by filling the recessed ends, using [^{32}P]dCTP (Dupont/NEN) and the Klenow fragment of DNA polymerase. The labeled fragment was purified by gel electrophoresis and electroeluted in the gel running buffer (6.7 mM Tris·HCl, pH 7.5/3.3 mM sodium acetate/1.0 mM EDTA). Porcine intestinal nuclear extract (PNE) was prepared as described (37) and used as a source of vitamin D receptor. Poly(dI-dC) (Boehringer Mannheim) was added at a concentration of 0.8 μg/μg of PNE protein in a buffer containing 5 mM Tris·HCl at pH 7.5, 20% (vol/vol) glycerol, bovine serum albumin at 10 mg/ml, 50 mM NaCl, and 5 mM dithiothreitol. The mixture was incubated for 15 min on ice, after which 2–3 ng of labeled probe was added. Incubation was continued for an additional 10 min at room temperature, and then the samples were electrophoresed on a 4% polyacrylamide gel for 3.5 hr at constant current (20 mA) at room temperature. In the competition mixture, the unlabeled probes were added at the indicated concentrations in the initial incubation mixture before the labeled probe was added. The specific monoclonal antibody directed against the vitamin D receptor (38) and a nonspecific antibody were mixed with the PNE prior to the addition of the labeled DNA.

DNA Sequencing. All sequencing of the 5′ upstream region of the calbindin D-9k gene and the clones were carried out by the dideoxynucleotide method (39).

RESULTS

Fig. 1 shows the sequence of the 5′ upstream region of the calbindin D-9k gene extending from nucleotide −1180 to +119. The position of the identified vitamin D-responsive sequence (see below) is underlined between nucleotide −489 and nucleotide −447 (43 nucleotides). This sequence is approximately 500 nucleotides away from the ERE sequence that is located at the 5′ end of the first intron at the border with the first exon (27). The region at the 5′ end of this sequence reveals an A+T-rich region between nucleotides −559 and −510.

To identify a possible vitamin D-responsive sequence in the 5′ upstream flanking region of this gene, several deletion constructs were generated to test their activity with the 1,25-$(OH)_2D_3$ receptor. The pSVAV2410 construct contains a DNA fragment extending from −2 kb to +88 nucleotides in the promoterless pSVOCAT vector as shown in Fig. 2. This construct contains the promoter of the calbindin D-9k gene. A truncated construct of this plasmid was generated by cloning a DNA fragment extending from nucleotide −431 to nucleotide +88 in the same CAT vector. Several other deletion constructs from the 5′ upstream region of the gene were generated in the pBLCAT2 vector as diagrammed in Fig. 2.

When the pSVAV2410CAT construct was cotransfected with the vitamin D receptor expression construct (pVDR) in

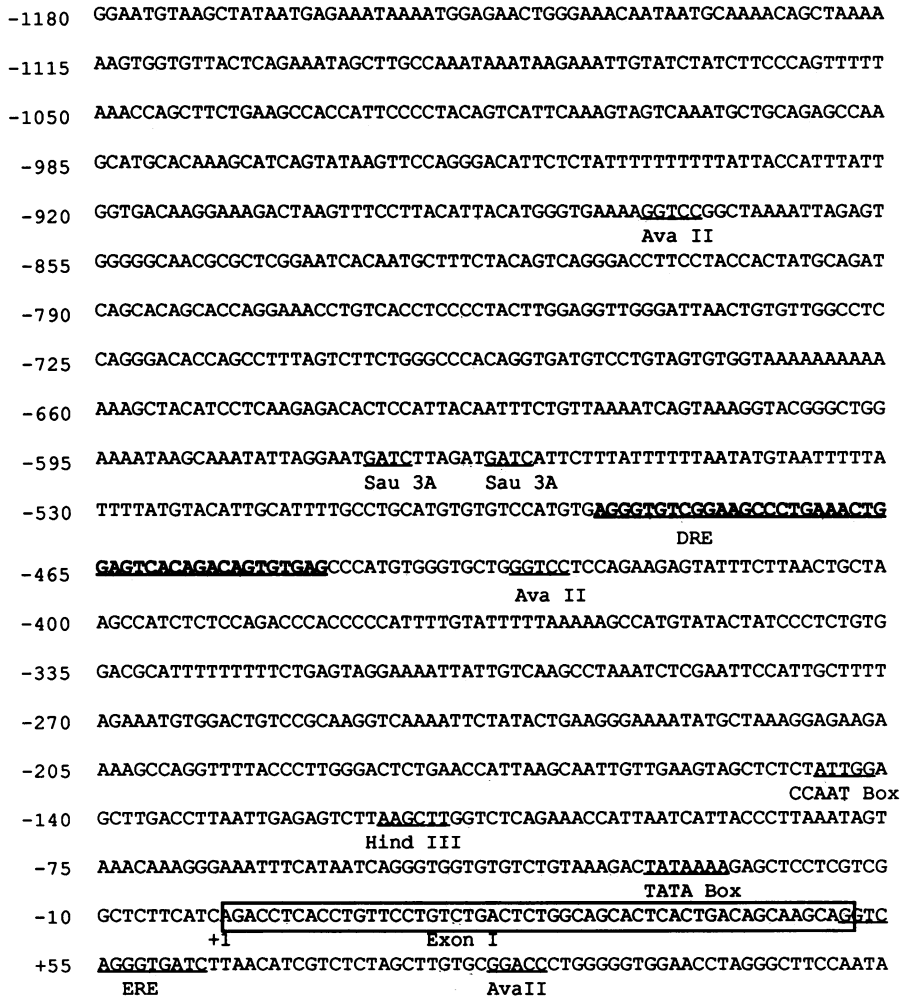

FIG. 1. Nucleotide sequence of the rat calbindin D-9k 5′-flanking region. The sequence from nucleotide −1180 to +119, which includes the first exon (box) and 95 nucleotides from the first intron, is shown. The TATA box and CCAAT boxes are shown and underlined. The ERE that is present at the border of the first exon with the first intron and the identified DRE (see below) in the 5′ region are marked and underlined. The restriction sites that were used for the deletion constructs are indicated, including *Hin*dIII, *Sau*3A, and *Ava* II sites. A+T-rich regions are noted at both the 5′ and 3′ sides of the putative DRE.

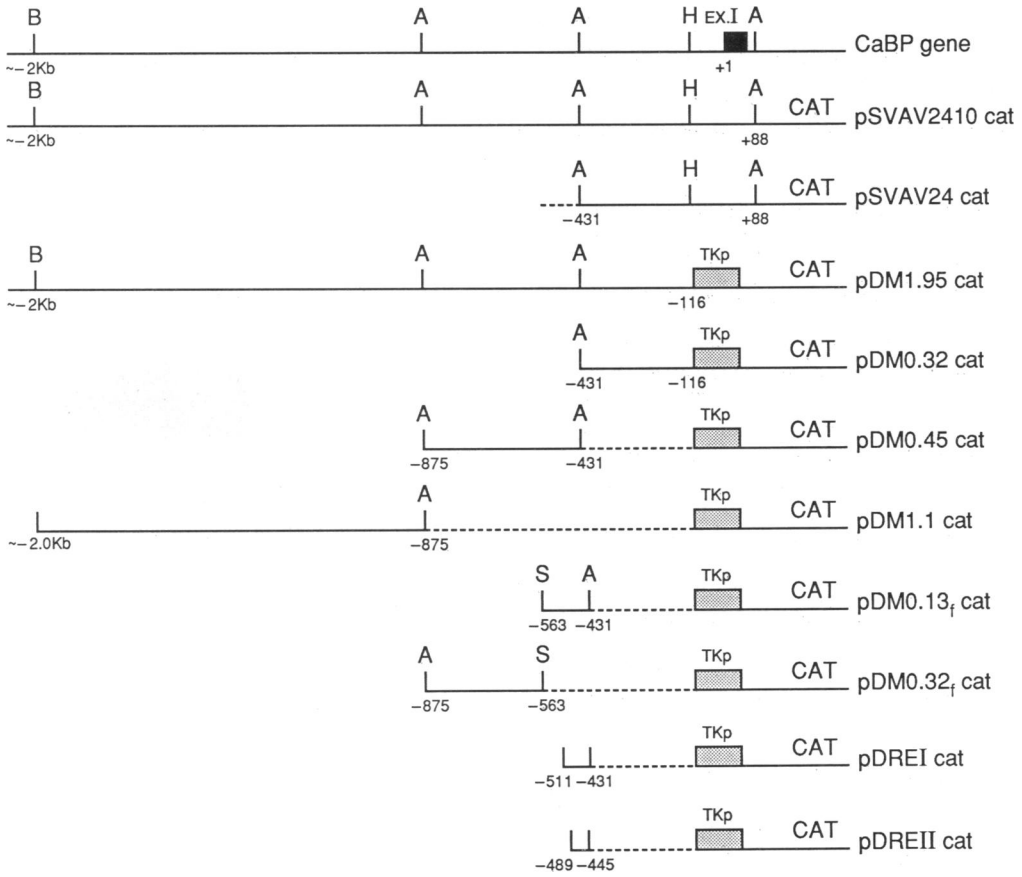

FIG. 2. Deletion constructs of the calbindin D-9k 5′-flanking region. A diagrammatic representation of the 5′-flanking region and a segment of the calbindin gene, marked CaBP gene, are shown with the position of some restriction sites, including *Bam*HI (B), *Ava* II (A), and *Hin*dIII (H) shown on the top. The deletion constructs pSVAV2410CAT and pSVAV24CAT were constructed in the pSVOCAT vector, while the rest of the deletion mutants were constructed in the pBLCAT2 vector. TKp, thymidine kinase promoter.

the CV-1 cells, an increase of approximately 3- to 4-fold of $1,25\text{-}(OH)_2D_3$-dependent CAT activity was detected (Fig. 3). This indicates that a $1,25\text{-}(OH)_2D_3$-responsive sequence does exist in the upstream region of the calbindin D-9k gene. This sequence seems to be localized between −2 kb and nucleotide −431, since no $1,25\text{-}(OH)_2D_3$-dependent CAT activity was detected from the pSVAV24CAT construct (Fig. 3). To further localize this sequence, the $1,25\text{-}(OH)_2D_3$-dependent activity of the deletion constructs in pBLCAT2 vector, which contains the viral TK promoter was evaluated in the same system. A 2- to 3-fold induction in CAT expression was detected with the pDM1.95CAT construct in response to treatment with $1,25\text{-}(OH)_2D_3$ (Fig. 4). This is in agreement with the results obtained with the pSVAV2410CAT construct, since this construct contains a DNA fragment from −2 kb to nucleotide −116 (Fig. 3). Furthermore, the sequence between nucleotides −431 and −116 does not seem to exert any inhibitory effect on the $1,25\text{-}(OH)_2D_3$-responsive sequence, which seems to be localized further upstream from nucleotide −431 (Fig. 4). As expected, the pDM0.32CAT

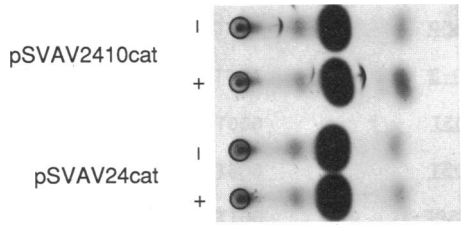

FIG. 3. Transcriptional activity of the deletion constructs in the pSVOCAT utilizing the native calbindin promoter. The plasmids were transfected into CV-1 cells and then dosed with 10 nM $1,25\text{-}(OH)_2D_3$ in ethanol (+) or ethanol alone (−). CAT activities were assayed 72 hr after transfection. The origins are on the left and the fastest-migrating species is diacetylated chloramphenicol.

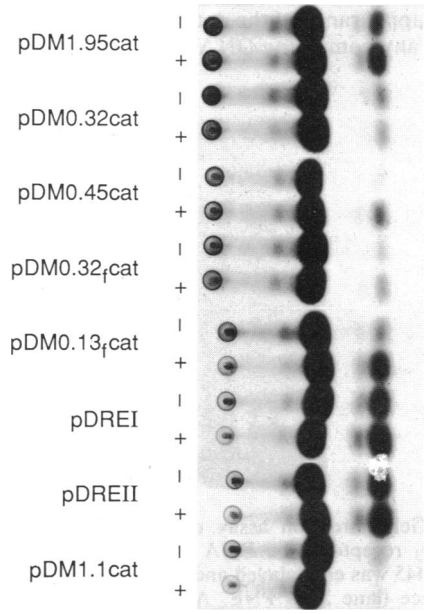

FIG. 4. Transcriptional activity of the deletion mutants constructed in the pBLCAT2 vector. Transfection and treatment were the same as for Fig. 3.

construct did not contain any responsive sequences, in agreement with the results obtained with the pSVAV24CAT construct. When the transcriptional activity of the sequence between nucleotides −875 and −431 was tested, a 1,25-$(OH)_2D_3$-dependent activity was apparent upon treatment with the hormone. This activity was further localized between nucleotides −563 and −431, since a 4- to 5-fold induction of the CAT level was detected with the PDMO.13fCAT construct (Fig. 4). The region between −2 kb and nucleotide −875 does not have a 1,25-$(OH)_2D_3$-responsive sequence, since no increase in the CAT levels was detected with the pDM1.1CAT construct.

Analysis of the nucleotide sequence between positions −563 and −431 reveals significant similarity with the other identified DREs in the region between −511 and −431 (see below). Therefore, the fragments between −511 and −431 and between −489 and −445 were cloned in the same vector and their activities were tested. Both the pDREICAT and the pDREIICAT constructs resulted in a slight (1.2- to 1.3-fold) induction of CAT in response to treatment with 1,25-$(OH)_2D_3$ (Fig. 4). Therefore, it seems that the presence of the sequence between nucleotides −563 and −511 contributes to the ability of the 1,25-$(OH)_2D_3$ receptor to induce transcription from this region.

To verify that this region contains a real 1,25-$(OH)_2D_3$-responsive sequence, the ability of the sequence between nucleotides −489 and −445 to bind the 1,25-$(OH)_2D_3$ receptor was tested by using the gel retardation assay. As shown in Fig. 5, when PNE was added to the labeled DNA fragment, a DNA–protein complex was detected (Fig. 5, lane 2) that was absent when no PNE was added (Fig. 5, lane 1). To verify that the observed complex contains a 1,25-$(OH)_2D_3$ receptor, a monoclonal antibody specific to the receptor (VD2F12) was added to the incubation mix. As is clear in Fig. 5, the addition of the antibody caused the complex to be retarded further (Fig. 5, lane 3), which was more obvious when more antibody was added (Fig. 5, lane 4). This effect is specific, since no effect on the complex was observed when a nonspecific antibody was used (Fig. 5, lane 5). As a further test for the specificity of the detected complex, the competition of unlabeled DNA with the labeled probe was tested. The addition of 50- (Fig. 6, lane 2) and 100- (Fig. 6, lane 3) fold excess of unlabeled probe to the incubation mixture resulted in a gradual disappearance of the detected complex formed in the absence of any competing DNA (Fig. 6, lane 1). Nonspecific

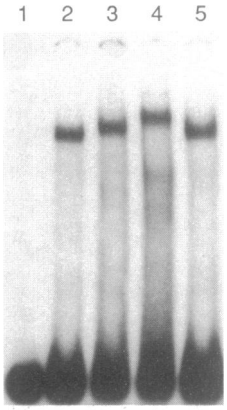

FIG. 5. Gel-retardation assay of the putative DRE with the 1,25-$(OH)_2D_3$ receptor. The DNA fragment between nucleotides −489 and −445 was end-labeled and incubated in the absence (lane 1) or presence (lane 2) of PNE. A specific monoclonal antibody against the 1,25-$(OH)_2D_3$ receptor was added to the mixture at 1:3 (vol/vol) antibody/PNA (lane 3) or at 1:1 (vol/vol) antibody/PNE (lane 4). Lane 5 shows the results obtained with a nonspecific antibody added at an excess of the antibody present in lane 3.

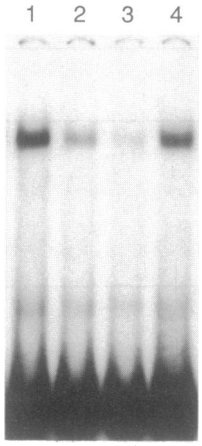

FIG. 6. Analysis of the DNA–protein complex by using unlabeled DNA probes. Labeled DNA probe (fragment −489 to −495) was incubated with PNE in the absence (lane 1) or presence of 20-fold excess (lane 2) or 100-fold excess (lane 3) of the same DNA probe, unlabeled. Lane 4 shows the result in the presence of 100-fold excess of unlabeled nonspecific DNA probe of the same size as the labeled DNA fragment.

DNA synthesized to the same size as the labeled probe was unable to affect the complex (Fig. 6, lane 4). These results indicate that the DNA sequence between nucleotides −489 and −445 is able to specifically bind the 1,25-$(OH)_2D_3$ receptor.

Fig. 7*A* shows the sequence between nucleotides −489 and −445, which was used as a probe in our binding studies. Fig. 7*B* shows that in a 15-nucleotide stretch at the 5′ end of this probe fragment between −488 and −474 there is a close similarity in the sequence of the DRE described here and the sequences of the other reported DREs as outlined by Umesono *et al.* (40).

DISCUSSION

In this report, we have identified a 1,25-$(OH)_2D_3$-responsive sequence in the 5′ upstream region of the calbindin D-9k gene. This sequence, which is located less than 1 kb upstream from the TATA box, lies between nucleotides −489 and −445 in a position similar to that of other identified DREs (28–31). In our studies, we have used CV-1 cells cotransfected with a 1,25-$(OH)_2D_3$ receptor expression vector with the various

A

A GGGTGTCGGAAGCCC TGAAACTGGAGTCACAGACAGTGTGAGCC

-489 - 445

B

CaBP	GGGTGT	CGG	AAGCCC
DR-3	AGGTCA	AGG	AGGTCA
rOST	GGGTGA	ATG	AGGACA
hOST	GGGTGA	ACG	GGGGCA
mSPP-1	GGTTCA	CGA	GGTTCA

FIG. 7. (*A*) Nucleotide sequence of the probe DNA fragment between −489 and −445. The sequence at the 5′ end of this fragment (box) is predicted to be the binding site of the 1,25-$(OH)_2D_3$ receptor. (*B*) Sequence comparison of the 15 nucleotides located at the 5′ end of the above probe (labeled CaBP) with the other identified DRE sequences as outlined by Umesono *et al.* (40).

deletion constructs of the 5′ upstream region of this gene, since no suitable intestinal cell line could be used in our investigation. Under the current conditions, the 1,25-$(OH)_2D_3$ receptor level was between 250 and 500 fmol/mg of protein (data not shown). This could be one reason why a 1,25-$(OH)_2D_3$-responsive region could be identified by using this system, since many cell types that were tried have a receptor level of less than 150 fmol/mg of protein, and the intestinal cells where this gene is expressed have receptor levels reaching approximately 1500 fmol/mg of protein as measured by an immunoradiometric assay specific to the 1,25-$(OH)_2D_3$ receptor (41). Other reasons could also include the presence of gene silencers in these cells or the lack of some nuclear factor(s) that is (are) required for the activity of this gene.

The 1,25-$(OH)_2D_3$ responsive sequence identified here appears to satisfy all criteria for a true vitamin D responsive element (DRE): (*i*) it confers 1,25-$(OH)_2D_3$ responsiveness with both its natural and a heterologous promoter; (*ii*) it binds specifically to the 1,25-$(OH)_2D_3$ receptor; and (*iii*) it is able to work in an orientation-independent fashion. Note, however, that this sequence has very little activity by itself, which could be due to its need to interact with other elements in the promoter region of the gene, as has been noted for the human osteocalcin gene DRE (29). This sequence contains a stretch of 15 nucleotides that is similar in sequence to the other identified DREs as was outlined by Umesono *et al.* (40). Therefore, this sequence could represent the DRE for this gene. Unlike the human osteocalcin DRE (42), no AP-1 binding site could be identified in the 1,25-$(OH)_2D_3$-responsive sequence of the calbindin D-9k gene.

It is interesting to note that A+T-rich regions are detected upstream of this element and also between this element and the TATA box. These sequences could be relevant to the activity of this sequence (43). However, at the present time we believe a DRE has been identified for the calbindin D-9k gene that presumably mediates the induction of this gene by 1,25-$(OH)_2D_3$ *in vivo*. This is the first step in understanding the detailed mechanism by which 1,25-$(OH)_2D_3$ regulates this gene. The interactions of this element with other positive and negative regulatory elements in the promoter region of this gene constitute an integral part in determining the tissue specificity of expression of this gene.

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