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Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D_3

(1α,25-dihydroxyvitamin D_3/human vitamin D_3 receptor/steroid response element/gene activation/osteocalcin gene promoter)

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ABSTRACT **Osteoblast-specific expression of the bone protein osteocalcin is controlled at the transcriptional level by the steroid hormone 1α,25-dihydroxyvitamin D_3. As this protein may represent a marker for bone activity in human disease, we examined the regulation of its expression at the molecular level by evaluating human osteocalcin gene promoter function. We describe regions within the promoter that contribute to basal expression of the gene in osteoblast-like cells in culture. Further, we define a 21-base-pair DNA element with the sequence 5′-GTGACTCACCGGGTGAACGGG-3′, which acts in cis to mediate 1α,25-dihydroxyvitamin D_3 inducibility of the osteocalcin gene. This response element bears sequence similarity with other short DNA segments, particularly those for estrogen and thyroid hormone, which act together with their respective trans-acting receptors to modulate gene transcription.**

Osteocalcin is an abundant osteoblast-specific noncollagenous bone protein that functions in mineralization under direct control of the vitamin D hormone 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] (1–3). Despite the controversy surrounding its function in bone, levels of this gene product in the circulation have been correlated with increased states of bone turnover, and thus osteocalcin may represent a marker for metabolic bone disease (4–6). In view of the importance of this protein as a potential marker for bone pathophysiology, knowledge of its transcriptional regulation in osteoblasts is essential.

The modulation of osteocalcin levels by hormones such as 1,25$(OH)_2D_3$ is widely believed to occur at the level of transcription (7, 8). The mechanism of vitamin D_3 induction likely involves the 1,25$(OH)_2D_3$ receptor (VDR), a 48-kDa protein whose recent cloning and structural characterization (9, 10) suggest a DNA-binding protein belonging to the steroid receptor gene superfamily (11). The molecular nature of this interaction, as well as that with other vitamin D_3-inducible genes, however, has not been clarified. In this report, we describe regions within the osteocalcin gene promoter that contribute to transcriptional activation and then map a region that confers vitamin D response. The steroid response element-related nature of this sequence provides evidence that 1,25$(OH)_2D_3$ operates through its intracellular receptor to activate gene transcription analogously to that of other small nonpeptide hormones (12–14).

MATERIALS AND METHODS

Reagents. [1,25$(OH)_2D_3$] was a kind gift of Milan Uskokovic (Hoffmann–La Roche). [^{14}C]Chloramphenicol was obtained from New England Nuclear. Molecular cloning reagents and modifying and restriction enzymes were obtained from Promega Biotec. Standard molecular biological techniques were as described by Maniatis *et al.* (15).

Plasmid Constructions. Osteocalcin gene fragments were isolated and cloned through standard molecular techniques as described (15). A *Sac* I/*Pvu* II fragment (−344/+10; nucleotide positions are numbered relative to the cap site, with negative numbers indicating sequence extending 5′) of the osteocalcin genomic locus was cloned initially into the chloramphenicol acetyltransferase (CAT) expression vector pBL-CAT3 (16). A *Bam*HI/*Sac* I fragment (−1339/−338) and a *Bam*HI/*Bam*HI fragment (−3900/−1339) were sequentially added to create −3900/+10 and −1339/+10). The fragment −3900/−1339 was cloned directly. The −1339/+10 fragment was cleaved with either *Pst* I, *Bsp*MII, or *Pvu* II to create fragments −838/+10, −568/+10, and −413/+10, respectively. The −344/+10 fragment was cleaved with *Apa* I or *Nco* I to create −193/+10 and −75/+10. The −1339/−338 fragment was isolated and cloned directly. All plasmids in these studies were isolated through two cesium chloride gradients (15). Complementary synthetic oligonucleotides were synthesized with *Xho* I or *Sal* I restriction ends, annealed following denaturation at 95°C, and cloned into pBL-CAT2 (16). All constructions were sequenced for verification by the dideoxy method (17) by utilizing denatured double-stranded pBL-CAT templates.

Cell Transfection Techniques. ROS 17/2.8 cells were maintained in culture utilizing standard techniques and grown in Ham's F12 medium supplemented with 10% (vol/vol) fetal calf serum. Cells were transfected with 10 μg of plasmid DNA per 60-mm plate 24 hr after passage with methods that employed Polybrene (18). Ethanol or 1,25$(OH)_2D_3$ in ethanol was added to the cells (0.1% final ethanol concentration) following brief shock with glycerol, and cells were harvested on ice 72 hr posttransfection. Protein concentration was determined in cell extracts lysed in 0.25 M Tris·HCl at pH 7.8 (19), and the CAT activity of 25 μg of cellular protein was assessed in a 1-hr assay at 37°C as described (20).

Primer Extension Analysis. Analysis of the start site of transcription within the osteocalcin promoter plasmid was performed as described (21) with 50 μg of total RNA. The latter was isolated by the method of Chomezynski and Sacchi (22). Primer extension was performed with an end-labeled 25-mer oligonucleotide corresponding to the CAT gene leader from 8 to 33 nucleotides 5′ to the ATG. The cap site of the authentic mRNA for the human osteocalcin gene represents unpublished data from this laboratory. The same oligonucleotide primer was used in a dideoxy sequencing reaction of the pBL-CAT3 template containing the −1339/+10 osteocalcin promoter construction to determine the extension product size.

Site-Directed Mutagenesis. The osteocalcin gene fragment −1339/+10 was cloned into the *Bam*HI and *Sal* I sites of M13mp18 and subjected to site-directed mutagenesis as described (23) by using four 25- to 27-base oligonucleotides,

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

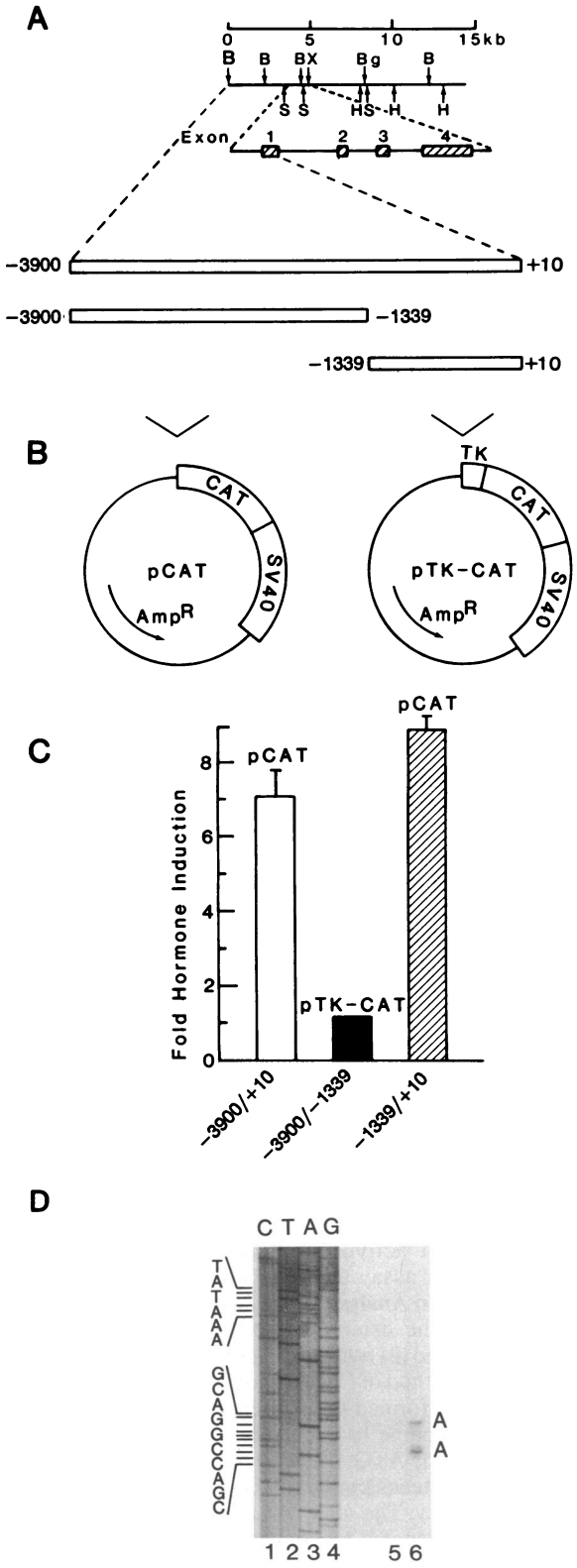

FIG. 1. Identification of a fragment of the human osteocalcin gene promoter, which is inducible by $1,25(OH)_2D_3$. (*A*) Organization of the cloned human osteocalcin gene (24) and identification of gene fragments −3900/+10, −3900/−1339 and −1339/+10 (numbers are given in bp). B, *Bam*HI; S, *Sac* I; Bg, *Bgl* II; H, *Hin*dIII; X, *Xba* I. Exons are indicated by hatched boxes. (*B*) CAT expression vectors with (pTK-CAT) and without (pCAT) the tk promoter (16). (*C*) Transcriptional activity of osteocalcin gene promoter fragments. CAT activity was assessed following incubation with or without $1,25(OH)_2D_3$ (10^{-9} M), and fold hormone induction represents the ratio of induced to uninduced chloramphenicol products. The degree of induction of the two pBL-CAT3 constructions was as follows: −3900/+10, 7.0 ± 0.68 (mean ± SEM), n = 3 and −1339/+10, 8.9 ± 0.46 (mean ± SEM), n = 9. (*D*) Messenger RNA transcription initiation site analysis. ROS cells were transfected with construction −1339/+10 and induced by $1,25(OH)_2D_3$, and total RNA was isolated 72 hr posttransfection. Lanes: 1–4, CTAG sequencing lanes; 5, cells transfected without −1339/+10; 6, cells transfected with −1339/+10. The two As indicate bands representing the expected primer extension product of 89 bases and a secondary product of 84 bases corresponding to the osteocalcin mRNA cap site within the sequence 5′-CA(89)GGCCA(84)GC-3′ upstream of the CAT initiation codon. The TATA homology is located 23 and 28 bp upstream. kb, Kilobases.

which were synthesized corresponding to the antisense sequence 5′ and 3′ of the deletion indicated. Four −838/+10 fragments, each bearing the specified deletion as ascertained by sequence analysis, were isolated with *Pst* I and cloned into the identical site in pBL-CAT3 for analysis. Quantitation of CAT activity was achieved by radioassay of isolated TLC spots.

RESULTS

To assess potential $1,25(OH)_2D_3$ regulation of osteocalcin, gene fragments obtained from the previously cloned human gene (ref. 24 and Fig. 1*A*) beginning 5′ at −3900 base pairs (bp) or −1339 bp and terminating at +10 bp relative to the transcription start site (nucleotide positions are numbered relative to the cap site, with negative numbers indicating sequence extending 5′) were fused to bacterial CAT gene expression vectors (Fig. 1*B*) and transfected into rat osteosarcoma (ROS 17/2.8) cells. These cells maintain an osteoblast-like phenotype and express both VDR and osteocalcin (25, 26). Each osteocalcin promoter construction exhibited both moderate basal promoter activity as well as a 7- to 9-fold elevation in activity following treatment with $1,25(OH)_2D_3$ (10^{-9} M) (Fig. 1*C*). Importantly, as assessed by primer extension analysis, transcripts derived from these constructions were correctly initiated, as seen for the −1339/+10 construct only in Fig. 1*D*. Since both fragments displayed similar activities in ROS 17/2.8 cells and the construction −3900/−1339 was not inducible, we conclude that significant control of osteocalcin gene expression, including response to $1,25(OH)_2D_3$, resides downstream of base pair −1339.

A low-resolution scan of the −1339/+10 osteocalcin promoter fragment for both changes in basal activity and $1,25(OH)_2D_3$ inducibility was achieved through unidirectional 5′ deletion analysis (Fig. 2*A*). Fragment −838/+10 alone exhibited enhanced basal and hormone-inducible activity (6.4 ± 1.3, mean ± SEM, n = 7) similar to that of larger gene fragments (−3900/+10 and −1339/+10, see Fig. 1). In contrast, fragments −568/+10 and −413/+10 each displayed a significant reduction in basal activity (5% of that seen in −1339/+10, see Fig. 2*B*), and only the larger fragment remained inducible in the presence of $1,25(OH)_2D_3$ (Fig. 2*B*). As the reduction in basal activity in the two constructions was generally reversed with fragments −344/+10, −193/+10, and −75/+10 (see Fig. 1*B*), these observations suggest that in the absence of strong basal enhancer activity lying upstream of −568, a dominant negative element may reside within the −413/+10 fragment. Most importantly, as fragment −413/+10 no longer retained hormone inducibility, a cis-acting vitamin D response element (VDRE) appears likely between nucleotides −568 and −413. These results suggest that both positive and negative cis-acting sequence elements may act in a combinatorial fashion to modulate basal transcription from the osteocalcin gene promoter in osteoblast-like ROS 17/2.8 cells. Certainly, however, other trivial explanations for changes in basal activity are possible, and

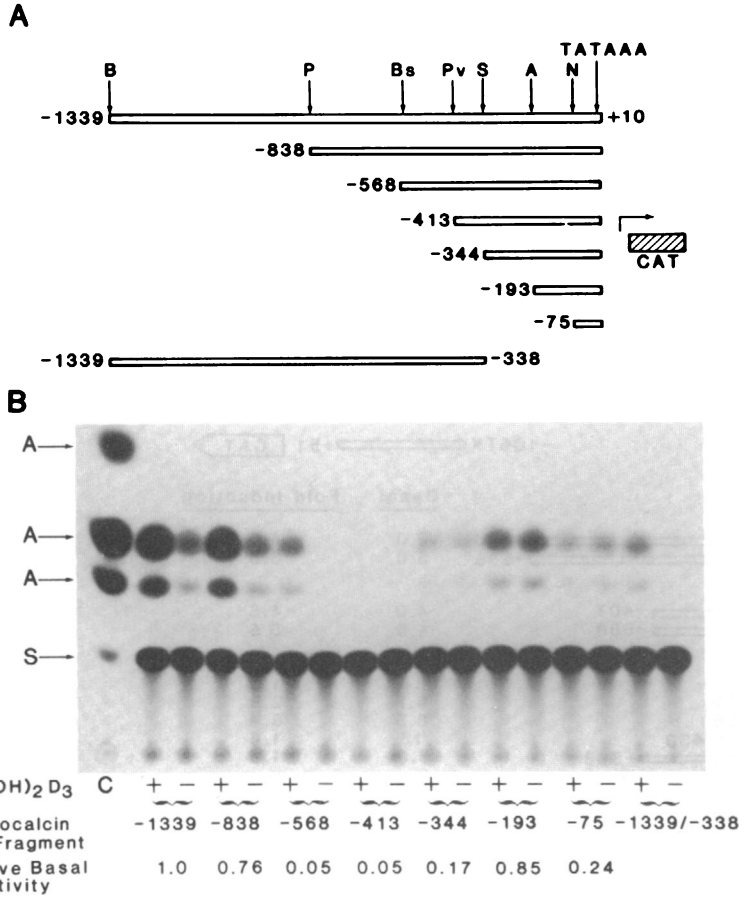

FIG. 2. Low-resolution scanning of the human osteocalcin gene promoter enhancer. (*A*) Isolated gene fragments that were tested for transcriptional activity. The fragment −1339/+10, as well as fragments whose 5′ termini are indicated, were constructed and fused to the promoterless CAT gene located in pBL-CAT3 (16). S, *Sac* I; B, *Bam*HI; P, *Pst* I; Bs, *Bsp*MII; Pv, *Pvu* II; A, *Apa* I; N, *Nco* I. (*B*) Transcriptional activity of osteocalcin gene fragments. Constructions were transfected into ROS 17/2.8 cells, treated with (+) or without (−) $1,25(OH)_2D_3$ (10^{-9} M) immediately after transfection, and evaluated for basal and $1,25(OH)_2D_3$-inducible response by CAT assay. Constructions are indicated by their 5′ terminal nucleotides when they end 3′ at +10 or by both coordinates (−1339/−338). Relative basal activity (percent conversion of input chloramphenicol substrate) of each of the 5′ constructions was normalized to the activity identified in the −1339/+10 construction. The latter's actual basal activity was 5% conversion of substrate to product. C, pure CAT control; S, substrate; A, mono- or diacetylated product. Assay shown is representative of six separate assays.

thus it will be necessary to map these sequences functionally. Nevertheless, these data clearly show that basal osteocalcin promoter activity is significantly enhanced in the presence of $1,25(OH)_2D_3$ through an element whose activity is lost following 5′ deletion at −413.

To localize more precisely the sequence(s) that confers $1,25(OH)_2D_3$ response, the osteocalcin gene fragment −1339/+10 was subjected to site-directed mutagenesis. Four 44- to 57-bp internal deletions were created, which together scanned the region from −571 to −397. Reporter gene activity (CAT) of these mutant osteocalcin gene fragment–CAT constructions indicated that, whereas the deletion of base pairs −571 to −525 or −454 to −397 had no effect on hormone inducibility, fragments containing deletions spanning −532 to −488 and −503 to −443 were inactive (Fig. 3*A*). As both deletions resulted in loss of response, these data suggest the presence of a VDRE located not only between nucleotides −532 and −443 but positioned to include base pairs −503 to −488, which had been deleted in both constructions. Interestingly, loss of vitamin D_3 response concomitantly resulted in a decrease in basal promoter activity (Fig. 3*A*). The cause of this decrease is unclear, but similar decreases have been noted during estrogen receptor induction of prolactin gene expression (27).

To examine whether sequences within the osteocalcin promoter could act as a $1,25(OH)_2D_3$-inducible enhancer, fragments −568/−338 and −568/−407 were fused to the herpes simplex virus thymidine kinase (tk) promoter (−105 to +51) located 5′ to the CAT gene and analyzed for activity. Both fragments, fused in either orientation relative to the tk promoter, were capable of conferring vitamin D_3 responsivity (Fig. 3*B*), although apparently to a slightly lesser degree with this heterologous promoter system (4.0- to 6.0-fold) than in the native gene (Figs. 1*C* and 2*B*). This observation suggests the possibility that less optimal positioning of the VDRE relative to components of the tk promoter may lead to a reduction in protein–protein interactions known to be required for productive transcriptional regulation (28, 29). In addition, although the −568/−338 construction was anticipated to contain silencing activity, its basal level was similar to that of −568/−407. Further experiments will be necessary to confirm the nature of the repression phenomenon within the native gene. Nevertheless, a DNA segment whose sequence characteristics are suggestive of a typical hormone-responsive element is clearly evident within the fragment −568/−407. This fragment includes base pairs −503 to −488, which were identified above to contain potentially a VDRE.

The ability of osteocalcin gene fragments to transfer hormone response to a heterologous promoter allowed us to test in ROS 17/2.8 cells a series of synthetic complementary overlapping oligonucleotides, which together spanned the region −568 to −368, and subsequently, a specific oligonucleotide suspected of bearing a VDRE sequence. We first examined the overlapping oligonucleotide series spanning the region of interest. Consistent with the results observed earlier, only the synthetic gene fragment −538/−469 contained sequences that conferred $1,25(OH)_2D_3$ response in either orientation (Fig. 3*B*). Since the synthetic fragment −503/−433 was inactive, this observation suggests that additional sequences 5′ to base pair −503 must comprise a portion of the VDRE essential for $1,25(OH)_2D_3$ activation. Finally, complementary oligonucleotides comprising the sequence between −509 and −489, which displayed apparent homology to other steroid response elements (30–32), were synthesized, and the double-stranded DNA fragment with the sequence 5′-GTGACTCACCGGGTGAACGGG-3′ was fused to the tk promoter in one, two, and three copies. Evaluation of reporter activity following transfection revealed increasing response to $1,25(OH)_2D_3$ as a function of VDRE copy number, with maximal response evident when

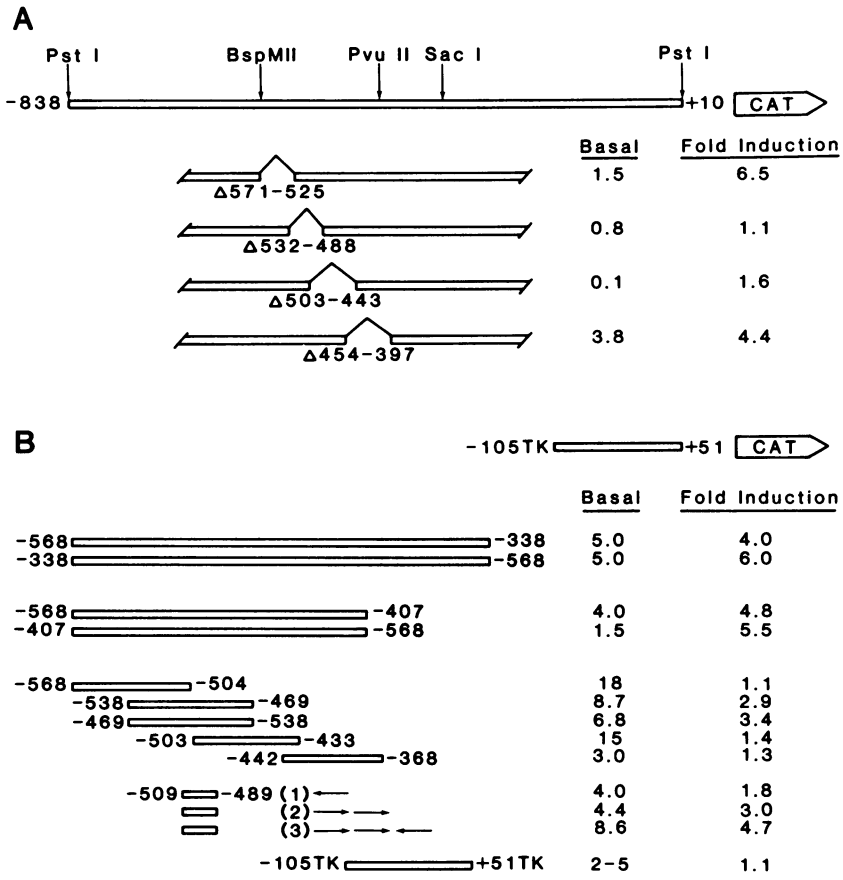

FIG. 3. Loss of $1,25(OH)_2D_3$ response in osteocalcin gene fragments bearing internal deletions and transfer of inducibility to the heterologous herpes simplex virus–tk promoter. (*A*) Osteocalcin promoter fragment −1339/+10 was subjected to site-directed mutagenesis to create four internally deleted constructions: Δ−571/−525, Δ−532/−488, Δ−503/−443, and Δ−454/−397. These modified DNA fragments were removed from M13mp18 at −838/+10, cloned, and evaluated. (*B*) Native osteocalcin fragments −568/−338 and −568/−407 were isolated from preexisting constructions and evaluated in the pBL-CAT2 vector (16). Complementary oligonucleotides spanning the osteocalcin sequence from −568/−504, −538/−469, −509/−489, −503/−433, and −442/−368 were synthesized, cloned into pBL-CAT2, and evaluated. Basal activity is as defined in Fig. 2. Basal and fold inductions indicated represent the averages of two to four separate experiments. pBL-CAT2 vector alone, whose basal activity ranged from 2 to 5% depending upon the experiment, is designated −105tk/+51tk. The arrows indicate the copy number and orientation of the sequence −509/−489.

three elements were evaluated (Fig. 3*B*). Inducible activity with the multiple copy VDRE plasmid (4.7-fold; Fig. 3*B*) was essentially equivalent to that achieved with fragments encompassing −568 to −407 in tk fusions. Although this observation is suggestive of several copies of the VDRE in this region, inspection of the nucleotide sequence between −568 and −407 does not support the presence of additional sequences related to base pairs −509/−489. Rather, the requirement for increased copy number may serve as replacement for other cis elements present in the natural gene but deleted in the synthetic 21-bp osteocalcin gene fragment–tk promoter fusion. This possibility has been explored in depth experimentally in recent reports (28, 29).

The sequence of the VDRE is identified in Table 1, where it is aligned relative to other known steroid response elements (SRE) (30–32). The core sequences of each are placed 5′ and 3′ of a gap of 2–6 nucleotides in the SREs and 5 bp in the VDRE. The role of these 5 bp in putative VDR sequence recognition is as yet undetermined, but it may be relevant that 4 of these 5 bp contribute to a perfect 5-bp palindromic sequence within the VDRE. This motif is a generalized feature of identified SREs and forms the basis for putative receptor homodimeric binding (33, 34). The VDRE contains features strongly in common with those of the other SREs. The VDRE differs in the 3′ half by only 1 or 2 bases relative to the thyroid and estrogen response element sequences, respectively, and the 5′ half also shares significant sequence similarity. The relationship of the VDRE with these specific elements strengthens the observation that the DNA-binding domain of the VDR displays highest homology within the receptor gene family to related domains of the estrogen and thyroid receptors (9–11).

DISCUSSION

The results presented here and which are summarized in the model of the osteocalcin gene promoter in Fig. 4 suggest that

Table 1. Sequence of the VDRE in the human osteocalcin gene and comparison with other SREs

Sequence			
5′	Gap	3′	Ligand
GACTCA	CCGGG	TGAACG	$1,25(OH)_2D_3$
AGGTCA	C A G	TGACCT	Estrogen
AGATCA	GGG ACG	TGACCG	Thyroxine
TGTACA	G G A	TGTTCT	Glucocorticoid/ progesterone

The sequence of the VDRE is indicated and aligned relative to response elements for estrogen (30–32), thyroxine (11, 32), and glucocorticoid/progesterone (33, 34). A dyad symmetry is apparent in all elements, and a gap of 2–6 bases is introduced between the partially symmetrical 5′ and 3′ halves. The underlined bases indicate components of the inverted repeat sequence, which contribute to the individual dyad symmetry of each element.

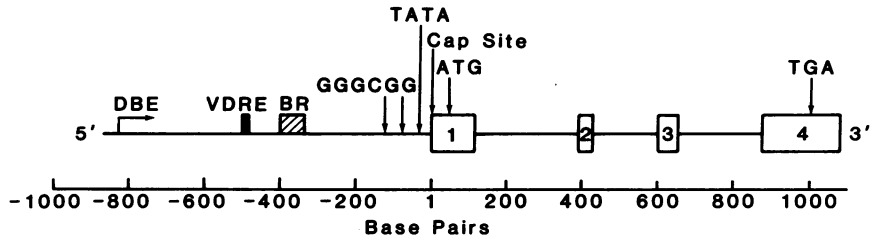

FIG. 4. Diagram of the human osteocalcin gene and its transcriptional control region. Features of the control region of the gene promoter including the cap site, TATA box homology, and VDRE, as well as the location of putative GC boxes, basal repressor (BR), and upstream basal enhancer (DBE) are indicated. Numbers 1–4 represent the four exons within the gene (24). Nucleotide base scale is indicated below the gene diagram.

human osteocalcin gene expression is controlled in osteoblasts principally by multiple elements lying less than a kilobase 5′ to the TATA homology. Recent investigations with the rat osteocalcin gene promoter arrive at similar but less detailed conclusions (35, 36). The present data define precisely the location and nature of a VDRE, an element that displays classical features of an SRE in its structure, ability to act in a position- and orientation-independent fashion, and in its nucleotide sequence, which bears regions of strong similarity to the DNA elements that confer steroid and thyroid hormone induction.

We have recently shown that osteocalcin promoter–enhancer inducibility is dependent upon the expression of the VDR (37). Thus, the availability of this sequence will now permit an analysis of the specific interaction that likely occurs between it and receptor. Importantly, the isolated VDRE requires several copies to function well and does not appear to confer the same degree of response to hormone that is seen in the native gene. Consequently, the element functions best within its own gene context, suggesting that interactions with other transcription factors must facilitate hormone inducibility. In this respect, identification of the VDRE in the osteocalcin gene clearly represents only a first step toward understanding the role of the VDR in modifying the transcriptional activity of the osteocalcin gene promoter. It does, however, provide the final evidence that 1,25-$(OH)_2D_3$ functions in a manner analogous to that of other steroid hormones. Analysis of interactions such as above at the molecular level should provide new insight, both into the mechanisms by which vitamin D operates as well as into the regulation and function of osteocalcin itself.

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