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Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression

(bone/hormone/transcription/calcification)

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ABSTRACT **The osteocalcin gene encodes a 6-kDa polypeptide, which represents one of the most abundant noncollagenous bone proteins, and the present studies establish that osteocalcin mRNA is detected only in bone tissue. An osteocalcin gene was isolated from a rat genomic DNA library, and sequence analysis indicated that the mRNA is represented in a 953-nucleotide segment of DNA consisting of four exons and three introns. A modular organization of the 5′ flanking sequences of the gene is reflected by the presence of at least three classes of regulatory elements, which include the following: (*i*) RNA polymerase II canonical sequences; (*ii*) a series of consensus sequences for hormone receptor binding sites and cyclic nucleotide responsive elements consistent with physiologic expression of the osteocalcin gene; and (*iii*) a 24-nucleotide sequence in the proximal promoter region with a CAAT motif as a central element. We have designated this highly conserved sequence as an "osteocalcin box" since only 2 nucleotide substitutions are found in the rat and human osteocalcin genes. We have demonstrated two factors regulating osteocalcin gene expression. First, a 200-fold increase occurs in normal fetal calvaria osteoblasts producing a mineralizing matrix, compared to confluent osteoblasts in a nonmineralizing matrix. Second, contained within the 600 nucleotides immediately upstream from the transcription start site are sequences that support a 10-fold stimulated transcription of the gene by 1,25-dihydroxyvitamin D.**

There has been much interest in the vitamin K-dependent protein of bone, osteocalcin (bone Gla protein), since its discovery over a decade ago (1). A distinguishing feature of this 5.7-kDa protein (46–50 amino acids, depending on the species), and of functional significance, are 3 residues of the calcium binding amino acid, γ-carboxyglutamic acid (Gla). Gla residues are posttranslationally synthesized from selected glutamic acid residues by a vitamin K- and CO_2-requiring enzyme complex (2). They are located at positions 17, 21, and 24 in all species from swordfish to mammals (1). This highly conserved sequence region from residues 20–34 in the central portion of the molecule, which also includes a disulfide loop (Cys-23–Cys-29), accounts for a structural conformation of the protein in the presence of calcium that promotes a tight binding of the protein to hydroxyapatite (1). The appearance of osteocalcin in embryonic bone coincident with mineral deposition (1), its association with the hydroxyapatite component of the matrix (3), its chemoattractant property for cells capable of bone resorption (4), and its modulated synthesis by the calcitrophic hormone 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] (5–7) suggest a role for the protein in bone turnover. Although many properties of the protein have been identified, the precise function of osteocalcin is still unknown.

Osteocalcin is synthesized *de novo* by osteoblasts as a 10,000-kDa precursor (8). While the majority of the processed osteocalcin peptide (5.7 kDa) is deposited in bone, nanogram levels are found in serum (9). Plasma levels of osteocalcin are now widely used as a clinical parameter of osteoblast activity (10). However, little is known regarding the factors regulating both osteocalcin gene expression and processing of the precursor. To approach an understanding of the factors modulating osteocalcin synthesis and to better define osteocalcin function, the present studies were initiated to isolate the rat osteocalcin gene. We have identified a vitamin D-responsive promoter region and find several consensus sequences for other hormones and second messengers that can regulate osteocalcin gene expression.‡

MATERIALS AND METHODS

Cloning of this Rat Osteocalcin Gene. Clones were obtained by screening a recombinant fetal rat liver DNA library cloned in λ Charon 4A that was provided to us by Thomas Sargent (National Institutes of Health, Bethesda, MD). The bacteriophage were grown in NC2YM medium in the LE 392 strain of *Escherichia coli*. Screenings were carried out in 15-cm Petri plates, each inoculated with 50,000 plaque-forming units of recombinant bacteriophage. Positive clones were selected as described by Benton and Davis (11) and plaque purified.

DNA Sequence Analysis. Overlapping segments of the osteocalcin gene were cloned into M13mp18 and sequenced by the dideoxynucleotide chain-termination method (12).

S1 Nuclease Hybridization Protection Analysis. A 207-base-pair *Pvu* II/*Hae* III fragment spanning nucleotides −143 to +64 of the rat osteocalcin gene was used as a radiolabeled probe coprecipitated with 25 μg of rat calvaria total cellular RNA for analysis of the transcription initiation site. Following hybridization and S1 nuclease digestion, the double-stranded protected probe fragments were analyzed by autoradiography following double-stranded electrophoretic fractionation in 8.3 M urea/6% polyacrylamide gels (13).

Construction of Promoter–Chloramphenicol Acetyltransferase (CAT) Fusion Genes. A vector was constructed to permit identification and to examine the regulation of promoter function, where expression of the bacterial CAT gene reflects the *in vivo* level of transcription. The 5′ flanking sequences of the rat osteocalcin gene were inserted into the *Sma* I site of pUC19. The 1.6-kilobase *Bgl* I/*Bam*HI fragment of pSV_2CAT (14) containing the CAT protein-coding sequence was cloned into the *Pst* I site of the resulting plasmid.

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Abbreviations: Gla, γ-carboxyglutamic acid; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CAT, chloramphenicol acetyltransferase.

‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04500).

The fusion construct was transfected into *E. coli* DH5 and the supercoiled form of plasmid DNA was isolated by centrifugation in cesium chloride/ethidium bromide equilibrium (15).

Transfection and Short-Term Transient Expression Assays of CAT Activity. ROS 17.2 cells kindly provided by Gideon Rodan (Merck Sharpe & Dohme Research Laboratories, Westpoint, PA) were maintained in monolayer culture in F12 medium supplemented with 5% fetal calf serum. Cells were transfected at confluency using the calcium phosphate coprecipitation method (16). Transfected cells were incubated in fresh medium for 12 hr, then treated for 24 hr with 10 nM $1,25(OH)_2D_3$ (a gift from Milan Uskokovic, Hoffmann-LaRoche, Nutley, NJ), and assayed for CAT activity (14).

RNA Isolation. RNA was isolated as described from individual calvaria, long bones, and other tissues (17) of 6-week-old rats and from ROS 17.2 cells (18). The integrity of each RNA preparation was confirmed by electrophoresis on 1% denaturing agarose gel and staining with ethidium bromide. Northern blots and slot blots were done with nitrocellulose or GeneScreen*Plus* membranes as described by the manufacturer (New England Nuclear, Boston, MA). Probes were labeled either by nick-translation (13) or random primer oligonucleotide labeling (19). Following hybridization (42°C–48°C) and washing, the membranes were exposed to Kodak X-Omat film. Quantitation of hybridization was done by scanning densitometry.

Osteocalcin. Medium and serum levels were determined by a radioimmunoassay (RIA) using species-specific antisera (goat anti-rat) and standards characterized in these laboratories (20).

RESULTS AND DISCUSSION

Organization of the Rat Osteocalcin Gene. An osteocalcin gene was isolated from a rat genomic DNA library cloned in λ Charon 4A using a ^{32}P-labeled rat osteocalcin cDNA as a hybridization probe. An *Eco*RI/*Bam*HI fragment containing the complete mRNA coding sequences, 800 nucleotides of 5′ flanking sequences, and 1300 nucleotides of 3′ sequences were subcloned in pUC19. Sequence analyses (Fig. 1) indicated that the mRNA encoding the 6-kDa osteocalcin polypeptide is represented in a 953-nucleotide segment of DNA containing four exons and three introns, which is schematically illustrated in Fig. 2.

The *in vivo* transcription initiation site was established by two methods. S1 nuclease analysis was carried out using RNAs isolated from rat long bones and a 207-nucleotide 5′-^{32}P-labeled *Pvu* II/*Hae* III segment of the gene, which spans the proximal promoter and the first 64 nucleotides of exon 1. As shown in Fig. 3, the principal start site is 36 nucleotides 5′ to the ATG initiation codon. Primer-extension analysis was carried out by hybridizing the BsbN1 *Ava* II segment of the gene to RNAs isolated from primary cultures of rat osteoblasts, rat calvaria, or rat long bone and transcribing with avian myeloblastosis virus RNA-dependent DNA polymerase. Transcripts that appear to initiate both upstream and downstream (at −59, −61, −65, −66, −67, and −68 5′ to ATG) are also detectable.

Exon 1 contains 36 nucleotides of nontranslated leader sequences and 64 nucleotides encoding the first 21⅓ amino acids of the signal peptide. Exon 2 encodes the remaining 1⅔ amino acids of the signal peptide and the initial 9⅓ amino acids of the osteocalcin propeptide. The remaining 16⅔ amino acids of the propeptide are encoded in exon 3. The third exon also encodes the first 6⅔ amino acids of the osteocalcin protein. The remaining 43⅓ amino acids of the osteocalcin protein are encoded in exon 4 followed by 136 nucleotides of nontranslated trailer sequence. Exon 4 additionally contains the canonical AATAAA poly(A) motif, which is located 115 nucleotides 3′ to the TAG stop codon.

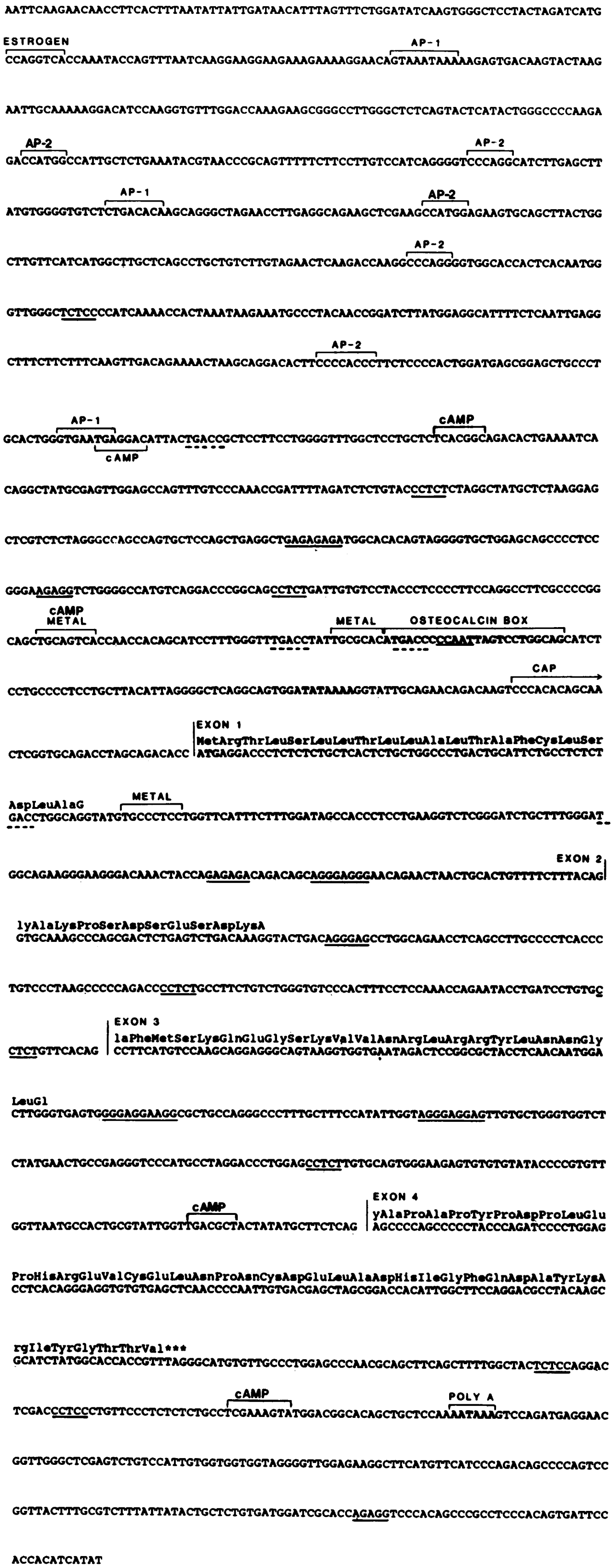

FIG. 1. Nucleotide sequence of the rat osteocalcin gene. Consensus regulatory elements in both the flanking and within the mRNA coding region of this gene are indicated (cAMP, metal). Underlined sequences contain GAGA and AGAGG motifs, which, although not restricted to the osteocalcin gene, have been suggested to be associated with vitamin D responsiveness. Dashed underlined sequences show the location of estrogen-responsive consensus elements. Also indicated is the principal transcription initiation site, CAAT and TATA boxes, and the polyadenylylation signal in boldface.

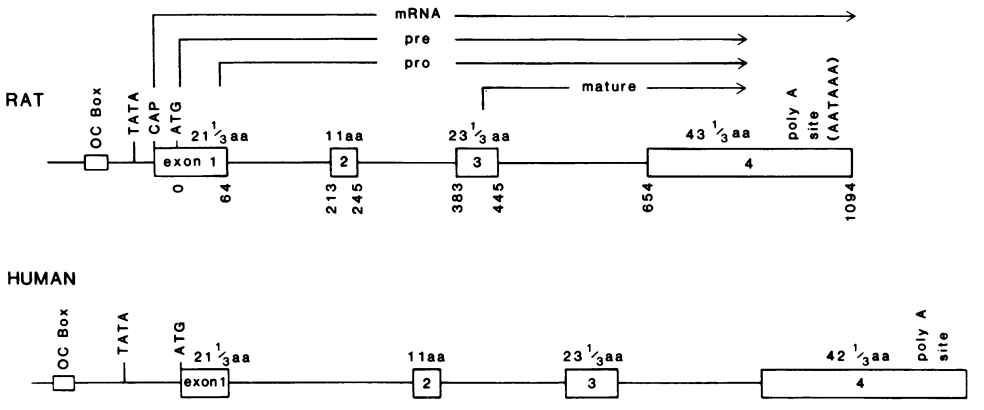

FIG. 2. General organization of the osteocalcin gene. The rat gene is compared to the human gene isolated by Celeste *et al.* (8). aa, Amino acids.

A comparison of the rat with the human osteocalcin gene indicates that the overall organization has been maintained in general, although the exons of the rat gene are larger. Both are single copy genes with 71.9–80.9% conservation of sequences among the 4 exons, which is consistent with the 73% homology of the rat and human osteocalcin protein. The non-amino acid coding sequences are far less conserved. Significantly greater variations are found in size and sequence in the three introns. Of functional significance, the sequences in exon 4 that encode the Gla residues of the calcium binding site are highly conserved (80.9%).

Osteocalcin Gene Expression. Using a radiolabeled hybridization probe derived from an mRNA coding region of the gene, we confirmed the tissue-specific expression of the osteocalcin gene (Fig. 4). In agreement with the presence of osteocalcin in bone and not in other tissues, detectable levels of osteocalcin gene transcripts were only observed in RNA isolated from bone (Fig. 4). Previously low or nondetectable levels of Gla or extractable osteocalcin from tissues other than bone led to the general assumption that osteocalcin is not produced in nonosseous tissues. This is a direct demonstration that osteocalcin gene expression is bone specific. Detectable levels of the amino acid Gla have been found in lung, placenta, cartilage, kidney, and liver (20); however, the absence of osteocalcin mRNA confirms that the Gla in those tissues derives from vitamin K-dependent proteins other than osteocalcin.

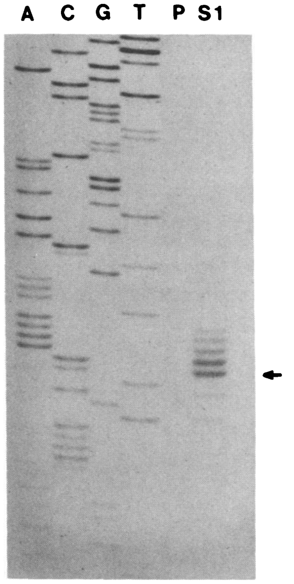

FIG. 3. S1 nuclease hybridization protection. RNA isolated from rat long bone was hybridized to a 207-base-pair *Pvu* II/*Hae* III fragment of the rat osteocalcin gene and subjected to S1 nuclease digestion, electrophoretic fractionation, and autoradiography. The S1 nuclease protected fragments are in lane S1. Sequence ladders (lanes A, C, G, T) were fractionated in the same gels for assignment of the transcription start sites at single nucleotide resolution. The primary initiation site is indicated by an arrow.

In an *in vitro* system in which osteoblasts isolated from fetal rat calvaria elaborate a matrix that mineralizes during a 30-day culture period, expression of the osteocalcin gene increased 3-fold between day 16 and day 20, a period when matrix mineralization is initiating (Table 1). In heavily mineralized cultures (day 28, calcium = 850 ± 72 μg; n = 3 35-mm wells) mRNA is 200-fold greater than in nonmineralized confluent osteoblast cultures (day 7, calcium = 6 ± 1 μg; n = 3 wells). Synthesized osteocalcin was 24 ± 3 ng per μg of DNA (n = 3 wells) on day 28 vs. 0.2 ± 0.1 ng per μg of DNA on day 7. These increases in rat osteocalcin during matrix mineralization are consistent with increases in osteocalcin synthesis also observed in mineralizing chicken osteoblast cultures (3). Whether induction of osteocalcin mRNA during mineralization directly involves the metal ion regulatory elements (see Fig. 1) or is related to osteoblast differentiation that occurs during mineralization of the extracellular matrix remains to be evaluated. Also shown in Table 1 and Fig. 5 are the increased cellular levels of osteocalcin mRNAs in the cultures following treatment with 1,25(OH)$_2$D$_3$. Interest-

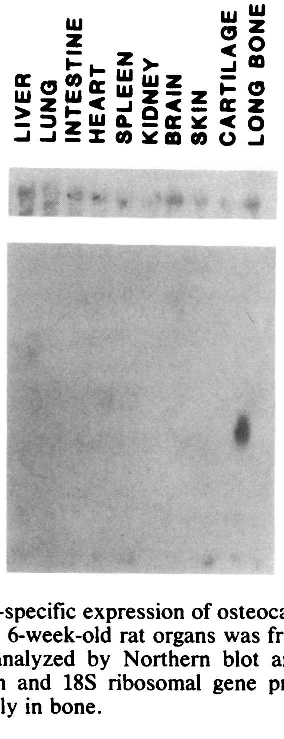

FIG. 4. Tissue-specific expression of osteocalcin mRNA. Total cellular RNA from 6-week-old rat organs was fractionated electrophoretically and analyzed by Northern blot analyses using ^{32}P-labeled osteocalcin and 18S ribosomal gene probes. Osteocalcin mRNA appears only in bone.

Table 1. Osteocalcin synthesis and gene expression in control and $1,25(OH)_2D_3$-treated rat osteoblast culture

Time in culture, days	Control	$1,25(OH)_2D_3$ (10 nM)	Ratio $1,25(OH)_2D_3$/control
		Synthesis, ng per μg of DNA	
16	2.4	16.3	6.8
20	7.4	23	3.1
		Gene expression	
16	0.033	0.227	6.7
20	0.109	0.714	6.6

Osteocalcin synthesis is measured as total (medium and cell layer) radioimmunoassayed protein (21) in 48 hr. Messenger RNA levels (gene expression) are represented as densitometric values from 2 μg of total cellular RNA immobilized onto nitrocellulose and hybridized to the osteocalcin gene probe.

ingly, there is a greater induction of osteocalcin expression related to mineralization than compared to vitamin D regulation. Osteocalcin mRNA is modulated from 6- to 7-fold by $1,25(OH)_2D_3$ but up to 200-fold during osteoblast matrix mineralization. Thus, control of osteocalcin expression appears to be mediated at several levels.

Of interest is the close correlation of stimulated osteocalcin protein synthesis (measured as secreted protein by RIA; Table 1) and osteocalcin mRNA levels. In control cultures, both parameters increase 3-fold between days 16 and 20 and also in $1,25(OH)_2D_3$-treated cultures when osteocalcin levels are low/moderate (on day 16). However, when osteocalcin synthetic levels are higher, as in day-20 cultures, $1,25(OH)_2D_3$-stimulated cultures are only 3-fold increased in total secreted protein but the mRNA levels are 6- to 7-fold elevated. Similarly in heavily mineralized cultures, mRNA is 200-fold greater than in nonmineralized cultures, whereas synthesized osteocalcin can vary from 20- to 100-fold increases. This disparity may result from several mechanisms including a feedback of osteocalcin peptide, transcriptional control, message processing, stability or turnover, and/or posttranslational modifications of the osteocalcin protein (for example, Gla synthesis).

In vivo osteocalcin expression is regulated by $1,25(OH)_2D_3$ as shown in Fig. 5. Vitamin D-deficient animals have decreased osteocalcin mRNA levels consistent with decreased bone and serum osteocalcin concentrations in these animals (6). Furthermore, rats injected with $1,25(OH)_2D_3$ showed increased mRNA levels. Serum osteocalcin levels, which reflect osteoblast synthesis of the protein (10), were as follows: control rats, 66 ± 12 ng/ml ($n = 3$); vitamin D-deficient rats, 34 ± 6 ng/ml ($n = 3$); $1,25(OH)_2D_3$-treated rats, 267 ± 11 ng/ml ($n = 3$). Therefore, osteocalcin mRNA exhibits a physiologic response to $1,25(OH)_2D_3$ consistent with increased serum osteocalcin values and similar to osteoblasts *in vitro*.

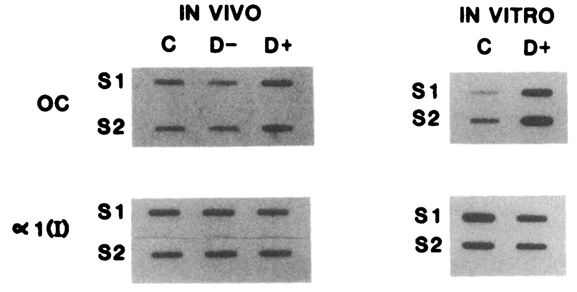

FIG. 5. Stimulation of osteocalcin mRNA by $1,25(OH)_2D_3$. (*Left*) *In vivo*. Osteocalcin mRNA in calvaria of normal, vitamin D-deficient, and $1,25(OH)_2D_3$-treated rats (S_1, S_2). mRNA was prepared from Holtzman male rats raised on a control diet (0.47% Ca/0.5% P, containing vitamin D_3; Bio-Serve, Frenchtown, NJ) (C) and on the same diet deficient in vitamin D (D−) for 7 days. Rats on a control diet were injected with 20 ng of $1,25(OH)_2D_3$ (Rocaltrol, Hoffmann-LaRoche) 72 hr prior to sacrifice (D+). Control and D+ rats weighed 31 ± 1.7 g at sacrifice, while D− rats weighed 21 ± 2 g. (*Right*) *In vitro*. Calvarial rat osteoblasts cultured 20 days in BGJb medium (with 25 μg of ascorbic acid per ml and 10% fetal calf serum) were treated 48 hr with 10 nM $1,25(OH)_2D_3$. Total cellular mRNA was isolated from bone and from control (C) and D+-treated cells and hybridized to slot blots with ^{32}P-labeled osteocalcin (OC) or collagen [$\alpha 1(I)$] gene probes. Collagen mRNA is significantly decreased by the hormone only *in vitro*.

The 5′ Regulatory Sequence of the Osteocalcin Gene. To directly assess the contribution of 5′ flanking sequences to vitamin D-modulated expression of the osteocalcin gene, the initial 600 nucleotides upstream from the transcription initiation site were fused to the mRNA coding sequences for bacterial CAT. The chimeric gene was transfected into ROS 17.2 cells and expression was assayed at 36 hr. As shown in Fig. 6 (*Left*), the ROS 17.2 cell line exhibits a 10-fold increase in osteocalcin mRNA following 10 nM $1,25(OH)_2D_3$ treatment for 24 hr. Fig. 6 (*Right*) shows that the proximal 600 nucleotides of the osteocalcin promoter support transcription as reflected by acetylation of chloramphenicol by lysates from transfected ROS 17.2 (8th and 9th lanes) cells. Moreover, as shown in 6th and 7th lanes, a 15-fold increase in CAT expression occurs in transfected cells treated with $1,25(OH)_2D_3$, indicating that a vitamin D-responsive element is present in the first 600 nucleotides of the 5′ flanking sequences. The absence of a vitamin D-induced increase in expression of the CAT gene under control of a simian virus 40 promoter and enhancer supports the specificity of the vitamin D-mediated response (lane pSV2 CAT D^+). However, these results do not preclude the possible contribution of additional sequences further upstream and/or within the osteocalcin mRNA coding sequences to vitamin D-induced expression. In fact, multiple vitamin D-responsive elements would provide a basis for modulating the extent to which vitamin D-responsive genes are expressed under different biological conditions. As indicated in Fig. 1, a series of putative vitamin D-responsive elements are underlined. Although not restricted to vitamin D-responsive genes, sequences rich in AGAGG and GAGA have been found in promoter and structural regions of genes encoding three vitamin D-dependent proteins—alkaline phosphatase (22), osteonectin (23), and calbindin (24). The osteocalcin gene has several such sequences and the complementary nucleotide sequences (CCTCT) located in the promoter region; in introns 1, 3, and 4; in exon 2; and in the 3′ flanking region (Fig. 1).

At least three classes of regulatory elements reside in the initial 800 nucleotides upstream from the rat osteocalcin mRNA coding sequences that have the potential for influencing the specificity and/or levels of transcription of the gene. First are consensus sequences for regulatory elements associated with most genes transcribed by RNA polymerase II (25). For example, a TATA box is found at −30, a CAAT motif, and AP1 sites (26) and AP2 (27) sites are present upstream from the start site as shown in Fig. 1. Second are a series of consensus sequences for the estrogen hormone receptor binding (28), metal binding sites (29), cAMP-responsive elements (30), and vitamin D as discussed above. These are indicated in Fig. 1. The presence of both estrogen and cAMP elements is consistent with observed changes in serum osteocalcin in estrogen-treated patients (31) and *in vitro* osteocalcin mRNA increases in response to parathyroid hormone and other cAMP-stimulating agents (21, 32). Thyroid hormone also increases mRNA in T4-treated rats, while hypothyroid rats have decreased levels (33). Although not indicated in Fig. 1, several AGGGAC sequences occur (in the promoter regions and exons 1 and 2). This is one of the thyroid hormone receptor binding sites identified in the rat growth hormone gene (34).

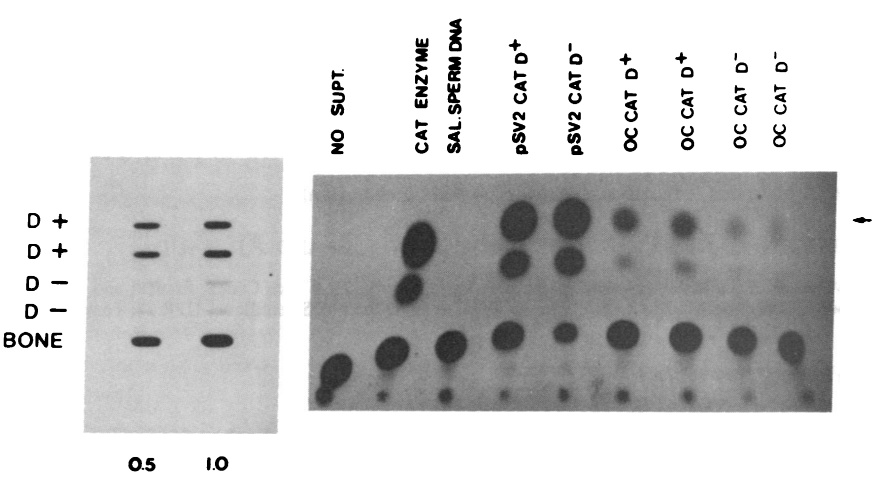

FIG. 6. (*Left*) Osteocalcin mRNA levels in ROS 17.2 cells pretreated with 10 nM 1,25(OH)$_2$D$_3$ (D+) and in untreated controls (D−). Total cellular RNA (0.5 or 1.0 μg) was subjected to slot blot analysis using a ^{32}P-labeled osteocalcin gene hybridization probe. (*Right*) CAT assay using cell extracts from ROS 17.2 cells, transfected with chimeric DNA consisting of 600 nucleotides of the rat osteocalcin 5′ proximal regulatory sequences fused to the CAT structural gene. Arrow designates acetylated forms of chloramphenicol resulting from CAT activity. Lanes: No supt., negative control for the CAT assay, no cell extract (supernatant) in the reaction mixture; CAT enzyme, positive control, 0.09 unit of CAT was added to the reaction mixture; Sal. sperm DNA, CAT activity extracts from cells transfected with salmon sperm DNA as a control; pSV2 CAT D$^+$, CAT activity in extracts from cells transfected with pSV2 CAT and pretreated with 1,25(OH)$_2$D$_3$; pSV2 CAT D$^-$, same as previous lane but without addition of 1,25(OH)$_2$D$_3$; OC CAT D$^+$, CAT activity in extract from cells transfected with osteocalcin–CAT chimeric DNA (described above) pretreated with 1,25(OH)$_2$D$_3$; OC CAT D$^-$, same as lanes OC CAT D$^+$ but from cells not pretreated with 1,25(OH)$_2$D$_3$.

Of particular interest is a third class of regulatory elements we have identified in the proximal promoter region and designated an osteocalcin box (Figs. 1 and 2). This 24-nucleotide sequence as shown below

Osteocalcin promotor box:
rat ATGACCCCCAATTAGTCCTGGCAG
human ATGACCCCCAAATAGCCCTGGCAG

contains a CAAT motif as a central core element. There are only two nucleotide substitutions between the rat and human osteocalcin gene. Despite the sequence conservation of this element in the osteocalcin gene promoter, a homologous element is not found in the proximal promoter of the vitamin D-responsive genes alkaline phosphatase (22) and calbindin (24), suggesting a gene-specific and/or tissue-specific role for this promoter sequence. Sequences of other vitamin D-responsive promoters and osteoblast synthesized proteins are not yet known.

In conclusion, we have identified multiple regulatory elements in the osteocalcin gene. There is evidence from *in vitro* and *in vivo* studies that these elements can potentiate expression of osteocalcin. Whether these consensus sequences function directly to affect osteocalcin gene expression or act synergistically needs to be investigated. Such an understanding of osteocalcin gene regulatory elements will both clarify existing observations and provide insight regarding osteocalcin function and the interpretation of serum osteocalcin measurements as a noninvasive bone-specific serum marker for evaluating and treating bone disorders.

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