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Vitamin D-mediated modifications in protein–DNA interactions at two promoter elements of the osteocalcin gene

(vitamin D-responsive element/bone/transcription factors)

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ABSTRACT **By the combined use of DNase I footprinting, electrophoretic mobility-shift assay, and methylation interference analysis, we have identified a series of sequence-specific protein–DNA interactions in the 5′ flanking region of the rat osteocalcin gene. Stimulation of osteocalcin gene expression by 1,25-dihydroxyvitamin D_3, a physiologic mediator of this bone-specific gene *in vitro* and *in vivo*, is associated with modifications in the binding of ROS 17/2.8 cell nuclear factors to two promoter segments that up-regulate transcription. One segment located between −462 and −437 exhibits a vitamin D-dependent increase in sequence-specific binding of nuclear factors. This element (CTGGGTGAATGAGGACATTACTGACC), identified at single nucleotide resolution, contains a region of hyphenated dyad symmetry and shares sequence homology with consensus steroid-responsive elements and with the sequence that has been identified as the vitamin D receptor binding site in the human osteocalcin gene. We have also observed that vitamin D stimulation of osteocalcin gene expression results in a 5-fold increase in protein binding to the region of the osteocalcin box, a 24-nucleotide segment in the proximal promoter with a CCAAT motif as the central core. Our results demonstrate protein–DNA interactions in a vitamin D-responsive element and in a second sequence, the osteocalcin box, both of which are involved in the physiologic regulation of the osteocalcin gene in response to 1,25-dihydroxyvitamin D_3.**

The osteocalcin gene encodes a 6-kDa polypeptide (1–5) that represents the most abundant noncollagenous protein of the bone matrix and the only known bone tissue-specific protein (reviewed in ref. 6). For the rat gene, the mRNA is transcribed from a 953-nucleotide segment of DNA containing 4 exons and 3 introns (2–5). The osteocalcin gene promoter has a modular organization reflected by the representation of RNA polymerase II canonical sequences and of a series of consensus sequences for hormone receptor binding sites and nucleotide-responsive elements, which are physiologic mediators of osteocalcin gene expression (2, 5).

The calcitrophic hormone 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] plays a key role in the transcriptional regulation of osteocalcin gene expression in osteoblasts both *in vitro* and *in vivo* (2–4, 7). Thus, experimentally addressing the mechanisms by which transcription of the osteocalcin gene is modulated by vitamin D is necessary for understanding regulation at the biochemical as well as at the molecular level. Our previous studies (4), and those of others (2, 3, 8), have established that sequences that reside within the 600 nucleotides immediately proximal to the transcription start site support a 10-fold stimulation of osteocalcin gene transcription by 1,25$(OH)_2D_3$ treatment of ROS 17/2.8 cells. We have also identified in the proximal promoter region a 24-nucleotide sequence, designated the osteocalcin box (−76 to −99), which contains a CCAAT motif as a central element and influences the level of osteocalcin gene transcription (4). Yet, to date, the interactions of nuclear factors with promoter elements that may modulate the transcriptional properties of the osteocalcin gene and the influence of vitamin D on such protein–DNA interactions have not been reported.

In this paper, we present results indicating that the stimulation of osteocalcin gene transcription by vitamin D is associated with modifications in sequence-specific binding of nuclear factors with two promoter elements. The element residing between nucleotides −462 and −437 shows >200-fold increase of protein–DNA interactions with extracts from vitamin D-treated cells; this element (CTGGGTGAATGAGGACATTACTGACC) contains hyphenated dyad symmetry and shares sequence homology with a steroid-responsive element and with the sequence (CACTGGGTGAA) that has been identified as a part of the vitamin D receptor binding site in the human osteocalcin gene (8). Vitamin D treatment additionally results in a 5-fold increase in protein binding to the osteocalcin box. This paper reports vitamin D-responsive protein–DNA interactions with the promoter of a gene that is physiologically regulated by 1,25$(OH)_2D_3$.

MATERIALS AND METHODS

Treatment of Cells and Extracts. ROS 17/2.8 cells, a gift from Gideon Rodan (Merck Sharp & Dohme), were grown to confluence in F12 medium supplemented with 5% (vol/vol) fetal calf serum. The medium was then exchanged for fresh medium containing 10 nM 1,25$(OH)_2D_3$ in 0.1% ethanol, and after 24 hr of treatment nuclei were isolated and nuclear extracts were prepared according to the method of Dignam *et al.* (9).

Probes and Competitor DNA Fragments. The probe for the proximal region of the osteocalcin promoter was the −141 to +39 fragment of the rat osteocalcin gene clone pOC3.4 (4). The osteocalcin box probe was the −99 to −76 sequence that includes the CCAAT box. The distal probe was the −527 to −344 fragment. For the competition experiments, a double-stranded oligonucleotide corresponding to sequences from −462 to −436, designated VDRE (vitamin D-responsive element) of the rat osteocalcin gene promoter, was synthesized. Competition experiments were also performed with sequences containing putative binding sites for general transcription factors; these oligonucleotides were derived from histone gene promoter DNA–protein interaction domains designated H3-site II (−148 to −122) and H4-site II (−91 to −64) and both include their respective CAAT box domains (10–12). The oligonucleotide designated H4-site I spans nucleotides −152 to −128 of the histone H4 gene promoter and contains a putative cAMP binding site. The Sp1 oligonucleotide contains two high-affinity Sp1 binding sites (13).

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Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VDRE, vitamin D-responsive element.

Gel Mobility Shift Analysis. All protein–DNA binding reactions contained ≈5000 cpm of a single end-labeled probe, 3 μg of poly(dI-dC)·poly(dI-dC), 50 mM KCl, 12 mM Hepes·NaOH (pH 7.9), 4 mM Tris·HCl (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, and 12% (vol/vol) glycerol. Various amounts of protein ranging from 2.5 to 12 μg were added and the incubation was carried out at 20°C for 15 min in a final reaction volume of 20 μl. The protein–DNA complexes were resolved on high ionic strength, 4% polyacrylamide (40:1, acrylamide/bisacrylamide) gels (14), which were dried and visualized by autoradiography.

DNase I Footprinting and Methylation Interference Analysis. DNase I footprinting was carried out essentially as described by Augereau and Chambon (15). The guanine residues in contact with the DNA binding factors were identified by methylation interference analysis (14).

RESULTS AND DISCUSSION

Identification of a VDRE: Characterization of Protein–DNA Interactions. Our experimental strategy for the identification of vitamin D response elements in the rat osteocalcin gene promoter was based on the presence within the initial 600 nucleotides of the 5′ flanking region of a series of sequences with dyad symmetry, resembling steroid-responsive elements. We and others had previously demonstrated that this 600-nucleotide region contains sequences that support a 10-fold stimulation of transcription by $1,25(OH)_2D_3$ (2–4, 8). Two segments in this region, at nucleotides −492 to −476 and −468 to −436 (Fig. 1), were identified as sharing sequence homology with consensus sequences for steroid receptor binding sites (16) and with the VDRE of the human osteocalcin gene reported by Kerner *et al.* (8). Regions of protein–DNA interactions were initially established by carrying out DNase I footprinting analyses with a 184-base-pair segment of the osteocalcin gene promoter that spans −527 to −344 and includes the two putative VDREs proposed by Minghetti *et al.* (16). As shown in Fig. 2*A*, 24 hr after treatment of ROS 17/2.8 rat osteosarcoma cells with 10 nM $1,25(OH)_2D_3$, there is a 10-fold stimulation of osteocalcin gene expression. Nuclear extracts prepared from these vitamin D-treated cells generated a specific region of protection from DNase I digestion corresponding to nucleotides −465 to −437, the more proximal of the two elements. The complete absence of nuclease protection in the distal consensus sequence (−492 to −476) (Fig. 2*B*) permitted us to distinguish between the functional properties of the two putative VDREs.

To further characterize sequence-specific protein–DNA interaction in the −527 to −344 region of the osteocalcin gene promoter, electrophoretic mobility-shift analysis was carried out with nuclear extracts prepared from control and vitamin D-treated (10 nM for 24 hr) ROS 17/2.8 cells. As shown in Fig. 3*A*, a protein–DNA complex designated V was the most prominent band produced by nuclear extracts from the vitamin D-treated cells but was not observed with extracts from the untreated control cells. When the protein–DNA complex V was further examined at single nucleotide resolution by methylation interference analysis, as shown in Fig. 3*B*, the G residues that interfered with the binding of the vitamin D-induced proteins were localized within sequences of the osteocalcin gene promoter (−465 to −437) also protected against DNase I digestion (Fig. 2*B*). These results confirmed the identification of the VDRE and the specificity of the observed protein–DNA interactions.

Sequence specificity of protein binding to the −527 to −344 fragment was additionally established by competition gel mobility-shift analyses. As indicated by the results in Fig. 3*C*, the vitamin D-dependent band, designated V, was effectively blocked by competition with an oligonucleotide representing

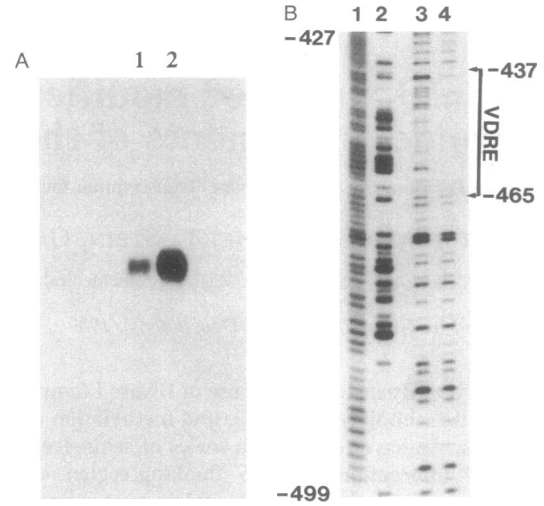

FIG. 2. (*A*) Northern blot analysis of osteocalcin mRNA from control and vitamin D-treated ROS 17/2.8 cells. Total cytoplasmic RNA was precipitated by the addition of LiCl/urea as described (17); 10 mM vanadyl–ribonucleoside complex was added as the RNase inhibitor. Ten micrograms of RNA from ROS 17/2.8 cells treated with $1,25(OH)_2D_3$ (lane 2) and from untreated controls (lane 1) was fractionated on a 6.6% formaldehyde/1.2% agarose gel, transferred to Zeta-Probe membrane (Bio-Rad) (18), and hybridized with the rat osteocalcin gene probe (4). (*B*) DNase I footprint analysis of protein–DNA interactions in the −527 to −344 promoter region of the rat osteocalcin gene. C + T (lane 1) and G + A (lane 2) sequencing reactions representing the coding strand were electrophoresed along with the DNase I footprint reactions. The control lane (lane 3) shows the DNase I digestion pattern of the probe incubated with 3 μg of bovine serum albumin. The pattern of digestion obtained with 13 μg of nuclear extract from $1,25(OH)_2D_3$-treated ROS 17/2.8 cells (lane 4) shows a specific region of protection indicated by solid line and designated VDRE.

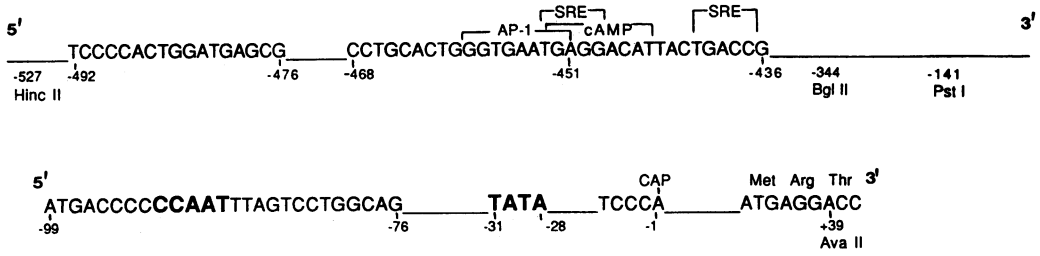

FIG. 1. Schematic organization of the proximal region of the osteocalcin gene promoter. Principal restriction sites used to generate sequence-specific probes for protein–DNA binding analysis are designated and nucleotides are numbered beginning from the transcription start site (CAP). Two primary regulatory elements are designated in boldface type—the TATA box and the osteocalcin box (−99 to −76) containing a CCAAT motif as the central core (4). Two putative triiodothyronine receptor binding sites are shown (−492 to −476 and −468 to −451). Within this segment and extending to −436 are other putative consensus transcription regulatory sequences for AP1, cAMP, and motifs for steroid-responsive elements (SRE).

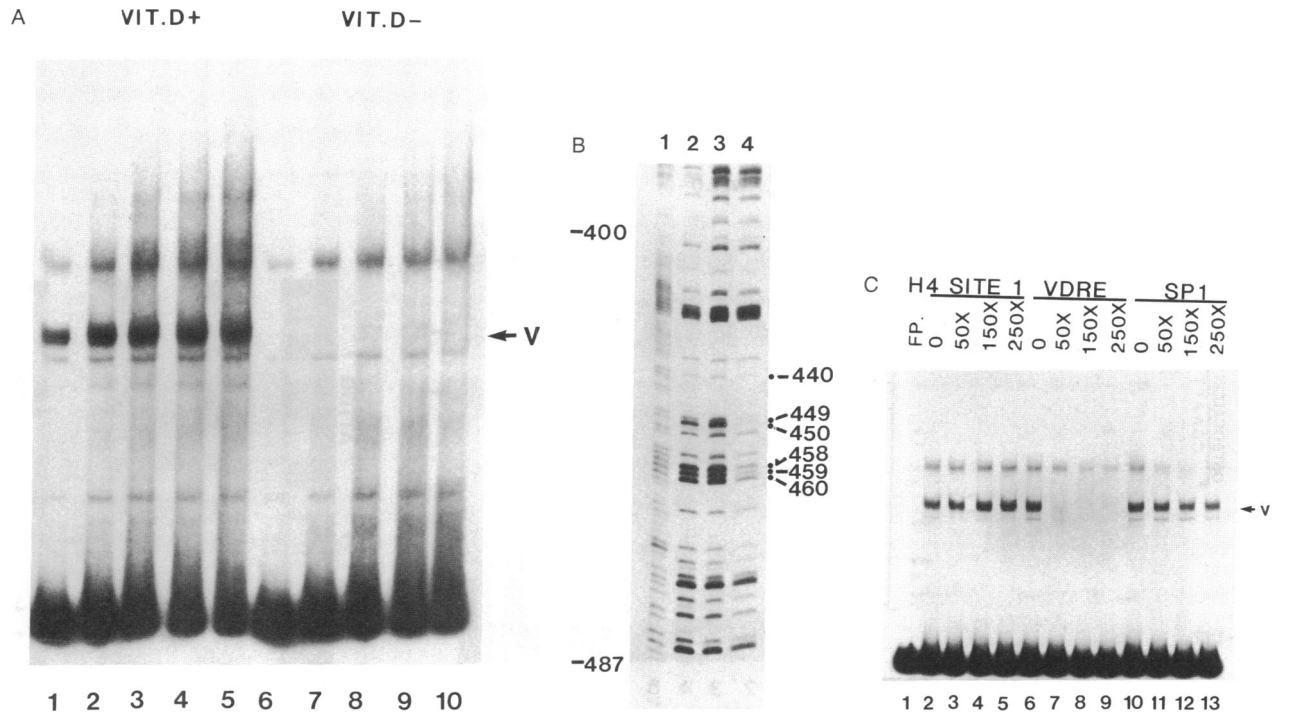

FIG. 3. (*A*) Detection of factors with affinity for the osteocalcin promoter region −527 to −344 by gel mobility-shift analysis. Increasing amounts of nuclear extracts (2.5, 5, 7.5, 10, and 12 μg) of 1,25(OH)$_2$D$_3$-treated (VIT.D+; lanes 1–5) and untreated (VIT.D−; lanes 6–10) ROS 17/2.8 cells were used in standard DNA binding reactions. The major protein–DNA complex that appears with vitamin D$_3$ treatment is denoted V. (*B*) Methylation interference analysis was performed to establish the contact sites of the vitamin D-induced factor. The ^{32}P-end-labeled probe (−527 to −344; pOC3.4) was partially methylated with dimethyl sulfate and used in standard DNA binding reactions. Nuclear extracts from vitamin D-treated ROS 17/2.8 cells were added and the DNA–protein complexes were resolved by native gel electrophoresis. DNA from the protein–DNA complex, indicated as V in *A*, and from free probe were eluted and cleaved with piperidine. An equal number of counts of free (lane 3) and bound (lane 4) DNA were electrophoresed on 8% polyacrylamide denaturing gels. Sequencing reactions C + T (lane 1) and G + A (lane 2) of the coding strand were electrophoresed alongside. The G residues that interfere with the binding of the protein are indicated by solid circles. (*C*) Competition analysis of DNA–protein complexes in the *Hin*cII/*Bgl* II (−527 to −344) fragment of the rat osteocalcin promoter. Five micrograms of nuclear extract from vitamin D-treated ROS 17/2.8 cells was added to standard binding reaction mixtures. Reaction mixtures also contained either no additional DNA (lanes 2, 6, and 10) or 50-fold (lanes 3, 7, and 11), 150-fold (lanes 4, 8, and 12), and 250-fold (lanes 5, 9, and 13) of an unlabeled synthetic oligonucleotide. The oligonucleotide used as the competitor in lanes 3–5, designated as H4 site I, contains a cAMP binding site. A partial homology to this consensus sequence is seen at −453 to −446 of the osteocalcin promoter. The oligonucleotide used as the competitor in lanes 7–9, designated VDRE, corresponds to −462 to −436 of the rat osteocalcin promoter. In lanes 11–13, the competitor used was the oligonucleotide synthesized to correspond to high-affinity binding sites for Sp1.

only the −462 to −436 sequence, the putative VDRE based on a region of homology with the human osteocalcin VDRE and a region containing hyphenated dyad symmetry (see Fig. 1). No competition was observed using an Sp1 consensus sequence or an oligonucleotide for the site I human H4 histone gene proximal promoter element (11), which contains a cAMP consensus sequence similar to that observed in the VDRE region of the osteocalcin gene (Fig. 1). The vitamin D-induced protein–DNA interactions, which are revealed by footprint analyses and defined at single nucleotide resolution by methylation interference (summarized in Fig. 4), allowed us to propose that the functional VDRE of the rat osteocalcin gene is located between nucleotides −462 and −437 of the 5′ flanking region.

Vitamin D-Dependent Protein–DNA Interactions in the Osteocalcin Box. Vitamin D-dependent modifications in protein–DNA binding were also observed in a second region of the rat osteocalcin gene promoter, the CAAT-containing osteocalcin box. As shown in Fig. 5*A*, a complex pattern of protein–DNA interactions was observed in the proximal 5′ flanking region of the gene. Using a radiolabeled probe for the −141 to +39 sequences, nuclear extracts from vitamin D-treated ROS 17/2.8 osteosarcoma cells generated several DNase I footprints. The strongest region of nuclease protection was found around the osteocalcin box (−113 to −85); other regions of protein–DNA interactions were reflected by weaker footprints between −67 and −46 immediately downstream from the osteocalcin box and also in the region of the TATA box (−39 to −11).

Focusing on the osteocalcin box, we carried out gel mobility-shift analyses with a probe corresponding to the −99 to −76 segment of the promoter flanked by pUC19 linker sequences, and we compared the binding of nuclear factors from control and 1,25(OH)$_2$D$_3$-treated ROS 17/2.8 cells. Three principal protein–DNA complexes, designated A, B,

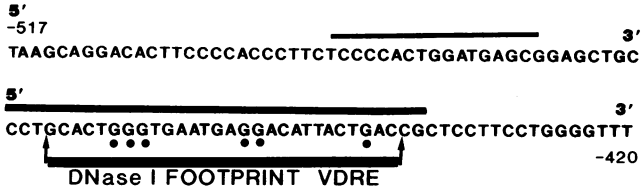

FIG. 4. Summary of the DNase I footprinting analyses and methylation interference analyses of the osteocalcin promoter from −517 to −420. Bars above the sequence from −492 to −476 and from −468 to −436 indicate two regions proposed to contain VDREs. Solid bar with arrows below the sequence indicates the limits of the footprint from −465 to −437 when DNase I digestion was done in the presence of nuclear extracts from vitamin D-induced ROS 17/2.8 cells. The G residues that interfered with the binding of the vitamin D-induced protein in the protein–DNA complex designated V (Fig. 3*A*) are shown by solid circles and correspond to positions −460, −459, −458, −450, −449, and −440.

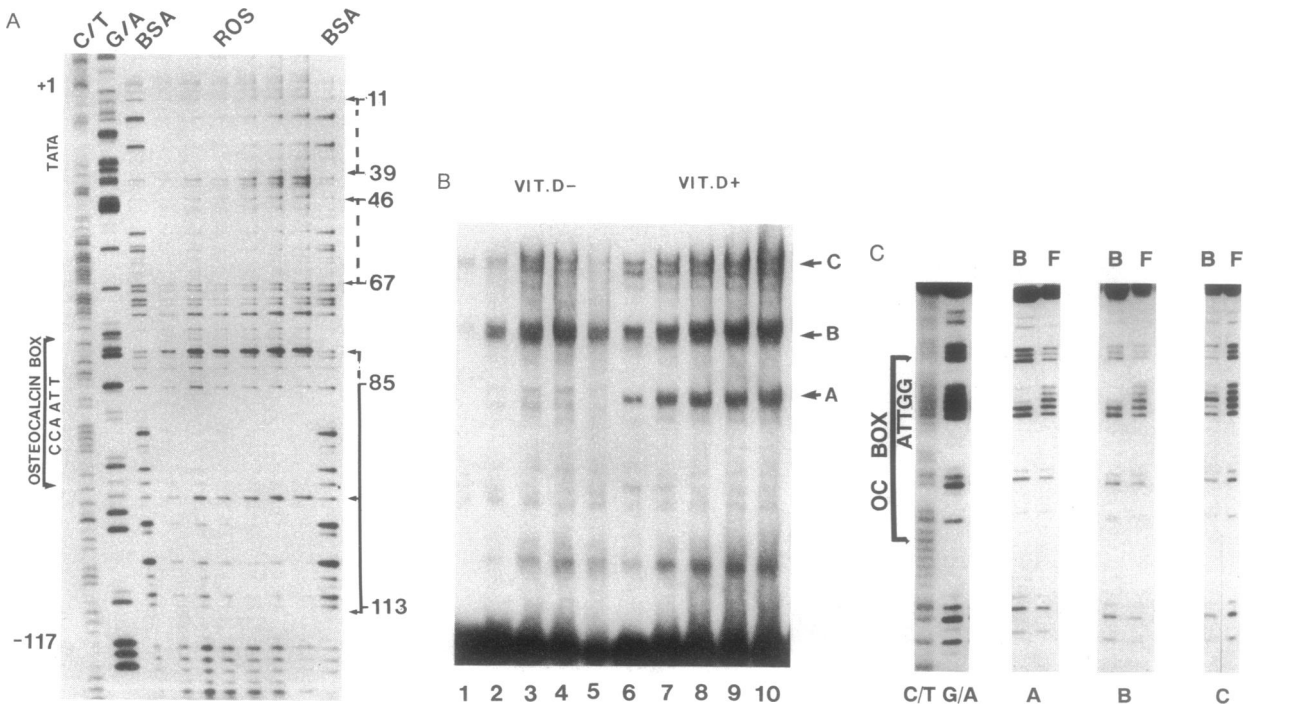

FIG. 5. (A) DNase I footprints generated by nuclear extracts from vitamin D-treated ROS 17/2.8 cells on the coding strand of the rat osteocalcin gene (pOC3.4) spanning −141 to +39. For the control lanes (BSA), the reactions contained 3 μg of bovine serum albumin instead of nuclear extract. In lanes 4–9 (ROS) DNase I digestions were carried out with 1, 2, 3, 4, 5, and 6 μl (5 μg/μl), respectively, of nuclear extract. Dashed lines, regions of weak protection; solid lines, regions of strong protection. (B) Nuclear extracts from $1,25(OH)_2D_3$-treated (VIT.D+) and control (VIT.D−) ROS 17/2.8 cells were analyzed by gel mobility-shift assay, for factors that bound to the osteocalcin box region (−99 to −76) of the rat osteocalcin gene promoter. The probe was incubated with 2.5, 5, 7.5, 10, and 12 μg of vitamin D-lacking (lanes 1–5) and vitamin D-containing (lanes 6–10) ROS cell extracts. The major DNA–protein complexes A, B, and C are indicated. (C) The G residue contacts of the protein–DNA complexes A, B, and C to the 75-base-pair fragment containing the osteocalcin (OC) box region (−99 to −76) were shown by methylation interference. Using a partially methylated probe and nuclear extracts from vitamin D-treated ROS 17/2.8 cells, standard binding reactions were scaled up 2-fold and analyzed on 4% polyacrylamide nondenaturing gels. The DNAs from complex A, complex B, and complex C (lanes B in each pair) and free probe (lanes F in each pair) were cleaved with piperidine and then electrophoresed on 8% polyacrylamide sequencing gels. The noncoding strand sequence was electrophoresed at the same time (C/T and G/A).

and C, were observed with both the control and vitamin D extracts; a selective 5-fold increase in representation of the A complex was associated with vitamin D-stimulated osteocalcin gene expression (Fig. 5B). Methylation interference analyses (Fig. 5C) indicated that both the A and B complexes map to the same sequences in the distal region of the osteocalcin box, despite the absence of an effect of vitamin D on formation of the B complex. It therefore appears that protein–DNA interactions in the 5′ region of the osteocalcin box may involve different proteins with similar binding sites or proteins with multiple subunits, such that formation of only the A complex is vitamin D dependent. The C complex, which spans the A and B binding sequences and extends several nucleotides both 5′ and 3′, is unaffected by vitamin D treatment. This finding supports the selectivity of the vitamin D effect (that is, on the A complex) on protein–DNA binding in the region of the osteocalcin box. Fig. 6 schematically

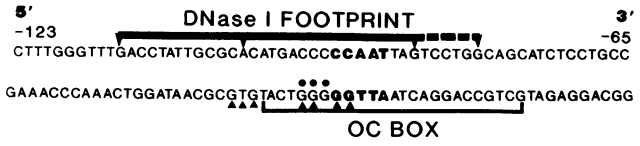

FIG. 6. Summary of the complex pattern of DNA–protein interactions around the osteocalcin (OC) box region of the osteocalcin promoter. An extensive DNase I footprint extending from −113 to −85 is indicated by a solid bar. Specific guanine residues that interfere with the binding of the factor(s) in complexes A and B are indicated by solid circles and those that interfere with the binding of complex C are indicated by solid triangles.

illustrates the multiple protein–DNA interactions formed in this proximal promoter region and reflects the complexity of regulation at this site.

Specificity of the observed protein–DNA interactions comprising the A, B, and C complexes associated with the osteocalcin box promoter elements is supported by results from competition gel mobility-shift analyses presented in Fig. 7. Sequence-specific competition for formation of the three complexes occurred when the unlabeled probe was used as competitor. In contrast, oligonucleotides for the site II elements of H3 (10) and H4 (11) histone gene proximal regulatory sequences, which contain CAAT consensus sequences, did not compete for binding of the ROS cell nuclear factors to the osteocalcin box. Similarly, an oligonucleotide for the H4 histone gene site I promoter element, which contains a cAMP consensus sequence, was ineffective as a competitor.

CONCLUSIONS

We have identified at single nucleotide resolution an element in the 5′ regulatory region of the rat osteocalcin gene (−462 to −437) that exhibits properties consistent with a binding site for the vitamin D receptor complex. This element was located by mapping the binding of vitamin D-stimulated nuclear factors from osteosarcoma cells to specific sequences in the osteocalcin gene promoter that contain steroid-responsive consensus sequences and that support a 10-fold vitamin D-dependent induction of transcription. We have additionally demonstrated that vitamin D stimulation of osteocalcin gene expression is associated with increased binding of nuclear factors to the osteocalcin box region (−113 to

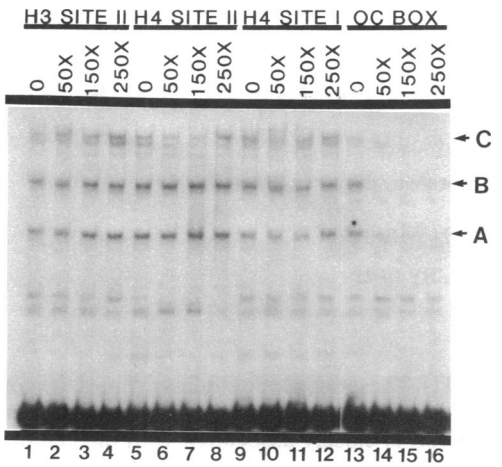

FIG. 7. Competition gel mobility-shift analysis of the specificity of protein–DNA complexes A, B, and C in the osteocalcin (OC) box. The single end-labeled *Hin*dIII/*Eco*RI fragment from pUC19, which contained the 24-base-pair osteocalcin box, was used as the probe. The same unlabeled fragment (50-fold, 150-fold, and 250-fold excess) was used as the OC box competitor (lanes 13–16). Other competitors used were H4 site II oligonucleotide (lanes 5–8) and H3 site II oligonucleotide (lanes 1–4), which contain the CAAT box regions of the human histone H4 and H3 genes and H4 site I oligonucleotide (lanes 9–12), which contains a putative cAMP binding site.

−85), a proximal regulatory element with a CCAAT motif as a central core.

The osteocalcin gene promoter exhibits a modular organization, with a complex series of consensus sequences bearing homology to response elements for several physiologic mediators of osteocalcin gene expression, including cAMP and estrogen, glucocorticoid, and thyroid hormone, in addition to $1,25(OH)_2D_3$. From our results, it is evident that at least one of these putative elements and the osteocalcin box exhibit vitamin D-dependent changes in protein–DNA interactions. However, the role of the vitamin D-induced changes in the osteocalcin box region is not clear, since gene constructs containing only this segment of the promoter have been shown to be unresponsive to vitamin D induction (2, 3, 8).

It remains to be established how the vitamin D-induced modifications in protein binding to both the regulatory elements of the osteocalcin gene are related, and how they individually or together contribute to the regulation of osteocalcin gene transcription. The observed changes in protein binding to these 5′ regulatory elements (which occur in association with modifications in transcription) may represent newly synthesized proteins, recruitment of proteins, or modifications of DNA-associated polypeptides; all are possibilities for establishing control mechanisms. However, the enhancer-like activity of steroid responsive elements and the primary regulatory roles of CCAAT-containing elements may provide a basis for modulating the extent to which the osteocalcin gene is transcribed in response to various concentrations of vitamin D both *in vitro* and *in vivo*. Mechanistically, this response could be achieved by modulating the complex series of protein–DNA interactions in the vitamin D-responsive element, the osteocalcin box, or in other promoter elements that control expression of this gene.

Note Added in Proof. After this paper was submitted, a vitamin D-responsive element between −462 and −437 nucleotides upstream from the transcription initiation site of the rat osteocalcin gene was also reported by Demay *et al.* (19).

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