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# Vitamin D-responsive protein–DNA interactions at multiple promoter regulatory elements that contribute to the level of rat osteocalcin gene expression

(transcription/differentiation/osteoblasts/hormone/gene regulation)

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**ABSTRACT** **The observation that vitamin D-mediated enhancement of osteocalcin (OC) gene expression is dependent on and reciprocally related to the level of basal gene expression suggests that an interaction of the vitamin D responsive element (VDRE) with basal regulatory elements of the OC gene promoter contributes to both basal and vitamin D-enhanced transcription. Protein–DNA interactions at the VDRE of the rat OC gene (nucleotides −466 to −437) are reflected by direct sequence-specific and antibody-sensitive binding of the endogenous vitamin D receptor present in ROS 17/2.8 osteosarcoma nuclear protein extracts. In addition, a vitamin D-responsive increase in OC gene transcription is accompanied by enhanced non-vitamin D receptor-mediated protein–DNA interactions in the "TATA" box region (nucleotides −44 to +23), which also contains a potential glucocorticoid responsive element. Evidence for proximity of the VDRE with the basal regulatory elements is provided by two features of nuclear architecture. (*i*) Nuclear matrix attachment elements in the rat OC gene promoter that bind nuclear matrix proteins with sequence specificity may impose structural constraints on promoter conformation. (*ii*) Limited micrococcal nuclease digestion and Southern blot analysis indicate that three nucleosomes can be accommodated in the sequence spanning the OC gene VDRE, the OC/CCAAT box (nucleotides −99 to −76), and the TATA/glucocorticoid responsive element, and thereby the potential distance between the VDRE and the basal regulatory elements can be reduced. A model is presented for the contribution of both the VDRE and proximal promoter elements to the enhancement of OC gene transcription in response to vitamin D. The vitamin D receptor plus accessory proteins may function cooperatively with basal regulatory factors to modulate the extent to which the OC gene is transcribed.**

Osteocalcin (OC) is a bone-specific protein that is expressed with the onset of mineralization of the collagenous bone extracellular matrix *in vivo* (1) and *in vitro* (2, 3). Normal diploid osteoblasts express the OC gene after proliferation has ceased during development of the bone tissue-like extracellular matrix, as reflected by transcription, cellular mRNA accumulation, and OC protein biosynthesis. In contrast, OC gene expression is constitutive in ROS 17/2.8 osteosarcoma cells during and after proliferation (4). In both normal diploid and transformed bone cells, vitamin D enhances OC gene expression at the three principal levels—transcription, mRNA accumulation, and protein synthesis (5, 6).

OC gene transcription is controlled by the combined activity of a series of positive and negative promoter elements that respond to physiological mediators. Basal and enhanced transcription represent key components of control that are regulated by sequence-specific protein–DNA interactions. Consequently, we examined two proximal basal promoter elements, the OC box and "TATA" box, as well as the vitamin D responsive element (VDRE). The OC box [nucleotides (nt) −99 to −76], which includes the CCAAT motif as a central core and is highly conserved between the rat and human OC gene promoters, serves as a proximal promoter element that in part controls basal transcription (7, 8). Another highly conserved basal regulatory domain in the OC gene proximal promoter is the TATA box region (nt −44 to +23), which contains a consensus glucocorticoid responsive element (GRE) (9). The VDRE provides a key component of steroid hormone-mediated transcriptional enhancement of OC gene expression and resides between nt −466 and −437 in the rat (10–12) and between nt −512 and −485 in the human OC gene promoters (13–15). *In vivo* binding of the 1,25-dihydroxyvitamin $D_3$ receptor (VDR) is at least in part regulated by nuclear accessory factors (15, 16), and several lines of evidence support the requirement of VDR phosphorylation for hormone binding (17, 18) and upregulation of transcription (H.D., unpublished data). We have demonstrated (10, 19) that both basal elements and the VDRE exhibit sequence-specific binding of transcription factors.

Defining molecular mechanisms that show the relationship of the contributions of basal regulatory elements and enhancer sequences is requisite to understanding physiological control of OC gene transcription. Our observation (6) that the vitamin D-mediated enhancement of OC transcription inversely correlates with the basal level of OC gene expression suggests that there is a coordinate transactivation involving the contribution of activities at the VDRE and basal elements. Here the possibilities include, but are not restricted to, coordinate occupancy of independent regulatory elements in proximal and distal domains of the OC gene promoter by the VDR and/or other sequence-specific factors. The VDR may also interact directly with one or more regulatory factors associated with proximal basal regulatory sequences. This interaction can be facilitated by chromatin structure when the three-dimensional organization of the nucleus is considered.

Here we present evidence supporting two classes of vitamin D-responsive modification in protein–DNA interactions in the OC gene promoter: (*i*) sequence-specific and antibody-sensitive *in vitro* binding of the VDR derived from bone-like ROS 17/2.8 osteosarcoma cells to the VDRE and (*ii*) vitamin D-dependent enhancement of protein–DNA interactions at the TATA box/GRE that are not directly VDR-mediated. Results

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Abbreviations: OC, osteocalcin; VDRE, vitamin D responsive element; nt, nucleotide(s); GRE, glucocorticoid responsive element; VDR, vitamin D receptor.

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are also presented that provide the basis for a model of the higher-order structure of the OC gene promoter, as reflected by interactions with the nuclear matrix and the presence of nucleosomes within the region of the OC promoter spanning the VDRE, the OC box, and the TATA/GRE. Such a model is consistent with functional cooperativity of the upstream VDRE and proximal regulatory elements via VDR and basal regulatory factor interactions that may support hormone-mediated enhancement of OC gene expression.

## MATERIALS AND METHODS

**Cell Culture.** Rat osteosarcoma-derived ROS 17/2.8 cells (gift of G. Rodan, Merck Sharp & Dohme, West Point, PA) were maintained as described (20). Cells were treated with 10 nM 1,25-dihydroxyvitamin $D_3$ (gift of M. Uskoković, Hoffmann–La Roche, Nutley, NJ), 0.1 $\mu$M dexamethasone, or vehicle (95% ethanol) for 24 hr prior to harvesting.

**Preparation of Soluble Nuclear Proteins and Nuclear Matrix Proteins.** Soluble nuclear proteins were prepared from ROS 17/2.8 cells on day 8 after plating and stored at −70°C in 2× binding buffer (see below) (21). Porcine intestinal extracts (22) and nuclear matrix proteins (23) were prepared as described.

**Gel Mobility Shift Assays.** All probes were labeled with $^{32}P$ using T4 polynucleotide kinase (New England Biolabs). Monoclonal antibodies against the porcine intestinal VDR (22) that cross-react with the rat VDR (antibody IVG8C11) or recognize only the porcine VDR (antibody XVIE6E6G10) were used in gel mobility shift assays. Oligonucleotides encoding the human H4 histone gene promoter nuclear matrix protein attachment site (NMP-1) (24) and the human OC VDRE (13–15) were used as unlabeled competitors. DNA binding reactions were performed as described (10).

**Nucleosome Organization Studies.** Nuclei were isolated from ROS 17/2.8 cells on day 7 (25), partially digested with micrococcal nuclease, resolved in 2% agarose gels, and transferred to Zeta-Probe membrane. Probes were prepared by restriction endonuclease digestion from pOC3.4 and labeled with [$^{32}P$]dCTP by the random-primer method (26).

## RESULTS AND DISCUSSION

**Protein–DNA Interactions at the VDRE.** Analysis of the transcriptional activity of reporter gene constructs establishes that the domain distal to nt −428 of the rat OC gene promoter is necessary for vitamin D-mediated enhancement of OC gene expression (11). We have reported (10) that increased OC gene transcription after treatment of ROS 17/2.8 osteosarcoma cells with 10 nM vitamin D results in the increased representation of two protein–DNA complexes. These interactions have been mapped at single nucleotide resolution to the VDRE that resides between nt −466 and −437 (10). Sequence specificity of the enhanced vitamin D-mediated protein–DNA interactions at the VDRE is further supported by the inability of synthetic oligomers with mutations in the imperfect direct repeat GGGTGANNNAGGACA (where N is any nucleotide) to compete for binding (data not shown). To definitively identify these enhanced protein–DNA interactions as directly involving the VDR, we used monoclonal antibodies directed at the VDR. Indeed, we show an antibody that recognizes the rat VDR, but not a porcine-specific or a nonspecific antibody, substantially reduces the binding of ROS 17/2.8 nuclear protein extracts to the VDRE (Fig. 1*A*). In previous reports, partially purified recombinant human VDR, alone or in combination with non-tissue-specific mammalian nuclear accessory factors, has been shown to bind the VDREs of both the human and rat OC promoters (27–29). The biological significance of our finding is the identification of the VDR as a component of an endogenous protein complex present in

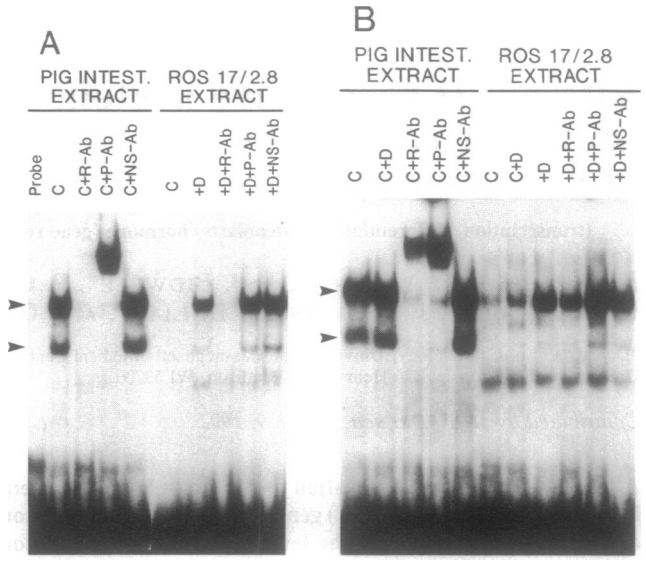


FIG. 1. Binding of ROS 17/2.8 or porcine intestinal (intest.) nuclear proteins to the VDRE of the rat OC gene. (*A*) Porcine intestinal nuclear extracts (lanes C) or nuclear extracts from untreated (lanes C) or vitamin D-treated (lanes +D) ROS 17/2.8 cells were incubated for 20 min with no antibody, antibodies against the porcine intestinal VDRs that recognize both porcine and rat (R-Ab) or only porcine (P-Ab) VDRs, or antibody against a nonrelated protein (NS-Ab). Labeled VDRE probe was added and the protein–DNA complexes were resolved by a gel mobility shift assay. (*B*) The same nuclear extracts were first incubated with labeled VDRE probe, the antibodies were added, and the protein–DNA complexes were resolved by a gel mobility shift assay. In some cases (lanes C+D), 10 nM vitamin D was added to the isolated extracts for 15 min prior to probe addition. Arrowheads indicate VDR antibody-sensitive protein–DNA interactions.

vitamin D-treated bone-like ROS 17/2.8 osteosarcoma cells that binds to the VDRE of the OC gene.

Bone-cell nuclear extracts incubated with VDR antibodies exhibited decreased binding to VDRE sequences rather than reduced electrophoretic mobility, as reported by other groups using these antibodies (11, 30). However, these investigators used porcine intestinal nuclear extracts as a source of proteins to examine vitamin D-mediated protein–DNA interactions at the VDREs. When we compared protein–DNA complexes formed by the VDRE and either porcine intestinal extracts or ROS 17/2.8 extracts for reactivity to VDR antibodies, we found striking differences (Fig. 1). The antibody that recognizes only the porcine VDR (XVIE6E6G10) caused a "supershift" of two protein–DNA interactions with the proteins present in the porcine intestinal extracts under conditions of either pre- or post-antibody incubation with DNA probe (Fig. 1). However, the antibody that recognizes both the rat and porcine VDRs (IVG8C11) caused both a supershift and a reduction in abundance of the protein–DNA complexes only under conditions of antibody postincubation (Fig. 1*B*). In contrast, preincubation of the antibody with the porcine intestinal extract completely blocked formation of the protein–DNA complexes (Fig. 1*A*).

When the ROS 17/2.8 extracts were preincubated with the species-cross-reactive VDR antibody, there was a dramatic reduction in the formation of two protein–DNA complexes (Fig. 1*A*). However, with addition of antibody after formation of the protein–DNA complex, the reduction was not as pronounced (Fig. 1*B*). Whereas the antibodies caused a supershift when incubated with the porcine intestinal extract, no evidence of a protein–DNA complex with altered mobility has been observed with the ROS 17/2.8 extracts under identical incubation conditions.

In both porcine and ROS 17/2.8 extracts, a dependency on vitamin D for formation of the VDR–VDRE protein–DNA complex is observed but to different extents. The control porcine extract contains a relatively high level of proteins that bind to the VDRE (Fig. 1*B*, lane 1) and addition of 10 nM vitamin D to the extracts increases the level of this interaction only slightly (Fig. 1*B*, lane 2). Similarly, when vitamin D is added to nuclear extracts from control ROS 17/2.8 cells (Fig. 1*B*, lanes 6 and 7), only a small increase in VDR–VDRE interactions is observed. In contrast, when intact ROS 17/2.8 cells were treated for 24 hr with vitamin D prior to extract preparation, the resulting nuclear extracts had a significantly higher representation of proteins that specifically bind to the OC VDRE (Fig. 1 *A*, lane 7, and *B*, lane 8). In both intestine *in vivo* and tissue culture, cells are constantly exposed to vitamin D, which is present in serum. This could account for the presence of vitamin D-dependent protein–DNA complexes in both the control porcine and ROS 17/2.8 extracts (Fig. 1*B*, lanes 1 and 6). These observations are consistent with current models of VDR action in which vitamin D binding to the VDR ultimately results in the VDRE-specific receptor–DNA interactions that affect gene transcription.

The differences in the interactions between the monoclonal antibodies and the hormone-dependent VDR complexes present in different tissues (intestine vs. bone) and/or from different species (pig vs. rat) illustrate that caution must be exercised in extrapolating general mechanisms of vitamin D-regulated gene expression from a single experimental system or tissue source. In a broader sense, however, these data are consistent with the potential for multiple combinations of nuclear factors to form complexes with specific promoter elements, but only a unique subset of these interactions contribute to tissue-specific gene expression.

**Vitamin D-Responsive Protein–DNA Interactions in Proximal Basal Regulatory Elements.** We have shown (6) that the extent to which vitamin D increases OC gene expression is dependent on the level of basal OC gene expression at the time of hormone addition. To experimentally address molecular mechanisms mediating the observed relationship between basal level of expression and the extent of vitamin D responsiveness, the proximal basal regulatory regions of the OC gene promoter were systematically examined to evaluate vitamin D-responsive changes in protein–DNA interactions associated with hormone-enhanced transcription. Focusing first on the area of the OC promoter containing the highly conserved OC box (nt −99 to −76), as can be seen in Fig. 2, a slight increase in one band was observed in extracts from vitamin D-treated cells using the segment from nt −141 to nt −44 as a probe. However, none of the protein–DNA complexes was affected by antibody to the VDR. We have previously reported that certain protein–DNA interactions at the OC box element were significantly enhanced by vitamin D (4, 10) when the cloned 24-base-pair oligonucleotide was used as a probe. Further analysis, however, indicated that the juxtaposition of two characteristic steroid half elements (one in the OC box and the other in the vector) results in the formation of an antibody-sensitive VDR binding site. Thus, a VDR binding site is not present in the OC box segment of the native OC gene promoter.

We next examined vitamin D-responsive protein–DNA interactions in the highly conserved TATA box region of the OC gene promoter. This basal regulatory region binds the general transcription factor TFIID (the TATA-binding factor) and associated proteins (31). In addition, two recent reports indicate the presence of a GRE based on (*i*) purified glucocorticoid receptor binding *in vitro* to a consensus GRE that overlaps the TATA box of the human OC promoter (9) and (*ii*) 5′ deletions of the human OC promoter linked to a chloramphenicol acetyltransferase reporter gene (14). A comparison of the rat and human OC promoters shows a high degree of homology in the TATA/GRE region (5, 9).

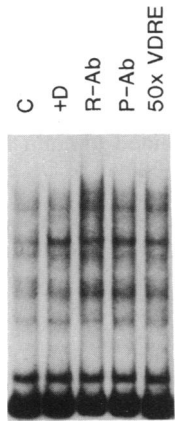


FIG. 2. Protein–DNA complexes in the OC box region formed using nuclear extracts from control (lane C) and vitamin D-treated (lane +D) ROS 17/2.8 cells. The vitamin $D_3$-treated extracts were incubated with antibodies against the porcine intestinal VDRs that cross-react with the rat VDR (R-Ab) or are specific for the porcine VDR (P-Ab), as described for Fig. 1, or with a 50-fold molar excess of the human OC gene VDRE oligonucleotide (lane 50× VDRE). Labeled OC box region DNA probe (nt −141 to −44) was then added to these extracts or to nuclear extracts from untreated ROS 17/2.8 cells (lane C), and protein–DNA complexes were resolved by a gel mobility shift assay.

By gel shift analysis, we demonstrate two vitamin D-enhanced protein–DNA interactions at the TATA box/GRE region (nt −44 to +23), a prominent enhancement of a rapidly migrating complex and a minor but reproducible increased representation of a lesser-mobility protein–DNA complex (designated by arrows in Fig. 3). However, formation of these protein–DNA complexes is not blocked by the VDR antibody nor is it competed by the VDRE oligonucleotide. It therefore appears that vitamin D-responsive protein–DNA interactions observed in the region from nt −44 to nt +23 are not directly mediated by the VDR. Interestingly, the observation that

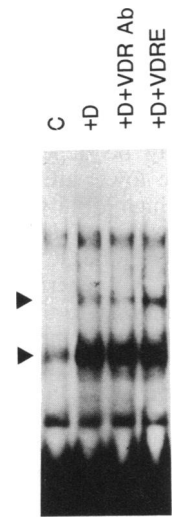


FIG. 3. Vitamin D-responsive protein–DNA interactions in the TATA box region of the rat OC gene promoter. Labeled TATA box region probe (nt −44 to +23) was added to nuclear extracts from untreated (lane C) or vitamin D-treated (lane +D) ROS 17/2.8 cells (10 nM, 24 hr), and protein–DNA complexes were resolved by gel mobility shift assay. Arrowheads indicate the two protein complexes that are enhanced as a result of vitamin D treatment of the cells. Nuclear extracts from vitamin D-treated extracts were incubated with antibody against the porcine intestinal VDR that recognizes the rat VDR (lane +VDR Ab) or with a 200-fold molar excess of the human OC gene VDRE oligonucleotide (lane +VDRE).

treatment of ROS 17/2.8 cells with the synthetic glucocorticoid dexamethasone (0.1 $\mu$M) enhances nuclear protein–DNA interactions in this proximal promoter region (data not shown) further suggests that this segment of the OC gene promoter contains a GRE. These results are consistent with reports in which combined vitamin D and dexamethasone treatment modulates the level of OC expression in a manner different from the effect of either hormone alone (14, 19, 32).

**A Model for Three-Dimensional Organization of the OC Gene Promoter Supporting Interaction of the VDRE and Proximal Basal Regulatory Domains.** Although the proximal basal regulatory region of the promoter alone is not capable of conferring vitamin D responsiveness to a heterologous reporter gene (11, 13), a potential role for these regulatory elements as a component of the mechanism through which vitamin D enhances OC gene expression is suggested. In a general biological context, these findings serve as the basis for postulating that basal transcription and enhancement are not independently controlled by unique promoter domains. Rather, our results are consistent with the multipartite organization of the OC gene promoter where the TATA/GRE element and the OC box/CCAAT element, together with the VDRE, synergistically contribute to the vitamin D-mediated enhancement of OC gene transcription. Such promoter domain interactions can respond to a series of regulatory signals and, thereby, accommodate the specific requirements for the extent to which the OC gene is expressed under various physiological conditions (2, 3, 6). The viability of this model is supported by two lines of evidence that provide a potential mechanism through which interactions between the VDRE and proximal promoter regulatory elements mediate transcriptional control within the three-dimensional context of nuclear architecture and chromatin organization.

Nuclear matrix association of the OC gene when actively transcribed may provide structural constraints on promoter conformation and may also facilitate colocalization of the gene and its transcription factors. A functional relationship between several actively transcribed genes and the nuclear matrix has been reported (33). Recent results indicate that the human H4 histone gene FO108, which is associated with the nuclear matrix when expressed, exhibits gene–nuclear matrix interactions at a strong positive promoter regulatory element designated NMP-1. An ATF-like factor that binds to this NMP-1 site is a nuclear matrix component (24). In addition, the overall association of other actively expressed genes with the nuclear matrix may be dependent on the combined interactions of multiple lower-affinity DNA binding sites, rather than on a single high-affinity site (34, 35). A possible role of the nuclear matrix in modulating the structural and/or transcriptional properties of the OC promoter was, therefore, suggested by the presence of a series of elements in the OC gene promoter with homology to the NMP-1 nuclear matrix binding domain of the histone gene (24).

As demonstrated by gel mobility shift assay in Fig. 4, a probe spanning the VDRE region of the OC gene promoter (nt −640 to −430) binds nuclear matrix proteins from ROS 17/2.8 cells expressing OC. Specificity of two nuclear matrix protein–DNA interactions with the OC gene promoter is indicated by competition with an oligonucleotide encoding the H4 histone NMP-1 binding site at a 360-fold or a 720-fold molar excess. The slight competition of the human OC VDRE at a 720-fold molar excess may be due to the close proximity of the VDRE and one of the putative nuclear matrix binding sites. Since these nuclear matrix interactions possibly involve multiple low-affinity DNA-binding sites (34, 35), the amounts of specific competition required to affect the protein–DNA interactions are higher than what would be required for a single high-affinity binding site.

The second line of evidence implicating nuclear structure in the potential interaction of the VDR with basal regulatory

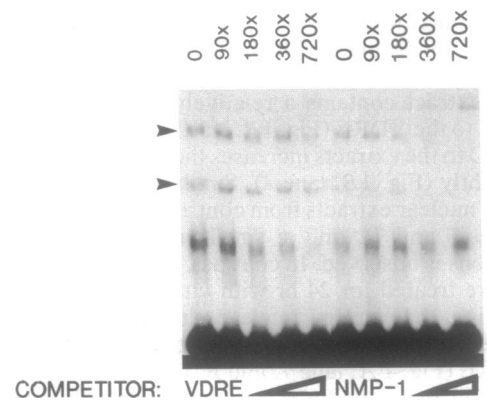


FIG. 4. Sequence-specific interactions of nuclear matrix proteins with the OC gene promoter. Gel mobility shift competition analysis of nuclear matrix proteins from vitamin D-treated (24 hr, 10 nM) ROS 17/2.8 cells binding to nt −640 to −430 (*Ban* I–*Bst*NI fragment) of the rat OC gene promoter. Arrowheads indicate sequence-specific protein–DNA interactions. Nuclear matrix proteins were preincubated with oligonucleotides corresponding to the human OC VDRE (VDRE) or the NMP-1 binding site from the human H4 histone gene FO108. Labels above the lanes indicate the fold molar excess of oligonucleotide included in the corresponding gel mobility shift binding reaction mixtures.

factors in vitamin D-enhanced OC gene transcription is the presence of nucleosomes in the region of the promoter where the VDRE, OC box, and TATA/GRE regulatory elements reside. As indicated in Fig. 5, when nuclei from ROS 17/2.8 cells expressing OC are subjected to limited digestion with micrococcal nuclease and Southern blots are probed with DNA segments spanning the OC gene VDRE or the OC box and TATA/GRE, the presence of nucleosomes is indicated. Three 180-bp nucleosomes can be accommodated within the promoter sequence spanning the VDRE, the OC box, and the TATA/GRE region, and thus the potential distance between these sequences can be reduced.

Thus these data raise the possibility of functional cooperativity between proteins interacting at both the basal regulatory sequences and the VDRE that, independently and/or together (15, 16), may contribute to enhancement of OC gene transcriptional activity, as schematically illustrated in Fig. 6. Although this model does not account for protein–protein interactions within the VDRE, it provides a basis for addressing relationships between multiple OC gene promoter elements. This model defines several elements where proteins bind and suggests a three-dimensional structure

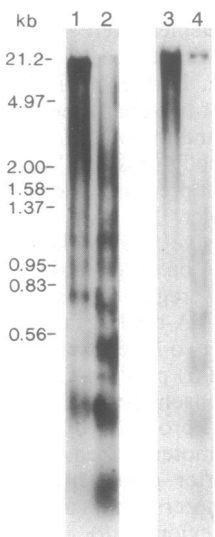


FIG. 5. Nucleosome organization in the rat OC gene promoter. Nuclei isolated from ROS 17/2.8 cells actively expressing the OC gene were subjected to partial digestion with micrococcal nuclease for 2 min (lanes 1 and 3), 5 min (lane 4), and 10 min (lane 2). DNA was isolated and fractionated electrophoretically in 2% agarose gels, and Southern blots were hybridized to probes representing the OC box and TATA/GRE (a *Sac* I–*Ava* I fragment, nt −309 to +146; lanes 1 and 2) or the VDRE (a *Hin*cII–*Bgl* II fragment, nt −527 to −344; lanes 3 and 4) regions of the OC gene promoter. Radiolabeled markers electrophoretically fractionated in adjacent lanes of the gel were *Eco*RI–*Hin*dIII λ phage DNA restriction fragments.

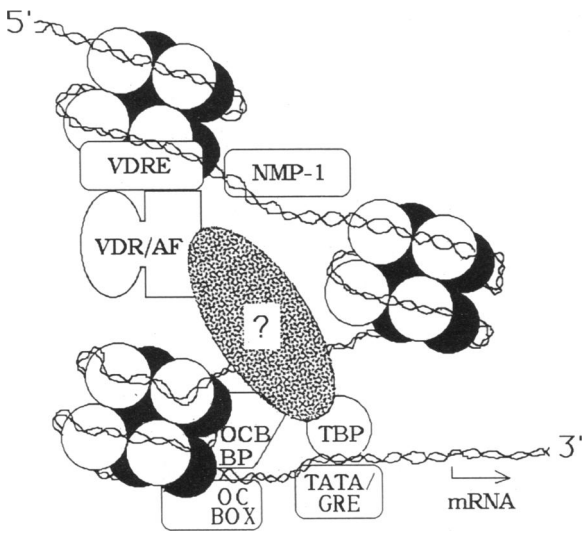


FIG. 6. Model for a three-dimensional configuration of the rat OC gene promoter showing interactions for the VDRE, the basal regulatory OC box, and TATA/GRE elements. Schematically illustrated are nuclear matrix protein–DNA interactions near the VDRE that could serve to structurally anchor this region of the promoter to impose conformational constraints on chromatin organization and/or to concentrate transcriptional factors that facilitate VDR binding to the VDRE. Association of the OC gene promoter with the nuclear matrix and the presence of nucleosomes with the VDRE, OC box, and TATA/GRE reduces the distance between these regulatory elements and thereby potentially modulates cooperative interactions that enhance OC gene expression. The question mark indicates the potential involvement of auxiliary proteins in mediating the interactions between these regulatory complexes. AF, accessory factor; OCB BP, OC box binding proteins; TBP, TATA/GRE binding proteins.

whereby these DNA-binding proteins may themselves interact by protein–protein interactions with other transcription factors. Remaining unanswered is whether the VDR/accessory factors (15, 16), CCAAT/OC box binding proteins, and TATA/GRE binding proteins interact directly or whether this occurs indirectly through other as-yet-unidentified factors. It must be emphasized that proof for such a model is required and, unquestionably, the proposed regulatory mechanisms are simplifications of extremely complex biological relationships. However, the viability of our model is that it spatially accounts for the potential contribution of interrelated activities from multiple elements in the OC gene promoter and, thereby, provides a basis for the regulation of transcription in relation to cell structure.

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