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Postproliferative transcription of the rat osteocalcin gene is reflected by vitamin D-responsive developmental modifications in protein–DNA interactions at basal and enhancer promoter elements

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ABSTRACT **In the osteocalcin (OC) gene promoter, both independent positive and negative regulatory elements, as well as others with contiguous [TATA/glucocorticoid-responsive elements (GRE)] or overlapping [TATA/GRE, vitamin D-responsive enhancer elements (VDRE)/AP-1, and OC box/AP-1] domains, are sites for modifications in protein–DNA interactions. In the present studies, we have examined nuclear protein extracts from fetal rat calvarial cells that undergo a developmental sequence of bone cell differentiation. Our results demonstrate modifications in protein–DNA interactions that relate to the developmental stages of the osteoblast and support developmental regulation of OC gene transcription. Basal expression of the OC gene is associated with sequence-specific protein–DNA interactions at the OC box, VDRE, and TATA/GRE box. Distinct differences are observed in proliferating osteoblasts, where the OC gene is not transcribed compared to postproliferative, differentiated osteoblasts that transcribe the OC gene. Furthermore, the protein–DNA complexes that reflect hormonal control are also developmentally regulated, mediating both the transcriptionally active and repressed states of the OC gene. For example, in proliferating osteoblasts, a vitamin D receptor–antibody-sensitive complex is formed that is different from the DNA binding complex induced by vitamin D postproliferatively when the OC gene is transcribed. Mutational analysis of the steroid hormone binding domain and the overlapping AP-1 site at the VDRE supports mutually exclusive occupancy by Fos–Jun heterodimers and vitamin D receptor. Such protein–DNA interactions at the VDRE are consistent with repression of competency for vitamin D-mediated transcriptional enhancement in proliferating osteoblasts expressing high levels of Fos and Jun.**

During development of the osteoblast phenotype, expression of the bone-specific osteocalcin (OC) gene is induced postproliferatively. The gene is then upregulated during the osteoblast differentiation sequence when the extracellular matrix (ECM) has matured sufficiently to support the ordered deposition of mineral (1, 2). The OC gene becomes responsive to steroid hormones [e.g., 1,25-dihydroxyvitamin D_3 (vitamin D) and glucocorticoids] only after initiation of basal expression (3–5).

Transcriptional control plays a key role in mediating OC gene expression during osteoblast phenotype development. Several lines of evidence have established the contributions of a series of promoter regulatory elements to both basal expression and vitamin D enhancement. We have previously shown that AP-1 sites, which exhibit sequence-specific binding of the Fos–Jun protein heterodimer, reside within the basal regulatory OC (a 24-nt sequence with a central CCAAT motif) box and the vitamin D-responsive enhancer element (VDRE) (6). The *in vitro* demonstration of binding competency for the oncogene-encoded Fos and Jun proteins by these promoter sites raises the possibility that they act as suppressors of basal OC gene transcription and hormone responsiveness until a specific time in the developmental sequence. However, the relationship of AP-1 binding to activity of OC gene promoter regulatory sequences under physiological conditions remains to be established. We therefore examined the occupancy of both basal and hormone-responsive elements in the rat OC gene promoter within the context of the nuclear proteins that form *in vivo* regulatory protein–DNA complexes.

We present results that demonstrate distinct differences in the transcription factor complexes formed at three key OC gene regulatory elements by nuclear proteins from proliferating osteoblasts that do not express OC compared to those from postproliferative osteoblasts actively expressing the bone-specific protein. Sequence-specific protein–DNA interactions were examined at two elements involved in control of basal transcription, the OC box and the TATA box, which is both contiguous to and overlapped by glucocorticoid-responsive elements (GRE) as well as at an enhancer sequence, the hormone-responsive VDRE. Our studies indicate that AP-1 binding activity is downregulated postproliferatively with concomitant modifications in transcription factor complexes at these regulatory elements. These results support a contribution of AP-1 activity to control of developmental expression of the OC gene.

MATERIALS AND METHODS

Normal diploid osteoblasts (ROB) were isolated from the calvaria of 21-day gestation fetal rats (1, 2). Cells were treated with 10 nM vitamin D, μM dexamethasone (Dex), or vehicle for 24 hr where indicated.

OC protein secreted into the culture medium was quantitated by radioimmunoassay (7). Transcription was determined by nuclear run-on (8). H4 histone and OC mRNA were isolated (9) and assayed by Northern blot using, respectively, pPS7 (10) and pOC3.4 (11) probes.

Nuclear proteins were prepared by the method of Dignam *et al.* (12) as described by Holthuis *et al.* (13). The following oligonucleotide were used in gel mobility-shift assays (steroid half elements are underlined and mutated sequences are boldface): rat OC VDRE oligonucleotide (nt −467 to −439; CTGCACTGGGTGAATGAGGACATTACTGA), VDRE steroid mutant (CTGCACTGTATGAATGACTACATTACTGA), VDRE AP-1 mutant (CTGCACTGGGTGATCGTGGACATTACTGA), OC box (nt −99 to −76); human OC VDRE (nt −506 to −480); metallothionein IIA

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Abbreviations: OC, osteocalcin; ECM, extracellular matrix; VDRE, vitamin D-responsive enhancer element(s); GRE, glucocorticoid-responsive element(s); Dex, dexamethasone; MT, metallothionein.

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(MT-IIA) (nt −103 to −88) (14, 15); and H4 histone nuclear matrix attachment site NMP-1 (16). The TATA box region probe from pOC3.4 (11) spanned nt −44 to +23. All probes were end-labeled with [^{32}P]ATP and gel purified. DNA binding of nuclear protein to the OC box, VDRE (17), TATA box region, and AP-1 (15) probes was assayed as described. Recombinant Fos–Jun protein binding to the human OC VDRE was as described by Owen *et al.* (6).

RESULTS AND DISCUSSION

Developmental Regulation of OC Gene Basal Transcription and Enhancement. Primary diploid rat calvarial osteoblasts in culture undergo a developmental sequence of gene expression resulting in formation of nodules of multilayered osteocytic cells in a mineralized ECM (1, 2, 18–21). Fig. 1*A* shows that the expression of OC, a bone-specific gene, is upregulated as expression of the proliferation-specific H4 histone gene (22, 23) is reciprocally downregulated. We therefore analyzed nuclear extracts from proliferating (day 5) and differentiated osteoblasts when OC gene expression is ongoing during active mineral deposition (day 14) or downregulated in mature osteocytic cells (day 28). OC gene expression is detectable at the protein (Fig. 1*B*), mRNA (Fig. 1*C*), and transcriptional (Fig. 1*D*) levels only in the mature osteoblasts and is not expressed in proliferating normal osteoblasts. While vitamin D enhances OC gene expression on days 14 and 28, the hormone cannot induce expression when the gene is suppressed at the basal level in proliferating cells (day 5). The parallel relationship between OC mRNA accumulation and protein biosynthesis suggests that cellular representation of OC mRNA is the rate-limiting step for biosynthesis and secretion of the protein.

The postproliferative transcriptional upregulation of OC gene expression raises the question of how the gene is maintained in a nontranscribed state during the proliferation period of osteoblast differentiation and is derepressed postproliferatively. Fig. 2 is a schematic diagram of the rat OC gene promoter (11) illustrating a modularly organized series

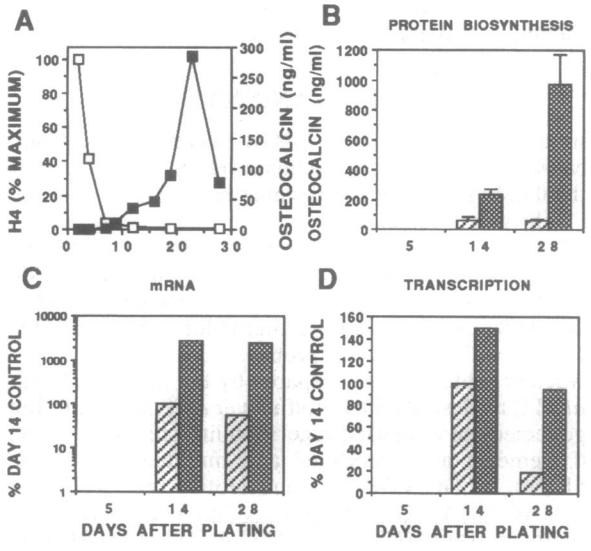

FIG. 1. Proliferation and phenotype-specific gene expression during differentiation of normal rat osteoblasts in culture. (*A*) ROB cells were harvested at the indicated times after plating and were analyzed for H4 histone mRNA (□) or for secreted OC protein (■). OC protein biosynthesis (*B*), mRNA accumulation (*C*), and transcription (*D*) were assayed during proliferation (day 5), ECM maturation (day 14), and after mineralization of the ECM (day 28) in control (hatched bars) or vitamin D-treated (cross-hatched bars) cells.

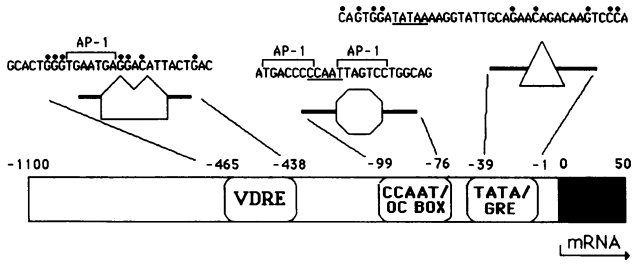

FIG. 2. Organization of rat OC gene promoter. Sequences of three regulatory regions—the VDRE, the OC box, and GRE—overlapping and adjacent to the TATA box are expanded. DNA contacts of the vitamin D (16) and glucocorticoid (A.H., R.B., J.L.S., G. Litwack, G.S.S. & J.B.L., unpublished data) receptors (●) and Fos–Jun dimers (6) (brackets) are indicated (2).

of promoter elements that independently and/or in combination contribute to OC gene expression (25, 26). Multiple activities within OC gene promoter regulatory elements are illustrated by a functional AP-1 site (6) overlapping the steroid-responsive half elements of the VDRE and by functional AP-1 sites flanking both the 5′ and 3′ ends of the CCAAT motif within the OC box (17, 27, 28).

AP-1 Activity During Osteoblast Differentiation. We have previously demonstrated by using purified recombinant Fos and Jun proteins that the AP-1 motifs within the basal regulatory OC (CCAAT) box and the VDRE are functional binding sites for the Fos–Jun protein heterodimer (6). Therefore, a principal question is whether these AP-1 sequences directly contribute to transcriptional suppression of the OC gene in proliferating osteoblasts. Fig. 3 shows that within the physiological context of the soluble nuclear proteins isolated from proliferating and postproliferative osteoblasts, the cessation of proliferation is accompanied by a dramatic decrease in AP-1 protein complex binding. In addition, treatment of proliferating osteoblasts with vitamin D results in an increased level of AP-1 binding activity (Fig. 3, day 5; compare lanes C and D) and, clearly, vitamin D treatment of postproliferative osteoblasts (day 14 or day 28) does not increase the minimal AP-1 activity that is present. These findings suggest that downregulation of AP-1 binding activity during the osteoblast developmental sequence could abrogate interactions of factors at AP-1 sites within critical basal regulatory and enhancer promoter elements of the OC gene that mediate transcriptional repression until completion of the proliferative period. Vitamin D stimulation of this AP-1 binding activity in proliferating osteoblasts is consistent with strengthening repression of the OC gene since vitamin D is a

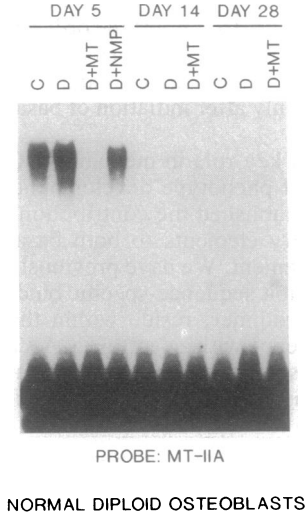

FIG. 3. Downregulation of AP-1 DNA binding activity with cessation of cell proliferation. Nuclear protein extract from untreated (lanes C) or vitamin D-treated (lanes D) cells was incubated with a probe representing the AP-1 site of the MT-IIA gene alone or in the presence of a 200-fold molar excess of unlabeled probe (lanes D+MT) or a nonspecific competitor oligonucleotide (lanes D+NMP).

strong enhancer of OC gene transcription, but only when the gene is basally transcribed (3). It is reasonable to anticipate that such a transcriptional repression mechanism is operative within the context of developmentally regulated expression of various members of the Fos and Jun superfamily of transactivation factors, posttranslational modifications in these protooncogene-encoded proteins, and interactions with other nuclear factors that bind within promoter regulatory elements that include AP-1 motifs.

Developmental Modifications in Protein–DNA Interactions at Basal Regulatory Elements. When we examined protein–DNA interactions at the OC box, which contains the CCAAT basal transcriptional element overlapped by two functional AP-1 sites during the osteoblast developmental sequence, we found that proliferating (day 5) osteoblasts support two major protein–DNA interactions within the OC box, one of which is retained and enhanced when the OC gene is transcribed postproliferatively (days 14 and 28) (Fig. 4). Proliferating cells contain several additional protein complexes (open arrowheads), which interact with the OC box but are represented in lower abundance. Importantly, these protein–DNA interactions are not detectable with nuclear protein extracts from postproliferative day-14 or day-28 osteoblasts expressing OC and potentially represent interactions involved in repressing the function of this basal transcriptional element in proliferating cells that are not expressing OC.

The other primary transcriptional element of the OC gene, the TATA box, is contiguous to and overlapped by GRE (refs. 29 and 30, and A.H., R.B., J.L.S., G. Litwack, G.S.S., & J.B.L., unpublished data). This relationship of a DNA binding hormone receptor with a primary basal transcriptional element may represent another mechanism through which the basal and hormone-modulated level of OC gene expression is controlled. The basis for such a regulatory mechanism is supported by vitamin D-mediated changes in protein–DNA interactions at the TATA box (26) and the potential for interactions between activities at proximal basal and upstream hormone-responsive enhancer elements by increasing the proximity of independent regulatory sequences within the three-dimensional context of nuclear architecture (e.g., chromatin structure, placement of nucleosomes, and gene–nuclear matrix interactions) (26). We examined the protein–DNA interactions at the TATA box/GRE in response to the addition of Dex, a synthetic glucocorticoid hormone, in proliferating osteoblasts (day 5) when the OC gene is transcriptionally inactive and in differentiated osteoblasts (day 28) when there are high levels of OC gene expression. In the proliferating cells, several high-mobility protein–DNA interactions were observed (Fig. 5 *Left*, lane C) and representation of these complexes did not change in abundance or mobility after Dex treatment (Fig. 5 *Left*, lane DEX). In contrast, mineralized (day 28) osteoblasts exhibit two prominent sets of protein–DNA interactions. The lower-mobility interaction (Fig. 5 *Right*, upper arrowhead) is decreased in abundance in response to Dex, while the higher-mobility group of interactions (Fig. 5 *Right*, lower arrowhead) exhibits increased abundance after Dex treatment of the cells.

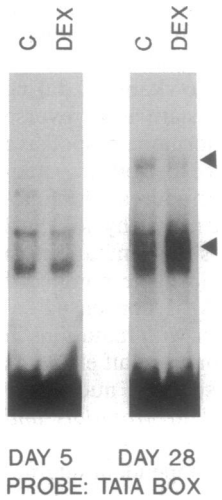

FIG. 5. Protein–DNA interactions at the TATA element in rat OC gene promoter change during differentiation of normal osteoblasts and are not Dex responsive in proliferating cells. Nuclear protein extracts were prepared from either control or Dex-treated proliferating (day 5) or differentiated (day 28) ROB cells and were analyzed by gel mobility-shift assay for binding to the TATA/GRE region of the OC gene promoter.

Developmental, Vitamin D-Responsive Transcription Factor Interactions at the VDRE Enhancer. To address mechanisms by which vitamin D increases OC gene transcription postproliferatively, we examined transcription factor binding at the VDRE in response to vitamin D. Fig. 6 shows vitamin D-responsive protein–DNA interactions during the three developmental periods. After vitamin D treatment of proliferating osteoblasts (day 5), two protein–DNA complexes become detectable (arrowheads), while after vitamin D treatment of postproliferative osteoblasts that are transcribing the OC gene (days 14 and 28), only the higher-mobility interaction indicated by the lower arrowhead is enhanced by vitamin D. The low-mobility vitamin D-induced protein–DNA complex present in the proliferating cells and indicated by the upper arrowhead is not detectable in either control or vitamin D-treated nonproliferating cells. We have established that both protein–DNA interactions represent different forms of the vitamin D receptor complex since formation of the two complexes are blocked by vitamin D receptor monoclonal antibodies (26).

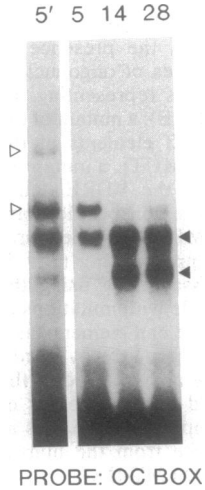

FIG. 4. Changes in protein–DNA interactions at the OC (CCAAT) element of the rat OC gene during differentiation of normal osteoblasts. Nuclear protein extracts were analyzed by gel mobility-shift assay for binding to the 24-nt OC box. All protein–DNA complexes are dependent on the CCAAT motif as shown by lack of binding to or competition with a CCAAT mutant probe (CGTGT) (data not shown). Lane 5′, 50-hr exposure, compared to an 18-hr exposure for other lanes, to demonstrate low abundance complexes. Protein–DNA interactions are increased (◂) or decreased (▷) in differentiated cells.

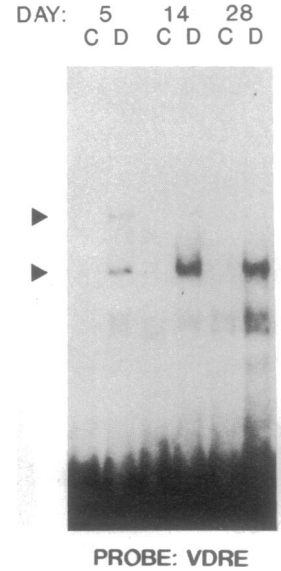

FIG. 6. Loss of a low-mobility and increased abundance of a higher-mobility protein–DNA interaction at the VDRE in response to vitamin D with the onset of OC gene expression. Gel mobility-shift assays were performed with nuclear proteins from untreated (lanes C) or 10 nM vitamin D-treated (lanes D) ROB cells and a rat OC VDRE probe.

The presence of a functional AP-1 site within the VDRE of the rat OC gene (6) provides the basis for postulating a component of the mechanism through which the gene is rendered nontranscribable and refractory to vitamin D during active cell proliferation until gene-specific activators reverse repression of basal transcription and hormone-responsive enhancement during the postproliferative period of osteoblast phenotype development. To further address the function of the AP-1 site within the VDRE in a more physiological context, we synthesized oligonucleotides with mutations in the steroid-responsive half elements to abrogate binding of the vitamin D receptor but retain the AP-1 consensus sequence (steroid mutant oligonucleotide) or with mutations in the AP-1 site that retain the steroid-responsive half elements (AP-1 mutant oligonucleotide). The specific nucleotide changes in these mutants are presented in *Materials and Methods*.

Fig. 7*A* shows that vitamin D receptor-dependent interactions at the rat OC gene VDRE are blocked by competition with the AP-1 mutant oligonucleotide (lanes D+AP-1 MUT) with an altered AP-1 site as effectively as by the wild-type VDRE (lanes D+VDRE). In contrast, the steroid mutant oligonucleotide (lanes D+ST MUT) does not compete significantly for these interactions, based on the modifications introduced in the steroid-responsive half element sequences. The very low level of competition observed is that seen with a nonspecific control competitor. In addition, when this AP-1 mutant VDRE oligonucleotide is used as a probe, binding of the vitamin D receptor does not occur, suggesting a specific requirement for spacing between the steroid-responsive half elements (31, 32) and that the spacer sequence may contribute to specificity of hormone receptor binding.

When the oligonucleotide containing mutations in the steroid-responsive half elements was used as a probe for protein–DNA interactions with nuclear proteins isolated from proliferating (day 5) cells, we found that the inability of the VDRE to bind the vitamin D receptor promotes binding of AP-1 activity (Fig. 7*B*). The protein–DNA interactions (arrowhead) detected with this DNA probe are of low mobility and are of increased abundance after vitamin D treatment, consistent with the properties of the AP-1 binding activity detected with an AP-1 consensus sequence probe (Fig. 3). More importantly, these interactions are blocked by competition with the OC gene VDRE steroid mutant, the OC gene wild-type VDRE, and consensus AP-1 site oligonucleotides, all of which contain an intact AP-1 binding site (Fig. 7*B*, lanes D+ST MUT, D+VDRE, and D+MT-IIA). Competition was not observed by the oligonucleotide containing intact steroid-responsive half elements with the internal AP-1 site mutated (Fig. 7*B*, lane D+AP-1 MUT). These results and identification (17) at single nucleotide resolution of protein–DNA contacts at the steroid-responsive half elements, but not at the internal AP-1 site, by nuclear extracts from cells expressing the OC gene suggest that the overlapping VDRE and AP-1 sites in the rat OC gene promoter are mutually exclusive transcription factor domains. Inability to bind the vitamin D receptor facilitates AP-1 binding and could represent a mechanism by which occupancy of the AP-1 site within the VDRE, together with occupancy of the AP-1 sites overlapping the CCAAT element in proliferating cells, is responsible for the inability of vitamin D to enhance OC gene transcription in the absence of basal expression.

Such a mechanism for regulation of OC gene expression may extend to the human OC gene. The highly conserved VDRE of the human OC gene contains an internal functional AP-1 site in a position analogous to that in the rat OC gene VDRE (Fig. 8*A*) and this site is competent to bind the Fos–Jun protein heterodimer (Fig. 8*B Left*). In addition, the human OC gene VDRE binds vitamin D-responsive protein complexes from mineralized osteoblasts (day 28) in a manner indistinguishable from the rat OC VDRE (Fig. 8*B Right*; compare to Fig. 5).

Recently, evidence has been presented that an AP-1 site upstream, but contiguous with the VDRE of the human OC gene, synergizes with the VDRE to elevate levels of OC gene transcriptional enhancement above those with the VDRE alone (33, 34). The rat OC gene similarly contains a second AP-1 consensus sequence at nt −485, only 25 nt 5′ to the VDRE; however, the activity of this upstream AP-1 sequence remains to be experimentally established. While "classical" AP-1 sites (TGACTCA) have a high affinity for the Fos–Jun protein complex, other sequences may preferentially interact with various members of the oncogene-encoded family of fos and jun transcription factors. Subtle variations in the sequences of AP-1 binding sites, and in the Fos–Jun family members that interact with each element or with other

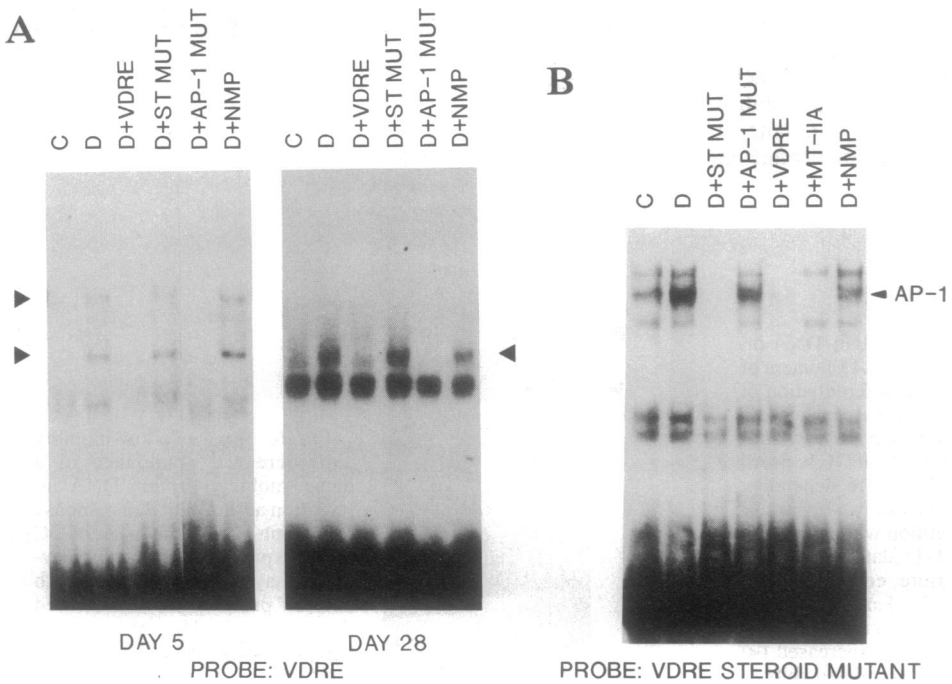

FIG. 7. Mutation of steroid-responsive half elements comprising the VDRE facilitates AP-1 binding to the internal AP-1 site. (*A*) Nuclear protein extracts were prepared from control (lanes C) or vitamin D-treated ROB cells and were assayed for binding to the rat OC VDRE. Extracts from the vitamin D-treated cells were also assayed in the presence of 200-fold molar excesses of oligonucleotide competitor DNAs representing the VDRE (lanes D+VDRE), a mutant of the steroid-responsive half elements of the VDRE (lanes D+ST MUT), a mutant of the AP-1 site within the VDRE (lanes D+AP-1 MUT), or a nonspecific competitor (lanes D+NMP). (*B*) Nuclear protein extracts from proliferating (day 5) osteoblasts were assayed by using the VDRE oligonucleotide with mutations in the steroid-responsive half elements as a probe. Extracts from the vitamin D-treated cells were also assayed in the presence of 200-fold molar excesses of oligonucleotide competitor DNAs, as in *A*, plus the AP-1 site from the human MT-IIA gene (lane D+MT-IIA).

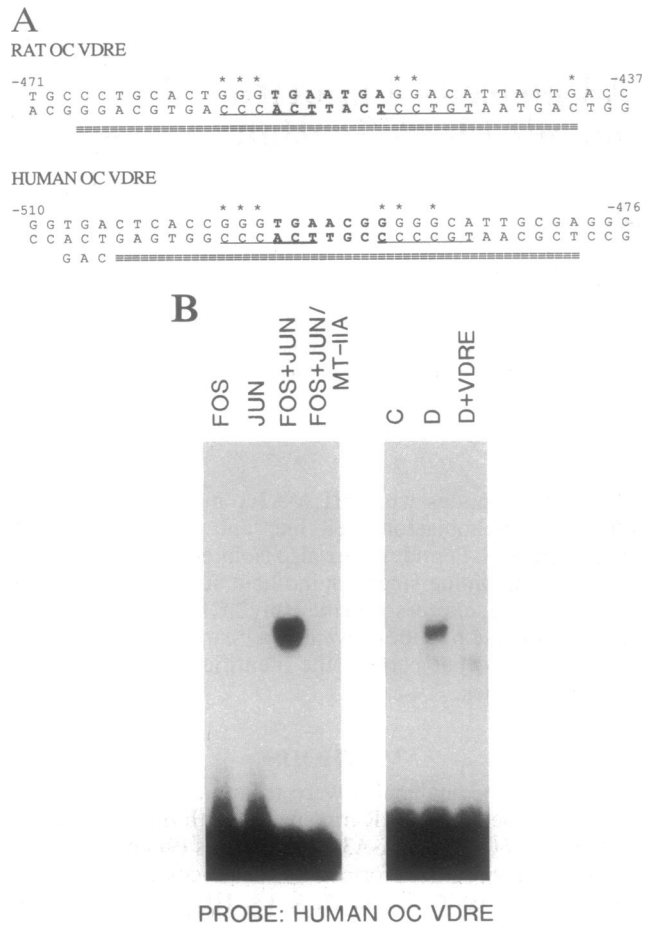

FIG. 8. Comparison of rat and human OC gene VDRE and demonstration of a functional AP-1 site in human OC VDRE. (*A*) Steroid-responsive half elements are single underlined, AP-1 consensus sequences are boldface, and DNA oligonucleotide probes used for gel mobility-shift assays are triple underlined. Nucleotides involved in vitamin D-responsive protein (rat) or vitamin D receptor (human) complexes are indicated with an asterisk. The 3 nt (GAC) at the 5′ end of the triple underline of the human OC VDRE probe were substituted for the endogenous sequence (TGA) to eliminate the second AP-1 consensus upstream but contiguous with the VDRE. (*B*) Gel mobility-shift assays using purified Fos and Jun proteins and human OC VDRE probe (*Left*) and blocked by competition with an AP-1 oligonucleotide (lane FOS+JUN/MT-IIA). (*Right*) Protein–DNA interactions of nuclear protein from day-28 osteoblasts with the human VDRE probe blocked by competition with a rat OC gene VDRE oligonucleotide (lane D+VDRE).

transacting factors (24, 35), could contribute to the mechanism controlling developmental expression of the OC gene. Such mechanisms may also be operative in regulating modifications in OC gene expression in response to a broad spectrum of physiological mediators affecting the bone cell phenotype.

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