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Identification of an Osteocalcin Gene Promoter Sequence That Binds AP1

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Abstract Osteoblasts are differentiated cells that produce bone matrix components including the bone-specific protein osteocalcin. The osteocalcin gene promoter has become a model for understanding how genes are regulated, specifically in osteoblasts. One model for cell-specific regulation suggests that osteoblast-expressed genes are regulated through common promoter sequences which bind osteoblast-specific transcriptional activators. The phenotype suppression model suggests osteoblast-specific promoters are switched off through the action of the common transcriptional activator AP1. We previously demonstrated that a short sequence element (OSCARE-2) in the osteocalcin promoter was homologous to a repressive element in the collagen type 1 (α1) promoters. In this paper we use electrophoretic mobility shift (EMS) assays to examine DNA–protein interactions in the OSCARE-2 sequence. In EMS assays, OSCARE-2 binds a complex of proteins, including AP1. This supports the role of AP1 sites in contributing to the regulation of the osteocalcin promoter. Exogenous c-JUN protein bound to OSCARE-2 and increasing c-JUN incubated with nuclear extract amounts caused a progressive increase in a higher-molecular-weight complex, consistent with c-JUN involvement in protein–protein as well as DNA–protein interactions. Anti-c-FOS antibody was capable of supershifting OSCARE-2 DNA–protein complexes produced using osteoblast-like cell nuclear extracts. In addition, EMS assays of nuclear proteins from osteoblast-like cells indicated that 1,25 $(OH)_2D_3$–inducible proteins are bound to OSCARE-2. Osteocalcin promoter constructs showed that OSCARE-2 contributed to the 1,25 $(OH)_2D_3$ response, albeit in a minor way. These data support the role of AP1 protein as a regulator of osteoblast-specific gene expression during osteoblast development.

Key words: osteocalcin promoter, AP1, osteoblast vitamin D induction, DNA binding

The osteoblast is a differentiated cell type that produces constituents of the extracellular matrix of bone. The protein osteocalcin is a marker of osteoblast function and the gene for osteocalcin is a model for understanding bone-specific expression. It is likely that the osteocalcin gene is controlled by both ubiquitous and specific factors. Sequential expression of osteoblast-specific genes occurs during the development of the osteoblast. One model suggests that the regulation of osteoblast-specific genes may occur through a regulatory element common to osteoblast-expressed promoters. The phenotype suppression model suggests that coordinate occupancy of AP1 sites in osteoblast-expressed genes, i.e., AP1 sites found adjacent to a steroid response element as in the osteocalcin, alkaline phosphatase, and collagen type I (α1) gene promoters, may mediate suppression of bone gene transcription [Lian et al., 1991]. It is possible that these two mechanistic models overlap: AP1 could act as a coregulator of osteoblast-specific factors independent of DNA binding, or AP1 could act at DNA elements that are homologous across bone-specific promoters where crucial transcriptional regulatory functions occur. Identification of AP1 binding sites in the osteocalcin promoter is a necessary prerequisite to understanding the role of AP1 in controlling osteocalcin expression.

In the rat osteocalcin promoter, it has been reported that there are functional AP1 elements positioned in the osteocalcin box (OC box) and the vitamin D response element (VDRE), and that these AP1 elements contribute to phenotype suppression [Owen et al., 1990]. In the human osteocalcin promoter, there are three functional elements that have adjacent AP1 sites,

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i.e., the VDRE, OC box, and TGFβ response elements, which may repress promoter activity through the adjacent AP1 site [Banerjee et al., 1994; Ozono et al., 1990; Schule et al., 1990].

In this study, we examine another potential AP1-like site (TGgCTCA) that, although within a highly conserved region, is not conserved in the rat promoter (TGtCctA) or mouse promoter (TGgCctc). This AP1-like site is in a region, OSCARE-2, previously examined because of strong homology to the collagen type I (α2) promoter [Goldberg et al., 1995a]. This 63 bp region between −225 and −163 has repressive activity demonstrable using osteocalcin promoter deletion constructs in NIH3T3 cells [Goldberg et al., 1995a]. Independent studies have shown that the species-conserved 5′ end of OSCARE-2 increases rat osteocalcin promoter activity in osteoblastic ROS17/2.8 cells [Towler et al., 1994].

Regulatory proteins that contribute to the function of human OSCARE-2 were sought using EMS assays with nuclear protein preparations from untreated and 1,25 $(OH)_2D_3$–treated human and rodent osteoblastic cells. Since 1,25 dihydroxyvitamin D3 treatment has been reported to increase AP1 binding activity to the VDRE sequence [Jaaskelainen et al., 1994; Owen et al., 1993], its effect on the AP1 binding activity to the OSCARE-2 sequence was examined. The contribution of OSCARE-2 to the 1,25 dihydroxyvitamin D3 response in these osteoblast-like cell lines was also examined using osteocalcin promoter deletion constructs. These data suggest that a 1,25 $(OH)_2D_3$–induced protein complex binds to OSCARE-2 and this sequence affects the 1,25 $(OH)_2D_3$ response of the osteocalcin promoter.

MATERIALS AND METHODS

All media and serum were obtained from ICN Flow Laboratories (Costa Mesa, CA). The human osteosarcoma cell lines SAOS-2, which does not express osteocalcin, and MG63, which does express osteocalcin under 1,25 $(OH)_2D_3$–induced conditions, were obtained from ATCC (American Type Culture Collection, Rockville, MD) and cultured in RPMI 1640 medium supplemented with 10% FCS, 6 mM glutamine (Cytosystems, Sydney, Australia), 0.2 U/ml human insulin (David Bull Labs, Mulgrave, Australia), 20 μg/ml gentamicin (David Bull Labs), 25 IU/ml penicillin, 25 μg/ml streptomycin, 14 mM sodium bicarbonate, and 28 mM HEPES (CSL, Melbourne, Australia). ROS17/2.8 and the UMR106.06 rat osteosarcoma cell lines, provided by S. Rodan (MSD Research Laboratories, West Point, PA) and T. J. Martin, respectively (St. Vincent's Institute of Medical Research, Melbourne, Australia), were cultured in Hams F12 medium supplemented with 7.5% fetal calf serum (FCS), 14 mM sodium bicarbonate, 1.5 mM glutamine, 0.8 mM $CaCl_2$, 10 μg/ml gentamicin, and 28 mM HEPES. The UMR106.06 and ROS17/2.8 cell lines are considered to represent earlier and later stages of osteoblast differentiation, respectively, since the latter but not the former expresses osteocalcin [Rodan and Noda, 1991].

Transient Transfection

DNA construct. The ΔOSCARE-2 constructs were derived from the human osteocalcin promoter constructs pOSCAT-2 and pOSCAT-3 previously described [Morrison et al., 1989], both of which have the intact VDRE (−513−−493); the OSCARE-2 sequence that lies between −225 and −163 was removed using PCR mutagenesis. Transient transfection assays were performed as previously described using calcium phosphate–DNA precipitation [Morrison et al., 1989]. Cells in exponential growth phase were cotransfected with CAT construct (20 μg) and pRSV-βgal (10 μg) as a control for transfection efficiency [Gorman et al., 1982]. ROS17/2.8 and UMR106.06 cells were transfected at 2.5 and 2×10^6 cells/cm^2, respectively. Cells were exposed to DNA for 16 h in DMEM with 2% charcoal-stripped (cs) FCS (DMEM–2% csFCS), which was then replaced by fresh DMEM–2% csFCS. Cells were replated into six-well plates, allowed to settle for 6 h before treatment for 16 h with 10^{-8} M 1,25$(OH)_2D_3$ or vehicle, and then cell lysates, obtained by freeze–thawing, were assayed. The β-galactosidase activity was assayed by cleavage of orthonitrophenyl-β-D-galactoside (Sigma, St. Louis, MO) and spectrophotometeric determination at 420 nm. A nonchromatographic, CAT extraction assay was used [Morrison et al., 1989], and CAT activity from the linear range of the assay in triplicate was normalized for transfection efficiency with respect to the β-galactosidase activity after correcting for background CAT and β-galactosidase values derived from mock transfections.

Electrophoretic mobility shift (EMS) assays. Nuclear extracts were prepared from confluent SAOS-2, MG63, ROS17/2.8, and UMR106.06 cells using a modification of Dignam et al. [1983]. The 1,25 $(OH)_2D_3$–treated ROS17/2.8 and UMR106.06 nuclear extracts were prepared from cells treated for 16 h with 10^{-8} M 1,25 $(OH)_2D_3$ or vehicle, and harvested at confluency. Nuclear extracts prepared from 10^7 cells were stored in buffer C (20 mM HEPES pH 7.9, 0.42 M NaCl, 3 mM $MgCl_2$, 0.5 mM DTT, and 0.5 mM PMSF) in 50 μl aliquots at −70°C. The 63 bp OSCARE-2 fragment was labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Du Pont, Boston, MA) by a fill-in reaction using the Klenow fragment of DNA polymerase 1 (Pharmacia, Sydney, Australia), and subsequently purified on 15% polyacrylamide gels. The binding reaction was based on Sagami et al. [1986]. EMS assays testing DNA–protein binding conditions showed the optimal binding capacity was at 50 mM NaCl and 10–25 mM $MgCl_2$. The labeled OSCARE-2 fragment (5 pmole, 4×10^5 cpm) was incubated with saturating quantities of nonspecific DNA [(poly(dI − dC) · (dI − dC), 10 μg] (Pharmacia, Sydney, Australia) in 10 mM HEPES, pH 7.9, 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, 2 mM $MgCl_2$, and 1 mM DTT. Nuclear extract protein (1–5 μg) was added to a total volume of 20 μl and the reaction incubated at room temperature or 4°C for 20 min. In competition experiments, the last addition was unlabeled competitor oligonucleotide. In the DNA binding reactions with recombinant human c-JUN protein (Promega, Sydney, Australia), the nuclear extract was pre-incubated with the c-JUN protein at room temperature for 15 min in the presence of 0.05 mM DTT and 1 μg nonspecific DNA [poly(dI − dC) · (dI − dC)] prior to the addition to reaction tubes. Reaction products were resolved on 4.5% polyacrylamide gels containing 5% glycerol at 30 mA for 3–4 h in 0.5 × Tris-Borate EDTA (25 mM Tris-Borate, 0.5 mM EDTA, pH 8.0) running buffer at 4°C. The dried gel was autoradiographed with intensifying screens at −70°C for 12–72 h. The EMS assay data was a representative of experiments performed at least twice.

Anti-c-FOS antibody was used to test for the presence of c-FOS in the gel shift complex. c-FOS is reported to be incapable of binding DNA by itself, requiring the presence of c-JUN or related proteins to form DNA binding heterodimers [Sambucetti and Curran, 1986]. The presence of an anti-c-FOS supershift is taken as evidence for the presence of a c-FOS-containing AP1-like complex in the DNA–protein complex. Anti-c-FOS was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and used according to the manufacturer's specifications. This antibody does not crossreact with other members of the AP1 family, including c-JUN, JUNB, FOSB, Fra-1, or Fra-2 (Santa Cruz Biotechnology technical report). A specific anti-c-JUN antibody that does not react with JUN-B and JUN-D [Santa Cruz Biotechnology Inc: c-JUN/AP-1 (N)] was also used. c-JUN peptide that complements the anti-c-JUN antibody, and c-FOS peptide that complements the anti-c-FOS antibody were both used as controls to neutralize the antibody according to the manufacturer's specifications (Santa Cruz Biotechnology Inc.).

Oligonucleotides

OSCARE-2. 5′-TCT GAT TGT GGC TCA CCC TCC ATC ACT CCC AGG GGC CCC TGG CCC AGC AGC ACG AGC TCC CAA-3′ was cross competed with oligonucleotides containing binding sites for general transcription factors (Strategene, La Jolla, CA) and the 70 bp VDRE derived from the human osteocalcin promoter.

The oligonucleotide sequences are as follows: **AP1,** 5′-CTA GTG ATG AGT CAG CCG GAT C-3′; **NF1/CTF,** 5′-ATT TTG GCT TGA AGC CAA TAT G-3′; **AP2,** 5′-GAT CGA ACT GAC CGC CCC GCG GCC CGT-3′; **AP3,** 5′-CTA GTG GGA CTT TCC ACA GAT C-3′; **SP1,** 5′-GAT CGA TCG GGG CGG GGC GAT C-3′; **VDRE,** 5′-GGC TGC CTT TGG TGA TCA CCG GGT GAA CGG GGG CAT TGC GAG GCA TCC CCT CCC TGG GTT TGG CTC TGC C-3′.

RESULTS

Sequence analysis of OSCARE-2 (−225−−163) showed it contained putative regulatory elements, some of which are in a region highly conserved in the rat osteocalcin promoter. The 5′ end of OSCARE-2 contains a 21 bp region that is highly conserved between the human (−225−−105), rat (−190−−170), and mouse promoter (−193−−173) (Fig. 1). Examination of the 21 bp sequence showed that a reverse CCAAT-like sequence (GtGTTA) is retained between species, whereas the AP1-like site (TGGCTCA) in the human sequence is not strongly retained in the rat or mouse. The human, rat, and mouse promoters have sequences

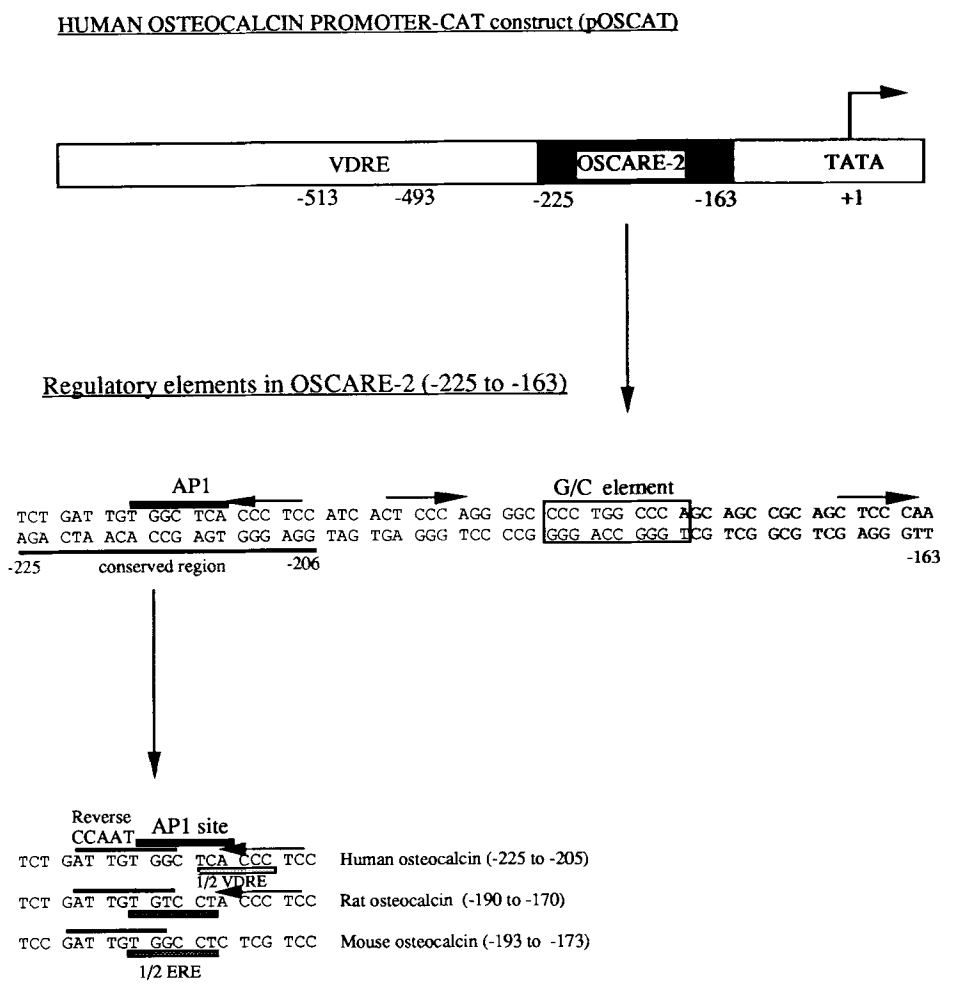

Fig. 1. Human osteocalcin promoter construct and regulatory elements in OSCARE-2. The human osteocalcin promoter construct (pOSCAT) used in this study contains the vitamin D response element (VDRE; −513−−493) and the 63 bp sequence (OSCARE-2; −225−−163) that lies within the more proximal promoter region and contains putative regulatory elements as indicated, some of which are in a region highly conserved in the rat and mouse promoters. The AP1-like site is not conserved in the rat or mouse promoter. There is a vitamin D half-site (1/2VDRE) in the rat promoter that is identical to that in the rat osteocalcin VDRE, and a similar site is conserved in the mouse promoter (underlined; ▨). In the human promoter there is a half-VDRE site (underlined; □) overlapping the AP1 site that is identical to that in the human osteocalcin VDRE. The reverse CCAAT-like motif is conserved in all three species (overlined). Further 3′ there is a G/C-rich element (boxed) with a possible repressive function in the human promoter [Goldberg et al., 1995a]. The arrows indicate 3 CTCCCA motifs that may play a yet unknown functional role.

highly homologous to zinc finger receptor response element half-sites [Schrader et al., 1995] (on the bottom strand; see Fig. 1): in the human the sequence is GGGTGA, which is identical to one of the half-sites of the upstream osteocalcin VDRE; in the rat the sequence is AGGACA, which again is identical to one of the rat VDRE half-sites; and in the mouse the sequence is AGGCCA, a 1 bp mismatch from the rat VDRE half-site.

If these putative DNA binding sites in the OSCARE-2 sequence are functional, then EMS assays should demonstrate a complex of proteins bound to the labeled OSCARE-2 fragment. The OSCARE-2 EMS assay with SAOS-2 nuclear extracts showed a complex banding pattern. As more nuclear protein was added, the intensity of the lower-molecular-weight complex was decreased as the upper band increased in intensity (Fig. 2A, lanes 1–6). Similarly, increasing the binding reaction temperature increased the level of bound protein and the proportion of oligonucleotide bound in the higher-molecular-weight complex. At lower temperatures, a faster-migrating complex D was formed (Fig. 2B, lanes 1, 2). At room temperature complex D was not pres-

ent, whereas the larger complex C was intensified (lane 3). Overall binding capacity was increased at 37°C (lane 4) with an array of faint complexes and a unique intense complex (asterisked) between C and D appearing. At higher temperatures (55°C and 65°C) the DNA–protein interaction was not apparent (lanes 5, 6).

Identifying the Factors Bound to the OSCARE-2 Fragment

Since sequence analysis of OSCARE-2 identified DNA binding sites for regulatory factors such as AP1, NF1, and SP1 (a G/C rich sequence), cross-competition studies were performed using SAOS-2 and UMR106.06 nuclear extracts to determine which of these factors may contribute to the multiple complexes bound to OSCARE-2 (Fig. 3A,B). In EMS cross-competition studies all the competitor oligonucleotides competed the higher-molecular-weight complex A. In SAOS-2 nuclear extracts the AP1 oligo fully competed the upper band (band A) at the lowest tested level of competitor, 20-fold molar excess. However, similar results were also observed using NF1/CTF, AP3, and AP-2 oligonucleotides, suggesting that the upper intense EMS assay band A could be composed of a number of factors. The SP1 oligonucleotide was tested at 100-fold molar excess only, where full competition of bands A and B was observed. The lower EMS band D was the only band that exhibited specific competition by the AP1 oligo, and it appeared unaffected by 100-fold molar excess of the other consensus factor binding oligos.

AP1 Analysis

AP1 binding to the human osteocalcin VDRE sequence has been reported by several authors [Jaaskelainen et al., 1994; Ozono et al., 1990; Schule et al., 1990]. Radioactively labeled VDRE was used in EMS assay with nuclear extract derived from MG63 cells, with AP1 oligonucleotide competition, resulting in the disappearance of an intense upper band (see Fig. 4A) exactly in accord with the results of Jaaskelainen et al. [1994]. This AP1-specific band comigrated with a faint band produced when 1.0 μg of purified recombinant c-JUN protein was incubated with the labeled VDRE (Fig. 4A, lane 4).

When 0.01 and 0.1 μg of c-JUN protein was incubated with labeled OSCARE-2 and SAOS-2 nuclear extract (Fig. 4B, lanes 2, 3), the intensity of the higher-molecular-weight complex increased (band A). This was associated with a

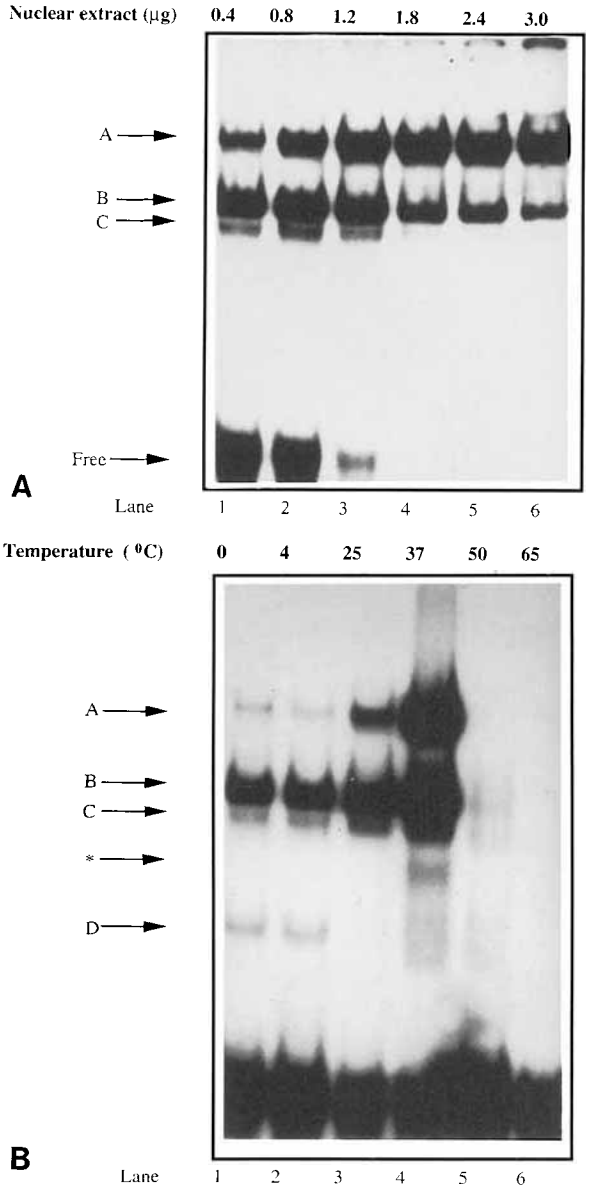

Fig. 2. Binding of nuclear proteins from SAOS-2 cells to OSCARE-2. The OSCARE-2 fragment was incubated at room temperature with increasing amounts of SAOS-2 nuclear extract (**A,** *lanes 1–6;* 0.4, 0.8, 1.2, 1.8, 2.4, and 3.0 μg). There is a progression to the higher-molecular-weight complex with increasing amounts of nuclear extract. The OSCARE-2 fragment was also incubated with SAOS-2 nuclear extract at increasing temperatures (**B,** *lanes 1–6;* 0, 4, 25, 37, 50, and 65 °C). The faster-migrating complex (*band D*) occurred at lower temperature and a triplet band (*asterisk*) was seen only at 37°C.

gradual decrease in the intensity of the AP1-specific complex D, apparent in longer exposure of the same gel (Fig. 4B, lanes 7, 8). The addition of more c-JUN, 1.0 or 2.5 μg, resulted in the appearance of faster-migrating bands (Fig. 4B, lanes 9, 10), with mobilities similar to the bands

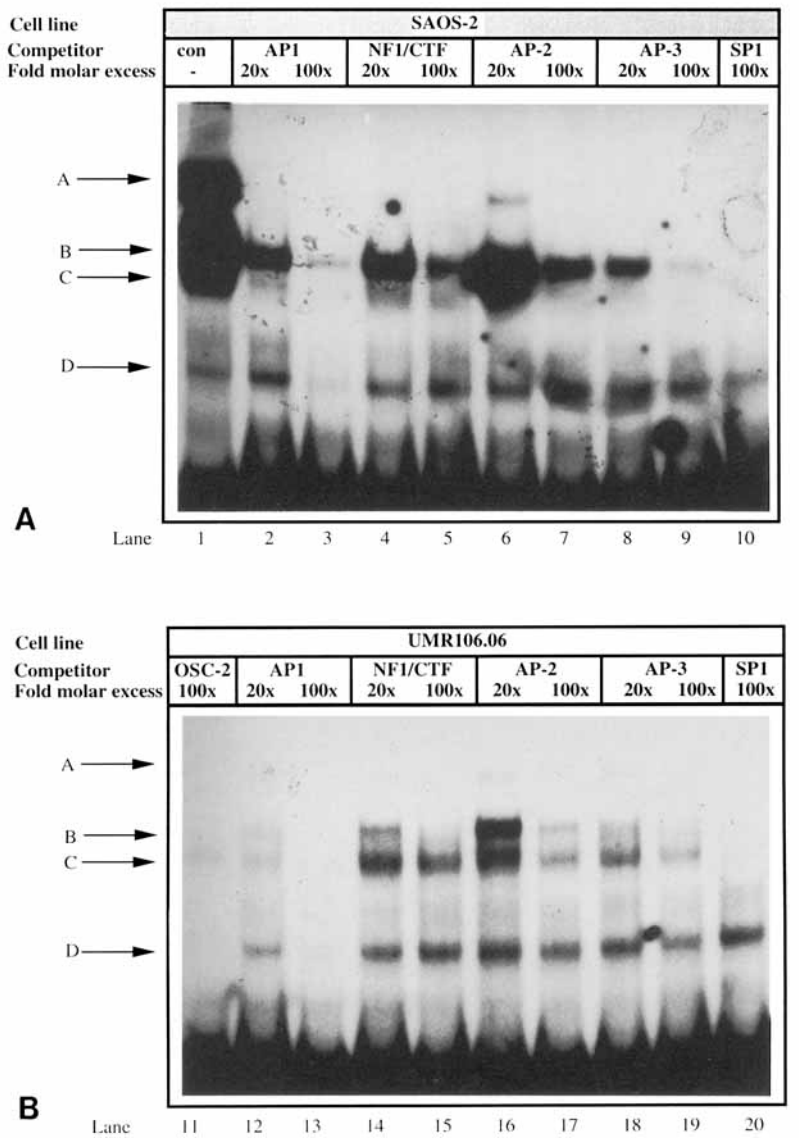

Fig. 3. Transcription factor sites using cross competition. The labeled OSCARE-2 fragment was incubated at 4°C with SAOS-2 (*lanes 1–10*) and UMR106.06 (*lanes 11–20*) nuclear extracts, and cross competed with unlabeled competitor oligonucleotides at 20- and 100-fold molar excess. Lane 1, no competitor; lanes 2 and 3, AP1 oligonucleotides; lanes 4 and 5, NF1/CTF oligonucleotides; lanes 6 and 7, AP2 oligonucleotides; lanes 8 and 9, AP3 oligonucleotides; and lane 10, SP1 oligonucleotides at 100-fold molar excess. The same order was followed in lanes 11–20 except lane 11 is cross competed with 100-fold unlabeled OSCARE-2. The UMR106.06 control is similar to lane 6 and is also the same as shown in Figure 5A, lane 3. Note that band D was competed by AP1 but no other oligonucleotide.

in EMS assays with the same amount of c-JUN protein alone (Fig. 4B, lanes 11, 12); these faster-migrating bands may represent c-JUN homodimers. The highest concentration of c-JUN caused a decrease in the higher-molecular-weight bound complexes and an increase in free probe (Fig. 4B, lanes 5, 10). The decrease in band A on addition of supraoptimal levels of recombinant c-JUN may reflect the depletion of heterodimer partner protein such as c-FOS in the extract, which is known to enhance the DNA binding ability of AP1 complexes. These data suggest that recombinant c-JUN could bind the OSCARE-2 element in the presence of nuclear extract.

c-FOS is not capable of binding DNA on its own, requiring a heterodimerization partner, which could be c-JUN, JUN-B, or JUN-D. c-FOS antibody produced a supershift of the OSCARE-2 element using SAOS-2 nuclear extract (Fig. 4C, lane 5). The antibody supershift experiments were done under conditions of high

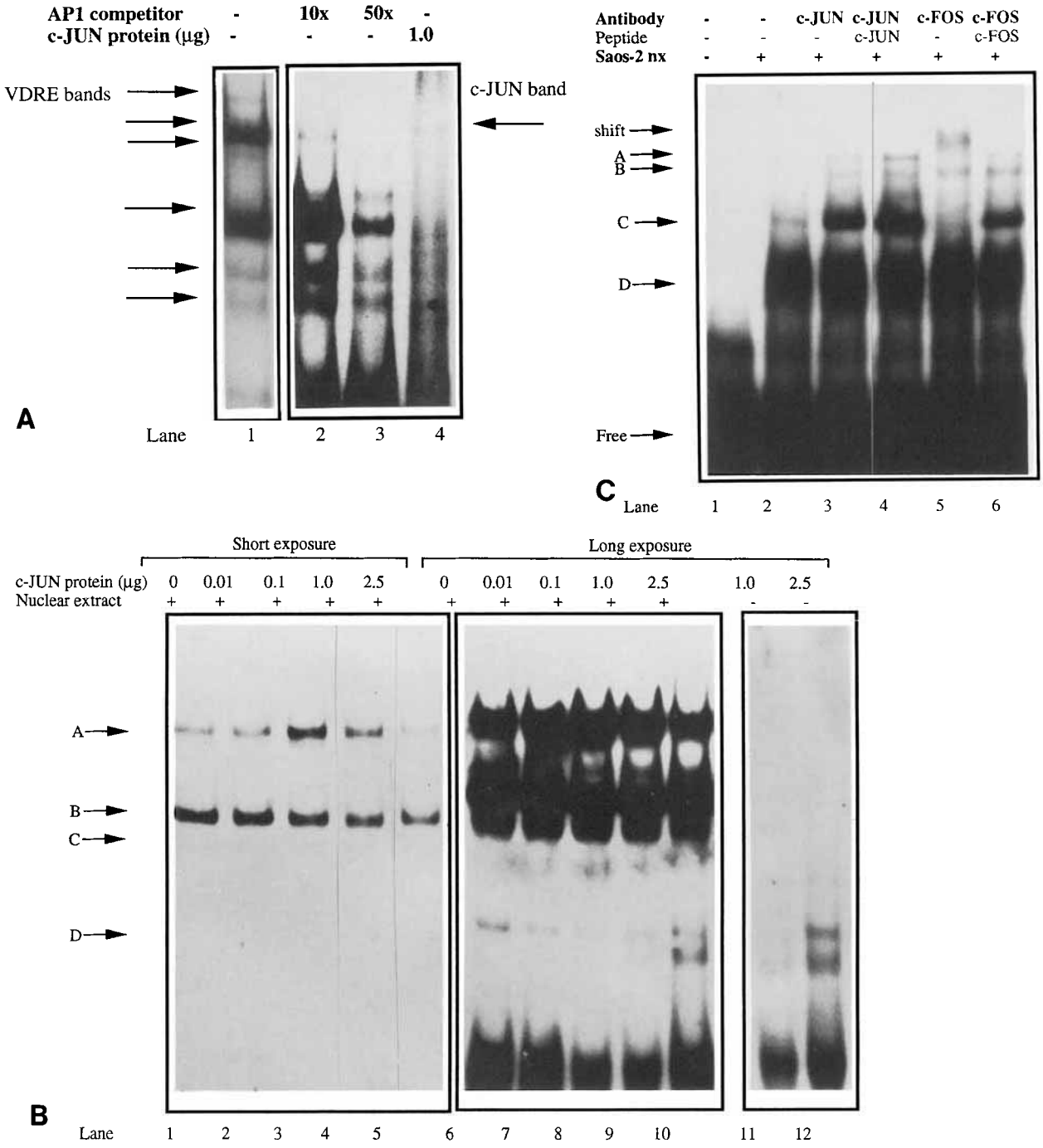

Fig. 4. Role of c-JUN protein in VDRE and OSCARE-2. c-JUN protein binds DNA sequences with an AP1 site, VDRE, and OSCARE-2, and contributes to the formation of protein complexes bound to OSCARE-2. **A:** Labeled VDRE incubated with MG63 nuclear extract (short exposure, *lane 1*) and cross competed with 10- and 50-fold molar excess AP1 oligonucleotide (*lanes 2, 3*). c-JUN protein (1.0 μg) was incubated with the labeled VDRE (*lane 4*). **B:** Increasing amounts of purified c-JUN protein were incubated with labeled OSCARE-2 and SAOS-2 nuclear extract; shown are the 24 hr (*lanes 1–5*) and 3 day (*lanes 6–10*) exposures. Lanes 1 and 6, no c-JUN protein; lanes 2 and 7, 0.01 μg; lanes 3 and 8, 0.1 μg; lanes 4 and 9, 1 μg; and lanes 5 and 10, 2.5 μg of c-JUN protein. In a simultaneous experiment the labeled OSCARE-2 was incubated with 1.0 and 2.5 μg of c-JUN protein without any nuclear extract (*lanes 11, 12*). **C:** Labeled OSCARE-2 (*lane 1*) was incubated with SAOS-2 nuclear extract (*lane 2*) and the c-JUN antibody (*lane 3*), and as a control to neutralize the anti-c-JUN antibody the c-JUN peptide was added (*lane 4*). The same combination of anti-c-FOS antibody and control c-FOS peptide was used to test for the presence of c-FOS (*lanes 5, 6*). The c-FOS antibody shifted the complex A to a higher molecular complex, whereas the anti-c-JUN antibody demonstrated an increase in the intensity of bound complexes.

free probe and low content of nuclear extract to ensure equilibrium, and the control c-FOS peptide confirmed the c-FOS interaction by neutralization of the shift (Fig. 4C, lane 6). The consensus AP1 oligo produced a single intense band in EMS assay using SAOS-2 extract (not shown). However, anti-c-JUN antibody was not capable of producing a clear antibody supershift as for anti-c-FOS antibody; rather, the anti-c-JUN experiments resulted in an increase in the intensity of band C (Fig. 4C, lane 3). This could be due to the comigration of a supershifted band with a pre-existing band. The band that was intensified by anti-c-JUN antibody was eliminated by anti-c-FOS antibody. Although the effects of anti-c-JUN antibody are not clear, the c-FOS supershift verifies that AP1 can bind the OSCARE-2. This may indicate that SAOS-2 has a low content of c-JUN.

1,25 $(OH)_2D_3$–Induced DNA–Protein Interactions on OSCARE-2

Since 1,25 $(OH)_2D_3$ treatment is reported to increase AP1 binding to the VDRE, AP1-binding activity to the OSCARE-2 sequence was examined using osteoblastic cell lines treated with 1,25 $(OH)_2D_3$. In addition, 1,25 $(OH)_2D_3$–induced osteocalcin promoter activity was examined in the absence of OSCARE-2 to determine its contribution to the 1,25 $(OH)_2D_3$ response. OSCARE-2 shifts with nuclear extracts from ROS17/2.8 cells showed the AP1-specific band D was significantly intensified upon 1,25 $(OH)_2D_3$ induction (Fig. 5A, lanes 1, 2). In ROS17/2.8 cell transfection studies, the osteocalcin promoter exhibited a decrease in 1,25 $(OH)_2D_3$ induction when OSCARE-2 was deleted (2.8 ± 0.2-fold to 2.2 ± 0.2, $P = 0.002$, n = 3; Fig. 5B).

UMR106.06 cells are in an earlier stage of differentiation to ROS17/2.8 and were also examined to determine if the role of OSCARE-2 was specific to the stage of osteoblast development. In contrast to 1,25 $(OH)_2D_3$-treated ROS17/2.8 cells that express osteocalcin, 1,25 $(OH)_2D_3$–treated UMR106.06 cells do not express osteocalcin. EMS assays using UMR106.06 cells showed band D was constitutively expressed at high levels relative to the other bands, and 1,25 $(OH)_2D_3$ treatment induced an additional minor band that migrated just above the AP1-specific band D (Fig. 5A, lanes 3, 4). The additional complex bound to OSCARE-2 from induced UMR106.06 cells was not evident in the EMS shifts from induced ROS17/2.8 cells. Furthermore, in UMR106.06 cell transfection studies the osteocalcin promoter displayed a contrasting increased 1,25 $(OH)_2D_3$ induction when OSCARE-2 was deleted (4.2 ± 1.0-fold to 5.4 ± 1.1, $P = 0.05$, n = 4; Fig. 5B).

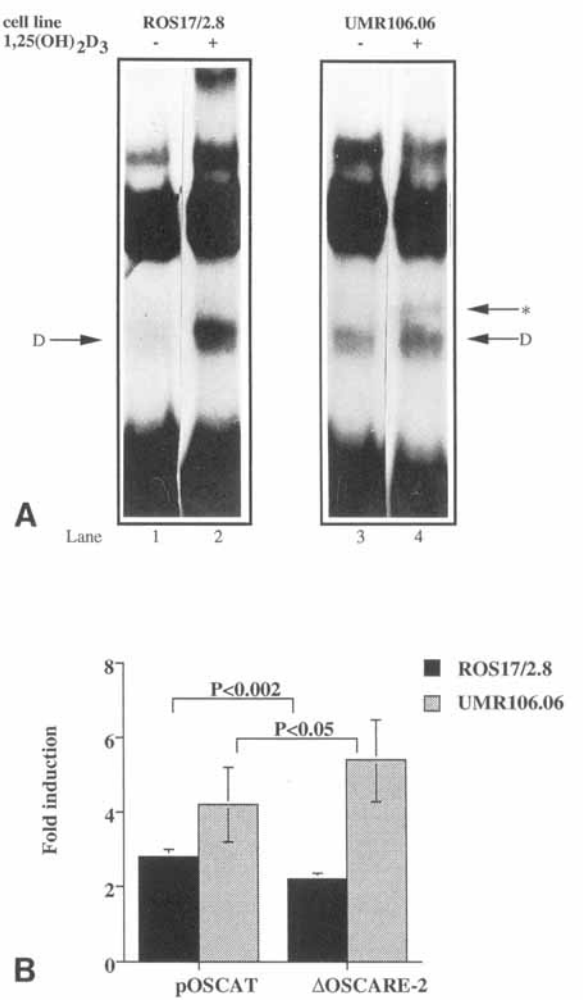

Fig. 5. Contribution of OSCARE-2 to vitamin D induction in ROS17/2.8 and UMR106.06 cells. **A:** Nuclear extracts from ROS17/2.8 and UMR106.06 cells; control and treated with 1,25 $(OH)_2$ D_3 were incubated with labeled OSCARE-2 in EMS assays. *Lane 1,* ROS17/2.8; *lane 2,* 1,25 $(OH)_2D_3$–treated ROS17/2.8; *lane 3,* UMR106.06; *lane 4,* 1,25 $(OH)_2D_3$–treated UMR106.06. The intensity of the lower-molecular-weight bands increased relative to the other bands. **B:** ROS17/2.8 and UMR106.06 cells were transiently transfected with the osteocalcin promoter reporter constructs containing the VDRE, with the OSCARE-2 sequence (pOSCAT) and without the OSCARE-2 sequence (ΔOSCARE-2). The promoter constructs were assessed for 1,25 $(OH)_2D_3$ fold induction. The mean fold induction (±SEM) was derived from three independent transfections in each cell line, and the significant differences are as shown (unpaired Student's *t* test).

DISCUSSION

A repressive region of the proximal osteocalcin promoter OSCARE-2 binds a complex of

proteins including an AP1-like protein. It has been suggested in the phenotype suppression model that the promoter is switched off through these AP1 sites [Lian et al., 1991; Owen et al., 1990]. Moreover, the OSCARE-2 sequence appears to contribute to the 1,25 $(OH)_2D_3$ response of the osteocalcin promoter through its similar AP1-like site. Clearly, antibody supershift experiments confirm that c-FOS is present in the SAOS-2 cell nuclear extract complex that binds OSCARE-2. Recombinant c-JUN is also capable of binding the sequence but it was not confirmed as a member of the actual complex by antibody supershift. Since EMS assays suggest more than one member of the AP1 family can bind to the OSCARE-2 sequence, it is possible that the overall contribution of OSCARE-2 is determined by the variant of AP1 to which it binds.

Multiple complexes from the SAOS-2 cells bound to OSCARE-2 in EMS assays, suggesting that different sites in the OSCARE-2 sequence may be active. However, the observation that higher-molecular-weight complexes are intensified and lower bands are reduced as more nuclear protein is added indicate that higher-molecular-weight complex formation is favored under these conditions (Fig. 1). When the reaction temperature is increased a similar phenomenon occurs (Fig. 2). Taken together, these data suggest that in addition to the DNA binding of a higher-molecular-weight protein complex, the increased protein–protein interactions between the bound factors may have caused the observed progression to a higher-molecular-weight complex (Figs. 1, 2).

In the OSCARE-2 sequence a hexamer sequence (bottom strand, Fig. 1; GGGTGA) is identical to the half-site of the VDRE found upstream in the same promoter. This half-site overlaps the 3′ end of the potential AP1 site (TGGCTCA). The upstream VDRE is also juxtaposed with an AP1 site in a sequence block that is highly homologous to the OSCARE-2 sequence. This may explain the contribution of OSCARE-2, although minor, to the induction of osteocalcin by 1,25 $(OH)_2D_3$. In the same relative position in the rat promoter a half-VDRE site exists (AGGACA), and in the mouse promoter a similar sequence exists (AGGcCA). Although autonomous hormone response elements require the presence of multimers of such half-sites [Schrader et al., 1995], the conservation of such half-sites between the human and the rat sequence suggests a regulatory role which is the subject of further study.

Of the multiple complexes, bands A and D were competed specifically by the AP1 oligonucleotide (Fig. 3). Since the upper band A was competed by the AP1 oligonucleotide as well as most general transcription factor oligonucleotides, band A presumably represents a complex of regulatory factors including AP1, whereas band D is specific to AP1 since it was competed only by the AP1 oligonucleotide and no other. Focusing on c-JUN binding activity, increasing c-JUN protein from 0 to 0.1 μg c-JUN caused an increase in the higher-molecular-weight complex, suggesting that c-JUN stabilized the higher complex through protein–protein interaction with other regulatory proteins already bound to OSCARE-2 (Fig. 4B, lanes 1–3). The addition of higher concentrations of c-JUN caused a gradual decreasing intensity of the higher-molecular-weight complex (A) (Fig. 4B, lanes 3–5) and increased intensity of band D (Fig. 4B, lanes 8–10). We suggest these observations may result from an increase in c-JUN homodimer bound to OSCARE-2, represented by band D, as well as the destabilization of the higher complex, represented by band A. Exogenous c-JUN protein binds to OSCARE-2 with an apparent biphasic response, and we suggest it is stabilized in the higher-molecular-weight complexes bound to OSCARE-2 by additional protein–protein interactions. Others have reported similar c-JUN protein–protein interactions; for instance, c-JUN interacts with the vitamin D receptor, preventing it from binding to the VDRE [Jaaskelainen et al., 1994], and in a similar manner the c-JUN prevents the glucocorticoid receptor binding to the GRE [Jaaskelainen et al., 1994; Yang-Yen et al., 1990]. Therefore, it is plausible that at optimal concentrations the c-JUN protein was stabilized in the OSCARE-2 complex through protein–protein interactions, possibly with endogenous c-FOS, which is present in the native complex.

EMS assay competition experiments suggest the presence of other factors binding to the OSCARE-2 sequence. We have previously presented evidence that regulatory repressor proteins bind an SP1-like, G/C-rich sequence [Goldberg et al., 1995b]. In addition Towler et al. [1994] report that the reverse CCAAT-like (GTgTTA) motif that overlaps an NF1/CTF-like (TgTGGCT) motif in the conserved OSCARE-2 region binds to the CCAAT/NFY protein. The

cross-competition studies with the NF1 and SP1 oligonucleotides suggest that these sites may be involved (Fig. 3).

1,25 $(OH)_2D_3$ treatment of ROS17/2.8 cells induced a factor with migration rates similar to the AP1-specific complex (band D) which binds to OSCARE-2 (Fig. 5A). Osteocalcin promoter deletion studies indicated that OSCARE-2 increased the magnitude of 1,25 $(OH)_2D_3$ induction in ROS17/2.8 cells (Fig. 5B). These data are consistent with EMS band D activity in ROS17/2.8 being regulated by 1,25 $(OH)_2D_3$.

The UMR106.06 cells are characterized as an osteoblast cell in an earlier stage of differentiation than the ROS17/2.8 cells [Rodan and Noda, 1991]. This cell line difference was reflected in the role of OSCARE-2 (Fig. 5A,B). According to our hypothesis, the band specifically induced in UMR106.06 cells may be an AP1 variant that is specific to the UMR106.06 osteoblast cells and contributes to the repression of the 1,25 $(OH)_2D_3$–induced osteocalcin promoter.

Other studies have demonstrated that 1,25 $(OH)_2D_3$ induces different members of the FOS and JUN protein family during osteoblast development [Bidwell et al., 1994; Candeliere et al., 1991] with the type of AP1 complex specific to the developmental stage of the osteoblast. More specifically, studies of the AP1 variant that binds the VDRE show it is present in osteoblast cells at only specific osteoblast developmental stages [Jaaskelainen et al., 1994; Owen et al., 1993]. It is consistent that the ROS17/2.8 cell line which expresses osteocalcin upon 1,25 $(OH)_2D_3$ treatment demonstrates increased promoter activity in the presence of OSCARE-2 and increased AP1-like binding to the OSCARE-2 sequence.

Possibly, AP1 that binds OSCARE-2 may also bind the AP1 site in the VDRE and work in combination to increase the 1,25 $(OH)_2D_3$ response. In summary, DNA–protein interactions indicate that the OSCARE-2 can bind to a complex of osteoblastic nuclear proteins, including an AP1 complex that has a c-FOS component. In ROS17/2.8 cells, 1,25 $(OH)_2D_3$ treatment induces an AP1 complex which correlates with an increased 1,25 $(OH)_2D_3$ response. In contrast, 1,25 $(OH)_2D_3$ induction of UMR106.06 cells displays a differing AP1-like complex which correlates with a decreased 1,25 $(OH)_2D_3$ response. The net effect of the OSCARE-2 site is determined by the complex interactions of a number of DNA binding activities, including c-FOS, regulation of the osteocalcin gene.

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