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Coordinate occupancy of AP-1 sites in the vitamin D-responsive and CCAAT box elements by Fos–Jun in the osteocalcin gene: Model for phenotype suppression of transcription

(oncogenes/collagen/growth control/proliferation/differentiation/transcription)

THOMAS A. OWEN*, RITA BORTELL*, SUE A. YOCUM*, STEVEN L. SMOCK*, MENG ZHANG*, CORY ABATE†, VICTORIA SHALHOUB*, NEIL ARONIN*, KENNETH L. WRIGHT*, ANDRE J. VAN WIJNEN*, JANET L. STEIN*, TOM CURRAN†, JANE B. LIAN*, AND GARY S. STEIN*

*Department of Cell Biology, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655; and †Roche Institute of Molecular Biology, Nutley, NJ 07110

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ABSTRACT **Osteocalcin, a bone-specific protein and marker of the mature osteoblast, is expressed only in nonproliferating osteoblasts in a mineralizing extracellular matrix, while type I collagen is expressed in proliferating cells. The nuclear proteins encoded by the c-*fos* and c-*jun* protooncogenes are expressed during the proliferation period of osteoblast phenotype development. We present evidence that AP-1 (HeLa cell-activating protein 1) sites residing within two promoter elements of the osteocalcin gene bind the Fos–Jun protein complex: the osteocalcin box (OC box; nucleotides −99 to −76), which contains a CCAAT motif as a central element and influences tissue-specific basal levels of osteocalcin gene transcription, and the vitamin D-responsive element (VDRE; nucleotides −462 to −440), which mediates enhancement of osteocalcin gene transcription. Gel electrophoretic mobility-shift analysis demonstrated high AP-1 binding activity in proliferating osteoblasts and dramatic changes in this activity after the down-regulation of proliferation and the initiation of extracellular-matrix mineralization in primary cultures of normal diploid osteoblasts. Methylation interference analysis established at single nucleotide resolution that purified recombinant Fos and Jun proteins bind in a sequence-specific manner to the AP-1 sites within the VDRE and OC box. Similarly, an AP-1 motif within a putative VDRE of the alkaline phosphatase gene, which is also expressed after the completion of proliferation, binds the Fos–Jun complex. These results support a model in which coordinate occupancy of the AP-1 sites in the VDRE and OC box in proliferating osteoblasts may suppress both basal level and vitamin D-enhanced osteocalcin gene transcription as well as transcription of other genes associated with osteoblast differentiation—a phenomenon we describe as phenotype suppression. This model is further supported by binding of the Fos–Jun complex at an AP-1 site in the type αI collagen promoter that is contiguous with, but not overlapping, the VDRE. Such a sequence organization in the collagen VDRE motif is compatible with vitamin D modulation of collagen but not with osteocalcin and alkaline phosphatase expression in proliferating osteoblasts.**

Development of the differentiated osteoblast phenotype both in culture and *in vivo* initially involves active proliferation, during which time genes associated with cell-cycle and cell-growth control as well as those related to the biosynthesis of the type I collagen extracellular matrix (ECM) are expressed (1, 2). Following the completion of proliferative activity, genes related to ECM maturation and specialization are expressed, leading to competency of the ECM for mineralization and the initiation of osteocalcin gene expression (1, 2). Osteocalcin is a bone-specific calcium-binding protein that in normal diploid osteoblasts is synthesized only by mature nonproliferating cells (1, 2). Hence, it is necessary to account for how the osteocalcin gene is rendered transcribable only after the down-regulation of proliferation and the onset of ECM mineralization. At the molecular level, this requires identification and characterization of transcription factors that interact in a sequence-specific manner with regulatory elements in the osteocalcin gene promoter to determine the level of gene transcription. Addressing this question will provide an understanding of the relationship between osteoblast proliferation and regulation of gene expression associated with differentiation of the osteoblast. In a broader biological context, such information has important implications for control of phenotype expression in general.

The rat osteocalcin gene promoter has a modular organization consisting of both positive and negative regulatory elements (3). The hormone 1,25-dihydroxyvitamin D_3 (vitamin D) plays a key role in the transcriptional regulation of osteocalcin gene expression in osteoblasts both *in vitro* and *in vivo* (3–7). We and others have demonstrated that vitamin D-mediated up-regulation of osteocalcin gene transcription is associated with binding of the vitamin D receptor complex to the vitamin D-responsive element (VDRE) (nucleotides −462 to −440) (5–8). Additionally vitamin D induces modifications in sequence-specific binding of nuclear factors to a proximal element designated the osteocalcin (OC) box (nucleotides −99 to −76). This element contains a CCAAT motif as a central element and influences the tissue-specific basal level of osteocalcin gene transcription (7). When we initially sequenced the osteocalcin promoter, we noted the presence of HeLa cell-activator protein AP-1 consensus sequences (3). These sites potentially bind proteins encoded by the c-*fos* and c-*jun* protooncogenes and related genes (9–11), all of which are expressed in proliferating osteoblasts (2). Subsequently the VDRE was identified in the rat and human osteocalcin gene promoters and found to overlap one of these AP-1 sites (5–8). Additionally, we have observed two potential AP-1 sites flanking the CCAAT motif. These findings, together with the observation that osteocalcin is not expressed in proliferating normal diploid osteoblasts, led us to address the possible role of the AP-1 sites in these important promoter regulatory sequences in the control of osteocalcin gene transcription.

In this report, we present evidence that the nuclear proteins encoded by the c-*fos* and c-*jun* protooncogenes bind to

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Abbreviations: vitamin D, 1,25-dihydroxyvitamin D_3; VDRE, vitamin D-responsive element; ECM, extracellular matrix; MT II_A, human metallothionein II_A; OC box, osteocalcin box.

the AP-1 sites in the VDRE and OC box of the rat osteocalcin gene promoter during the proliferation period of osteoblast phenotype development and to an AP-1 site within the VDRE of the alkaline phosphatase gene (12) that is expressed in osteoblasts after completion of proliferation. These results are consistent with a model in which coordinate occupancy of the AP-1 sites within the VDREs of the osteocalcin and alkaline phosphatase gene promoters and in the osteocalcin gene OC box in proliferating osteoblasts may suppress both basal level and vitamin D-induced transcription—a phenomenon we describe as *phenotype suppression*. This model of suppression of gene expression and steroid modulation of phenotype markers during cellular proliferation is further supported by the organization of a putative VDRE we identified in the promoter of the rat type αI collagen gene (13), which is expressed in proliferating osteoblasts (2). We observed binding of the Fos–Jun complex to an AP-1 site that is contiguous with but not overlapping the vitamin D receptor binding domain in the type αI collagen promoter. Such a sequence organization of the collagen VDRE motif is compatible with and may support expression and vitamin D regulation of the type αI collagen gene in proliferating osteoblasts, in contrast to osteocalcin and alkaline phosphatase.

MATERIALS AND METHODS

Primary diploid rat osteoblasts were isolated from fetal rat calvaria (1), and ROS 17/2.8 cells (from S. Rodan; Merck Sharpe & Dohme) were maintained as reported (8). ROS 17/2.8 cells were plated at a density of 5×10^5 cells per 100-mm dish on day 0 and harvested 3 or 8 days later or were treated 7 days later with 10 nM vitamin D for 24 hr and harvested. DNA synthesis was monitored by [^{3}H]thymidine incorporation, (2) and osteocalcin was measured by RIA (14).

Nuclear run-on transcription assays were performed (15), and total cellular RNA was isolated and analyzed for osteocalcin mRNA (2) as described.

Double-stranded oligonucleotides for the rat osteocalcin VDRE spanning nucleotides −462 to −440 and for the rat OC box spanning nucleotides −99 to −76 were synthesized (8). For the rat type αI collagen gene (13) and the human liver/bone/kidney alkaline phosphatase gene (12), regions of each promoter with high degrees of homology to the rat osteocalcin VDRE (6, 8) were identified, and double-stranded oligonucleotides spanning nucleotides −2957 to −2912 for type I collagen and −954 to −922 for alkaline phosphatase were synthesized. In addition, double-stranded oligonucleotides spanning the AP-1 binding site of the human metallothionein II_A (MT II_A) gene promoter (DAP-1) (9, 16) or comprised of three repeats of the potential AP-1 site either within the rat osteocalcin gene VDRE (TGAATGA) or adjacent to the putative VDRE in the rat type αI collagen gene (TGAGTGA) were synthesized. To use oligonucleotides as probes, each strand was labeled with [^{32}P]ATP with phage T4 polynucleotide kinase, and the strands were annealed.

Nuclear proteins were extracted by the method of Dignam *et al.* (17) as modified by Holthuis *et al.* (18). Gel mobility shift assays for analysis of nuclear extract protein–DNA interactions were performed as described by Markose *et al.* (8) for the osteocalcin gene OC box and VDRE and by van Wijnen *et al.* (19) for the H4 histone promoter site II.

Rat c-*fos*- and c-*jun*-encoded proteins purified from *Escherichia coli* were those described by Abate *et al.* (20). Gel mobility-shift (9) and methylation-interference (21) assays were performed as described.

RESULTS AND DISCUSSION

Osteocalcin Gene Expression in Normal Diploid Osteoblasts and Osteosarcoma Cells. Transcriptional activation and direct effects of vitamin D on the osteocalcin gene were examined during the developmental sequence of primary cultures of diploid calvarial-derived osteoblasts. During the initial 10 days in culture, the cells actively proliferate and no detectable levels of osteocalcin protein, mRNA, or transcription are observed (Fig. 1). After the down-regulation of proliferation and initiation of extracellular matrix mineralization, nuclear run-on transcription assays (performed on day 23) indicate that the osteocalcin gene becomes actively transcribed and Northern blot analysis demonstrates cellular osteocalcin mRNA (Fig. 1*C*). Osteocalcin protein is detected in the culture medium, increasing from 0.2 ng/μg of DNA on day 13 to 21 ng/μg of DNA on day 20 (data not shown). Fig. 1 also indicates that 10 nM vitamin D increases osteocalcin gene expression 5- to 10-fold, but only when the gene is actively transcribed, reflecting two components of control at the transcriptional level—activation of basal expression and vitamin D-mediated enhancement. The VDRE has previously been identified by analysis of promoter deletion mutants and direct determinations of protein–DNA interactions in the rat osteocalcin gene 5′ regulatory sequences (6, 8).

In contrast to normal diploid osteoblasts, osteocalcin is expressed in proliferating osteosarcoma cells. However, the relationship between suppression of osteocalcin gene expression in proliferating cells and expression after the down-regulation of cell growth found in normal osteoblasts is generally maintained, although not stringently controlled. The ROS 17/2.8 osteosarcoma cell line expresses osteocalcin only at low levels during proliferation but exhibits a 10-fold increase with the cessation of cell growth at confluency (Fig. 2*C*). This reciprocal relationship between cell growth and osteocalcin gene expression is reflected at the transcriptional level by changes in sequence-specific binding of nuclear factors at rate-limiting proximal promoter elements of the cell cycle-regulated H4 histone gene (site II) and the differentiation-associated osteocalcin gene (OC box). Fig. 2 shows factor binding to a regulatory site of the H4 histone gene promoter (18, 19) in proliferating ROS cells (day 3) and the absence of a specific protein–DNA interaction at the OC box

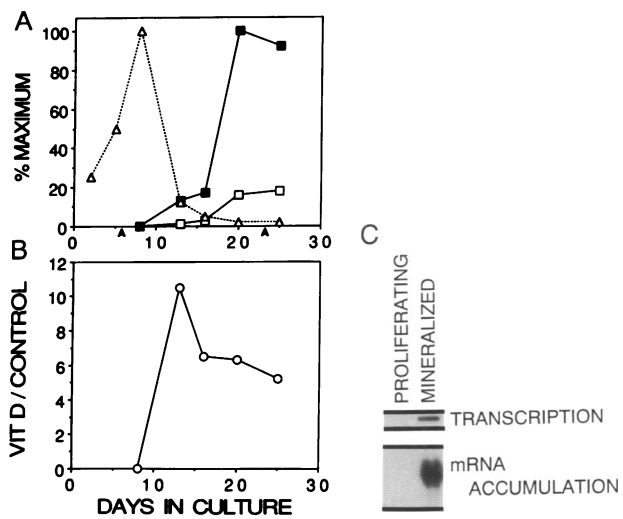

FIG. 1. Expression and vitamin D (Vit D) modulation of the osteocalcin gene during development of the mineralized ECM in normal diploid rat osteoblasts. (*A*) Normal osteoblasts in culture proliferate during the initial period after isolation as determined by [^{3}H]thymidine incorporation (△) and subsequently express osteocalcin as determined by RIA (□). Vitamin D at 10 nM for 48 hr enhances osteocalcin expression (■). Two arrowheads on the abscissa indicate the times at which cells were harvested for transcriptional and mRNA analyses described below (*C*). (*B*) Ratio of vitamin D-stimulated osteocalcin to basal osteocalcin mRNA. (*C*) Osteocalcin gene transcription and cellular mRNA levels.

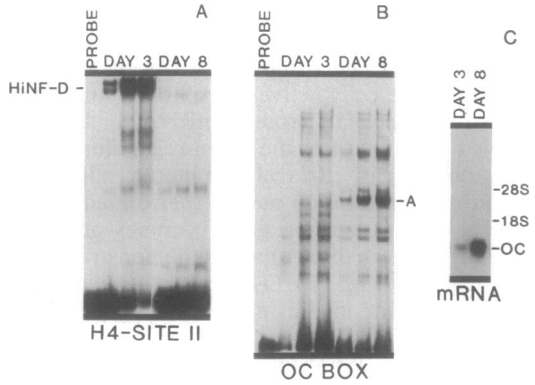

FIG. 2. Reciprocal relationship between the binding of nuclear factors to promoters of a proliferation-coupled H4 histone gene and the differentiation-associated osteocalcin gene. ROS 17/2.8 osteosarcoma cells were harvested while proliferating (day 3) or following confluence (day 8), and nuclear-protein extracts and total cellular RNA were prepared. (*A*) Binding of nuclear factor HiNF-D to the site II region of the F0108 H4 histone gene promoter as determined by gel retardation assay greatly decreased on day 8. (*B*) Binding of nuclear proteins to the osteocalcin gene promoter OC box determined by gel retardation assay shows an increased protein–DNA interaction (band A, characterized in ref. 7) upon confluency at day 8. For *A* and *B*, the three lanes for each day represent 2.5, 5, or 7.5 μg of nuclear protein per lane. (*C*) Osteocalcin mRNA (OC) as determined by Northern blot analysis.

of the osteocalcin gene. At confluency on day 8, binding of nuclear factors to the H4 histone gene promoter site is greatly decreased, and the protein–DNA interaction at the OC box is prominent (Fig. 2*B*).

The vitamin D-mediated up-regulation of osteocalcin gene expression was further pursued by assaying mRNA levels, nuclear run-on transcription, and protein–DNA interactions in the VDRE and the OC box of nonproliferating ROS 17/2.8 cells expressing high levels of osteocalcin. Osteocalcin mRNA levels and secreted protein were not significantly elevated at 1 hr after vitamin D treatment (Fig. 3*A*); however, a 10-fold increase in osteocalcin gene transcription was observed 1 hr after treatment (Fig. 3*B*) and was paralleled by increased factor binding at the VDRE (data not shown). No

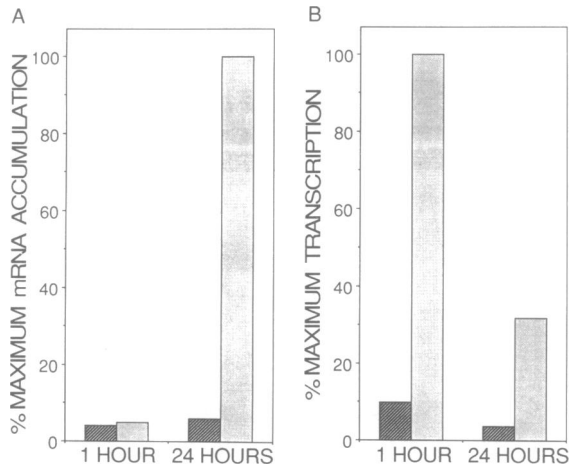

FIG. 3. Onset of osteocalcin gene transcription and mRNA accumulation after treatment with vitamin D. Confluent (day 8) ROS 17/2.8 cells were stimulated with vitamin D for 1 or 24 hr. At each time, cells were harvested, and nuclei, nuclear proteins, and mRNA were prepared. (*A*) Osteocalcin mRNA accumulation was assayed by Northern blot, quantitated by densitometry, and expressed relative to maximum expression. (*B*) Osteocalcin gene transcription was determined by nuclear run-on assay. Hatched bars represent control cells, and gray bars represent vitamin D-treated cells.

increase in protein binding at 1 or 24 hr was observed at the OC box, reflecting the maximal basal levels of osteocalcin gene expression already present in nonproliferating, confluent cultures, which are not further influenced by vitamin D. These results suggest a two-step vitamin D-mediated enhancement mechanism; initially transcription is up-regulated, and subsequently the osteocalcin mRNA is stabilized and/or accumulated.

Interaction of Fos–Jun with Two Osteocalcin Gene Regulatory Elements. The sequences of the VDRE and OC box of the rat osteocalcin gene promoter elements (3, 6, 8), which both contain potential AP-1 binding sites, are shown below and compared with the AP-1 site of the human metallothionein II_A gene promoter (9, 16). The AP-1 consensus sequences are indicated by solid lines:

Rat osteocalcin

VDRE	−462 CTGGGTGAATGAGGACATTACTG	−440
OC box	−99 ATGACCCCCAATTAGTCCTGGCAG	−76
Human MT II_A gene	−102 GTGACTCAGCGCG	−90

Therefore, the possibility can be considered that the Fos–Jun proteins that are expressed in proliferating osteoblasts contribute to negative regulation of osteocalcin gene expression. This concept is supported by decreased levels of nuclear protein binding to the osteocalcin VDRE AP-1 site following the down-regulation of proliferation in normal diploid osteoblasts (data not shown).

The nuclear protooncogene-encoded proteins Fos and Jun form, via a leucine zipper, a stable heterodimeric complex that interacts with the AP-1 binding site (9, 20, 22–24). To directly establish that the AP-1 consensus sequences within the VDRE and OC box support sequence-specific binding of the Fos–Jun heterodimer, we assayed the ability of purified recombinant Fos–Jun proteins (20) to interact with these regulatory elements. Gel retardation assays (Fig. 4) establish unequivocally that the Fos–Jun complex binds to the AP-1 sites within the VDRE and OC box. DNA binding was dependent on both Fos and Jun and was competed by an

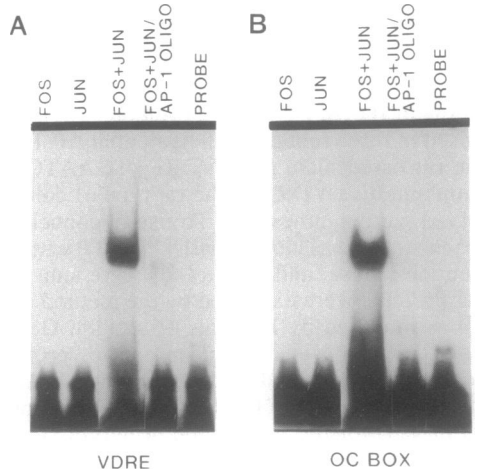

FIG. 4. Binding of purified recombinant Fos and Jun proteins to rat osteocalcin VDRE (*A*) or OC box (*B*) probes as assessed by gel retardation assay. Protein–DNA complexes were resolved on 4.5% native polyacrylamide gels. For each probe, the binding of the Fos–Jun complex was specifically in competition with an oligonucleotide spanning the human MT II_A gene AP-1 site in lane FOS + JUN/AP-1 OLIGO. The probe incubated without protein is in lane "PROBE."

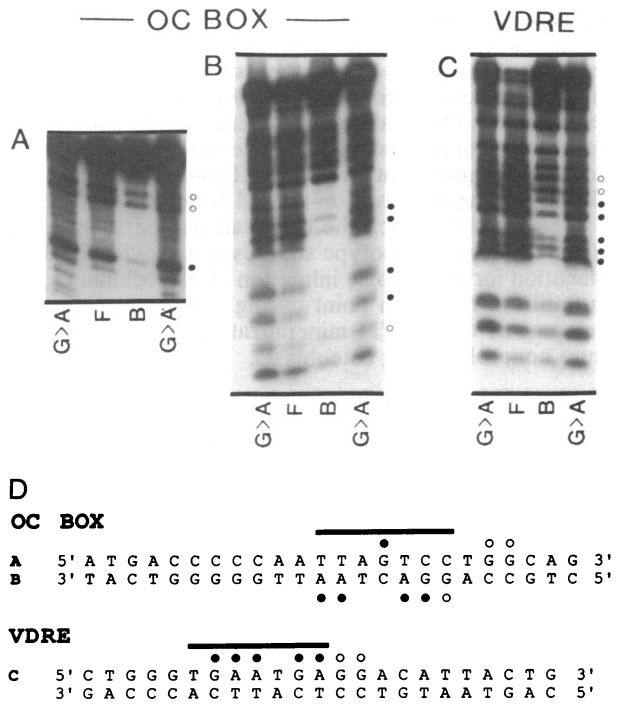

FIG. 5. Methylation interference analysis of the contact sites of the purified recombinant Fos–Jun complex within the rat osteocalcin gene OC Box and VDRE. The ^{32}P-labeled OC box (*A* and *B*) and VDRE (*C*) oligonucleotides were partially methylated and used in binding reactions with purified Fos and Jun proteins as described. An equal number of cpm of free (lanes F) and bound (lanes B) probe were electrophoresed on a 15% polyacrylamide denaturing gel, and sequencing reactions (G > A) were electrophoresed in adjacent lanes. ●, G and A residues that strongly interfere with binding of the Fos–Jun complex; ○, those that partially interfere. (*D*) Sequences of the double-stranded OC box and VDRE oligonucleotides are shown with the G and A contacts indicated by closed and open circles as described above. Solid bars indicate the sequences similar to the human MT II_A gene AP-1 site (TGACTCA) to which the Fos–Jun complex bound. Although in the OC box, the CCAAT element is flanked by AP-1 consensus sequences; the probe used (nucleotides −99 to −76) permits identification of only the proximal AP-1 site.

oligonucleotide from the human MT II_A gene promoter, which contains an AP-1 binding site (9, 16). Sequence-specific interactions with the AP-1 motifs are demonstrated by dimethyl sulfate interference patterns, which define protein–DNA contacts at guanine and adenine residues corresponding to the AP-1 consensus sequence (Fig. 5).

We have identified an analogous VDRE sequence in an alkaline phosphatase gene promoter (see below) that is expressed immediately after the down-regulation of proliferation during development of the osteoblast phenotype. The AP-1 motif within the alkaline phosphatase VDRE binds the Fos–Jun complex (Fig. 6*A*). We additionally have identified a VDRE sequence in the rat type αI collagen gene promoter (13) which has an AP-1 consensus sequence contiguous to but not within the VDRE (shown below). The alkaline phosphatase (AP; nucleotides −948 to −923) and type I collagen (Col.; nucleotides −2957 to −2912) gene promoter regions with homology to the rat osteocalcin VDRE (OC; nucleotides −462 to −439) are shown below (bullets indicate sequence identity to the OC VDRE; AP-1 consensus sequences are indicated by bold print).

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AP   GGGGGTGACTGATGGT AACCTGATT
          .  .  .  .  .  .  .
OC   CTGGGTGAATGA-GG--ACATTACTG
     .  .  .  .  .  .  .  .  .
Col.CTGGGGGCAGAA-GA--ACTTT-CTGGAGGATTTGAGTGA
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Fig. 6*B* clearly demonstrates the ability of the AP-1 site in the type αI collagen promoter to bind the Fos–Jun complex. Thus it appears that subtle variations in the organization of the VDRE and AP-1 motifs in the osteocalcin and AP genes compared with that in the type αI collagen gene promoters may contribute to their differential expression during the osteoblast developmental sequence.

Model for Phenotype Suppression of Osteocalcin Gene Transcription. Our results indicate that two primary regulatory elements, the OC box, which mediates the bone-specific basal levels of osteocalcin gene transcription, and the VDRE, which mediates vitamin D enhancement of expression, both contain bona fide AP-1 sites. AP-1 binding activity in proliferating osteoblasts are consistent with a model (Fig. 7) in which Fos and Jun and related proteins are synthesized during proliferation, thus providing the basis for coordinate occupancy of the AP-1 sites in the VDRE and OC box. This suppresses both the basal level and the vitamin D-enhanced transcription in proliferating osteoblasts that have not achieved competency for the final stages of differentiation. We describe this phenomenon as phenotype suppression. Osteocalcin gene expression is induced and responsive to enhancement by vitamin D only after the completion of proliferative activity at the onset of ECM mineralization. At this time AP-1 binding activity distinct from that in proliferating cells is detected. While this manuscript was in preparation, Schule *et al.* (25) reported an AP-1 site within the VDRE of the human osteocalcin gene promoter. Their results indicate that Fos–Jun binding can down-regulate osteocalcin gene transcription, corroborating our findings and working model.

The presence of the AP-1 site within the VDRE of the osteocalcin gene and its interaction with the Fos–Jun heterodimer in proliferating osteoblasts could prevent occupancy by the vitamin D receptor complex. Similarly, the AP-1 site within the VDRE of the alkaline phosphatase gene could block occupancy of the VDRE by the vitamin D–receptor complex. In contrast, the AP-1 site adjacent to but not overlapping the putative VDRE in the type αI collagen gene promoter appears to be compatible with and possibly facilitates collagen gene expression and regulation by vitamin D in proliferating osteoblasts. This observation is consistent with the concept of AP-1 site regulation of phenotype suppression of osteocalcin and alkaline phosphatase in that the type αI collagen gene is actively transcribed in proliferating osteoblasts and is also vitamin D responsive. Suppression of

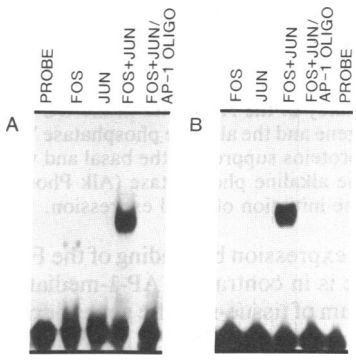

FIG. 6. Binding of purified recombinant Fos and Jun proteins to the regions of the human alkaline phosphatase and rat type αI collagen promoters analogous to the rat osteocalcin VDRE. Fos–Jun protein complexes with the regions of the human alkaline phosphatase (*A*) and rat type αI collagen (*B*) promoters analogous to the rat osteocalcin VDRE were resolved in 4.5% native polyacrylamide gels. Binding of the Fos–Jun complex was specifically in competition with an oligonucleotide spanning the human MT II_A gene AP-1 site in lane FOS + JUN/AP-1 OLIGO. The probe incubated without protein is in lane "PROBE."

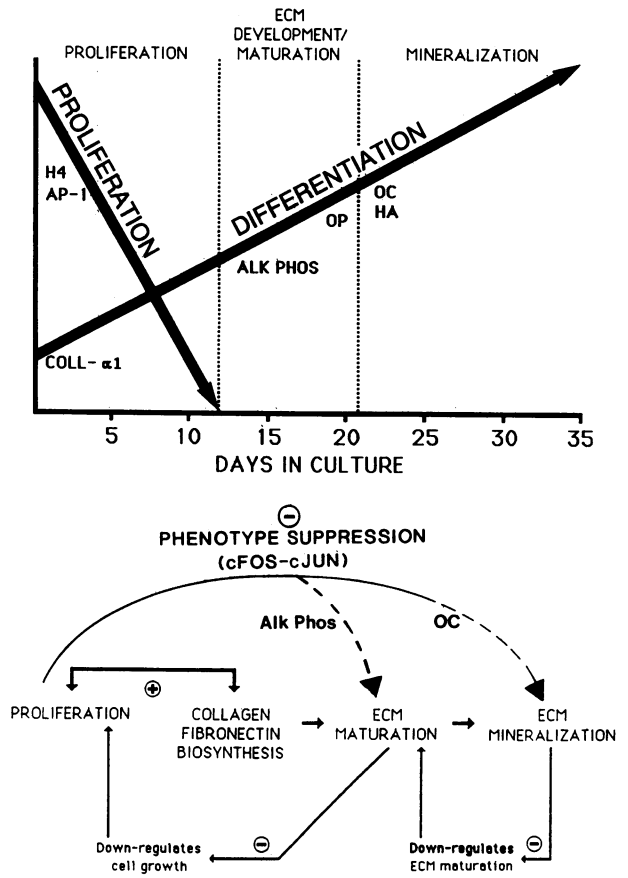

FIG. 7. Model for suppression of a marker gene of the mature osteoblast phenotype in actively proliferating cells by protein binding to the osteocalcin VDRE and OC Box AP-1 sites. The relationship between proliferation and differentiation is schematically illustrated within the context of down-regulation of proliferation (H4 histone and AP-1 binding activity) and the up-regulation of genes associated with the maturation and mineralization (hydroxyapatite deposition) of the osteoblast ECM (type αI collagen, alkaline phosphatase, osteopontin, and osteocalcin). The three principal periods of the osteoblast developmental sequence are designated with broken vertical lines (proliferation, matrix development and maturation, and mineralization). AP-1, AP-1 binding activity; H4, H4 histone; COLL-αI, type αI collagen; ALK PHOS, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; HA, total accumulated hydroxyapatite (calcium + phosphate). (*Lower*) The proliferation period supports the synthesis of a type I collagen/fibronectin ECM, which continues to mature and mineralize. The formation of this matrix down-regulates proliferation, and matrix mineralization down-regulates the expression of genes associated with the formation–maturation period. The occupancy of the AP-1 sites in the OC box and VDRE of the osteocalcin gene and the alkaline phosphatase VDRE by Fos–Jun and/or related proteins suppresses the basal and vitamin D-induced expression of the alkaline phosphatase (Alk Phos) and osteocalcin genes prior to the initiation of basal expression.

tissue-specific expression by binding of the Fos–Jun complex to an AP-1 site is in contrast to AP-1-mediated activation of a broad spectrum of tissue-specific genes during the course of cellular differentiation (10, 26–28). However, this may in part reflect the location of the AP-1 site within the responsive element influenced by Fos–Jun binding.

A key question that remains unresolved is the mechanism by which the osteocalcin and alkaline phosphatase genes are rendered transcribable and vitamin D-responsive following down-regulation of proliferation and onset of ECM mineralization. However, our results are consistent with a common mechanism for suppressing expression of certain osteoblast genes by Fos–Jun when the cells are actively proliferating and a gene-specific mechanism for the sequential activation of these genes during the subsequent expression of the osteoblast phenotypes. Here, the possibilities include: (*i*) release of the Fos–Jun complex from the AP-1 sites to permit the sequences to be available for occupancy by the vitamin D–receptor complex and/or by tissue-specific transcription factors or (*ii*) modifications of the Fos–Jun complex that facilitate binding of activation-related factors. Regardless of the mechanism by which the osteocalcin gene is transcriptionally activated, phenotype suppression provides a viable explanation for physiologic inhibition of osteocalcin expression until the transition point during osteoblast phenotype development, when ECM mineralization is initiated by the mature nonproliferating osteoblasts.

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1. Aronow, M. A., Gerstenfeld, L. C., Owen, T. A., Tassinari, M. S., Stein, G. S. & Lian, J. B. (1990) *J. Cell Physiol.* **143,** 213–221.
2. Owen, T. A., Aronow, M. A., Shalhoub, V., Barone, L. M., Wilming, L., Tassinari, M. S., Kennedy, M. B., Pockwinse, S., Lian, J. B. & Stein, G. S. (1990) *J. Cell Physiol.* **143,** 420–430.
3. Lian, J. B., Stewart, C., Puchacz, E., Mackowiak, S., Shalhoub, V., Collart, D., Zambetti, G. & Stein, G. S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1143–1147.
4. Yoon, K., Rutledge, S. J., Buenaga, R. F. & Rodan, G. A. (1988) *Biochemistry* **27,** 8581–8526.
5. Kerner, S. A., Scott, R. A. & Pike, J. W. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4455–4459.
6. Demay, M. B., Gerardi, J. M., DeLuca, H. F. & Kronenberg, H. M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 369–373.
7. Morrison, N. A., Shine, J., Fragonas, J.-C., Verkest, V., McMenemy, M. D. & Eisman, J. A. (1989) *Science* **246,** 1158–1161.
8. Markose, E. R., Stein, J. L., Stein, G. S. & Lian, J. B. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1701–1705.
9. Rauscher, F. J., III, Voulalas, P. J., Franza, B. R., Jr., & Curran, T. (1990) *Genes Dev.* **2,** 1687–1699.
10. Cohen, D., Ferreira, C. P., Gentz, R., Franza, B. R. & Curran, T. (1989) *Genes Dev.* **3,** 173–184.
11. Rauscher, F. J., III, Sambucetti, L. C., Curran, T., Distel, R. J. & Spiegelman, B. M. (1988) *Cell* **52,** 471–480.
12. Matsuura, S., Kishi, F. & Kajii, T. (1990) *Biochem. Biophys. Res. Commun.* **168,** 993–1000.
13. Lichtler, A., Stover, M. L., Angilly, J., Kream, B. & Rowe, D. W. (1989) *J. Biol. Chem.* **264,** 3072–3077.
14. Gundberg, C. M., Hauschka, P. V., Lian, J. B. & Gallop, P. M. (1984) *Methods Enzymol.* **107,** 516–544.
15. Shalhoub, V., Gerstenfeld, L. C., Collart, D., Lian, J. B. & Stein, G. S. (1989) *Biochemistry* **28,** 5318–5322.
16. Lee, W., Haslinger, A., Karin, M. & Tjian, R. (1987) *Nature (London)* **325,** 368–372.
17. Dignam, J., Lebovitz, R. & Roeder, R. (1983) *Nucleic Acids Res.* **11,** 1475–1489.
18. Holthuis, J., Owen, T. A., van Wijnen, A. J., Wright, K. L., Ramsey-Ewing, A., Kennedy, M. B., Carter, R., Cosenza, S. C., Soprano, K. J., Lian, J. B., Stein, J. L. & Stein, G. S. (1990) *Science* **247,** 1454–1457.
19. van Wijnen, A. J., Wright, K. L., Lian, J. B., Stein, J. L. & Stein, G. S. (1989) *J. Biol. Chem.* **264,** 15034–15042.
20. Abate, C., Luk, D., Gentz, R., Rauscher, F. J., III, & Curran, T. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1032–1036.
21. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds. (1989) *Current Protocols in Molecular Biology* (Greene Publ. Assoc. and Wiley-Interscience, New York), Vol. 2, pp. 12.2.1–12.3.4.
22. Kouzarides, T. & Ziff, E. (1988) *Nature (London)* **336,** 646–651.
23. Turner, R. & Tjian, R. (1989) *Science* **243,** 1689–1694.
24. Gertz, R., Rauscher, F. J., III, Abate, C. & Curran, T. (1989) *Science* **243,** 1695–1699.
25. Schule, R., Kazuhiko, U., Mangelsdorf, D. J., Bolardo, J., Pike, J. W. & Evans, R. M. (1990) *Cell* **61,** 497–504.
26. Schonthal, A., Herrlich, P., Rahmsdork, H. J. & Ponta, H. (1988) *Cell* **54,** 325–334.
27. Setoyama, C., Hatamochi, A., Peterkofsky, B., Pranther, W. & de Crombrugghe, B. (1986) *Biochem. Biophys. Res. Commun.* **136,** 1042–1048.
28. Lucibello, F. C., Neuberg, M., Hunter, J. B., Jenuwein, T., Shuermann, M., Wallich, R., Stein, B., Schonthal, A., Herlich, P. & Muller, R. (1988) *Oncogene* **3,** 43–51.