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# The CCAAT Displacement Protein/*cut* Homeodomain Protein Represses Osteocalcin Gene Transcription and Forms Complexes with the Retinoblastoma Protein-related Protein p107 and Cyclin A[1]

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## ABSTRACT

**Developmental control of bone tissue-specific genes requires positive and negative regulatory factors to accommodate physiological requirements for the expression or suppression of the encoded proteins. Osteocalcin (OC) gene transcription is restricted to the late stages of osteoblast differentiation. OC gene expression is suppressed in nonosseous cells and osteoprogenitor cells and during the early proliferative stages of bone cell differentiation. The rat OC promoter contains a homeodomain recognition motif within a highly conserved multipartite promoter element (OC box I) that contributes to tissue-specific transcription. In this study, we demonstrate that the CCAAT displacement protein (CDP), a transcription factor related to the *cut* homeodomain protein in *Drosophila melanogaster*, may regulate bone-specific gene transcription in immature proliferating osteoblasts. Using gel shift competition assays and DNase I footprinting, we show that CDP/*cut* recognizes two promoter elements (TATA and OC box I) of the bone-related rat OC gene. Overexpression of CDP/*cut* in ROS 17/2.8 osteosarcoma cells results in repression of OC promoter activity; this repression is abrogated by mutating OC box I. Gel shift immunoassays show that CDP/*cut* forms a proliferation-specific protein/DNA complex in conjunction with cyclin A and p107, a member of the retinoblastoma protein family of tumor suppressors. Our findings suggest that CDP/*cut* may represent an important component of a cell signaling mechanism that provides cross-talk between developmental and cell cycle-related transcriptional regulators to suppress bone tissue-specific genes during proliferative stages of osteoblast differentiation.**

## INTRODUCTION

Bone tissue formation occurs late in fetal development and is mediated by osteoblasts that secrete bone-related extracellular matrix proteins (*e.g.,* collagen type I and OC[3]) that support mineral deposition (1, 2). The temporal appearance and location of preosseous tissues is controlled in part by homeodomain transcription factors and developmental morphogens (3–5). The classical Hox homeodomain proteins represent 13 paralogous classes of related proteins, which share a 60-amino acid protein segment (homeobox) that contacts DNA (6, 7). Several Hox genes (*e.g.,* Hoxa-10, Hoxa-11, and Hoxa -13, as well as Hoxd-10, Hoxd-11, Hoxd-12, and Hoxd-13) participate in the formation of axial and appendicular skeletal elements (3). However, whereas Hox and other developmental mediators primarily control positional information along the anterior/posterior body axis, osteoblast maturation appears to involve other classes of ("non-Hox") homeodomain proteins that are expressed at later stages of fetal development. For example, expression of the Dlx-5 and Msx-2 homeodomain proteins is cell type restricted and confined to specific stages of skeletal development and bone cell differentiation. Furthermore, mutations in the Msx-2 and Dlx-5 genes have been implicated in genetic skeletal abnormalities (8, 9). Both transcription factors are capable of repressing bone tissue-related gene expression (10–14) and are differentially expressed during osteoblast differentiation. These data suggest that Dlx-5 and Msx-2 represent tissue-specific components of a homeodomain-mediated signaling cascade that may determine the progression of osteoprogenitors into mature osteocytic cells. Although the activities of Msx-2 and Dlx-5 appear to control bone tissue-specific genes during late developmental stages, other homeodomain proteins may regulate osteoblast-related genes during earlier stages of osteoblast phenotype commitment.

Osteogenesis proceeds by developmental transitions of pluripotent progenitor cells of mesenchymal origin into precommitted osteoprogenitor cells that eventually mature into osteoblasts that support the extracellular matrix (1, 2). Osteoblast differentiation *in vitro* proceeds by a three-stage developmental sequence and occurs concomitantly with formation of a mineralized bone-like extracellular matrix similar to bone *in vivo* (1, 2). The OC gene represents a paradigm for understanding transcriptional control of osteoblast maturation (15). The OC gene is transcriptionally inactive in nonosseous cells and immature osteoprogenitor cells. The gene is up-regulated during osteoblast differentiation when osteoblasts cease to divide and is expressed maximally in mineralizing osteoblasts. Because OC is a major noncollagenous bone protein and biosynthesis is primarily restricted to mature mineralizing osteoblasts, expression of the OC gene appears to be positively and negatively controlled to accommodate physiological requirements for the encoded protein in mineralized bone. One principal tissue-specific element of the OC promoter regulating these transcriptional modulations is a multipartite transcriptional element designated OC box I. Both Msx-2 and Dlx-5 functionally interact with a homeodomain motif (5′-CTAATT) that overlaps a CCAAT-like element within OC box I (10–14). Because Dlx-5 and Msx-2 are not expressed in many nonosseous cells, we postulate that OC gene transcription may be in part actively repressed by general rather than tissue-specific transcription factors.

CDP/*cut* is a ubiquitous homeodomain protein homologous to the *Drosophila cut* gene (16–18) and represents an active repressor of differentiation-specific gene expression in many vertebrate cell types (19–27). CDP/*cut* contains four conserved DNA binding domains, *i.e.,* three cut repeats and a single homeodomain, which each recognize redundant but related sequence elements (28, 29), including CCAAT and TAAT motifs identical to those present in OC box I of the rat and mouse OC genes (10, 11). In addition, interactions of CDP/*cut* with gene promoters can occur in conjunction with pRB and related tumor suppressor proteins (27, 30, 31). Hence, CDP/*cut* may represent an important component of a cell signaling mechanism that provides cross-talk between developmental and cell growth-related transcriptional regulators. Despite these interesting biological proper-

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[3] The abbreviations used are: OC, osteocalcin; CDP, CCAAT displacement protein; pRB, retinoblastoma protein; nt, nucleotide(s); GST, glutathione *S*-transferase; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase; poly (G/C) DNA, poly (dG−dC)·(dG−dC); poly (I/C) DNA, poly (dI−dC)·(dI−dC).

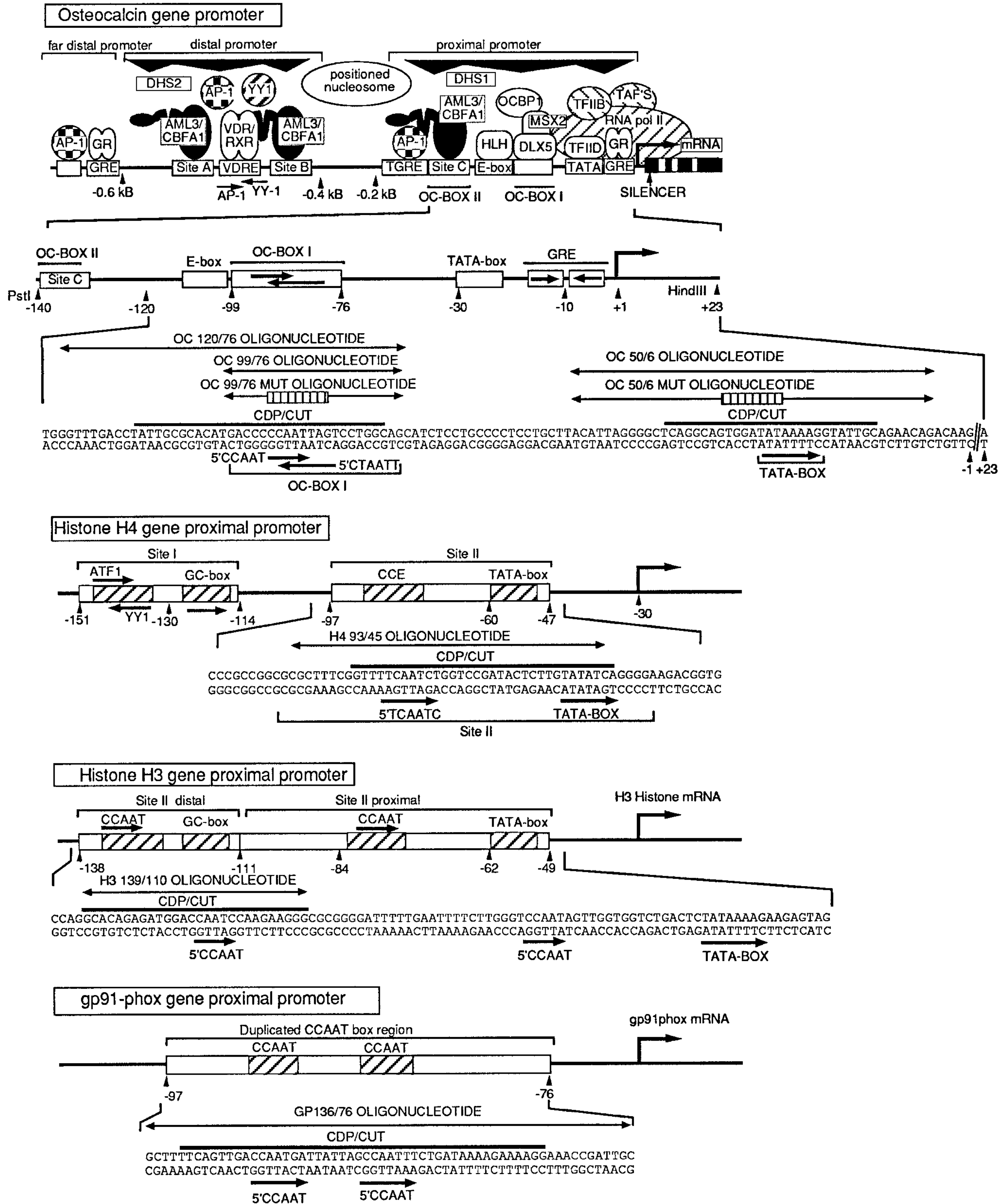



Fig. 1. Summary of DNA probes, oligonucleotides, and OC promoter mutations. The *top portion* shows a schematic representation of the OC promoter including key gene-regulatory elements as described in detail previously (15). The proximal promoter contains a series of regulatory elements including the TATA box and OC box I, as well as sites for AML/CBFA-related runt-homology proteins (*Site C*), HLH proteins (*E-box*), and the glucocorticoid receptor (*GRE*). OC box I is a principal tissue-specific element that contains a CCAAT-like motif (5′-CCAAT, sense strand) and an overlapping homeodomain recognition motif (5′-TCAATT, antisense strand; Refs. 11 and 12). The DNA probe used for protein/DNA interaction studies is indicated immediately below the OC promoter diagram and spans nt −140/+23 (Pst/*Hin*dIII fragment). OC promoter oligonucleotides used as specific competitor DNAs in this study are indicated by *lines with arrowheads*. The OC 99/76 MUT oligonucleotide contains an 8-nt mutation (*box with vertical stripes*) in which the 5′-CCAATTAG element is mutated to 5′-GACTGCTC. Additional oligonucleotides containing established CDP/*cut* binding sites (*lines with arrowheads on each end*) were derived from the promoters of the gp91phox (17, 22), histone H4 (34, 36), and histone H3 (51) genes. Key regulatory elements (*boxes with diagonal stripes*) and relevant promoter domains (*horizontal brackets*) for each of these genes are indicated to show the relative locations of CDP/*cut* sites. DNase I footprints for CDP/*cut* have been established for all four promoters and are indicated by *thick lines above the sequences* (the DNase I footprint data for the OC gene were determined as a component of this study and are presented in Fig. 3). Relevant motifs related to CCAAT and TATA boxes that are located within the DNase I footprints for each gene are indicated with *arrows below the sequences*.

ties, functional involvement of CDP/*cut* in regulating osteoblast-related gene expression has not been explored.

In this study, we show that CDP/*cut* functionally interacts with OC box I and the TATA box and is capable of repressing OC promoter activity. Furthermore, our data demonstrate that CDP/*cut* interacts with the OC promoter in a proliferation-specific manner and forms a higher order protein/DNA complex with the pRB-related protein p107 and cyclin A. Therefore, in a broader context, these data may provide support for the concept that the CDP/*cut* homeodomain protein is a component of an intricate transcriptional mechanism that suppresses differentiation-specific gene expression in actively dividing cells by forming specific protein/DNA complexes with cell growth regulatory factors.

## MATERIALS AND METHODS

**Protein/DNA Interaction Assays.** Nuclear proteins from ROS 17/2.8 osteosarcoma cells and calvarial rat osteoblasts were obtained by salt extraction (0.42 M KCl; Ref. 32) using a procedure that has been documented in detail previously (33). Nuclear extracts from HeLa cells were prepared as described previously (34). The bacterially expressed GST/CDP (CR2-Cterm) fusion protein (17) was purified using glutathione-resin as described by the manufacturer (Pharmacia). All protein preparations were quantitated by Coomassie Blue dye binding assays.

Gel shift assays with nuclear extracts were performed as described previously (34) in a volume of 20 $\mu$l and contained 10 fmol of a $^{32}$P-labeled DNA fragment spanning the promoters of the genes for rat OC (nt −140/+23; *Pst*I/*Hin*dIII fragment of pOCZCAT; Ref. 11), human histone H4 (nt −97/−38; *Eco*RI/*Hin*dIII fragment of pFP201; Ref. 34), or human gp91phox (nt −136/−76; *Eco*RI/*Hin*dIII fragment of pFPUC; Ref. 17). Binding reactions with nuclear extract protein contained 2 $\mu$g of poly(G/C) DNA and 0.2 $\mu$g of poly(I/C) DNA, whereas reactions with purified GST/CDP(CR2-Cterm) contained 0.2 $\mu$g of poly(G/C) DNA. Standard competition assays were performed by adding a 100-fold molar excess (1 pmol) of unlabeled oligonucleotides to the DNA probe before adding protein. Gel shift immunoassays were performed as described previously (27) with antibodies against p107 and p130 (Santa Cruz Biotechnology) or with hybridoma supernatants and antisera as indicated in the figure legends. Gel shift assays aimed at determining the molecular weight of the CDP/*cut* protein/DNA complexes were performed as described by Orchard and May (35) using $\alpha$-macroglobulin as a standard. To facilitate measurements (in mm) of the migration of the CDP/*cut* complex in gel shift assays, electrophoresis times were extended to a total of 4–8 h at 200 V.

DNase I footprinting analyses of GST/CDP(CR2-Cterm) binding were performed with probes that were labeled at an artificial *Hin*dIII site (nt +23) present in pOCZCAT (11) using either [$^{32}$P]g-ATP and T4 polynucleotide kinase (for labeling the antisense strand) or [$\alpha$-$^{32}$P]dCTP and Klenow polymerase (for labeling the sense-strand). Binding reactions were performed with the GST/CDP(CR2-Cterm) protein in a 50-$\mu$l volume using 10 fmol of probe and 500 ng of poly(G/C) DNA. DNase I digestions were performed by diluting samples to 100 $\mu$l while adding DNase I (1 Kunitz unit) and $MgCl_2$ (5 mM, final concentration). Reactions were allowed to proceed for 1 min at ambient temperature (20°C) and terminated by the addition of 50 ml of stop-mix (10 mM EDTA, 0.1% SDS, and 0.2 $\mu$g/$\mu$l salmon sperm DNA). Samples were then subjected to phenol/chloroform extraction, followed by ethanol precipitation. DNA pellets were quantitated by Cerenkov counting and dissolved in 90% formamide. Samples were electrophoresed in sequencing gels and analyzed by autoradiography.

**Transient Expression Assays.** Functional evaluation of the effects of CDP/*cut* on OC promoter activity was performed in ROS 17/2.8 osteosarcoma cells by cotransfecting a CMV promoter-driven construct containing the full-length CDP/*cut* cDNA (17) with wild-type or mutant OC promoter/CAT fusion construct (11) using calcium phosphate-mediated gene transfer (32). Variation in transfection efficiency was accounted for by cotransfecting a SV40 promoter-driven luciferase construct. Samples were harvested 2 days after transfection and evaluated for CAT and luciferase activity. CAT values were normalized for luciferase activity and evaluated by ANOVA of repeated measures for statistical significance ($P < 0.01$; Dr. Stephen Baker, University of Massachusetts Biocomputing Department, Worcester, MA).

## RESULTS

**The CDP/*cut* Homeodomain Protein Interacts Specifically with the Proximal Promoter of the OC Gene.** The proximal promoter of the rat OC gene (Fig. 1) contains at least one CCAAT-like element and a 5′-TCAATT homeodomain motif, both of which reside in OC box I, a principal tissue-specific promoter element of the OC gene (1). Both elements are among a variety of sequence motifs recognized by CDP/*cut* (28, 29). We directly addressed the question of whether CDP/*cut* is capable of interacting with the proximal OC promoter. Binding of purified recombinant GST-CDP/*cut* fusion protein [GST/CDP(CR2-Cterm)] was assessed in gel shift assays using a probe spanning nt −140 to +23 of the OC gene (Fig. 2*A*). In addition, we analyzed the binding of endogenous CDP/*cut* complexes in nuclear extracts from ROS 17/2.8 osteosarcoma cells (Fig. 2*B*). The GST/CDP(CR2-Cterm) protein mimics the DNA binding properties of the full-length CDP/*cut* protein and spans the COOH-terminal portion of CDP/*cut*, encompassing cut repeats 2 and 3 and the homeodomain (22). As a positive control, we used a probe spanning the duplicated CCAAT box region of the myeloid gp91-phox gene that represents a prototypical high affinity CDP/*cut* site (Refs. 17 and 19; data not shown). The results show that the GST/CDP(CR2-Cterm) fusion protein (Fig. 2*A*) and endogenous CDP/*cut* proteins (Fig. 2*B*) form electrophoretically stable complexes with the OC gene promoter.

To establish specificity of the CDP/*cut* protein/DNA interactions, we performed gel shift competition assays with a panel of oligonucleotides (Fig. 1) spanning wild-type and mutant sequences of the OC promoter that encompass the OC box. DNA segments comprising previously characterized binding sites in the gp91-phox (17, 19) and histone H4 and histone H3 genes[4] (27) were also analyzed. The data show that complexes mediated by recombinant GST/CDP(CR2-Cterm) protein (Fig. 2*A*) and endogenous CDP/*cut* proteins (Fig. 2*B*) with the OC probe (nt −140/+23) are competed by OC promoter segment nt −120/−76. In contrast, the OC segment nt −99/−76 competes with very low efficiency. No competition is observed with a mutant oligonucleotide OC nt −99/−76, which contains mutations in OC box I that alter the overlapping CCAAT and CTAATT motifs. These findings suggest that the interaction of CDP/*cut* with the OC gene requires the CCAAT/homeodomain motif of OC box I and adjacent sequences between nt −120 and −76.

For comparison, the site in the gp91-phox gene that binds CDP/*cut* with very high affinity (estimated $k_d = 10^{-11}$ M; Ref. 22) competes strongly for recombinant GST/CDP(CR2-Cterm) binding to the OC gene. Segments spanning the promoters of the histone H4 (estimated $k_d = 10^{-9}$ M)[4] and histone H3 genes compete with moderate efficiency (Fig. 2*B*). Comparison of the oligonucleotide competition data suggests that the relative strength of GST/CDP(CR2-Cterm) binding to different genes is gp91-phox $\gg$ OC > H4 > H3.

DNase I footprinting results (see the data presented below) indicate that both OC promoter regions are important for binding. Competition experiments using ROS 17/2.8 nuclear proteins were also performed with wild-type and OC box mutant nt −120/−76 oligonucleotides (OC 120/76), as well as with wild-type and mutant oligonucleotides spanning nt −50 to −6 of the OC promoter that encompasses the TATA box region (OC 50/6; Fig. 2*C*). The competition data (Fig. 2*C*) show that the OC 120/76 and OC 50/6 oligonucleotides both compete for CDP/*cut* binding, albeit with modest efficiency, but the corresponding mutant oligonucleotides show reduced competition potential. We then performed gel shift experiments with the wild-type OC 120/76 and OC 50/6 oligonucleotides in comparison to the plasmid-derived OC −140/+23 fragment (OC 140/+23) as probes (Fig. 2*D*).

[4] Unpublished data.

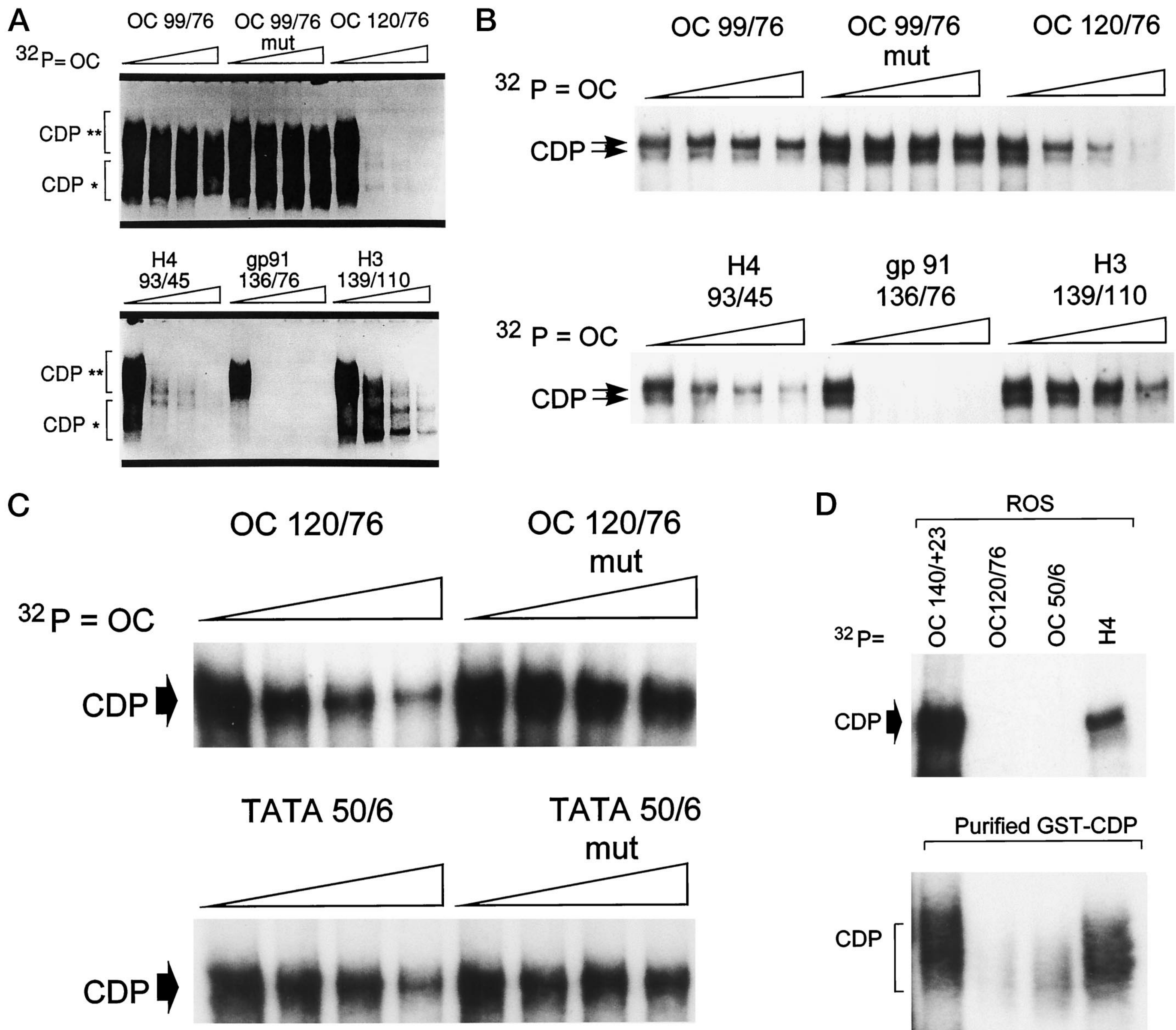



Fig. 2. Sequence-specific binding of CDP/*cut* to the OC promoter. *A*, gel shift assays were performed with purified recombinant GST-CDP/*cut* fusion protein [GST/CDP(CR2-Cterm)] to a probe spanning nt −140/+23 of the OC gene (*top* and *bottom panels*). Oligonucleotide competition analysis of GST/CDP-Cterm binding to the OC probe was performed using increasing amounts of unlabeled competitor DNA oligonucleotides (*sloped triangle*). The amounts added were, respectively, 0, 1, 2, or 5 pmol of oligonucleotide per lane. Because each reaction contains 10 fmol (0.5 nM) of probe, these amounts correspond with, respectively, 0-, 100-, 200-, and 500-fold molar excess of unlabeled competitor oligonucleotide. The oligonucleotides span portions of the OC gene promoter (*top panel*) or the promoters of the histone H4, gp91-phox, and histone H3 genes (*bottom panel*). The OC 99/76 mut fragment contains an 8-nt mutation of the internal region of the OC box that abolishes the overlapping CCAAT-like element and CTAATT homeodomain motif (11, 12). The OC probe mediates both high (*) and low (**) mobility complexes with recombinant CDP/*cut* protein. The high mobility complexes increase with increasing amounts of protein (data not shown) and may reflect binding of more than one CDP protein per probe molecule. *B*, gel shift oligonucleotide competition assays were performed with nuclear protein (3 μg) from ROS 17/2.8 cells and the OC probe (nt −140/+23) using the same increasing amounts of competitor DNA oligonucleotides (*sloped triangle*) as described in *A*. The *double arrowhead* indicates the endogenous CDP/*cut* complex that appears as a closely spaced doublet. The origin of electrophoretic migration is just above the border of the panel. The results show that endogenous CDP/*cut* complexes in osteoblast and osteosarcoma cells have a binding specificity similar to that of recombinant GST/CDP/*cut*. We note that the electrophoretic mobilities of endogenous CDP/*cut* protein/DNA complexes (*B*) are significantly slower than those mediated by recombinant CDP/*cut* protein (*A*); the difference in mobility may be due to the absence or presence of cofactors (see Fig. 5). *C*, oligonucleotide competition assays were performed using the OC promoter fragment nt −140/+23 as a probe and 45-mer oligonucleotides spanning the wild-type or mutant OC box I region from nt −120 to −76 (*OC 120/76*) or the TATA box region from nt −50 to −6 (*OC 50/6*). These experiments were performed using approximately 6 μg of ROS 17/2.8 nuclear protein, and the *sloped triangle* represents increased competitor quantities (0, 2.5, 5, and 10 pmol, respectively). We note that the CDP/*cut* complex is detected as a single band (rather than a doublet, see *B*) in these assays, which may be due to subtle differences in experimental procedures. *D*, gel shift assays were carried out with ROS 17/2.8 nuclear protein (6 μg; *top panel*) and recombinant GST/CDP(CR2-Cterm protein) (*lower panel*) using radiolabeled OC promoter fragments spanning nt −140 to +23 (*OC 140/+23*), −120 to −76 (*OC 120/76*) and −50/−6 (*OC 50/6*), as well as a probe spanning a CDP/*cut* binding site in the histone H4 gene (52).

Using nuclear protein from ROS 17/2.8 cells under stringent conditions to suppress the binding of nonspecific proteins present in nuclear extracts, *i.e.,* using relatively high nonspecific DNA amounts [2 μg of poly(G/C) DNA and 200 ng of poly(I/C) DNA] per 20 μl reaction), we observed the formation of an electrophoretically stable complex containing CDP/*cut* only for the −140/+23 fragment (OC 140/+23) and not for either of the 45-mer oligonucleotides (OC 120/76 and OC 50/6; Fig. 2*D, top panel*). Gel shift experiments were performed in parallel using GST/CDP(CR2-Cterm) protein (Fig. 2*D, lower panel*). We note that these binding reactions with affinity-purified protein are performed with less nonspecific DNA [200 ng of poly(G/C) DNA] than used with nuclear extracts, because there are few nonspecific proteins

present. Under these more relaxed binding conditions, we observed that the full-length OC −140/+23 promoter probe mediates the expected strong binding of CDP, but CDP also exhibits weak binding to the radiolabeled OC 120/76 and OC 50/6 oligonucleotides (Fig. 2*D, lower panel*). Thus, whereas the ability to detect low-affinity interactions of CDP/*cut* with the 45-mers is influenced by the stringency of the binding reactions, the formation of electrophoretically stable CDP/*cut* complexes is significantly increased when the full-length OC promoter fragment nt −140/+23 (OC 140/+23) is used in gel shift assays.

Interestingly, the TATA box oligonucleotide nt −50/−6 spans the segment of the OC promoter that is more strongly protected from DNase I in the presence of CDP/*cut* than the OC box region (see below). However, either oligonucleotide is relatively inefficient in forming electrophoretically stable protein/DNA complexes (Fig. 2*D*) and in competition for protein binding (Fig. 2*C*). These findings suggest that efficient binding of CDP/*cut* with the OC promoter requires that the OC box and TATA box regions are linked within the same DNA fragment.

**The Tissue-specific OC Box and the Basal Transcription-related TATA Box in the OC Gene Are Recognition Targets for CDP/*cut* Binding.** To determine the sites of interaction of CDP/*cut* with the rat OC promoter, we performed DNase I footprinting analyses on the sense and antisense strands (Fig. 3). On both strands, two DNase I protected regions were observed in the presence of recombinant GST/CDP(CR2-Cterm) protein. Region 1 (nt −100/−81, sense; nt −112/−82, anti-sense; Fig. 3) overlaps OC box I nt −99/−76. This result corroborates the results of the oligonucleotide competition assays (Fig. 2) that suggest that OC box I is important for CDP/*cut* binding. Rat OC box I, which contains a binding site for the Msx-2 (11) and Dlx-5 (13) homeodomain proteins (5′-CCAATTAG; nt −92/−85), may provide a recognition motif for the homeodomain portion of CDP/*cut* (28). Region 2 (nt −39/−11, sense; nt −37/−11, anti-sense) encompasses the TATA box of the OC gene. Because the GST/CDP(CR2-Cterm) protein contains three intrinsic DNA binding domains (a single homeodomain and two cut repeats, *i.e.,* cut 2 and cut 3), protection of the TATA box may reflect interactions of secondary DNA binding domains within the same CDP/*cut* molecule. However, equally viable is the possibility that a second CDP/*cut* protein may associate with the OC promoter, as suggested by the formation of putative oligomeric GST/CDP(CR2-Cterm) complexes with the OC promoter (Fig. 2). Based on gel shift data (see Fig. 2, *C* and *D*), efficient binding of CDP/*cut* occurs only when the OC box I and TATA box regions are present within the same DNA fragment. Because CDP/*cut* specifically recognizes the OC box I and TATA box element of the rat OC gene, it appears that CDP/*cut* targets two key elements essential for tissue-specific basal levels of OC gene transcription (1).

**CDP/*cut* Represses OC Gene Transcription, Which Requires the CCAAT/Homeodomain Motif of the OC Box.** To assess the functional consequences of CDP/*cut* interactions with the OC promoter in osseous cells, we performed transient cotransfection experiments with the full-length CDP/*cut* protein and an OC promoter/CAT reporter gene construct in ROS 17/2.8 osteosarcoma cells (Fig. 4). Normal diploid osteoblasts do not transcribe the OC gene during proliferative stages. However, ROS 17/2.8 osteosarcoma cells display abrogation of cell growth and differentiation interrelationships resulting in basal transcription of the bone differentiation-specific OC gene (1). Because nonosseous cells do not activate the OC promoter and putative *trans*-activators that operate via OC-box I remain to be identified (10–14), ROS 17/2.8 cells represent a viable cell culture model for assaying repressive effects of CDP expression on OC promoter activity.

Transient expression of CDP/*cut* in ROS 17/2.8 cells reduces OC promoter activity by approximately 2-fold, which is significant as

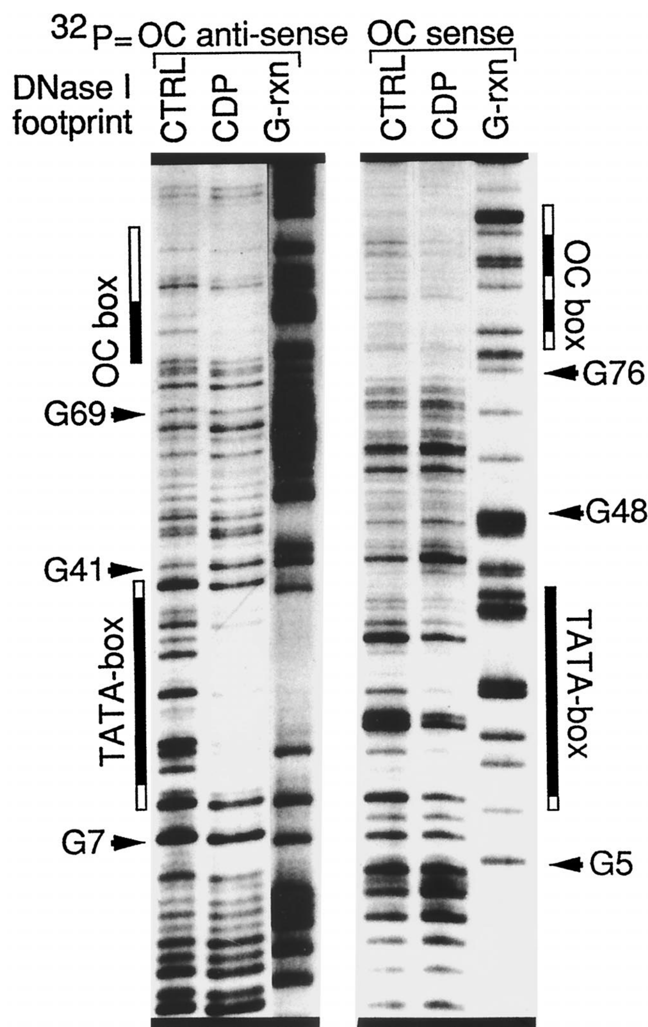



Fig. 3. CDP/*cut* binds to the TATA box and OC box of the OC gene. DNase I footprinting reactions were performed with the sense (*left*) and antisense strands (*right*) in the absence (*CTRL*) or presence (*CDP*) of GST/CDP(CR2-Cterm) protein. Electrophoresis of DNase I-digested samples was performed in parallel with a chemical sequence ladder (*G-rxn*). The locations of representative guanine residues in the OC promoter (*e.g.,* G69) are indicated by *arrowheads*. DNase I protected regions reflected by reduced DNase I cleavage in the presence of protein are indicated by ■ along the autoradiograms. □, areas of uncertainty in determining footprint boundaries due to bp preferences of DNase I and infrequent cleavage of sequences near OC box I. Differences in the relative strength of the DNase I footprints for the sense *versus* antisense strand may be due to experimental variation in probe quantity and specific activity related to the labeling procedure (T4 kinase *versus* Klenow polymerase). However, similar results were obtained in three separate experiments, and the locations of footprints on the sense and antisense strands occur within overlapping regions of the OC promoter.

determined by computer-assisted statistical evaluation using ANOVA ($P < 0.01$). In contrast, expression of CDP/*cut* does not influence OC promoter activity when the CCAAT/homeodomain motif of OC box I is mutated. We conclude that CDP/*cut* specifically represses OC gene transcription by direct interactions with the OC promoter involving OC box I.

Although loss of CDP/*cut* repression is abrogated by the OC box I mutation, this result does not preclude a role for the CDP/*cut* recognition motif in the TATA box region. However, assessing the relative importance of the TATA box within the context of the native OC promoter is technically difficult because of the overlap between the CDP/*cut* site and the TATA element that is required for transcriptional initiation. In an extensive series of studies with the histone H4 gene in which CDP/*cut* (also known as HiNF-D) interacts with CAAT and TATA-like elements, we have not been able to design subtle muta-

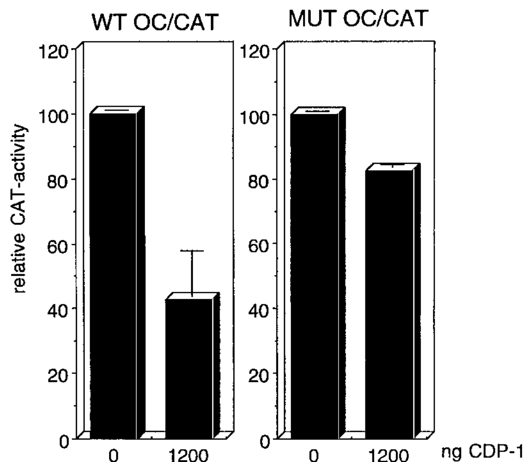



Fig. 4. Transient cotransfection analysis of CDP/*cut* effects on OC gene transcription. Transfection experiments were performed with a CMV construct driving the expression of the full-length CDP/*cut* protein and OC promoter/CAT reporter gene in ROS 17/2.8 osteosarcoma cells. The wild-type OC/CAT construct (pOCZCAT) spans 1.1-kb of the 5′-flanking region, and the mutant OC/CAT construct is based on pOCZCAT but contains an 8-nt mutation in OC box I that abrogates the CTAATT homeodomain motif (11). Each bar graph represents the average values of CAT activity corrected for luciferase activity (internal standard) of three independent experiments, each performed in triplicate, with the indicated amount of CMV/CDP construct. Statistical significance ($P < 0.01$) of CDP/*cut* effects on the wild-type but not the mutant OC promoter activity was established by ANOVA.

tions that abolish CDP/*cut* binding but do not affect binding of the TATA-binding protein (Refs. 34 and 36 and references therein). Thus, the role of the TATA region in CDP/*cut*-dependent regulation of OC gene expression remains to be established.

Results from gel shift immunoassays indicate that p107 and cyclin A interact with CDP/*cut* (see the data presented below). To assess whether these cofactors can influence OC promoter activity, we performed two independent transfection experiments in triplicate in which CDP/*cut* was coexpressed with p107 or cyclin A in osteoblastic ROS 17/2.8 cells to evaluate responsiveness of the wild-type and mutant OC promoter (data not shown). The results indicate that expression of p107 or cyclin A does not influence OC promoter activity and does not affect suppression by CDP/*cut*. These findings suggest that the endogenous levels of p107 and cyclin A are not rate-limiting for the activity of CDP/*cut* in this cell type. Consistent with this suggestion, we have not been able to detect CDP/*cut* containing protein/DNA complexes at the OC promoter that lack p107 or cyclin A by gel shift assays (data not shown).

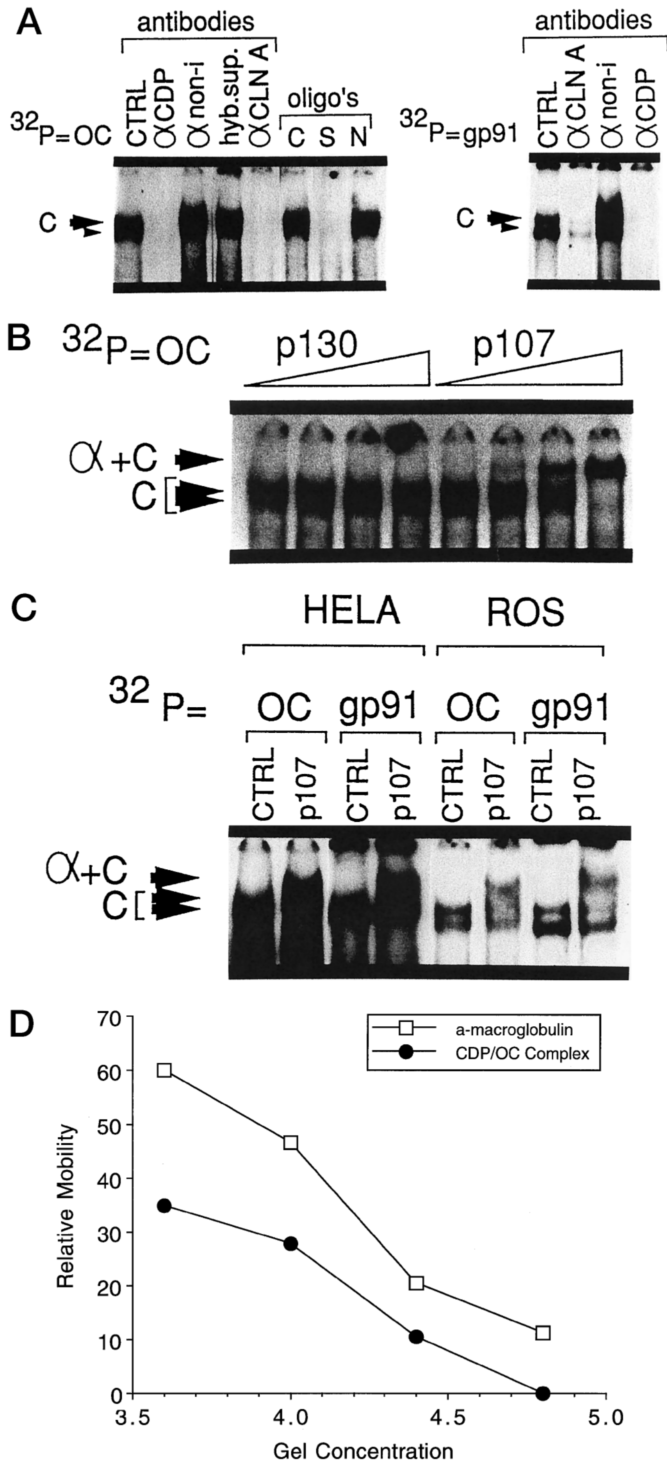
**CDP/*cut* Interacts in Conjunction with the pRB-related Protein p107 and Cyclin A to Form a Large ($M_r$ > 340,000) Protein/DNA Complex at the OC Gene Promoter.** Nuclear proteins from ROS 17/2.8 cells mediate a closely spaced gel shift doublet representing two types of protein/DNA complexes in 4% (80:1) polyacrylamide gels (Figs. 2*B* and 5), although the resolution of this doublet is dependent on experimental conditions (*e.g.,* see Fig. 2*C*). The very low mobility of these complexes is a signature property of CDP/*cut* complexes isolated from mammalian cells (16, 27). Preincubation of nuclear proteins from ROS 17/2.8 cells with a polyclonal CDP/*cut* antibody (16, 17) results in inhibition of CDP/*cut* binding to the OC promoter (Fig. 5*A*). No immunoreactivity is observed with control antisera (nonimmune or preimmune guinea pig serum; Fig. 5*A* and data not shown). These data establish that there are at least two types of CDP/*cut* complexes in ROS 17/2.8 cells.

We have previously shown that CDP/*cut* protein/DNA complexes with cell cycle-controlled histone H4, H3, and H1 genes contain a pRB-related protein and cyclin A (27, 30). We assessed whether CDP/*cut* interactions with the OC promoter involve similar higher order protein/DNA complexes. Gel shift immunoassays were performed using antibodies against the pRB-related proteins p130 and p107 (Fig. 5*B*), as well as cyclin A (Fig. 5*A*). Nuclear proteins from ROS 17/2.8 cells were preincubated with specific polyclonal antibodies directed against COOH-terminal residues of p107 or p130. The results show quantitative formation of a ternary antibody/protein/DNA complex ("supershift") at the OC promoter in the presence of the p107 antibody, but not in the presence of the p130 antibody (Fig. 5*B*). In addition, CDP/*cut* complexes with the OC promoter are completely inhibited in the presence of a monoclonal cyclin A antibody, but not in the presence of a control hybridoma supernatant (Fig. 5*A*). Thus, the immunoreactivity of CDP/*cut* complexes at the OC promoter with p107 and cyclin A antibodies indicates that CDP/*cut* forms higher order protein/DNA complexes in conjunction with p107 and cyclin A.

We determined whether CDP/*cut* complexes with p107 are unique to osseous cells or the bone-related OC promoter. We directly compared immunoreactivity of CDP/*cut* complexes with p107 antibodies using nuclear proteins from ROS 17/2.8 osteosarcoma and HeLa cervical carcinoma cells with DNA probes spanning the promoters of the OC and myeloid gp91phox gene (Fig. 5*C*). The results demonstrate that CDP/*cut* complexes from both osseous (ROS 17/2.8) or nonosseous (HeLa) cells form supershift complexes in the presence of the p107 antibody with probes spanning the proximal promoters of either the OC or gp91phox gene. For comparison, we have previously shown that CDP/*cut* complexes with cell cycle-controlled histone H4, H3, and H1 genes are immunoreactive with a panel of monoclonal antibodies against pRB, but not with antibodies against p107 or p130 (27). Therefore, formation of CDP/*cut* complexes with pRB-related proteins is not unique to osteoblastic cells and appears to be promoter selective.

As a technical note, the ability of antibodies to show immunoreactivity in gel shift assays is dependent on intrinsic properties of the antibody (*e.g.,* titer, avidity, and specificity for native protein) and protein/DNA complex (*e.g.,* accessibility of the epitope and conformation of the protein on DNA). Gel shift immunoassays monitoring interactions of cyclins and pRB-related proteins with CDP/*cut* have been performed with distinct cyclin A antibodies and a series of different pRB-related antibodies (see Refs. 27, 30, and 31). Immunoreactivity of the CDP/*cut* complex at the OC gene promoter in gel shift assays was only observed with the p107 antibody (SC-X-318) used in this study. However, this same antibody does not shift the CDP/*cut* complex observed for the histone H4 gene involving a pRB(p105) interaction with CDP/*cut* (27), which demonstrates the molecular specificity of the p107 antibody. Further definition of protein/protein interactions of CDP/*cut* with cyclin A and pRB-related proteins and the involvement of DNA recognition in modulating these interactions is currently under way.

To begin assessing the size of the CDP/*cut* complex on the OC promoter (−140/+23), we performed molecular weight analysis in gel shift assays according to the method of Orchard and May (35). This method involves parallel electrophoresis of the CDP/*cut* complex and a series of protein molecular weight markers in polyacrylamide gels with different percentages (Fig. 5*D*). CDP/*cut* protein/DNA complexes migrate very slowly (<5 mm/h) in standard 4% (80:1) polyacrylamide gels. For comparison, we have previously shown that higher order E2F protein/DNA complexes (containing pRB and cyclin A) have a much higher mobility (>20 mm/h) than CDP/*cut* protein/DNA complexes under very similar experimental conditions (31). The mobility of CDP/*cut* in gels of different polyacrylamide concentrations is significantly lower than that of all protein markers tested, including $\alpha$-macroglobulin ($M_r$ 340,000; Fig. 5*D*). Furthermore, CDP/*cut* protein/DNA complexes are virtually immobilized at the origin of



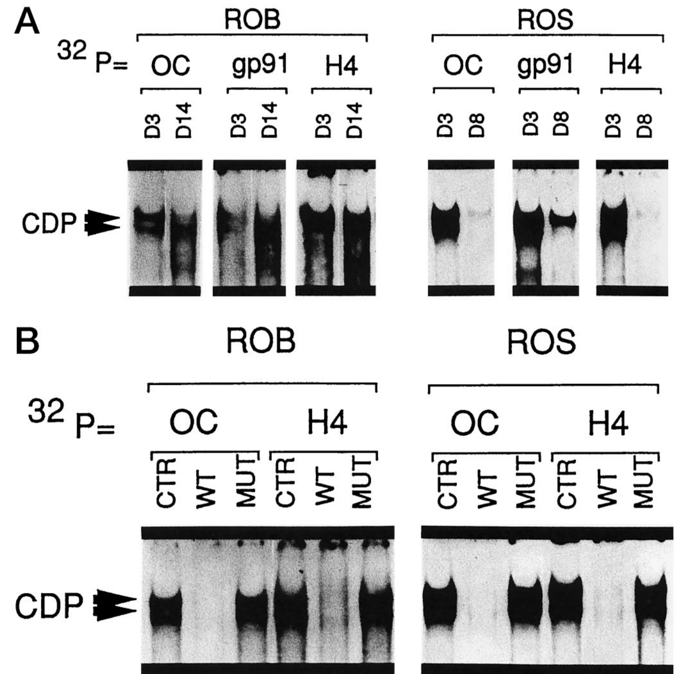




Fig. 6. Endogenous CDP/*cut* complexes in osteoblast-related cells are most abundant in proliferating cells. *A*, gel shift assays using nuclear protein (3 $\mu$g) from proliferating (*D3*) and postproliferative (*D14*) ROB cells, as well as proliferating (*D3*) and confluent (*D8*) ROS 17/2.8 cells incubated with the OC probe. *Double arrowheads* indicate the positions of the CDP/*cut* complexes. *B*, gel shift competition assays demonstrating the sequence specificity of the CDP/*cut* complexes shown in *A* using nuclear protein from ROB (*D3*) and ROS 17/2.8 (*D3*) cells. Competition assays were performed with a 100-fold excess (1 pmol) of oligonucleotides (37) spanning wild-type (*Lane S*, TM-3) and mutant (*Lane N*, NH-6) CDP/*cut* binding sites.

migration when the percentage of polyacrylamide is increased to 4.8% (constant cross-linking ratio of 80:1 acrylamide to bis-acrylamide; Fig. 5*D*) or when the cross-linking ratio is increased from 80:1 to 20:1 (constant polyacrylamide percentage of 4%; Ref. 34). Taken together, these data suggest that CDP/*cut* forms macromolecular protein/DNA complexes that are considerably larger than $M_r$ 340,000.

**Endogenous CDP/*cut* Complexes in Osseous Cells Are Proliferation-specific and Down-Regulated at the Cessation of Cell Growth.** The association of cell growth-regulatory factors p107 and cyclin A with CDP/*cut* complexes at the OC promoter suggests that these complexes may be restricted to dividing cells. We assessed whether endogenous CDP/*cut* complexes with the OC promoter are regulated in relation to proliferation. The levels of CDP/*cut* binding activity were examined in normal diploid ROB osteoblasts and ROS 17/2.8 osteosarcoma cells during the proliferative period and postproliferatively in confluent cell cultures. Similar to ROS 17/2.8 cells, ROB cells display two CDP/*cut* complexes (Fig. 6*A*), which are specifically competed by a wild-type CDP/*cut* binding site oligonucleotide (Fig. 6*B*). The level of the CDP/*cut* complex with reduced mobility is high in proliferating normal diploid ROB cells and ROS

Fig. 5. Osteoblast-related CDP/*cut* complexes contain p107 and cyclin A. *A*, gel shift immunoassays were performed using ROS 17/2.8 nuclear proteins (3 $\mu$g) that were preincubated with polyclonal CDP/*cut* guinea pig antiserum (17), nonimmune guinea pig serum ($\alpha$ *non-i*), control hybridoma supernatant (*hyb.sup.*), and monoclonal cyclin A hybridoma supernatant; *Lanes CTRL* and *C* represent standard binding reactions in the absence of antibodies. The *left panel* shows results obtained for binding reactions containing the OC probe, and the *right panel* shows the results obtained for binding reactions with the gp91-phox probe for comparison. Immunoreactivity is reflected by inhibition of the CDP/*cut* complex. The last three lanes of the *left panel* represent a competition assay with unlabeled DNA fragments (1 pmol) spanning wild type (*Lane S*, TM-3) and mutant (*Lane N*, NH-6) CDP/*cut* binding sites that were established previously (34). These competition assays were routinely performed as parallel reactions to confirm sequence-specific binding of CDP/*cut* in our assays. *B*, same as *A*, using increasing amounts (*sloped triangle*; 0, 1, 2, and 4 $\mu$l of antibody, respectively) of polyclonal antipeptide antibodies directed against p130 (C-20, SC-X-317; Santa Cruz Biotechnology) and p107 (C-18, SC-X-318; Santa Cruz Biotechnology). The *double arrowhead* indicates the CDP/*cut*-complex (doublet), and $\alpha$ + *C* points at the supershift complex of CDP/*cut* and the p107 antibody. *C*, same as in *B*, using the OC and gp91-phox probes in combination with nuclear protein from human HeLa S3 cells and rat ROS 17/2.8 cells. *D*, the relative molecular weight of CDP/*cut* protein/DNA complexes was analyzed by the method of Orchard and May (35). The relative mobility (*vertical axis*) of the CDP/*cut* complex (●) was determined by autoradiography, and the relative mobility of the protein marker ($\alpha$-macroglobulin, $M_r$ 340,000; □) was detected by Coomassie Blue staining after electrophoresis in polyacrylamide gels with different percentages (3.6–4.8% polyacrylamide with a cross-linking ratio of 80:1 acrylamide to bis-acrylamide), as indicated on the *horizontal axis*. The results show that the CDP/*cut* complex is substantially larger than the $M_r$ 340,000 protein marker and is virtually immobilized at the origin of migration in high percentage gels (*e.g.*, 4.8%). These properties are consistent with CDP/*cut* forming a high molecular weight protein/DNA complex.

17/2.8 osteosarcoma cells but barely detectable in confluent ROB and ROS 17/2.8 cells (Fig. 6*A*). Similarly, CDP/*cut* complexes with the gp91-phox and histone H4 genes are detectable at high levels only in proliferating ROB and ROS 17/2.8 cells.

To control for quantitation and integrity of the nuclear protein preparations, this same panel of ROS 17/2.8- and ROB-derived nuclear protein preparations was also evaluated for levels of several other transcription factors including SP-1, YY-1, ATF, AP-1, and AML-3/CBFA1-related DNA binding activities (data not shown; see Ref. 33). In these assays, we observed that the levels of SP-1 and AML are highest in nondividing ROB and ROS 17/2.8 cells. The levels of YY-1- and ATF-related proteins were not significantly different between proliferating and nonproliferating cells. Furthermore, modulations in the levels of AP-1 activity were found to parallel those of CDP/*cut* complexes (33). Thus, it appears that the levels of CDP/*cut* complexes with pRB-related proteins and cyclin A are selectively down-modulated at the cessation of cell proliferation.

## DISCUSSION

In this study, we have shown that the ubiquitous homeodomain transcription factor CDP/*cut* interacts with two separate recognition motifs (OC box I and TATA box) in the proximal promoter of the bone-related OC gene. Gel shift immunoassay data indicate that CDP/*cut* forms proliferation-specific complexes with cell growth-regulatory factors p107 and cyclin A on the OC promoter. Furthermore, using transient cotransfection assays, we have shown that CDP/*cut* is capable of transcriptionally repressing the differentiation-specific OC gene in osteoblastic cells, which requires sequences in rat OC box I. We note that the rat, mouse, and human OC genes display differences in transcriptional regulation (37–39). These differences are reflected by subtle nucleotide variations in the sequences spanning OC box I and the TATA box in the three species. The conservation of the functional role of CDP/*cut* in OC gene expression in these three species remains to be addressed.

The repression of OC gene promoter activity by the CDP/*cut* homeodomain protein extends previous observations indicating that two other non-Hox homeodomain proteins, Msx-2 (10, 11) and Dlx-5 (13, 14), can repress OC gene transcription via the homeodomain recognition element of OC box I. The physiological role for each of these factors in transcriptional control of osteoblast-specific genes must be addressed. Differentiation of precommitted but immature osteoblasts into mature and mineralizing osteocytic cells in culture occurs by a multistage developmental sequence characterized by modifications in gene expression of developmental stage-specific markers (*e.g.,* histone H4, alkaline phosphatase, osteopontin, and OC; Ref. 1). The role of Msx-2 in osteoblast differentiation is indicated by results showing that: (*a*) Msx-2 is expressed *in vivo* at several locations involving epithelial/mesenchymal interactions (40, 41); (*b*) mutations in the Msx-2 gene result in craniosynostosis (8); (*c*) antisense inhibition of Msx-2 in calvarial osteoblasts prevents osteoblast maturation (12); and (*d*) maximal expression of Msx-2 is restricted to proliferating osteoblasts and is down-regulated at the maturation transition (11). Functional involvement of Dlx-5 in osteoblast maturation is suggested by the following observations: (*a*) Dlx-5 and Dlx-6 are the only Dlx family members expressed in the perichondral region of developing fetal skeletal elements (42, 43); and (*b*) Dlx-5 gene expression is dramatically up-regulated at the onset of extracellular matrix mineralization in calvarial osteoblast cultures (13). Differences and similarities in expression of Dlx-5 and Msx-2 *in vivo* may accommodate ossification of different skeletal elements and are consistent with a role for Msx-2 and Dlx-5 in progression of osteoblast differentiation.

In contrast to the tissue-specific roles of Msx-2 and Dlx-5 in skeletal differentiation, CDP/*cut* and related factors are involved in transcriptional suppression of differentiation-specific marker genes in a broad spectrum of tissues and cell types (17, 19–27). Although high-level expression of CDP/*cut* is restricted to specific regions during embryonic or fetal development *in vivo* (44–48), CDP/*cut* DNA binding activity can be detected in a plethora of proliferating cells in vertebrates (summarized in Refs.22 and 34). The proliferation-specific nature of CDP/*cut* protein/DNA complexes and the association of CDP/*cut* with cell growth-regulatory factors p107 and cyclin A in a large ($M_r > 340,000$) multimeric complex binding to the bone-related OC promoter suggest that CDP/*cut* may represent a general suppressor of tissue-specific gene expression in response to cell cycle-regulatory signals.

It has been well established that a subset of the E2F class of cell cycle-regulatory transcription factors forms complexes with p107 and cyclin A. Recently, we have shown that CDP/*cut* is the DNA binding subunit of the histone gene-regulatory protein HiNF-D that contains cyclin A and a pRB-related protein as auxiliary subunits (27, 30). The interaction of CDP/*cut* with the cell cycle-related proteins p107 and cyclin A as components of transcription factor complexes at the differentiation-specific OC and gp91-phox promoters observed in this study suggests a novel and CDP/*cut*-dependent role for pRB-related proteins in gene regulation. Interestingly, Webster *et al.* (49) have presented data suggesting that the development of uterine leiomyomas in transgenic mice expressing the polyomavirus-encoded large T antigen (a known pRB-binding protein) may be causally related to molecular interactions between large T, pRB, and CDP/*cut*. Furthermore, Wiggan *et al.* (50) have observed interactions between p107 and paired-like homeodomain proteins (*e.g.,* MHox and Pax-3). Thus, interactions of pRB-related proteins with distinct classes of homeodomain proteins may represent a general mechanism for coupling cell growth-related signals with developmental cues regulating tissue-specific gene expression.

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## REFERENCES

1. Stein, G. S., and Lian, J. B. Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr. Rev., *14:* 424–442, 1993.
2. Rodan, G. A., and Noda, M. Gene expression in osteoblastic cells. Crit. Rev. Eukaryotic Gene Expression, *1:* 85–98, 1990.
3. Tickle, C., and Eichele, G. Vertebrate limb development. Annu. Rev. Cell Biol., *10:* 121–152, 1994.
4. Tabin, C. The initiation of the limb bud: growth factors, hox genes, and retinoids. Cell, *80:* 671–674, 1995.
5. Erlebacher, A., Filvaroff, E. H., Gitelman, S. E., and Derynck, R. Toward a molecular understanding of skeletal development. Cell, *80:* 371–378, 1995.
6. Gehring, W. J., Affolter, M., and Burglin, T. Homeodomain proteins. Annu. Rev. Biochem., *63:* 487–526, 1994.
7. Ruddle, F. H., Bartels, J. L., Bentley, K. L., Kappen, C., Murtha, M. T., and Pendleton, J. W. Evolution of Hox genes. Annu. Rev. Genet., *28:* 423–442, 1994.
8. Jabs, E. W., Muller, U., Li, X., Ma, L., Luo, W., Haworth, I. S., Klisak, I., Sparkes, R., Warman, M. L., Mulliken, J. B., Snead, M. L., and Maxson, R. A mutation in the homeodomain of the human MSX2 gene in a family affected with autosomal dominant craniosynostosis. Cell, *75:* 443–450, 1993.
9. Scherer, S. W., Poorkaj, P., Massa, H., Soder, S., Allen, T., Nunes, M., Geshuri, D., Wong, E., Belloni, E., Little, S., Zhou, L., Becker, D., Kere, J., Ignatius, J., Niikawa, N., Fukushima, Y., Hasegawa, T., Wessenbach, J., Boncinelli, E., Trask, B., Tsui, L-C., and Evans, J. P. Physical mapping of the split hand/split foot locus on chromosome 7 and implication in syndromic ectrodactyly. Hum. Mol. Genet., *3:* 1345–1354, 1994.
10. Towler, D. A., Rutledge, S. J., and Rodan, G. A. Msx-2/Hox 8.1: a transcriptional regulator of the rat osteocalcin promoter. Mol. Endocrinol., *8:* 1484–1493, 1994.

11. Hoffmann, H. M., Catron, K. M., van Wijnen, A. J., McCabe, L. R., Lian, J. B., Stein, G. S., and Stein, J. L. Transcriptional control of the tissue-specific, developmentally regulated osteocalcin gene requires a binding motif for the Msx family of homeodomain proteins. Proc. Natl. Acad. Sci. USA, *91:* 12887–12891, 1994.
12. Hoffmann, H. M., Beumer, T. L., Rahman, S., McCabe, L. R., Banerjee, C., Aslam, F., Tiro, J. A., van Wijnen, A. J., Stein, J. L., Stein, G. S., and Lian, J. B. Bone tissue-specific transcription of the osteocalcin gene: role of an activator osteoblast-specific complex and suppressor hox proteins that bind the OC box. J. Cell. Biochem., *61:* 310–324, 1996.
13. Ryoo, H-M., van Wijnen, A. J., Stein, J. L., Lian, J. B., and Stein, G. S. Detection of a proliferation specific gene during development of the osteoblast phenotype by mRNA differential display. J. Cell. Biochem., *64:* 106–116, 1997.
14. Newberry, E. P., Latifi, T., and Towler, D. A. Reciprocal regulation of osteocalcin transcription by the homeodomain proteins msx2 and dlx5. Biochemistry, *37:* 16360–16368, 1998.
15. Stein, G. S., Lian, J. B., van Wijnen, A. J., and Stein, J. L. The osteocalcin gene: a model for multiple parameters of skeletal-specific transcriptional control. Mol. Biol. Rep., *24:* 185–196, 1997.
16. Barberis, A., Superti-Furga, G., and Busslinger, M. Mutually exclusive interaction of the CCAAT-binding factor and of a displacement protein with overlapping sequences of a histone gene promoter. Cell, *50:* 347–359, 1987.
17. Neufeld, E. J., Skalnik, D. G., Lievens, P. M., and Orkin, S. H. Human CCAAT displacement protein is homologous to the *Drosophila* homeoprotein, cut. Nat. Genet., *1:* 50–55, 1992.
18. Blochlinger, K., Bodmer, R., Jack, J., Jan, L. Y., and Jan, Y. N. Primary structure and expression of a product from cut, a locus involved in specifying sensory organ identity in *Drosophila*. Nature (Lond.), *333:* 629–635, 1988.
19. Skalnik, D. G., Strauss, E. C., and Orkin, S. H. CCAAT displacement protein as a repressor of the myelomonocytic-specific gp91-phox gene promoter. J. Biol. Chem., *266:* 16736–16744, 1991.
20. Andres, V., Chiara, M. D., and Mahdavi, V. A new bipartite DNA-binding domain: cooperative interaction between the cut repeat and homeo domain of the cut homeo proteins. Genes Dev., *8:* 245–257, 1994.
21. Dufort, D., and Nepveu, A. The human cut homeodomain protein represses transcription from the c-myc promoter. Mol. Cell. Biol., *14:* 4251–4257, 1994.
22. Lievens, P. M., Donady, J. J., Tufarelli, C., and Neufeld, E. J. Repressor activity of CCAAT displacement protein in HL-60 myeloid leukemia cells. J. Biol. Chem., *270:* 12745–12750, 1995.
23. El-Hodiri, H. M., and Perry, M. Interaction of the CCAAT displacement protein with shared regulatory elements required for transcription of paired histone genes. Mol. Cell. Biol., *15:* 3587–3596, 1995.
24. Mailly, F., Berube, G., Harada, R., Mao, P. L., Phillips, S., and Nepveu, A. The human cut homeodomain protein can repress gene expression by two distinct mechanisms: active repression and competition for binding site occupancy. Mol. Cell. Biol., *16:* 5346–5357, 1996.
25. Luo, W., and Skalnik, D. G. CCAAT displacement protein competes with multiple transcriptional activators for binding to four sites in the proximal gp91phox promoter. J. Biol. Chem., *271:* 18203–18210, 1996.
26. Pattison, S., Skalnik, D. G., and Roman, A. CCAAT displacement protein, a regulator of differentiation-specific gene expression, binds a negative regulatory element within the 5′ end of the human papillomavirus type 6 long control region. J. Virol., *71:* 2013–2022, 1997.
27. van Wijnen, A. J., van Gurp, M. F., de Ridder, M. C., Tufarelli, C., Last, T. J., Birnbaum, M., Vaughan, P. S., Giordano, A., Krek, W., Neufeld, E. J., Stein, J. L., and Stein, G. S. CDP/*cut* is the DNA-binding subunit of histone gene transcription factor HiNF-D: a mechanism for gene regulation at the $G_1$/S phase cell cycle transition point independent of transcription factor E2F. Proc. Natl. Acad. Sci. USA, *93:* 11516–11521, 1996.
28. Aufiero, B., Neufeld, E. J., and Orkin, S. H. Sequence-specific DNA binding of individual cut repeats of the human CCAAT displacement/cut homeodomain protein. Proc. Natl. Acad. Sci. USA, *91:* 7757–7761, 1994.
29. Harada, R., Berube, G., Tamplin, O. J., Denis-Larose, C., and Nepveu, A. DNA-binding specificity of the cut repeats from the human cut-like protein. Mol. Cell. Biol., *15:* 129–140, 1995.
30. van Wijnen, A. J., Aziz, F., Grana, X., De Luca, A., Desai, R. K., Jaarsveld, K., Last, T. J., Soprano, K., Giordano, A., Lian, J. B., Stein, J. L., and Stein, G. S. Transcription of histone H4, H3, and H1 cell cycle genes: promoter factor HiNF-D contains CDC2, cyclin A, and an RB-related protein. Proc. Natl. Acad. Sci. USA, *91:* 12882–12886, 1994.
31. van Wijnen, A. J., Cooper, C., Odgren, P., Aziz, F., De Luca, A., Shakoori, R. A., Giordano, A., Quesenberry, P. J., Lian, J. B., Stein, G. S., and Stein, J. L. Cell cycle-dependent modifications in activities of pRb-related tumor suppressors and proliferation-specific CDP/cut homeodomain factors in murine hematopoietic progenitor cells. J. Cell. Biochem., *66:* 512–523, 1997.
32. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. Current Protocols in Molecular Biology. New York: John Wiley & Sons, Inc., 1987.
33. Lindenmuth, D. M., van Wijnen, A. J., Hiebert, S., Stein, J. L., Lian, J. B., and Stein, G. S. Subcellular partitioning of transcription factors during osteoblast differentiation: developmental association of the AML/CBFα/PEBP2α-related transcription factor-NMP-2 with the nuclear matrix. J. Cell. Biochem., *66:* 123–132, 1997.
34. van Wijnen, A. J., van den Ent, F. M., Lian, J. B., Stein, J. L., and Stein, G. S. Overlapping and CpG methylation-sensitive protein-DNA interactions at the histone H4 transcriptional cell cycle domain: distinctions between two human H4 gene promoters. Mol. Cell. Biol., *12:* 3273–3287, 1992.
35. Orchard, K., and May, G. E. An EMSA-based method for determining the molecular weight of a protein-DNA complex. Nucleic Acids Res., *21:* 3335–3336, 1993.
36. van Wijnen, A. J., Ramsey-Ewing, A. L., Bortell, R., Owen, T. A., Lian, J. B., Stein, J. L., and Stein, G. S. Transcriptional element H4-site II of cell cycle regulated human H4 histone genes is a multipartite protein/DNA interaction site for factors HiNF-D, HiNF-M, and HiNF-P: involvement of phosphorylation. J. Cell. Biochem., *46:* 174–189, 1991.
37. Aslam, F., McCabe, L., Frenkel, B., van Wijnen, A. J., Stein, G. S., Lian, J. B., and Stein, J. L. AP-1 and vitamin D receptor (VDR) signaling pathways converge at the rat osteocalcin VDR element: requirement for the internal activating protein-1 site for vitamin D-mediated *trans*-activation. Endocrinology, *140:* 63–70, 1999.
38. Heinrichs, A. A. J., Banerjee, C., Bortell, R., Owen, T. A., Stein, J. L., Stein, G. S., and Lian, J. B. Identification and characterization of two proximal elements in the rat osteocalcin gene promoter that may confer species-specific regulation. J. Cell. Biochem., *53:* 240–250, 1993.
39. Javed, A., Gutierrez, S., van Wijnen, A. J., Stein, G. S., Stein, J. L., and Lian, J. B. Cbfa binding sites are required for hormonal responsiveness in the osteocalcin promoter. Bone (NY), *23:* S196, 1998.
40. Mina, M., Gluhak, J., Upholt, W. B., Kollar, E. J., and Rogers, B. Experimental analysis of *Msx-1* and *Msx-2* gene expression during chick mandibular morphogenesis. Developmental Dynamics, *202:* 195–214, 1995.
41. Liu, Y. H., Kundu, R., Wu, L., Luo, W., Ignelzi, M. A. Jr, Snead, M. L., and Maxson, R. E., Jr. Premature suture closure and ectopic cranial bone in mice expressing Msx2 transgenes in the developing skull. Proc. Natl. Acad. Sci. USA, *92:* 6137–6141, 1995.
42. Simeone, A., Acampora, D., Pannese, M., D'Esposito, M., Stornaiuolo, A., Gulisano, M., Mallamaci, A., Kastury, K., Druck, T., Huebner, K., and Boncinelli, E. Cloning and characterization of two members of the vertebrate Dlx gene family. Proc. Natl. Acad. Sci. USA, *91:* 2250–2254, 1994.
43. Ferrari, D., Sumoy, L., Gannon, J., Sun, H., Brown, A. M., Upholt, W. B., and Kosher, R. A. The expression pattern of the Distal-less homeobox-containing gene Dlx-5 in the developing chick limb bud suggests its involvement in apical ectodermal ridge activity, pattern formation, and cartilage differentiation. Mech. Dev., *52:* 257–264, 1995.
44. Blochlinger, K., Jan, L. Y., and Jan, Y. N. Postembryonic patterns of expression of cut, a locus regulating sensory organ identity in *Drosophila*. Development, *117:* 441–450, 1993.
45. Vanden Heuvel, G. B., Quaggin, S. E., and Igarashi, P. A unique variant of a homeobox gene related to *Drosophila* cut is expressed in mouse testis. Biol. Reprod., *55:* 731–739, 1996.
46. Vanden Heuvel, G. B., Bodmer, R., McConnell, K. R., Nagami, G. T., and Igarashi, P. Expression of a cut-related homeobox gene in developing and polycystic mouse kidney. Kidney Int., *50:* 453–461, 1996.
47. Quaggin, S. E., Heuvel, G. B. V., Golden, K., Bodmer, R., and Igarashi, P. Primary structure, neural-specific expression, and chromosomal localization of Cux-2, a second murine homeobox gene related to *Drosophila* cut. J. Biol. Chem., *271:* 22624–22634, 1996.
48. van Wijnen, A. J., Choi, T. K., Owen, T. A., Wright, K. L., Lian, J. B., Jaenisch, R., Stein, J. L., and Stein, G. S. Involvement of the cell cycle-regulated nuclear factor HiNF-D in cell growth control of a human H4 histone gene during hepatic development in transgenic mice. Proc. Natl. Acad. Sci. USA, *88:* 2573–2577, 1991.
49. Webster, M. A., Martin-Soudant, N., Nepveu, A., Cardiff, R. D., and Muller, W. J. The induction of uterine leiomyomas and mammary tumors in transgenic mice expressing polyomavirus (PyV) large T (LT) antigen is associated with the ability of PyV LT antigen to form specific complexes with retinoblastoma and CUTL1 family members. Oncogene, *16:* 1963–1972, 1998.
50. Wiggan, O., Taniguchi-Sidle, A., and Hamel, P. A. Interaction of the pRB-family proteins with factors containing paired-like homeodomains. Oncogene, *16:* 227–236, 1998.
51. van Wijnen, A. J., Lian, J. B., Stein, J. L., and Stein, G. S. Protein/DNA interactions involving ATF/AP1-, CCAAT-, and HiNF-D-related factors in the human H3-ST519 histone promoter: cross-competition with transcription regulatory sites in cell cycle controlled H4 and H1 histone genes. J. Cell. Biochem., *47:* 337–351, 1991.
52. Ginty, D. D., Bonni, A., and Greenberg, M. E. Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. Cell, *77:* 713–725, 1994.