0888-8809/03/$15.00/0
*Printed in U.S.A.*

Molecular Endocrinology 17(4):743–756

doi: 10.1210/me.2002-0122

# Transcriptional Induction of the Osteocalcin Gene During Osteoblast Differentiation Involves Acetylation of Histones H3 and H4

JIALI SHEN, HAYK HOVHANNISYAN, JANE B. LIAN, MARTIN A. MONTECINO, GARY S. STEIN, JANET L. STEIN, AND ANDRE J. VAN WIJNEN

*Department of Cell Biology and Cancer Center (J.S., H.H., J.B.L., G.S.S., J.L.S., A.J.V.), University of Massachusetts Medical School, Worcester, Massachusetts 01655-0106; and Departamento de Biologia Molecular (M.A.M.), Universidad de Concepcion, Casilla 160-C Concepcion, Chile*

**The remodeling of chromatin is required for tissue-specific gene activation to permit interactions of transcription factors and coregulators with their cognate elements. Here, we investigate the chromatin-mediated mechanisms by which the bone-specific osteocalcin (OC) gene is transcriptionally activated during cessation of cell growth in ROS 17/2.8 osteosarcoma cells and during normal osteoblast differentiation. Acetylation of histones H3 and H4 at the OC gene promoter was assayed during the proliferative and postproliferative stages of cell growth by using chromatin immunoprecipitation assays with antibodies that recognize different acetylated forms of histones H3 or H4. The results show that the promoter and coding regions of the OC gene contain very low levels of acetylated histones H3 and H4 during the proliferative period of osteoblast differentiation when the OC gene is inactive. Active expression of the OC gene in mature osteoblasts and confluent ROS 17/2.8 cells is functionally linked to preferential acetylation of histone H4 and, to a lesser extent, to acetylation of histone H3. Histone acetylation at the loci for RUNX2 (CBFA1), alkaline phosphatase, bone sialoprotein, osteopontin, and the cell growth regulator p21, which are expressed throughout osteoblast differentiation, is not altered postproliferatively. We conclude that acetylation of histones H3 and H4 is functionally coupled to the chromatin remodeling events that mediate the developmental induction of OC gene transcription in bone cells. (*Molecular Endocrinology* 17: 743–756, 2003)**

BONE FORMATION *in vivo* is regulated by osteogenic factors that mediate the staged expression of bone phenotypic genes during differentiation of precommitted osteoprogenitor cells to mature osteoblasts. At each stage of osteoblast differentiation, a subset of genes is transcriptionally activated to accommodate developmental stage-specific physiological requirements for bone-specific gene products (1, 2). This activation requires the conversion of transcriptionally inactive chromatin to transcriptionally competent chromatin in which regulatory sequences of bone-related genes have been rendered accessible to the cognate transcription factors (3, 4). Indeed, recent studies have shown that transcriptional control of osteogenesis is intricately associated with modifications in the chromatin organization of the bone-specific osteocalcin (OC) gene, which represents a key paradigm for understanding the molecular mechanisms of bone cell differentiation (5–10).

Covalent modifications of core (H2A, H2B, H3, and H4) and linker (H1) histone proteins (*e.g*. acetylation, methylation, phosphorylation, and ubiquitination) are known to influence the higher order organization of chromatin (11–14). Furthermore, in recent years it has become apparent that many coactivators and corepressors that interface with sequence-specific transcription factors and catalytic components of gene transcription represent chromatin-modifying enzymes with the capability to acetylate or deacetylate the N termini of histones H3 and H4 (14). The lysine-rich N termini of H3 and H4 are thought to protrude from the histone octamer and are reversibly acetylated during gene activation and repression (11–15). The functional coupling between histone modifications, chromatin remodeling, and transcription has been extensively studied in yeast cells and a limited number of mammalian cell types (*e.g*. Refs. 16–21; reviewed in Ref. 14). There is a paucity of experimental data for osteogenic cells, which represent specialized mesenchymal cells that develop late during gestation in mammals and have the ability to mineralize their extracellular matrix. Bone cell differentiation reflects a relatively recent adaptation during vertebrate evolution. Establishing whether chromatin remodeling proceeds by the same molecular mechanisms during osteogenic development as observed in other mammalian cell types or yeast will provide an important test for the universality of the chromatin remodeling principles that have emerged to date.

Abbreviations: AP, Alkaline phosphatase; BSP, bone sialoprotein; HA, hemagglutinin; HDAC, histone deacetylase; His4, rat histone 4 gene; OC, osteocalcin; OP, osteopontin; P/CAF, p300/CBP-associated factor; ROB, rat calvarial osteoblast; SDS, sodium dodecyl sulfate; SSC, NaCl-sodium citrate; SWI/SNF, switch/sucrose nonfermenting; VD3, 1,25-dihydroxyvitamin $D_3$.

Modifications in higher order chromatin structure and nucleosomal organization accompany activation of OC gene transcription during osteoblast differentiation (3–9). Our current focus is to define the mechanisms that mediate chromatin remodeling of the OC gene during bone cell differentiation. The promoter of the bone-specific OC gene contains a proximal region (−0.2–0.0 kbp) that is responsible for tissue-specific basal levels of transcription and a distal enhancer region (−0.6 to −0.4 kbp) that contains a 1,25-dihydroxyvitamin $D_3$ (VD3)-responsive element (reviewed in Refs. 3 and 4). These proximal and distal promoter regions correspond with two nuclease-sensitive domains that are present in chromatin only in cells that actively transcribe the OC gene (5, 6). The region between the proximal and distal promoter is associated with a positioned nucleosome (5–7, 9). The localized nucleosome reduces the distance between proximal and distal gene-regulatory sequences and may promote protein-protein interactions between tissue-specific basal transcription factors and vitamin D receptor-retinoic X receptor containing activator complexes at the distal VD3-responsive element (reviewed in Refs. 3 and 4). Hence, the active chromatin conformation of the OC gene is optimized for the molecular integration of cell signaling events responding to developmental, tissue-specific, and/or steroid hormone-dependent regulatory cues.

One key question that emerges from our previous studies is what are the biochemical events that mediate the formation of active chromatin at the OC gene? We have shown that the osteogenic transcription factor RUNX2/CBFA1 interacts with three distinct sites in the OC promoter that are located near the boundaries of the two nuclease-sensitive domains (5, 9, 22). Ablation of these RUNX2 sites abolishes nuclease sensitivity and VD3 responsiveness (8). Furthermore, RUNX2 interacts with a series of transcriptional coregulators, including Groucho/transducin-like enhancer (TLE; Ref. 23), yes-associated protein (24, 25), and Smad (26, 27) that, together with other factors, may form multimeric complexes exhibiting histone acetyltransferase or deacetylase activities (28). Based on these data, it appears that RUNX2 may mediate bone-specific activation of OC gene expression by supporting posttranslational modifications of histones H3 and H4.

We have previously shown that acetylation of histones H4 and H3 is coupled to the vitamin D-dependent transcription of the OC gene in two osteosarcoma cell culture models (ROS17/2.8 and ROS 24/1) that differ in their ability to express bone-specific markers (10). Both osteosarcoma cell types exhibit deregulation of normal cell growth and differentiation interrelationships and cannot provide insight into events that occur during normal osteoblast phenotype development. Therefore, in this study we used chromatin immunoprecipitation assays to assess the functional coupling between histone acetylation and developmental activation of OC gene expression during cessation of cell growth in osteosarcoma cells and during differentiation of normal diploid primary osteoblasts. Here we demonstrate that acetylation of histone H4 and, to a lesser extent, acetylation of histone H3 at the OC gene promoter accompany the developmental induction of OC gene expression in mature osteoblasts. These histone modifications may support the temporal changes in higher order chromatin structure that are required for bone-specific gene activation during osteoblast phenotype development.

## RESULTS

### Elevated Levels of Acetylated Histones H4 and H3 Accompany Enhanced Expression of the OC Gene in Confluent ROS17/2.8 Cells

The OC gene is a well established bone-specific marker and its expression is phenotypic for late stages of osteoblast maturation. The OC gene is expressed in ROS17/2.8 osteosarcoma cells that exhibit loss of stringent cell growth control and are not capable *in vitro* of differentiating into osteoblasts with a mineralized extracellular matrix (29). However, consistent with previous data from our laboratory (30, 31), OC expression is up-regulated in ROS17/2.8 cells concomitant with down-regulation of cell growth as cells become confluent and more densely packed at late stages of the culturing period (data not shown). Increased OC gene expression is reflected by increased accumulation of OC mRNA and secretion of OC in the culture media. The cell cycle-regulated rat histone 4 gene (His4) is expressed reciprocally with OC (30) and is down-regulated when cells become confluent (data not shown). We used this biological model to assess histone acetylation at the OC locus when the tissue-specific basal level of OC gene transcription is selectively increased during cell density-dependent inhibition of cell growth.

Chromatin immunoprecipitations were performed with proliferating and confluent ROS 17/2.8 cells using antibodies against acetylated histones (*i.e.* H3-Ac; H4-Ac-1 and H4-Ac-2). The immunoprecipitates were analyzed by PCR using primers spanning the OC locus −1377 and +466 (Fig. 1), and the PCR products were separated by agarose gel electrophoresis (Fig. 2). We find that one of the acetylated histone H4 antibodies (*i.e.* H4-Ac-1) precipitates more DNA from the OC promoter when ROS 17/2.8 cells become confluent (Fig. 2), whereas comparable amounts of the His4 gene are precipitated. These data indicate that the increased expression of OC is reflected by increased acetylation of histone H4 at the OC locus. Strikingly, another acetylated histone H4 antibody (*i.e.* H4-Ac-2) does not show a difference in precipitation of OC gene-derived fragments, but instead reveals a decrease in the signal of DNA derived from the His4 locus (Fig. 2A). These results indicate that the antibodies recognize different types of histone H4 acetylation and detect locus-specific changes in chromatin.

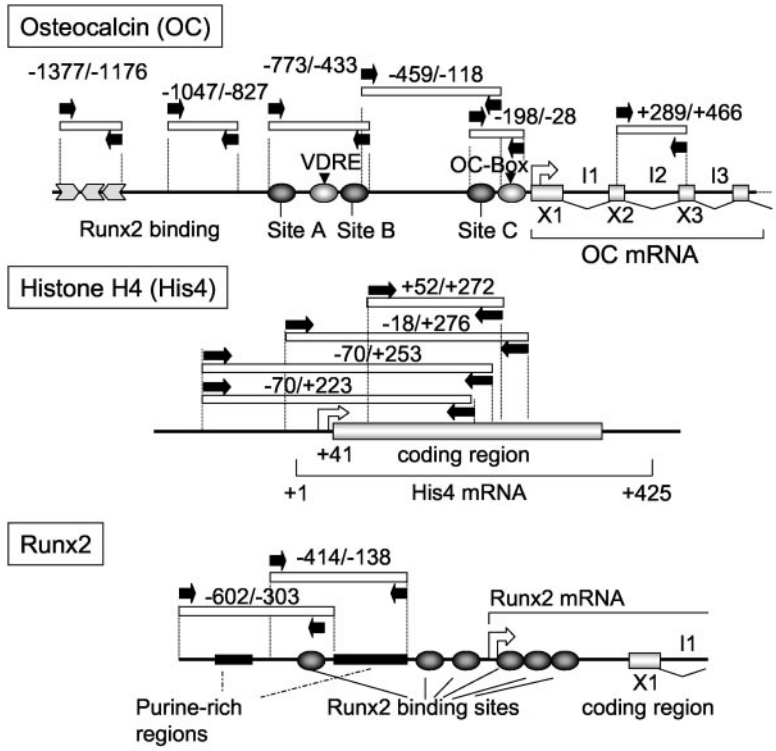


**Fig. 1.** Primers Used for Chromatin Immunoprecipitation Assays

PCR primer pairs used for the detection of DNA segments of the rat genes for OC (*top*), His4 (*middle*), and Runx2 (*bottom*) are schematically illustrated. The *top panel* shows the PCR primer pairs for the OC locus *above* a diagram that depicts gene-regulatory elements (*filled* and *open ovals*) B1/B2 repetitive sequences (*chevrons*), exons (X; *boxes*) and introns (I). The *middle panel* shows the PCR primer pairs for the His4 gene. His4 primer pair +52/+272 was used with OC primer pairs −773/−433 and −459/−118; His4 −18/+272 with OC −1377/−1176, −198/−28, and +289/+466; His4 −70/+223 with OC −1047/−827. The *bottom panel* shows the PCR primer pairs used for detection of sequences derived from the Runx2 locus. The Runx2 primers were used in combination with the His4 −70/+253 primer pair.

The acetylated histone H3 antibody used in our studies (*i.e.* H3-Ac) efficiently precipitates fragments from the His4 locus and OC fragments between −459 to +466 but detects only low levels of OC gene-derived segments upstream of −459. The amount of His4- or OC-derived DNA upstream from −28 that is precipitated with the H3-Ac antibody remains essentially similar in both proliferating and confluent ROS 17/2.8 cells (Fig. 2). We note that there is a clear change in H3 acetylation within the coding region (+289/+466), but the physiological relevance of this change is unclear at present. The nonspecific antibody [against the hemagglutinin (HA)-epitope] does not precipitate appreciable amounts of DNA. Taken together, results presented in Fig. 2 suggest that 1) active expression of the OC gene is functionally linked to preferential acetylation of histone H4 and, to a lesser extent, to acetylation of H3, and 2) that postproliferative up-regulation of OC gene expression in ROS 17/2.8 cells involves increased acetylation of histone H4.

To address whether changes in histone H4 acetylation at the OC locus reflect global changes in histone acetylation during cell growth inhibition, we assessed levels of acetylated histone H3, as well as a panel of histone-modifying enzymes by Western blot analysis (Fig. 3). The results show that the levels of acetylated histone H3, the acetyltransferases p300 and p300/CBP-associated factor (P/CAF), as well as the histone deacetylase HDAC1 are similar in ROS 17/2.8 cells grown at different densities (Fig. 3A and data not shown). Similarly, the levels of the cyclin-dependent kinase 2 and lamin B are comparable in all samples tested. These findings, combined with the data presented in Fig. 2, indicate that increased histone acetylation at the OC locus in confluent osteosarcoma cells may reflect a gene-specific alteration in the post-translational modification of nucleosomes, rather than an overall cellular response.

Strikingly, we observed a major decline in the levels of phosphorylated histone H3 as the proliferative potential of ROS 17/2.8 cells decreases in more confluent cultures (Fig. 3). This result indicates a correlation between osteosarcoma cell proliferation and histone H3 phosphorylation and is consistent with mitosis-related phosphorylation of histone H3 (32–34). The decrease in H3 phosphorylation parallels the loss of mitotic ROS 17/2.8 cells as they cease division due to contact inhibition of cell growth.

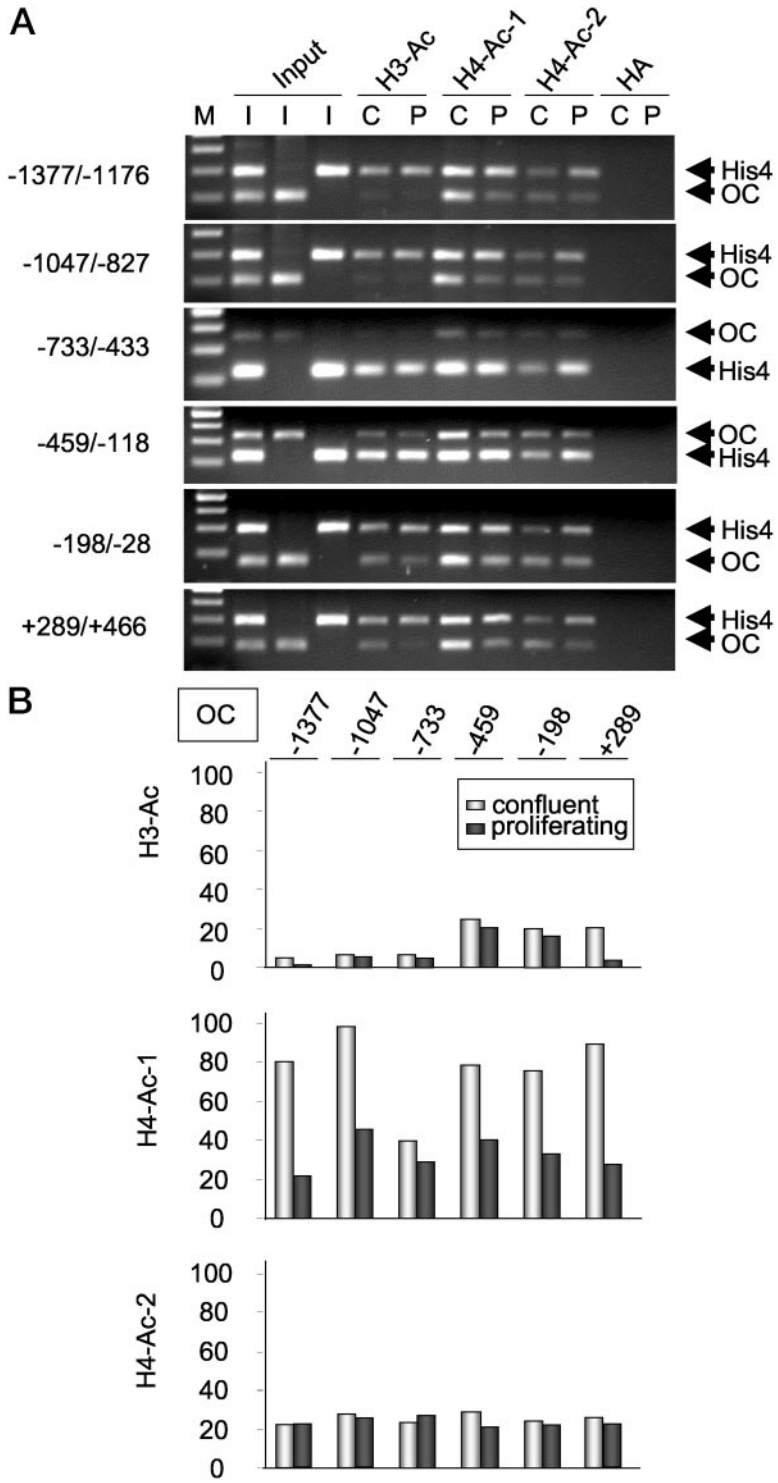


**Fig. 2.** Increased Acetylation of Histones H4 and H3 in Postproliferative ROS 17/2.8 Cells

Immunoprecipitation assays were performed with formaldehyde cross-linked chromatin isolated from confluent (C) or proliferating (P) ROS 17/2.8 cells using antibodies against acetylated histones H3 or H4. A, Ethidium bromide-stained agarose gels of PCR products obtained with OC primer pairs shown in Fig. 2, in chromatin immunoprecipitates with the indicated antiacetylated histone antibodies (H3-Ac, H4-Ac-1, H4-Ac-2), or an unrelated antibody (HA). Input DNAs (I) were used for PCR with the His4 and/or OC primer pair to show the relative locations of the PCR products (indicated by *arrowheads*). Lane M contains a 100-bp repeat ladder. B, Quantitation of signal intensities of the PCR products from representative experiments that were digitally recorded with a charge-coupled device camera and analyzed using Image J 1.22d (http://rsb.info.nih.gov/ij/). For each lane, the values were expressed as relative densitometric units. The data are taken from a representative experiment, and similar results were obtained in three independent experiments.

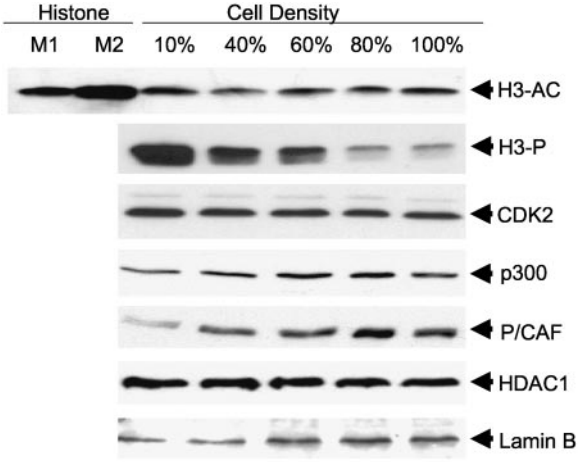


**Fig. 3.** Western Blot Analysis of Nuclear Proteins During Growth Inhibition of ROS 17/2.8 Cells

Protein blots were prepared with cell lysates from ROS 17/2.8 cells harvested at different densities and analyzed using antibodies against acetylated histone H3 (H3-Ac), phosphorylated H3 (H3-P), cyclin-dependent kinase 2, p300, P/CAF, HDAC1, or lamin B. The *left two lanes* show purified histone proteins (M1; Sigma-Aldrich Corp., St. Louis, MO) or histones from butyrate-treated ROS 17/2.8 cells (M2).

### Acetylation of Histones H3 and H4 Accompanies Induction of OC Gene Expression During Osteoblast Maturation

OC gene expression is induced by transcriptional mechanisms when committed osteoblast precursors differentiate into mature osteoblasts embedded in a mineralized extracellular matrix (1, 6, 29). To investigate whether the levels of acetylated histones H3 and H4 at the OC locus are modulated during osteoblast differentiation, we harvested rat calvarial osteoblasts (ROB cells) at different stages of bone cell maturation. We first validated the biological parameters of the culture system (Fig. 4). Functional expression of the OC gene at d 11, but not d 7, was evidenced by RIA with an OC-specific antibody that monitors OC levels in the culture medium (Fig. 4A) and Northern blot analysis (data not shown). The RIA results show that OC synthesis is not detected in proliferating ROB cells (d 7; Fig. 4A). Low levels of soluble OC are detected at d 11, and OC is actively secreted in differentiated ROB cells (d 16; Fig. 4A). Thus, under our experimental conditions the OC gene is inactive at d 7 and OC gene expression is initiated at d 11.

Osteoblast maturation involves significant changes in gene expression profiles (1), and these changes require major alterations in chromatin organization as proliferation-specific genes are silenced and bone-specific genes are up-regulated. Therefore, we analyzed the cellular levels of histone acetylation and histone-modifying enzymes. We find that the levels of P/CAF, p300, and lamin B each exhibit subtle decreases during osteoblast differentiation (Fig. 4B). More importantly, the acetylation of histones H3 and

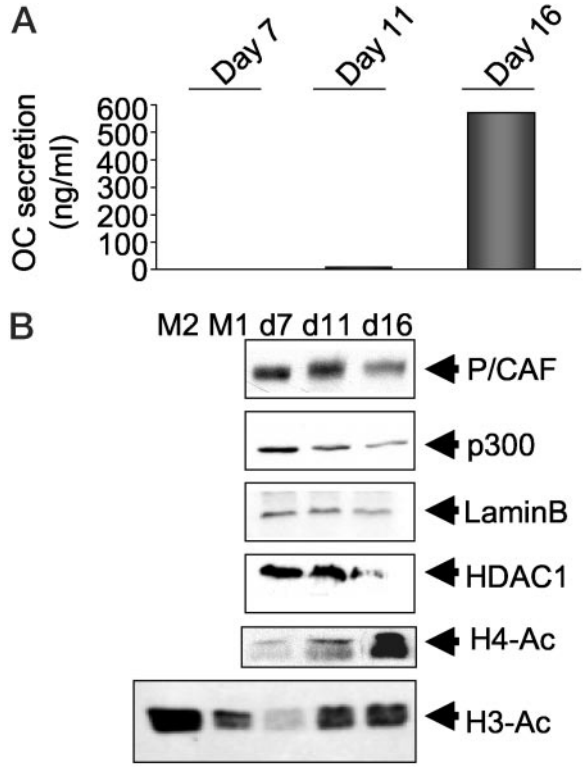


**Fig. 4.** Induction of OC Gene Expression During Rat Osteoblast Differentiation

A, OC secretion (ng/ml) as measured by RIA using samples of the culture media that were collected at the indicated days of the culture period. B, Western blot analyses with cell lysates from proliferating (d 7) or differentiated (d 11, d 16) osteoblasts using antibodies against acetylated histones H4 and H3, P/CAF, p300, and HDAC. The antibody against lamin B was used as a control for protein loading. The *left two lanes* in panel B show histones isolated from butyrate-treated ROS17/2.8 cells (M2) and purified histone proteins (M1).

H4 is up-regulated postproliferatively, whereas the histone deacetylase HDAC1 is decreased (Fig. 4B). Hence, there are limited modulations in the cellular levels of these chromatin-related proteins during progression of osteoblast maturation.

To address OC gene-specific changes in posttranslational modifications of chromatin during bone cell differentiation, we performed immunoprecipitation assays with chromatin isolated from immature (d 7) and differentiated (d 11) osteoblasts (Fig. 5). Genomic DNA associated with acetylated histones was precipitated with the H3-Ac, H4-Ac-1, and H4-Ac-2 antibodies and analyzed by PCR using primers specific for various segments of the OC and His4 genes. The results show that OC-derived fragments are either below the level of detection or present at very low levels (*e.g*. the −459/−118 region) compared with His4 fragments in samples from proliferating cells (Fig. 5A). Thus, there is minimal presence of acetylated histones H3 and H4 at the OC gene in proliferating (d 7) osteoblasts. Strikingly, high levels of histone acetylation are observed

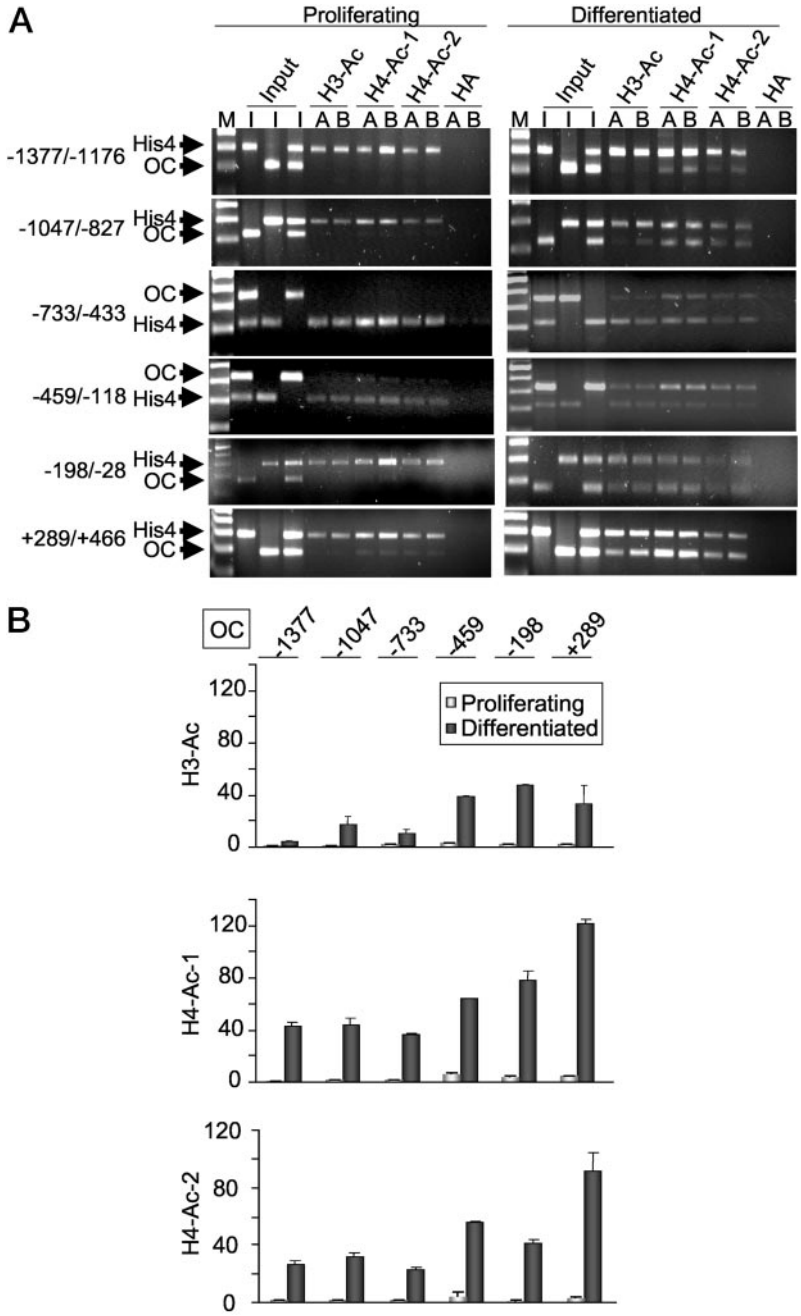


**Fig. 5.** Increased Acetylation of Histones H3 and H4 at the OC Gene Locus during Bone Cell Maturation

Chromatin immunoprecipitation assays were performed with antibodies against acetylated histones using chromatin from proliferating (*left panel*) or differentiated osteoblasts cells (*right panel*) as described in Fig. 3. Each panel shows ethidium bromide-stained agarose gels of PCR products obtained with different OC and His4 primer pairs (see Fig. 2) from immunoprecipitates. The *left lane* in each gel represents a 100-bp repeat DNA marker (M). The *letters A* and *B above* the *lanes* refer to two distinct biological samples used in our analysis (A = control; B = treatment with VD3 for 24 h). B, Densitometric analyses, which quantitate the levels of acetylated histones H3 and H4 in differentiated osteoblasts (d 11; *dark column*) as compared with proliferating osteoblasts (d 7; *light column*). The quantitation of signal intensities of the PCR products was performed as described in Fig. 2, and data are based on values from one of three very similar experiments.

across the OC locus in differentiated (d 11) osteoblasts (Fig. 5A). Quantitation of the chromatin immunoprecipitation assay results from a representative experiment reveals that there are significant differences in the relative levels of acetylated histones H3 and H4 at the proximal segments of the OC gene in differentiating compared with proliferating cells (Fig. 5B). Three independent chromatin imunoprecipitation experiments yielded similar quantitative changes. Our experiments were performed under two biological condi-

tions (A = normal culture conditions; B = cultures supplemented for 24 h with VD3), but unlike previous results that were obtained with ROS17/2.8 cells (10), we did not observe reproducible changes in acetylation of histone H3 or H4 in response to VD3 at the OC locus in these normal diploid osteoblasts. Thus, the developmental modifications in histone acetylation that we observe during differentiation of normal diploid osteoblasts occur irrespective of the presence of VD3 (see *Discussion*).

To assess whether changes in histone acetylation occur at other bone-related genes, we first analyzed histone acetylation at the promoter of the RUNX2 gene, which encodes an osteogenic transcription factor that is expressed in both proliferative and nonproliferative stages of bone cell differentiation (Fig. 6). The results show that the RUNX2 locus is associated with acetylated histones in both proliferating and mature osteoblasts. Furthermore, levels of histone acetylation are comparable during osteoblast differentiation. Thus, constitutive expression of the RUNX2 gene throughout the osteoblast developmental sequence is reflected by the persistent presence of acetylated histones H3 and H4 at the RUNX2 locus.

### Histone Modifications at Osteoblast-Related Genes During Bone Cell Differentiation Are Locus Specific

Several bone-related markers including alkaline phosphatase (AP), bone sialoprotein (BSP), osteopontin (OP), and p21 are known to be expressed in proliferating osteoblasts, and expression of these genes is further modulated from these basal levels in osteoblasts during differentiation. Figure 7A shows a representative Northern blot that exemplifies developmental modulations of preexisting basal levels of expression for the AP and OP genes. In contrast, the OC gene exhibits a developmental induction from levels that are well below background under our experimental conditions (Fig. 7A). Cells were harvested after treatments with or without VD3 for 45 min. The total cellular levels of histone acetylation are elevated in the presence of VD3 (10), but modulations in the mRNA levels of AP, OC, and OP are not apparent after short-term treatment with VD3 except for a modest increase in OC at a late stage of osteoblast differentiation (d 20; Fig. 7A).

We correlated the level of histone acetylation at the loci for AP, OP, BSP, and p21 with the stage of osteoblast differentiation by performing chromatin immunoprecipitations with cells harvested during the proliferative period (d 3) or the differentiated period (d 12). Chromatin immunoprecipitation analyses were performed with one or two primer pairs for each gene (Figs. 7B and 8). Figure 8A shows chromatin immunoprecipitation results for samples in which the OP and p21 genes were analyzed in parallel with the His4 gene within the same PCR. Figure 8B presents data for the AP and BSP genes that were performed in the absence of the His4 primers because they interfere with detection of AP- or BSP-related signals. The OC gene is included in both experiments to facilitate comparison of results presented in Fig. 8, A and B. We find that the genes for OP and p21 are each associated with acetylated histone H3 and H4 in proliferating cells and exhibit elevated association with acetylated histones in differentiated cells (Fig. 8A). In contrast, histone acetylation at the AP and BSP loci remains unchanged. Furthermore, we did not observe reproducible VD3-dependent modifications in histone acetylation for any of the genes. We conclude that acetylation of histones associated with bone-related promoters is functionally coupled to activation and enhancement of gene expression during osteoblast maturation.

## DISCUSSION

The main finding of our study is that acetylation of histones H3 and H4 at the OC locus is dramatically increased when OC gene transcription is induced postproliferatively in osteosarcoma cells and during differentiation of calvaria-derived osteoblasts. These results provide a mechanistic link to the increased nuclease sensitivity of chromatin structure at the OC promoter during osteoblast differentiation, as reflected by enhanced cleavage by deoxyribonuclease I, micrococcal nuclease, and restriction enzymes that we observed in previous studies (5–7). Taken together, our data constitute compelling experimental evidence that chromatin-related mechanisms mediate the developmental regulation of the bone-specific OC gene in osteoblasts. Thus, bone cells activate osteoblast-related genes by selectively remodeling chromatin in gene-regulatory regions and by acetylating histones H4 and H3. We propose that increased histone acetylation marks the transition from a transcriptionally silent and closed state in immature bone cells to an active open chromatin conformation in mature osteoblasts. Consistent with this concept, active expression of other bone-related genes we have analyzed (*i.e.* RUNX2, AP, OP, BSP, and p21) is linked to association with acetylated histones.

Upon establishment of basal tissue-specific levels of transcription in an open chromatin context, the OC gene is rendered competent for VD3-dependent enhancement of transcription. We have previously shown that VD3 enhancement of OC gene transcription in ROS17/2.8 osteosarcoma cells consistently occurs concomitant with increased acetylation of histones H3 and H4 (10). This VD3-responsive acetylation of histones associated with the OC gene in osteosarcoma cells is time dependent, and the effect increases steadily until at least 24 h after treatment. In the current study, we show that neither the OC gene nor other osteoblast-related genes exhibit reproducible VD3-dependent changes in histone acetylation status in the

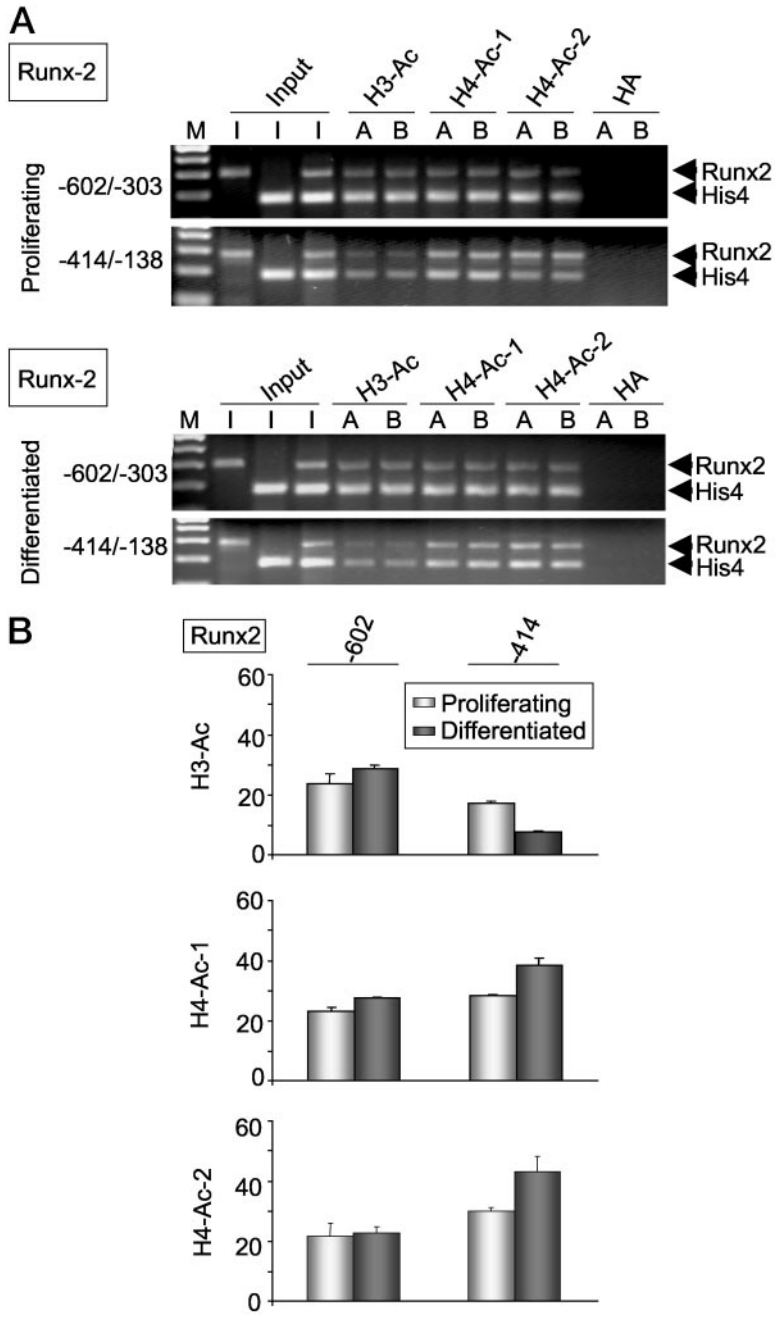


**Fig. 6.** Acetylated Histones H3 and H4 Are Present at the Runx2 Locus in Both Proliferating and Differentiated Osteoblasts

PCR reactions were performed with samples from chromatin immunoprecipitation assays using proliferating and differentiated osteoblasts as described in Fig. 5. A, PCR products from proliferating osteoblasts (*top panels*) or differentiated osteoblasts (*bottom panels*) using different Runx2 primer pairs (see Fig. 1) as revealed by ethidium bromide staining of 2% agarose gels. The *arrowheads* indicate the locations of the Runx2 and His4-derived DNA fragments. B, Quantitation of the data presented as described in Fig. 2. Values for proliferating (*light column*) and differentiated (*dark column*) osteoblasts are expressed as relative densitometric units and are based on one of three representative experiments.

proliferative and differentiated stages of normal diploid osteoblast. We do not yet understand the basis for the differences in the histone acetylation response after vitamin $D_3$ treatment in normal diploid osteoblasts and osteosarcoma cells, which we have observed in parallel with the same set of reagents. One possible explanation is that VD3-dependent changes in histone acetylation occur only at a stage of normal osteoblast differentiation much later than we examined here. However, there are technical barriers that preclude performing chromatin immunoprecipitations with cells from heavily mineralized osteoblast cultures (*e.g.*

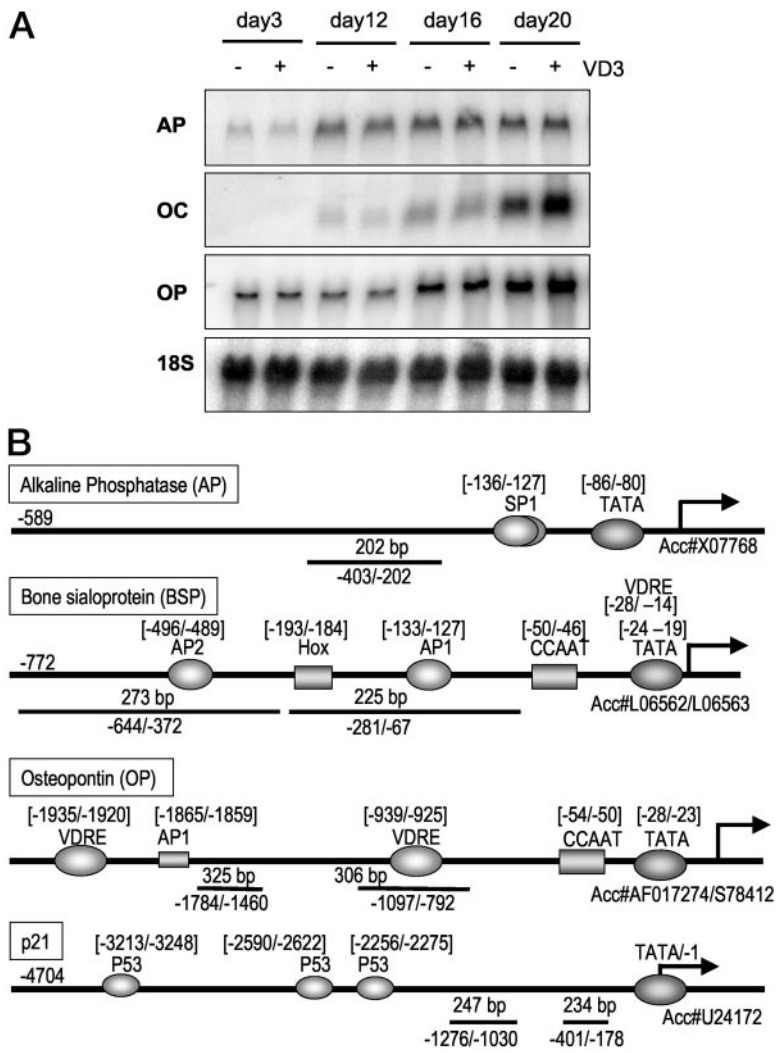


**Fig. 7.** Selective Induction of OC Gene Expression Relative to Representative Bone-Related Marker Genes during Normal Osteoblast Differentiation

A, Northern blot analysis was performed with RNA samples harvested at the indicated days of the osteoblast culturing period after a 45-min treatment with or without VD3. RNA signals for OC are below the level of detection during the proliferative phase (d 3) of osteoblast differentiation, whereas other bone-related genes are already expressed at detectable levels at this stage. B, Diagram showing the regulatory regions from bone-related loci that are amplified by the PCR primers used in our study. Also indicated are the approximate locations of regulatory elements (*ovals and boxes*) and the accession numbers for each gene.

formaldehyde cross-linking of cells and nuclei to the dense extracellular matrix). Furthermore, antibodies against acetylated histones H3 and H4 are sensitive to other posttranslational modifications and/or epitope masking by different promoter-associated cofactors. While our current data do not yet permit us to favor these or alternative interpretations with respect to VD3-dependent mechanism of chromatin modifications, it is clear that gene-selective changes in the acetylation of histones H4 and H3 accompany the developmental induction of OC gene transcription during osteoblast differentiation.

During differentiation of erythrocytes, it has been shown that histones H3 and H4 are dynamically acetylated at the $\beta$-globin gene locus (35). Histones H3 and H4 are rapidly converted into mono- and diacetylated isoforms, which are slowly deacetylated near transcriptionally competent genes. In contrast, transcriptionally active genes are associated with labile hyperacetylated histone isoforms (35). Consistent with this concept, our data show that acetylation of histones H3 and H4 at the OC promoter accompanies the onset of transcriptional competency and activity during osteoblast maturation. Spencer and Davie (35) have proposed that the reversible acetylation of the globin gene locus may involve structural association with histone acetyl transferases and deacetylases, which are integral components of the nuclear matrix subnuclear compartment. Similarly, we have obtained evidence suggesting that the OC gene may be transiently tethered to the nuclear matrix through interactions of RUNX2 (also referred to as NMP2) and histone-modifying cofactors (3,

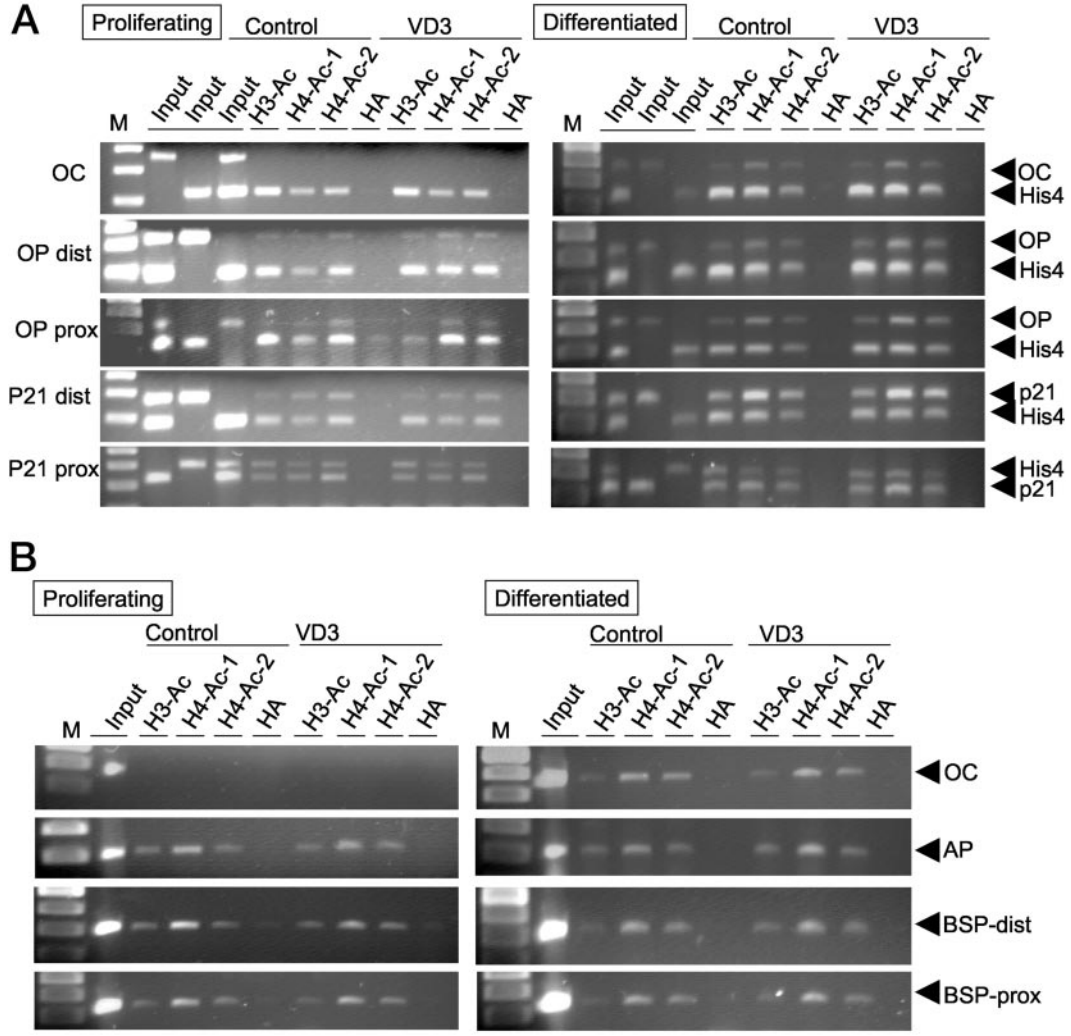


**Fig. 8.** Acetylated Histone H3 and H4 Are Associated with the Promoters of Genes Expressed during Bone Cell Growth and Differentiation

Chromatin immunoprecipitation assays were performed with antibodies against acetylated histones in proliferating (*left portion*) and differentiated osteoblasts (*right portion*). A, Ethidium bromide-stained agarose gels were used to detect PCR products generated with primers spanning the distal (OP-dist, −1784/−1460) or proximal (OP-prox, −1097/−792) promoter of the OP gene, the distal promoter (p21-dist, −1276/−1030) or proximal (p21-prox, −401/−178) promoter of the p21 gene, and primer pairs spanning the rat His4 gene, which were used in the same PCR reactions. B, The ethidium bromide-stained agarose gels detect PCR products amplified with primer pairs spanning the AP gene, or the distal (BSP-dist, −644/−372) or proximal (BSP-prox, −281/−67) promoter regions of the BSP gene. Primer pairs for the rat His4 gene interfere with detection of the AP and BSP genes and were not included in the same PCRs in these experiments. Samples in panels A and B were also analyzed with primer pairs against a representative segment of the OC gene (−459 to −118) to permit a direct comparison of results.

28, 36). The macromolecular assembly of complexes containing bone-specific transcription factors and chromatin remodeling complexes at specific locations within the nucleus may represent a key rate-limiting step for transcriptional control *in situ*.

Bone-specific remodeling of the OC gene critically depends on the activity of the osteoblast lineage-specific factor RUNX2 (8). RUNX is capable of interacting with, and thus presumably mediating, promoter recruitment of several cofactors capable of modifying chromatin, including HDACs, histone acetyltransferases, and switch/sucrose nonfermenting (SWI/SNF) components (28). Chromatin remodeling also accompanies promoter activation of tissue-specific genes during both adipocyte and myogenic differentiation (37, 38). One principal regulator of lineage commitment in adipocytes is the transcription factor CCAAT enhancer binding protein-$\alpha$, which interacts with the SWI/SNF chromatin remodeling complex (37). Mammalian SWI/SNF complexes also cooperate with the transcription factor myoblast differentiation (MyoD) to mediate myoblast differentiation (38). Based on genetic evidence, the common denominator for CCAAT enhancer binding protein-$\alpha$, myoblast differentiation

(MyoD), and RUNX2 is that all three factors are essential for lineage commitment. The emerging concept in the field is that factors genetically rate limiting for phenotypic maturation are essential for chromatin remodeling during mammalian development. Future studies therefore should focus on the role of RUNX2 and its cofactors in histone acetylation and chromatin remodeling in the activation of bone-specific genes during osteoblast differentiation. Our working model is that the developmental modifications in histone H3 and H4 acetylation we observed in this study may represent a key component of a RUNX2-dependent mechanism that renders the proximal basal promoter and the distal VD3-responsive enhancer domain of the OC gene competent for dynamic protein-DNA and protein-protein interactions that support physiological responsiveness.

## MATERIALS AND METHODS

### Cell Culture

ROS17/2.8 cells were cultured in F-12 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% fetal calf serum. Cells were initially plated at a density of $0.2 \times 10^6$ cells per 100-mm dish and harvested at d 3 as actively proliferating cells (~ 20–30% confluent). Cells were also seeded at a density of $0.5 \times 10^6$ and harvested at d 6 as confluent cells or at d 11 as tightly packed and partially multilayered cells. The culture medium of each sample was collected for RIA with an OC antibody to measure secreted OC levels.

Normal osteoblasts (ROB cells) were isolated from calvaria of fetal rats at d 21 of gestation by sequential digestion for 20, 40, and 90 min at 37 C in 2 mg/ml collagenase P (Roche Molecular Biochemicals, Indianapolis, IN) and 0.25% trysin (Life Technologies, Inc.). Cells released in the initial digests were discarded, and those released from the third digestion were plated at a density of $4 \times 10^5$ cells/100-mm dish. Cells were fed every 2 d with MEM (Life Technologies, Inc.) supplemented with 25 $\mu$g/ml ascorbic acid. All subsequent feedings used medium supplemented with 10% fetal calf serum, 50 $\mu$g/ml ascorbic acid, and 10 mM $\beta$-glycerol phosphate. Culture conditions for differentiation and mineralization were reported previously (39, 40). The culture medium was analyzed by RIA to monitor the expected increase in OC levels during progressive stages of osteoblast differentiation (*i.e.* the early proliferation period and the matrix maturation stage when nodules start to form that eventually become mineralized).

### Northern Blot Analysis

Total cellular RNA was extracted with Trizol (Life Technologies, Inc.) according to the instructions of the manufacturer. ROB cells were harvested at different days of the developmental sequence (*e.g.* d 3, d 7, d 11, and d 16) and resuspended in Trizol solution to extract the RNA. RNA was resuspended in diethylpyrocarbonate-treated water. Total RNA (10–15 $\mu$g) was separated by electrophoresis in 1% agarose-17.6% formaldehyde gels and transferred overnight onto a Zetaprobe membrane (Bio-Rad Laboratories, Inc., Hercules, CA) by capillary action using 10× SSC buffer (3 M NaCl, 0.3 M sodium citrate). The RNA was cross-linked to the filter by UV irradiation for 20 sec using a UV Crosslinker (Spectronics Corp., Westbury, NY). The blots were prehybridized in 5× SSC, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 10 $\mu$g/ml sonicated salmon sperm DNA for 5 h at 65 C. Probes specific for the OC gene were heat denatured at $10^7$ cpm/ml radiolabeled cDNA generated by random priming with $^{32}$P-dCTP (30,000 Ci/mmol, NEN Life Science Products, Boston, MA) and added to the prehybridization buffer for hybridization overnight at 65 C. Other bone-specific markers were detected by overnight hybridization with $^{32}$P-labeled DNA probes for rat AP (*Eco*RI fragment), rat OP (*Eco*RI fragment), rat OC (*Eco*RI-*Bam*HI fragment), and the human 18S ribosomal gene at 65 C. RNA blots were washed in buffers of progressive stringencies starting at 2× SSC (0.1% SDS), followed by 1× SSC and a final rinse in 0.1× SSC (0.1% SDS) at 65 C. Blots were subjected to autoradiographic exposure overnight at −70 C or analyzed by using a STORM PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

### Antibodies

Chromatin immunoprecipitation assays were performed with antibodies recognizing acetylated histones. The antiacetylated histone H4 antibody, which we refer to as H4-AC-1 (Upstate Biotechnology, Inc., Lake Placid, NY; catalog no. 06866) is a rabbit antiserum containing a polyclonal IgG directed against a tetraacetylated peptide (AGG [K*] GG [K*] GMG [K*] VGA [K*] RHSC; K* is acetylated lysine) that spans the highly conserved residues 1–19 of His4 (41). The antiacetylated histone H3 antibody (H3-Ac) (Upstate Biotechnology, Inc., catalog no. 06599) is a protein A-purified rabbit polyclonal IgG that is directed against the diacetylated peptide (ARTKQTAR [K*] STGG [K*] APRKQLC), which spans the highly conserved N-terminal residues 1–20 of histone H3. Phosphorylation of Ser 10 has been reported to decrease the affinity of this antibody for diacetylated histone H3 (42). The antibody that recognizes hyperacetylated histone H4 (H4-Ac-2; Upstate Biotechnology, Inc., catalog no. 06946) is a rabbit antiserum containing a polyclonal IgG directed against a synthetic substrate related to residues 2–19 of tetrahymena histone H2A (43, 44). Western blot analyses were performed with the same antibodies described above, as well as with an antibody recognizing phosphorylated histone H3 or acetylated histone H4. These antibodies are protein A-purified rabbit polyclonal IgG directed against a peptide phosphorylated on serine 10 and spanning amino acids 7–20 of histone H3 (45, 46) or acerylated histone H4.

### Western Blot Analysis

Whole-cell lysates were prepared from ROS17/2.8 and ROB cells by washing monolayers in ice-cold PBS and resuspending cells in SDS-lysis buffer (0.5% SDS; 50 mM Tris-Cl, pH 8.0; and 1 mM dithiothreitol). Cell pellets were boiled and the lysates subjected to SDS-PAGE in 18% (40:1) polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes using a semidry electroblotter. After transfer, nonspecific antibody binding sites were blocked with 5% nonfat milk powder in PBS (8.1 mM $Na_2HPO_4$; 1.9 mM $NaH_2PO_4$; 0.137 M NaCl; and 2.7 mM KCl, pH 7.4) containing 0.1% Tween 20 for 1 h at 4 C. Primary antibodies (as indicated in the figure legends) were added in PBS containing 0.1% Tween 20 buffer at a 1:10,000 dilution, and the membrane was incubated overnight at 4 C. Bound antibody was detected using a second antirabbit Ig coupled to horseradish peroxidase at a 1:10,000 dilution, and immunoreactive bands were visualized using the substrate 4-chloro-1-naphthol by autoradiography.

### Chromatin Immunoprecipitations

Chromatin immunoprecipitations were performed as we described in detail previously (10). ROS 17/2.8 and ROB cells

were grown with or without 1,25-$(OH)_2$-$D_3$ and incubated for 10 min at room temperature in incomplete F-12 medium containing 1% formaldehyde. This step produces reversible cross-links of proteins with DNA. Cells were harvested and lysed in SDS lysis buffer (1% SDS; 10 mM EDTA; 50 mM Tris-Cl, pH 8.1; 10 mM sodium butyrate; 1 mM phenylmethylsulfonylfluoride; 1 $\mu$g/ml pepstatin A) for 10 min on ice. Samples were sonicated to reduce the DNA length to 0.1–0.6 (average size 0.3 kbp). Cellular debris was removed by centrifugation and chromatin solutions were distributed into multiple 1-ml aliquots that were used as the starting material of all subsequent steps.

Chromatin aliquots were precleared with 100 $\mu$l of a 25% (vol/vol) suspension of DNA-coated protein A/G agarose in the absence of antibody as described (10). Samples were used directly for immunoprecipitation experiments with a 1:200 dilution of antiacetyl histone H3, antiacetyl histone H4, or antihyperacetylated histone H4 (Upstate Biotechnology, Inc.). A nonspecific (HA) antibody against the unrelated HA-epitope tag was used as a negative control. Chromatin immunoreactions were allowed to proceed overnight at 4 C on a rotating wheel.

Immune complexes were mixed with 100 $\mu$l of a 25% precoated protein A/G agarose suspension followed by incubation for 1 h at 4 C while rotating. Beads were collected by brief centrifugation, and the bead pellets were sequentially washed with buffers containing different NaCl and LiCl concentrations (10). The beads were then washed twice using 1 ml of buffer (Tris-HCl, 1 mM EDTA at pH 8.0).

The immune complexes were eluted by adding two consecutive 250-$\mu$l aliquots of a freshly prepared solution of 1% SDS/0.1 M $NaHCO_3$. During each step, the sample was briefly vortexed and incubated at room temperature for 15 min with rotation. Samples were consecutively digested with ribonuclease A (10 mg/ml) and proteinase K (20 mg/ml), respectively, to remove RNA and protein. The cross-linking reaction was reversed by overnight incubation of the solutions at 68 C, and the DNA was recovered by phenol-chloroform extractions and ethanol precipitations. DNA pellets were dissolved in 30 $\mu$l Tris-HCl-EDTA buffer (designated the "bound DNA fraction"). Approximately 10% of each DNA fraction was used for quantitative PCR to detect the presence of specific DNA segments.

### PCR and Quantitation

PCR primer pairs were generated to detect DNA segments located between nucleotide −1377 and +466 of the rat OC locus (nucleotide +1 is the mRNA cap site; Fig. 1). We also designed PCR primers to detect the rat histone H4 (His4) gene coding region (Fig. 1) for comparison with PCR signals obtained with OC-derived primers. The sequences for the OC and His4 primer pairs were previously presented (10), and the approximate locations of these primers is shown in Fig. 1. Each OC primer pair was matched with a His4 primer pair of the same Tm as described previously (10). A panel of primer pairs was used to detect segments of bone-related promoters. Runx2-distal: forward, 5′-AAC GCC GCA CTC ACT TGA AA; reverse, 5′-TGA CAA AGG CTT GTG GTG A; AP: forward, 5′-GGC GTA CAT GGT TAC AGA CAA GGA; reverse, 5′-CCC GTA CTG GCA CAG GCA GGT TAG; BSP-distal: forward, 5′-GTA TAC AGC GCT TGA TGA CCT CAC; reverse, 5′-CAC AGC ACT GCC CGA GCC TAA AAG; BSP-proximal: forward, 5′-GCT TAG TCG TTT GCC ATT TAG TT; reverse, 5′-GGT CAC GGC CGG TGC GAC GTC AGG; OP-distal: forward, 5′-GCA CAG CGC TGA AAA CAA ACT CAT; reverse, 5′-GCC ATC CGT TGC CAC CTA CAA AAT; OP-proximal: forward, 5′-AAA TCA CGG CTC CTT GCT CAT GCT; reverse, 5′-TAA TGG CCA CAT AGA ACA GAA GTG; p21-distal: forward, 5′-GTC TGT CCG CAG CAT CAG TAG ATA; reverse, 5′-AGA CAG CAT GCG GAC ATT TCT CAC; p21-proximal: forward, 5′-GGA GAT CCA ACC AGG GGC TTC CAA; reverse, 5′-CCC CAC ACC TGC TTG TCA CGC AGC.

Different rat His4 primer pairs were used in combination with bone-related primers. His4 primer pair 1 (forward, 5′-GCC CAC TCC CTG CTG TTT TCA AAC; reverse, 5′-CTC GGA GAA CCT TGC GGT GTC GCT) was used with the Runx2-distal, Runx2-proximal, OP-distal, and p21-distal primers. His4 primer pair 2 (forward, 5′-AGG GAA AGG CGG CAA AGG TCT G; reverse, 5′-TGG CGT GCT CGG TGT AGG TGA C) was used with the OP-proximal primers. His4 primer pair 3 (forward, 5′-GTC AAG CGT ATC TCG GGT CTT ATC; reverse, 5′-TAG AGA CTA AAT GTC CAC AGC TCC) was used with the p21-proximal primers.

Each PCR was performed with a 10% aliquot (3 $\mu$l) of the bound DNA fraction from the chromatin precipitate or a 1% aliquot (1 $\mu$l) of the unbound fraction. DNA was amplified as described previously (10). The PCR products were separated in 2.0% agarose gels containing 0.5 $\mu$g/ml ethidium bromide. DNA bands were visualized using UV light and recorded as tagged image format files using a high-resolution charge-coupled device camera linked to an AlphaImager gel documentation system (Alpha Innotech, San Leandro, CA). Tagged image format files of ethidium bromide-stained gels were subject to densitometric analysis by ImageJ 1.22d (http://rsb.info.nih.gov/ij/). Values were imported in Microsoft Excel for numerical analysis and data plotting. Chromatin immunoprecipitation experiments were repeated at least three times and yielded very similar results. Representative experiments are presented in the figures.

## Acknowledgments

The authors thank Soraya Gutierrez and Amjad Javed for experimental advice and Beata Paluch for assistance with cell culture.

Received March 28, 2002. Accepted January 2, 2003.

Address all correspondence and requests for reprints to: Andre van Wijnen and Janet Stein, Department of Cell Biology, Room S3-310, 55 Lake Avenue North, Worcester, Massachusetts 01655. E-mail: andre.vanwijnen@umassmed.edu; E-mail: janet.stein@umassmed.edu.

This work was supported by NIH Grants AR-45689, AR-39588, 4TW-00990, and DE-12528.

The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

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