Histone Acetylation in Vivo at the Osteocalcin Locus Is Functionally Linked to Vitamin D-dependent, Bone Tissue-specific Transcription*

Received for publication, December 28, 2001, and in revised form, February 13, 2002
Published, JBC Papers in Press, March 13, 2002, DOI 10.1074/jbc.M112440200

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The accessibility of regulatory elements in chromatin represents a principal rate-limiting parameter of gene transcription and is modulated by enzymatic transcriptional co-factors that alter the topology of chromatin or covalently modify histones (e.g. by acetylation). The bone-specific activation and 1,25-dihydroxyvitamin D₃ enhancement of osteocalcin (OC) gene transcription are both functionally linked to modifications in nucleosomal organization. The initiation of tissue-specific basal transcription is accompanied by the induction of two DNase I hypersensitive sites, and this chromatin remodeling event requires binding of the key osteogenic factor RUNX2/CBFA1 to the OC promoter. Here, we analyzed the acetylation status of histones H3 and H4 when the OC gene is active (in osteoblastic ROS17/2.8 cells) or inactive (in fibroblastic ROS24/1 cells) using chromatin immunoprecipitation assays. We find that acetylated histone H3 and H4 proteins are associated with the OC promoter only when the gene is transcriptionally active and that the acetylation status is relatively uniform across the OC locus under basal conditions. Acetylation of H4 at the OC gene is selectively increased following vitamin D₃ enhancement of OC transcription, with the most prominent changes occurring in the region between the vitamin D₃ enhancer and basal promoter. Thus, our results suggest functional linkage of H3 and H4 acetylation in specific regions of the OC promoter to chromatin remodeling that accompanies tissue-specific transcriptional activation and vitamin D enhancement of OC gene expression. These findings provide mechanistic insights into bone-specific gene activation within a native genomic context in response to steroid hormone-related regulatory cues.

Steroid hormones and retinoids (e.g. 1,25)-dihydroxyvitamin D₃, glucocorticoid, retinoic acid) modulate bone formation and remodeling in vivo at least in part by controlling proliferation and/or differentiation of osteoblasts (1–3). These ligands regulate transcription of genes in osseous cells through receptors that bind as heterodimers to cognate response elements in the promoters of bone related genes (4–6). The vitamin D₃ receptor (VDR) is a principal regulator of the bone-related osteocalcin (OC) gene and interacts together with the retinoid X receptor to a vitamin D₃ response element (VDRE) located in a distal enhancer region (7–14). Transcriptional enhancement of OC gene expression in response to vitamin D₃ occurs only after basal tissue-specific transcription is initiated in osteoblasts through bone-specific factors (1, 15, 16). The basal activation of the OC gene involves conversion of silent closed chromatin to active open chromatin concomitant with increased accessibility of the OC gene promoter to cognate transcription factors at the final stages of osteoblast differentiation (17, 18). Vitamin D₃ enhancement of OC gene transcription is accompanied by changes in genomic protein/DNA and protein/protein interactions and further modifications in chromatin structure (10, 16, 17, 19). Hence, chromatin remodeling is intricately associated with modulations in OC gene transcription in response to bone-related physiological regulatory cues.

The remodeling of chromatin structure is mediated in part by enzymes that topologically alter the interactions of DNA with histone octamers or that covalently modify the core histone proteins H3 and H4 (20–24). Many co-activators and co-repressors that interact with promoter-bound transcription factors represent chromatin-modifying enzymes capable of acetylating or deacetylating lysine residues in the N termini of histones H3 and H4 (25–29). Therefore, we have assessed in this study whether acetylation or deacetylation of histones H3 and H4 in the regulatory regions of a bone-related mammalian gene is linked mechanistically to steroid hormone-dependent modifications in osteoblast-specific gene expression.

Steroid hormone-dependent transcriptional control of bone-specific genes necessitates functional modifications in chromatin organization (4, 30). Understanding chromatin structure of the osteocalcin gene is fundamental in characterizing the molecular and genetic mechanisms of bone cell differentiation. We have previously shown that developmental modifications in the nucleosomal organization of the OC gene are functionally coupled to the establishment of a local promoter architecture containing two nucleosome hypersensitive sites separated by a position nucleosome (17, 18). These sites span the distal VD3 enhancer and the proximal basal promoter, and increased nucleosome hypersensitivity of both sites accompanies vitamin D₃ enhancement of OC gene transcription (17, 18, 31). In this study, we have addressed the mechanisms by which vitamin D₃ mediates chromatin remodeling of the OC gene to promote steroid hormone enhancement of OC gene transcription. The vitamin D₃ response element; VD3, vitamin D₃; OC, osteocalcin; ChIP, chromatin immunoprecipitation; PBS, phosphate-buffered saline; TPCK, 1-1-tosylamido-2-phenylethyl chloromethyl ketone; H4-Ac, acetylated histone H4; H3-Ac, acetylated histone H3; nt, nucleotide(s); CBP, CREB-binding protein; CCD camera, charge-coupled device camera.
Histone Acetylation at the Osteocalcin Gene

key results of this study are that the OC gene promoter is associated with acetylated histones H3 and H4, when transcriptionally active, and that the relative level of histone H4 acetylation is increased during vitamin D₃ enhancement of transcription.

MATERIALS AND METHODS

Cell Culture—ROS 17/2.8 and ROS 24/1 osteosarcoma cells (32) were plated at 0.5 × 10⁶ cells per 100-mm dish and grown in F-12 medium plus 5% fetal calf serum. Cells reached confluence at approximately day 6. Cells were treated with 10⁻⁶ M 1,25-­(OH)₂D₃ (a gift from Dr. M. Uskokovic, Hoffmann-La Roche) in F-12 medium containing 2% fetal calf serum, which was growth factor- and steroid hormone-depleted Uskokovic, Hoffmann-La Roche) in F-12 medium containing 2% fetal calf serum, growth factor- and steroid hormone-depleted medium after 2 min at 3,000 rpm in an Eppendorf microcentrifuge at 4 °C followed by four sequential washes of the beads with dilution buffer. Prior to chromatin immunoprecipitations, the 1-mL chromatin aliquots were pre-cleared with 100 μL of a 25% (v/v) suspension of DNA-coated protein A/G-agarose in the absence of antibody. The supernatant was recovered following brief centrifugation at 3,000 rpm in the Eppendorf microcentrifuge and was used directly for immunoprecipitation experiments with a 1:200 dilution of the acetylated histone H3 or H4 antibodies described above. A nonspecific antibody against the unrelated hemagglutinin 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 μL of a 25% pre-coated protein A/G-agarose suspension followed by incubation on a rotating wheel. We also observed neutralization of histones H4, H4-acetyl. After washing in ice-cold PBS and centrifugation for 2 min at 3,000 rpm for 2 min at 4 °C. The supernatant of this step in the procedure is designated the "unbound DNA fraction." The bead pellets were sequentially washed with 1 mL each of the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 500 mM NaCl), and LiCl wash buffer (0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-Cl, pH 8.1). The beads were then washed twice using 1 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The immunocomplexes were eluted by adding a 250-μL aliquot of a freshly prepared solution of 1% SDS, 0.1 M NaHCO₃. The sample was briefly vortexed and incubated at room temperature for 15 min with rotation. Following recovery of the supernatant by microcentrifugation, the beads were washed with a second aliquot, and the resulting supernatant was carefully pooled with the first aliquot. Samples were sequentially digested with RNase A (10 mg/mL) at 37 °C for 1 h and proteinase K (20 mg/mL) at 42 °C for 2 h to remove RNA and protein. The cross-linking reaction was reversed by overnight incubation of the sample at 68 °C, and the DNA was recovered by phenol/chloroform extraction. The DNA was precipitated with two volumes of ethanol containing 20 mg/mL glycogen as carrier. Each DNA pellet was dissolved in 30 μL of TE buffer (designated the "bound DNA fraction"). An aliquot (3 μL) of each DNA fraction was used for quantitative PCR to detect the presence of specific DNA segments.

For each immunoprecipitation, an aliquot of the starting material was digested with RNase A (10 mg/mL) and proteinase K (20 mg/mL) and used as a negative control. The beads were washed with a second aliquot, and the resulting supernatant was carefully pooled with the first aliquot. Samples were sequentially digested with RNase A (10 mg/mL) at 37 °C for 1 h and proteinase K (20 mg/mL) at 42 °C for 2 h to remove RNA and protein. The cross-linking reaction was reversed by overnight incubation of the sample at 68 °C, and the DNA was recovered by phenol/chloroform extraction. The DNA was precipitated with two volumes of ethanol containing 20 mg/mL glycogen as carrier. Each DNA pellet was dissolved in 30 μL of TE buffer (designated the "bound DNA fraction"). An aliquot (3 μL) of each DNA fraction was used for quantitative PCR to detect the presence of specific DNA segments.

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RESULTS

The OC Promoter Is Associated with Acetylated Histones H3 and H4 in Osseous Cells Actively Expressing the OC Gene—The osteocalcin gene is constitutively expressed in ROS 17/2.8 rat osteosarcoma cells, and its promoter displays an active chromatin conformation reflected by the presence of two DNase I hypersensitive sites (17, 18). To assess whether acetylated histones are associated with the OC gene when it is transcriptionally active, we performed ChIP assays with antibodies recognizing mono- and diacetylated histone H3 (H3-Ac) and multiple acetylated forms of histone H4 (H4-Ac1,2 and H4-Ac3,4). These antibodies were used in whole cell lysates to precipitate sonicated chromatin that was obtained following formaldehyde cross-linking of DNA to histones in vivo. DNA fragments that co-precipitated with acetylated histones were purified upon reversal of histone/DNA cross-links and used as the template for PCR reactions with gene-specific primers (Fig. 1). We ad-
FIG. 2. Association of acetylated histones H3 and H4 with the promoter of the osteocalcin gene in ROS 17/2.8 osteosarcoma cells that actively express osteocalcin. Immunoprecipitation assays were performed with formaldehyde-cross-linked chromatin isolated from ROS 17/2.8 cells and antibodies against acetylated histones H3 and H4. Panel a shows ethidium bromide-stained agarose gels of PCR products obtained with OC primer pairs −773/−433 and −459/ −118 in chromatin immunoprecipitates with the indicated antibodies (H3-Ac, H4-Ac1,2, H4-Ac3,4, NS). The arrows on the right indicate the relative locations of the PCR reaction products. The His4 gene signals (primer pairs +52/+272) observed in the left and right lanes for each panel should be equal, and any differences in intensities reflect experimental variation. Panel b shows the quantitation of signal intensities of the PCR products from three separate experiments that were digitally recorded with a CCD camera and quantitated using ImageJ, version 1.22d (developed by Wayne Rasband, National Institutes of Health). For each lane, the values were expressed as the ratio of the OC signal versus the His4 signal (which represents a parallel parameter for comparison) and plotted for each antibody. Values for the nonspecific antibody in panel a (NS) are not included in the graph. Panels c and d are the same as Panels a and b, respectively, except that different primer pairs were used and the size of the PCR product for the His4 primer pair is larger than that for the OC primer pairs.

justed the number of PCR cycles to remain within the linear range of PCR amplification. The resulting DNA products were resolved by agarose electrophoresis, visualized by ethidium bromide staining, and quantitated by digital image analysis (Fig. 2). Chromatin immunoprecipitates were analyzed with a series of PCR primers covering the OC locus between −1.4 and +0.5 kb relative to the mRNA cap-site (Fig. 1, a and c). In each experiment, the specificity of the PCR procedure was assessed by appropriate positive and negative control reactions in which either specific primers or templates were omitted. Furthermore, the authenticity of the PCR products was validated by Southern blot analysis with probes spanning the OC gene (data not shown). For comparison, we also tested PCR primers spanning the coding region of a cell growth regulated rat histone H4 (His4) gene (Fig. 1b), which is expressed in a broad range of proliferating cells and tissues (39).

We first analyzed the acetylation status of histones within the region of the OC promoter that contains the distal nuclease hypersensitive site and the vitamin D enhancer (nt −773/ −433), as well as the nucleosomal region between the proximal and distal promoters (nt −459/−118) (Fig. 2a). The results show that all three acetylated histone antibodies (H3-Ac, H4-Ac1,2, and H4-Ac3,4) precipitate fragments from the OC gene and also the His4 gene. However, no signal was detected in precipitates obtained with an antibody recognizing the unrelated hemagglutinin epitope (panel NS, Fig. 2a), which provides a negative control for our chromatin immunoprecipitations. These results indicate that different acetylated forms of histones H3 and H4 are specifically associated with nuclesomes in the vicinity of the transcriptionally active OC and His4 genes.

The PCR signals for the His4 gene are relatively uniform for the three acetylated histone antibodies (Fig. 2a). Thus, we normalized the values for the OC primers with those for the His4 primers and expressed our results as OC/His4 ratios (Fig. 2b). We find that the OC/His4 ratios for the OC −773/−433 and OC −459/−118 primer pairs are lowest for the H3-Ac antibody (OC/His4 ratios of 0.2 to 0.4) and ~2-fold higher for the H4-Ac1,2 and H4-Ac3,4 antibodies (OC/His4 ratios of 0.4 to 0.8). These results indicate quantitative differences in the relative representation of acetylated histones H4 and H3 at the OC gene as compared with the His4 locus. These differences suggest that the OC gene promoter is preferentially associated with acetylated histone H4 compared with the His4 gene.

Differential Association of Acetylated Histones H3 and H4 across the OC Locus When the OC Gene Is Transcriptionally Active—To understand the relative distribution of acetylated histones across the OC gene promoter, we performed additional PCR experiments using primers upstream from the vitamin D-dependent enhancer region and downstream at the proximal promoter and OC coding region (Fig. 2c). Immunoprecipitations with the H3-Ac, H4-Ac1,2, and H4-Ac3,4 antibodies using chromatin isolated from ROS 17/2.8 cells. The results indicate that the OC promoter regions −1047/−827 and −198/ −28 contain on the average 1.5–3-fold higher levels of acetylated histones H3 and H4 than the OC segments in the far distal promoter (−1377/−1176) and the OC coding sequence (−289/+466) (Fig. 2d). Thus the relative level of acetylation of histones H3 and H4 gradually declines in chromatin upstream from −1.0 kb and downstream from the mRNA cap-site. The levels of acetylated forms of H4 are significantly higher than those for acetylated histone H3 throughout the OC locus. The level of histone H4 acetylation is highest in the −1047/−827 and −198/−28 regions and is reduced by 1.5–2-fold in the −1377/−1176 and +289/+466 regions (Fig. 2, c and d). These data indicate that there are transitions in the acetylation status of histones H3 and H4 that correspond with the regulatory boundaries of the OC gene promoter. Our findings further indicate that the promoter region from approximately −1.0 to 0.0 kb, which encompasses the principal regulatory elements and two major nuclease hypersensitive domains of the OC promoter, contains relatively high levels of acetylated histones H3 and H4.

Absence of Acetylated Histones H4 and H3 in ROS 24/1 Osteosarcoma Cells That Do Not Express Mature Bone Phenotypic Markers—To gain insight into the acetylation status of histones at the OC locus when the gene is transcriptionally inactive, we carried out ChIP assays with histone-DNA complexes isolated from both ROS 17/2.8 and ROS 24/1 osteosarcoma cells. ROS 24/1 cells do not express markers of the mature bone phenotype including osteocalcin (40–42). In addition, the
OC promoter adopts a closed and transcriptionally silent chromatin conformation in ROS24/1 cells that do not express the OC gene. Chromatin immunoprecipitation assays are presented as described in Fig. 2 using samples isolated from ROS 17/2.8 and ROS 24/1 cells as indicated. Panels a and c show ethidium bromide-stained agarose gels of PCR products obtained with the indicated OC primer pairs and antibodies. The arrows on the right indicate the relative locations of the PCR reaction products. The OC signal is above the His4 signal in a and below the His4 signal in c. Panels b and d show quantitations of the experiments presented in a and c as described in Fig. 2. The ratio of the OC versus the His4 signals observed for each of the three acetylated histone antibodies (i.e., H3-Ac, H4-Ac_{1,2}, and H4-Ac_{3,4}) was plotted for each region of the OC promoter to permit direct comparison of the results for ROS17/2.8 and ROS 24/1 cells.

Histone Acetylation at the OC Promoter Is Selectively Increased following Vitamin D_{3} Enhancement of OC Gene Transcription in ROS17/2.8 Cells—Steroid hormone dependent up-

**Fig. 3.** Reduced levels of histones H3 and H4 acetylation at the OC locus in fibroblastic ROS 24/1 cells that do not express the OC gene. Chromatin immunoprecipitation assays are presented as described in Fig. 2 using samples isolated from ROS 17/2.8 and ROS 24/1 cells as indicated. Panels a and c show ethidium bromide-stained agarose gels of PCR products obtained with the indicated OC primer pairs and antibodies. The arrows on the right indicate the relative locations of the PCR reaction products. The OC signal is above the His4 signal in a and below the His4 signal in c. Panels b and d show quantitations of the experiments presented in a and c as described in Fig. 2. The ratio of the OC versus the His4 signals observed for each of the three acetylated histone antibodies (i.e., H3-Ac, H4-Ac_{1,2}, and H4-Ac_{3,4}) was plotted for each region of the OC promoter to permit direct comparison of the results for ROS17/2.8 and ROS 24/1 cells.
regulation of OC gene expression is mediated at least in part by transcriptional mechanisms and occurs concomitant with structural changes in chromatin organization (17, 43). Administration of vitamin D₃ strongly enhances OC gene expression in ROS 17/2.8 cells but not in ROS24/1 cells, which lack a functional vitamin D receptor (44). In our experiments, the enhancement of OC gene expression was evident at 2.5 h after VD₃ treatment and increased until 24 h after treatment (data not shown), in agreement with our previous analysis of transcription by nuclear run-on assays (10). In contrast, the levels of glyceraldehyde-3-phosphate dehydrogenase and histone H4 mRNAs remained relatively constant or decreased modestly throughout the time course (data not shown). These data provide the basis for assessing whether VD₃-dependent modulation of OC gene expression involves modifications in histone acetylation. Western blot analysis reveals that the levels of acetylated histones H4 and H3 are increased modestly within 45 min following VD₃ induction but do not change significantly thereafter (Fig. 4 and data not shown). Interestingly, the levels of phosphorylated histone H3 are rapidly increased by 45 min after VD₃ addition. Consistent with large pre-existing pools of glyceraldehyde-3-phosphate dehydrogenase and histone H4 mRNAs, total cellular levels of histones packaged as nucleosomes, total cellular levels of histone proteins remain similar based on Coomassie Blue staining (Fig. 4). Major changes are also not apparent in the levels of CDK2 and lamin B or in the levels of the histone acetyltransferases CBP (CREB-binding protein) and P/CAF, HDAC1, or CDK2. Whole cell lysates were prepared at the indicated times after vitamin D treatment. The left lane shows purified histone proteins from butyrate-treated ROS 17/2.8 cells. The top panel shows Coomassie Blue staining of the gel and reveals that total cellular levels of histone proteins are similar following vitamin D treatment.

We therefore investigated whether VD₃-dependent enhancement of OC gene transcription is accompanied by modifications in the acetylation status of histones H3 and H4 associated specifically with the OC gene locus (Figs. 5 and 6). ROS 17/2.8 cells were cultured for 12 h in vitamin D₃-depleted medium, and vitamin D₃-dependent effects on histone acetylation were monitored by ChIP assays in cells harvested at 45 min and 2.5 and 24 h following the administration of 10 nM (1,25)-dihydroxyvitamin D₃. We found that acetylation of histone H3 or H4 proteins associated with the −773/−433 and −459/−118 regions of the OC gene is not significantly enhanced after short term treatment (i.e., 45 min) with vitamin D3 (Fig. 5a, top). However, the levels of acetylated histone H4 were modestly up-regulated at 2.5 h after treatment in the −459/−118 segment of the OC gene promoter (Fig. 5a, center). Histone H4 acetylation was further enhanced by 24 h after vitamin D₃ treatment (Fig. 5a, bottom). The levels of acetylated histone H3 at the −773/−433 and −459/−118 regions of the OC gene remained relatively constant during the first 2.5 h but were modestly increased by 24 h (Fig. 5a). In contrast, the levels of acetylated histones H3 and H4 in the Hist4 gene remained constant or were down-regulated following vitamin D₃ administration. Our results are expressed quantitatively and summarized in Fig. 5c. We conclude that vitamin D₃ enhancement of OC gene transcription in ROS 17/2.8 cells is accompanied by a selective increase in acetylation of histone H4 and, to a lesser extent, by acetylation of histone H3 associated with the −773/−433 and −459/−118 regions of the OC gene.

Analysis of chromatin from ROS 24/1 cells treated with the same concentration of VD₃ and for the same duration as ROS17/2.8 cells (Fig. 5b) reveals that VD₃ does not induce acetylation of either histone H3 or histone H4 proteins associated with the OC gene. These data demonstrate that steroid hormone-dependent modifications in histone acetylation at the OC gene promoter are absent in ROS 24/1.

We also analyzed vitamin D effects on histone acetylation of OC gene segments upstream from −773 and downstream from −198 (Fig. 6 and data not shown). The data reveal that the proximal promoter (−198/+28) and coding region (+289/+466) each exhibit major changes in histone H3 and H4 acetylation at 24 h after vitamin D₃ treatment of ROS 17/2.8 cells (Fig. 6a).
Similar to the results obtained for the −773/−433 and −459/−118 regions of the OC gene (Fig. 5a), changes in histone acetylation status in the −198/−28 and +289/+466 regions are already apparent but less dramatic at 2.5 h (data not shown). In contrast, the −1047/−827 region does not exhibit major changes in histone acetylation at either 2.5 or 24 h after vitamin D₃ treatment (Fig. 6a and data not shown). Furthermore, similar to results presented in Fig. 5b, no modifications in histone acetylation at the −1047/−827, −198/−28 or +289/+466 regions were observed at any time after vitamin D₃ treatment of ROS 24/1 cells (Fig. 6b and data not shown). Taken together, our findings indicate that increased acetylation of the OC promoter in response to vitamin D₃ does not occur upstream from −827 but is evident at the VDRE (−773/−433) and downstream at the proximal promoter (−198/−28) and coding (+289/+466) segments of the OC gene, as well as in the segment (−459/−118) spanning the positioned nucleosome between the proximal and distal regulatory regions. Therefore, our results establish that there are selective changes in histone acetylation of the OC gene that accompany and may be mechanistically linked to chromatin remodeling and vitamin D₃-dependent enhancement of transcription.

**DISCUSSION**

In this study, we experimentally addressed the functional coupling between histone acetylation and osteocalcin gene expression.
transcription in osseous cells in the presence or absence of vitamin D₃ using chromatin immunoprecipitation assays. We find that the OC gene promoter is associated with acetylated histones H3 and H4 when transcriptionally active and that acetylation of histone H4 is increased in response to vitamin D₃. Furthermore, these results together with previous data from our laboratory (17, 18, 31) indicate that enhancement of OC gene transcription is mediated by specific changes in higher order chromatin structure and modifications in the acetylation status of histones H3 and H4.

Our previous studies demonstrated that cells expressing the bone-specific OC gene exhibit two DNase I hypersensitive sites spanning the proximal (nt −170 to −70) and distal (nt −600 to −400) promoter regions and a nucleosome positioned between these hypersensitive sites (17, 18). In other studies, we found that treatment of ROS 17/2.8 cells with the histone deacetylase inhibitor sodium butyrate blocks vitamin D stimulation of OC transcription (31). The figure shows multiple ethidium bromide-stained gels with PCR products from precipitates analyzed with different OC primer pairs as indicated. The arrowheads on the right mark the relative locations of the PCR products.

The association of histone acetylation with transactivation and deacetylation with repression was first suggested in 1964 by Allfrey et al. (45). Many studies subsequently supported the correlation between histone acetylation and active gene transcription (46, 47). In the chicken β-globin locus, hyperacetylated histones are present when the locus is transcriptionally active (48). In the ε-globin promoter, a very specific and directed acetylation of histone H3 at a TATA-proximal nucleo-

some occurs during transcriptional activation, whereas acetylation of H4 appears to be more widespread and involves two nucleosomes spanning almost 400 bp of the ε-globin 5’ flanking sequence (49). Analysis of the histone acetylation status of X chromosomal genes showed that the promoter regions of actively expressed genes (e.g. OCRL, PGK1, and XIST) are hyperacetylated regardless of whether they are located on the inactive (Xı) or active (Xa) chromosome. In contrast, the promoters of silent genes (e.g. ODS, XPC7, and NDF) on the Xi chromosome are associated with hypoacetylated histone H4 (50). Our findings demonstrate that histone H3 and H4 acetylation at the bone-tissue specific OC locus occurs only when the gene is transcriptionally active.

Several hormone-responsive genes have been shown to undergo changes in histone acetylation upon ligand treatment. Four estrogen-responsive genes (pS2, EB1, c-myc, and CTD) were shown to exhibit preferential acetylation of H4 versus H3 in the promoter regions following estrogen treatment (51). The pS2 and CTD genes initially showed low levels of histone H4 acetylation, which increased in response to estradiol and reached maximal levels at 60 min in a receptor-dependent manner. These results and our data, which demonstrate vitamin D-dependent modifications in histone acetylation at the OC gene, suggest that histone H4 acetylation is functionally coupled with steroid hormone-dependent transcriptional activation of cellular genes.

There are several distinct genes involved in metabolic regulation or cell cycle control for which acetylation of histone H3, but not H4, correlates with increased transcription. Acetylated histone H3, but not H4, is associated with the promoter region of the StAR (steroidogenic acute regulatory protein) gene when it is transcriptionally active (52). Acetylated histone H3, but not H4, increased in chromatin at the low density lipoprotein receptor and hydroxymethylglutaryl-CoA reductase promoters (53). Selective hyperacetylation of H3 is associated with cell cycle regulation of the p21WAF1 (54) and histone H4 genes.² We conclude that differential acetylation of histones H3 and H4 at gene promoters is involved in selective changes in chromatin in response to physiological signals.

The variety and dynamics of histone protein acetylation observed at the promoters of different genes suggest that acetylation of histones functions in transcriptional regulation through highly intricate mechanisms. One mechanism may involve electrostatic repulsion between acetylated histones and DNA. More recently, it has become apparent that acetylation modulates interactions among adjacent nucleosomes (21, 22, 24, 55). In addition, a histone code of post-translational modifications may direct transcription factors and co-factors to the promoter (20, 56, 57). Further, the association of SWI/SNF chromatin remodeling complexes with chromatin is stabilized by histone acetylation (23, 58). Accumulating evidence suggests that acetylation of the N-terminal tails of H3 and H4 may be a principal regulator of transcription factor access to nucleosomal DNA and the establishment of transcription initiation complexes at gene promoters (49, 59).

In summary, our data support the concept that bone tissue-specific activation of the osteocalcin gene requires acetylation of histones H3 and H4 to establish an open chromatin conformation and basal levels of transcription. Subsequent vitamin D-dependent enhancement of OC gene transcription involves acetylation of histone H4 and increased accessibility of proximal and distal promoter regions. These findings provide the basis for future studies on the sequential order of events re-

² B. Cho, H. Hovhannisyan, M. Montecino, J. B. Lian, A. J. van Wijnen, J. L. Stein, and G. S. Stein, unpublished data.
quired for chromatin remodeling that occurs during normal osteoblast differentiation.

Acknowledgments—We thank Judy Rask for preparation of this manuscript and Hayk Hovhannisyan and Soraya Gutierrez for helpful discussions.

REFERENCES

1. Stein, G. S., Lian, J. B., Stein, J. L., van Wijnen, A. J., and Montecino, M. (1996) Physiol. Rev. 76, 583–629
2. Aubin, J. E. (1998) J. Cell Biol. 139, 805–808
3. Lian, J. B., Stein, G. S., Stein, J. L., and van Wijnen, A. J. (1999) in Vitamins and Hormones (Lutwack-Gould, ed) pp. 433–509, Academic Press, San Diego
4. Barone, L. M., and Frenkel, B. (2000) Gene 246, 9–21
5. Tsai, M.-J., and O'Malley, B. W. (1994) Annu. Rev. Biochem. 63, 451–486
6. MacDonald, P. N., Dowd, D. R., Nakajima, S., Galligan, M. A., Reeder, M. C., Haussler, C. A., Ozato, K., and Haussler, M. R. (1998) Mol. Cell. Biol. 13, 5907–5917
7. Morrison, N. A., Shine, J., Fragonas, J. C., Verkest, V., McMeneny, L., and Eisma, J. J. (1989) Science 246, 1158–1161
8. Demay, M. B., Gerardi, J. M., DeLuca, H. F., and Kronenberg, H. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 201–205
9. Markose, E. R., Stein, J. L., Stein, G. S., and Lian, J. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1701–1705
10. Bannister, A. J., and Kouzarides, T. (1996) Nature 380, 286–289
11. Bannister, A. J., and Kouzarides, T. (1999) Nature 398, 212–215
12. Bannister, A. J., and Kouzarides, T. (2001) Nature Rev. Mol. Cell Biol. 2, 381–390
13. Bannister, A. J., and Kouzarides, T. (2002) Nature 418, 490–497
14. Bannister, A. J., and Kouzarides, T. (2003) Nat. Rev. Mol. Cell Biol. 4, 49–55
15. Bannister, A. J., and Kouzarides, T. (2004) Nat. Rev. Mol. Cell Biol. 5, 79–88
16. Bannister, A. J., and Kouzarides, T. (2005) Nat. Rev. Mol. Cell Biol. 6, 301–310
17. Bannister, A. J., and Kouzarides, T. (2006) Nat. Rev. Mol. Cell Biol. 7, 104–113
18. Bannister, A. J., and Kouzarides, T. (2007) Nature Rev. Mol. Cell Biol. 8, 495–506
19. Bannister, A. J., and Kouzarides, T. (2008) Nat. Rev. Mol. Cell Biol. 9, 233–244
20. Bannister, A. J., and Kouzarides, T. (2009) Nat. Rev. Mol. Cell Biol. 10, 57–63
21. Bannister, A. J., and Kouzarides, T. (2010) Nat. Rev. Mol. Cell Biol. 11, 334–348