Chromatin Remodeling and Transcriptional Activity of the Bone-specific Osteocalcin Gene Require CCAAT/Enhancer-binding Protein β-dependent Recruitment of SWI/SNF Activity*§

Received for publication, October 27, 2005, and in revised form, June 7, 2006. Published, JBC Papers in Press, June 13, 2006. Published, JBC Papers in Press, June 13, 2006. DOI 10.1074/jbc.M511640200

Alejandro Villagra†,1, Fernando Cruzat†, Loreto Carvallo†, Roberto Paredes†, Juan Olate†, Andre J. van Wijnen§, Gary S. Stein§, Jane B. Lian§, Janet L. Stein§, Anthony N. Imbalzano§, and Martin Montecino‡

From the †Department of Bioquimica y Biologia Molecular, Facultad de Ciencias Biologicas, Universidad de Concepcion, Casilla 160-C, 4079100 Concepcion, Chile and the §Department of Cell Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

Tissue-specific activation of the osteocalcin (OC) gene is associated with changes in chromatin structure at the promoter region. Two nuclease-hypersensitive sites span the key regulatory elements that control basal tissue-specific and vitamin D3-enhanced OC gene transcription. To gain understanding of the molecular mechanisms involved in chromatin remodeling of the OC gene, we have examined the requirement for SWI/SNF activity. We inducibly expressed an ATPase-defective BRG1 catalytic subunit that forms inactive SWI/SNF complexes that bind to the OC promoter. This interaction results in inhibition of both basal and vitamin D3-enhanced OC gene transcription and a marked decrease in nuclease hypersensitivity. We find that SWI/SNF is recruited to the OC promoter via the transcription factor CCAAT/enhancer-binding protein β, which together with Runx2 forms a stable complex to facilitate RNA polymerase II binding and activation of OC gene transcription. Together, our results indicate that the SWI/SNF complex is a key regulator of the chromatin-remodeling events that promote tissue-specific transcription in osteoblasts.

Within the eukaryotic nucleus, the packaging of DNA into nucleosomes and higher order chromatin structures have been implicated in the regulation of key cellular events, such as replication and transcription. During the last decade, a large family of protein complexes that promote transcription by altering chromatin structure have been described (1–3). Among them is the SWI/SNF complex subfamily that remodels chromatin in an ATP-dependent manner (1–3). SWI/SNF complexes are composed of several subunits, which have been implicated in a wide range of cellular events, including gene regulation, cell cycle control, development, and differentiation (1, 3). The mammalian SWI/SNF complexes contain a catalytic subunit that can be either BRG1 or BRM, which includes ATPase activity. Mutations in the ATPase domain of BRG1 or BRM that abrogate the ability of these proteins to bind ATP result in the formation of inactive SWI/SNF complexes (4–6). Furthermore, expression of mutant BRG1 or BRM proteins in NIH3T3 cells impairs the ability of these cells to activate endogenous stress response genes in the presence of arsenite (5) and to differentiate into muscle or adipocytic cells (4, 5, 7). In addition, we have recently shown that the presence of the mutant BRG1 protein in these NIH3T3 cell lines inhibits BMP2-induced differentiation into the osteoblast lineage (8).

The rat osteocalcin (OC)3 gene encodes a 10-kDa bone-specific protein that is expressed at late stages of osteoblast differentiation, concomitant with the mineralization of the extracellular matrix (9). Osteoblast-specific transcription of the OC gene is controlled by modularly organized basal and hormone-responsive elements located within two DNase I-hypersensitive sites (distal site –605 to –400 and proximal site –170 to –70; see Fig. 1) that are present only in osteoblastic cells expressing this gene (10). Thus, chromatin remodeling of the OC gene promoter accompanies transcriptional activity during osteoblast differentiation.

Bone-specific expression of the OC gene is principally regulated by the Runx2 transcription factor (11, 12). The rat OC gene promoter contains three recognition sites for Runx2 interaction (see Fig. 1), site A (–600 to –595), site B (–435 to –430), and site C (–138 to –130). Mutation of all three Runx2 sites results in altered chromatin structure reflected by loss of the DNase I-hypersensitive sites and by inhibition of OC promoter transcriptional activity (13–15). Another key regulatory element that controls OC gene expression is recognized by the 1α,25-dihydroxyvitamin D3 (vitamin D3) receptor (VDR) complex upon ligand activation. This vitamin D3-responsive ele-
ment (VDRE) is located in the distal region of the rat OC promoter (−465 to −437; see Fig. 1) and functions as an enhancer to increase OC transcription (9). Runx2 interacts with the VDR within the context of the OC promoter, and together these factors up-regulate OC gene expression in vitamin D₃-treated osteoblastic cells (15). In addition, Runx2 recruits chromatin-remodeling complexes with histone acetyltransferase (HAT) activity (p300/P/CAF), which enhance both basal and vitamin D₃-stimulated OC promoter activity (16).

C/EBPβ is also a principal transactivator of the OC gene that binds within the proximal promoter (−106 to −99; see Fig. 1) and synergizes with Runx2 to enhance basal and tissue-specific OC gene transcription (17). C/EBPβ interacts with chromatin-remodeling complexes such as those including histone acetyltransferase (HAT) activity (p300/P/CAF) or ATP-dependent activities (SWI/SNF) (18, 19). Hence, C/EBPβ has been proposed to recruit these chromatin-remodeling complexes to tissue-specific gene promoters, leading to transcriptional activation during cell differentiation.

Because there is a tight relationship between chromatin remodeling at the rat OC gene promoter and transcriptional activity of this gene, it is necessary to address the molecular mechanisms responsible for these cellular events. Here, we have determined that SWI/SNF activity is required for the chromatin remodeling that accompanies transcription of the OC gene in osteoblastic cells. We find that the SWI/SNF complex is associated with the OC promoter in osteoblastic cells expressing OC and that in the presence of inactive SWI/SNF, the OC gene is neither remodeled nor transcribed. Interestingly, the inhibition in OC transcription is associated with a drastic reduction in the level of RNA polymerase II bound to the proximal promoter. In addition, we determined that SWI/SNF is recruited to the proximal OC promoter region by the transcription factor C/EBPβ and, once bound to this region of the promoter, interacts also with the bone-specific transcription factor Runx2. Thus, our results indicate that the activity of the SWI/SNF complex at the proximal OC promoter is a requirement for both formation of the proximal nuclease-hypersensitive site and the transcriptional activation of this gene in differentiated osteoblastic cells.

**EXPERIMENTAL PROCEDURES**

Expression Constructs—Luciferase reporter constructs driven by the wild type full-length (1.1 kb, pOC-Luc) or deleted forms (208 bp, p208OC-Luc) of the rat OC gene promoter were described previously (16, 17). The p208OC-Luc/C/EBPm construct carries a mutated C/EBP site that prevents binding of the C/EBPβ transcription factor (17). The pCMVβ plasmid coding for the enzyme β-galactosidase and driven by the cytomegalovirus (CMV) promoter was purchased from BD Biosciences (Palo Alto, CA). The pCEP-4 plasmid encoding the hygromycin B phosphotransferase gene was obtained from Invitrogen. The constructs pBJ5-BRG1 and pBJ-BRG1K-R coding for HA-tagged BRG1 wild type and HA-tagged BRG1 carrying a mutation in the ATP-binding site, respectively, were reported previously (20). pTSCeBRG1K-R carries the FLAG-tagged mutated version of BRG1 under the control of the tetracycline-inducible promoter system were reported previously (5).

**Cell Cultures, Transient Transfections, and Reporter Assays—**

ROS17/2.8 osteoblastic cells were cultured as described previously (21). These cells respond to 10⁻³ m 1α,25-dihydroxyvitamin D₃ (vitamin D₃) with a 3–5-fold increase in OC expression. Cells were plated in 6-well plates (35-mm diameter wells) and transiently transfected with 1 μg of pOC-Luc, p208OC-Luc, or p208OC-LUC-C/EBPm and 0.5 μg of CMV empty vector using the Lipofectamine Plus reagent (Invitrogen). In the BRG1, Runx2, and C/EBPβ forced expression experiments, cells were co-transfected with the corresponding expression vector as described before (15, 16) at the concentrations detailed in the legends to Figs. 2 and 9. The total amount of exogenous DNA was maintained at 5 μg/well with salmon sperm DNA (Invitrogen). The pCMVβ plasmid was included as internal control for transfection efficiency. Cells were harvested at 24 h after transfection and assayed for luciferase (Promega, Madison, WI) and β-galactosidase (BD Biosciences) activities in a luminometer.

**Generation and Selection of Stably Transfected ROS17/2.8 Cell Lines—**

ROS17/2.8 cells grown in 6-well plates were transfected with 5 μg of the p-TetR-VP16 plasmid and 0.25 μg of the pCEP4 plasmid using Lipofectamine Plus (Invitrogen) and were then selected for their ability to grow in the presence of 200 μg/ml hygromycin. Selected cell lines were evaluated for expression of the tetracycline-VP16 regulator by transiently transfecting the plasmid pUHC13.3, which encodes the luciferase reporter gene under the control of a promoter containing tetracycline operator sites (22). A clone expressing high levels of luciferase activity in the absence but not in the presence of tetracycline was chosen for further studies. These cells were then stably transfected with 5 μg of the pTSCeBRG1K-R plasmid encoding an ATPase-defective BRG1 (5) and 0.25 μg of the plasmid pRSV-Neo and selected for growth in 400 μg/ml Geneticin (Invitrogen), 50 μg/ml hygromycin, and 10 μg/ml tetracycline. Once generated, the ROSBRG1TA cell lines were maintained in 50 μg/ml hygromycin, 100 μg/ml Geneticin, and 10 μg/ml tetracycline. All selected cell lines were evaluated for their ability to express FLAG-tagged mutant BRG1 (ROSBRG1TA) proteins by Western blot using anti-FLAG antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Expression of the endogenous genes osteocalcin, alkaline phosphatase, VDR, Runx2, and β-actin (and others not shown) was determined by reverse transcription-PCR using specific primers.

**Immunoprecipitations—**

Nuclear extracts from wild type ROS17/2.8 or ROSBRG1TA cell lines cultured with or without tetracycline and in the presence or absence of 10⁻⁸ m vitamin D₃ for 4 h were prepared by the Dignam method (23). Co-immunoprecipitation experiments were performed as described before (16). FLAG-tagged BRG1 mutant proteins were immunoprecipitated with a Sepharose-bound anti-FLAG mouse antibody (Sigma). C/EBPβ was immunoprecipitated with an antibody from Santa Cruz Biotechnology. The co-immunoprecipitated proteins were detected by Western blot using specific antibodies. Anti-BAF170, -C/EBPβ, and -Runx2 antibodies were purchased from Santa Cruz Biotechnology. Anti-BAF170, anti-BAF57 antibodies were generously donated by Dr. Weidong Wang. Anti-BAF170, anti-BAF57, and anti-FLAG antibodies were described previously (5).
**SWI/SNF Activity in Osteoblast-specific Transcription**

**FIGURE 1. Schematic representation of the rat OC promoter in osteoblastic cells expressing the OC gene.**

In the diagram, key transcriptional regulatory elements of the rat OC gene are shown: Runx2 sites A and B (−605 to −595 and −440 to −433, respectively), the VDRE (−467 to −437), and the YY1 site (−449 to −445) localized within the distal DNase I-hypersensitive site (dDHS; −600 to −400). The Runx2 site C (−138 to −130), C/EBP site (−106 to −99), and OC box (−99 to −76) localized within the proximal DNase I-hypersensitive site (pDHS; −170 to −70). The figure also shows the TATA box and a nucleosome (filled circle) positioned between both hypersensitive sites. The black arrow marks the direction of transcription. The primer pairs utilized to amplify the different regions of the OC promoter in the ChIP experiments are also shown below.

**Nuclease-hypersensitive Analyses—Restriction endonuclease accessibility analyses were performed as described before (10, 24).** Nuclei were isolated from wild type ROS17/2.8 cells and from the ROSBRG1TA cell line incubated with the restriction endonucleases in the specific buffer conditions provided by the supplier (New England Biolabs, Beverly, MA). The mixture was incubated for 30 min at 37 °C, and the genomic DNA was purified. After complete cleavage with Apal or BamHI, the samples were electrophoresed in 1.2 or 2% (w/v) agarose gels, blotted, and hybridized with probes shown in Fig. 7A, as indicated in the legend to Fig. 7. The intensities of the bands were determined by using a molecular imager System (BIO-RAD). The percentage of digestion was quantitated as the fraction of the signal of the band compared with the total signal of the bands within a given lane on the gel.

**Chromatin Immunoprecipitation (ChIP) and Re-ChIP Assays—ChIP studies were performed as described earlier (15, 16) with modifications.** All of the steps were performed at 4 °C. ROS17/2.8 cell cultures (100-mm diameter plates) were incubated for 10 min with 1% formaldehyde and gentle agitation. The cross-linking was stopped by the addition of 0.125 M glycine for 5 min. The cells were then washed with 10 ml of PBS, scraped off in the same volume of PBS, and collected by centrifugation at 1,000 g for 5 min. The cell pellet was resuspended in 3 ml of lysis buffer (50 mM Hepes, pH 7.8, 20 mM KCl, 3 mM MgCl2, 0.1% Nonidet P-40, and 0.1% deoxycholic acid), and incubated for 10 min on ice. The cell extract was collected by centrifugation at 1,000 g for 5 min, resuspended in 3.0 ml of sonication buffer (50 mM Hepes, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate acid, 0.1% SDS, and a mixture of proteinase inhibitors), and incubated for 10 min on ice. To reduce the length of the chromatin fragments to −500 bp or smaller (confirmed by electrophoretic analysis and PCR), the extract was sonicated with a Misonix sonicator (model 3000), using 10 15-s pulses at 30% power. After centrifugation at 16,000 g, the supernatant was collected, frozen in liquid nitrogen, and kept at −80 °C. An aliquot was used for A_{260} measurements. Cross-linked extracts (10 A_{260} units) were resuspended in sonication buffer to a final volume of 500 μl. The samples were precleared by incubation with 30 μl of protein A/G-agarose beads preblocked with bovine serum albumin (Santa Cruz Biotechnology) for 15 min at 4 °C with agitation. After centrifugation at 1,000 × g for 5 min, the supernatant was collected and immunoprecipitated with either an anti-FLAG antibody (Sigma), an anti-VDR monoclonal antibody (Merck), anti-RXRα polyclonal antibody (Santa Cruz Biotechnology), anti-C/EBPβ polyclonal antibody (Santa Cruz Biotechnology), anti-YY1 polyclonal antibody (Santa Cruz Biotechnology), anti-Runx2 polyclonal antibody (Santa Cruz Biotechnology), anti-RXRα polyclonal antibody (Santa Cruz Biotechnology), anti-BRG1 polyclonal antibody (5), an anti-BAF57 polyclonal antibody (donated by Dr. Borja Belandia), or an anti-IN1 polyclonal antibody (5). The immunocomplexes were recovered with the addition of 30 μl of protein A-agarose beads and subsequent incubation for 1 h at 4 °C with agitation. The complexes were washed twice with sonication buffer, twice with sonication buffer plus 500 mM NaCl, twice with LiCl buffer (100 mM Tris–HCl, pH 8.0, 500 mM LiCl, 0.1% Nonidet P-40, and 0.1% deoxycholic acid), and twice with dialysis buffer (2 mM EDTA and 50 mM Tris–HCl, pH 8.0), with the solution incubated at each washing for 5 min at 4 °C. The protein-DNA complexes were then eluted by incubation with 100 μl of elution buffer (50 mM NaHCO₃ and 1% SDS) for 15 min at 65 °C. After centrifugation at 1,000 × g for 5 min, the supernatant was collected and incubated with 10 μg of RNase A/ml for 1 h at 37 °C. Then NaCl was added to the mixture to a final concentration of 200 mM and incubated at 65 °C to reverse the cross-linking. The proteins were then digested with 200 μg/ml of proteinase K for 2 h at 50 °C. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation using tRNA (5 μg/ml) as a precipitation carrier. The PCR conditions and primers used to evaluate the OC proximal and distal promoter regions (see Fig. 1) were reported previously (15, 16). To amplify the upstream OC promoter sequence, the following primers were used: forward, 5′-GTCCTACAGGTTAGGGGAAT-3′; reverse, 5′-GTCCCCAATCTCCCTAGACA-3′. The oligonucleotide used as a reverse primer in the amplification of the p208OC-Luc constructs was 5′-CGTATCTCTTCATAGCCCTA-3′. To amplify a histone H4 gene promoter sequence (see supplemental data), we used the following primers: forward, 5′-GATAGACACAGAGACAAAA-GAAC-3′; reverse, 5′-CCAGGCAAGAGCTTATATTCC-3′. The re-ChIP assays were performed as described earlier (15). The immunoprecipitated complexes obtained by ChIP were eluted by incubation for 30 min at 37 °C in 25 μl of 10 mM dithiothreitol. After centrifugation, the supernatant was diluted 20 times with sonication buffer and subjected to the ChIP procedure.

**RESULTS**

**BRG1-containing SWI/SNF Complexes Stimulate Basal and Vitamin D₃-enhanced Osteocalcin Promoter Activity—Chromatin remodeling accompanies basal tissue-specific and vitamin D₃-enhanced transcription of the OC gene in osteoblastic
overexpression in ROS17/2.8 cells were confirmed by Western blot analyses using antibodies that recognize the HA epitope present in this protein (Fig. 2B).

Previous reports indicated that whereas transient overexpression of wild type BRG1 can increase activity of some reporter genes, expression of BRG1 mutated in the ATP-binding domain does not, since it forms inactive SWI/SNF complexes (20, 25). To show that the effect of BRG1 on the OC promoter requires its ATPase activity and therefore formation of active SWI/SNF complexes, we transiently co-expressed the BRG1K798R mutant protein (Fig. 2B), which is unable to bind ATP (20), and determined its effect on OC promoter activity. As shown in Fig. 2, BRG1K798R functions as a dominant negative mutant, by both inhibiting OC promoter activity and preventing BRG1-dependent enhancement of this promoter (Fig. 2, lanes 5–10).

To determine whether SWI/SNF complexes interact with the OC gene promoter in ROS17/2.8 osteoblastic cells, we carried out ChIP assays, using antibodies against SWI/SNF components and specific transcriptional regulators of the OC gene. We find that BRG1, INI1, and BAF57 (all key SWI/SNF components) bind strongly to the proximal (Fig. 3, A and B) but only weakly to the distal (Fig. 3, E and F) OC promoter regions. Binding of BRG1, INI1, or BAF57 was not affected by treatment of the cells with vitamin D3 (4 h), suggesting that the interaction of SWI/SNF complexes is independent of the liganded VDR/RXR heterodimer. As described previously, Runx2, a major regulator of the OC gene in bone-derived cells, binds strongly to both proximal (Fig. 3, A and B) and distal (Fig. 3, E and F) OC promoter regions (15). Also, the transcription factor YY1 binds strongly to the distal OC promoter in the absence of vitamin D3 (Fig. 3, E and F), whereas the VDR/RXR heterodimer binds to the distal OC promoter only in cells incubated with vitamin D3 (Fig. 3, E and F). Additional controls further indicated that these protein-DNA interactions are specific, since other unrelated DNA sequences were not amplified from our immunoprecipitates (Fig. 3I; see supplemental Fig. 1S). Taken together, our results indicate that the SWI/SNF complex binds primarily to the proximal OC promoter in the osteoblastic cell line ROS17/2.8 to stimulate OC gene transcription.

Transcription of the Osteocalcin Gene in Osteoblastic Cells Requires SWI/SNF Activity—We next determined whether transcription of the endogenous OC gene in osteoblastic cells requires SWI/SNF activity. We generated ROS17/2.8 cell lines that inducibly express FLAG-tagged BRG1 mutated in the ATP binding site. It has previously been shown that these mutant forms are competent for the assembly of nonfunctional SWI/SNF complexes (5). We utilized the tetracycline-inducible expression system (22) with modifications (26) following a protocol previously described (5). ROS17/2.8 cells were stably transfected with the tetracycline-VP16 regulator and the gene encoding hygromycin resistance. Drug-resistant colonies were screened for the ability to activate a transiently transfected luciferase reporter construct under the control of tetracycline operator elements (22). A clone expressing high levels of luciferase only in the absence of tetracycline was selected for further manipulations. This cell line was then stably transfected with the gene encoding neomycin resistance and a tetracycline oper-
ator-controlled vector encoding for FLAG-tagged BRG1 mutated at the ATP-binding site. Several drug-resistant clones were selected and analyzed for expression of FLAG-tagged proteins. A time course of FLAG-tagged protein expression was carried out for each of the cell lines by incubating in the absence of tetracycline and evaluating the protein levels by Western blot with anti-FLAG antibodies. Fig. 4A shows a representative cell line that expresses increasing concentrations of the mutant BRG1 protein (ROSBRG1-TA) after removal of tetracycline. We chose day 4 after removal of tetracycline as the time point at which to perform our experiments, since expression peaked at this time. This period also provided sufficient time for the mutant proteins to be incorporated into nuclear SWI/SNF complexes (see below).

To demonstrate that the BRG1 mutant protein is incorporated into SWI/SNF complexes, we immunoprecipitated nuclear extracts isolated from ROSBRG1-TA cell lines cultured with or without tetracycline for 4 days and in the presence or absence of vitamin D₃ for 4 h (see description in the legend to Fig. 4B), using anti-FLAG antibodies bound to Sepharose beads. The precipitated material was then fractionated by SDS-PAGE, and the co-immunoprecipitated proteins were confirmed by Western blot using specific antibodies. As shown in Fig. 4B, BRG1 mutant proteins in the ROSBRG1-TA cell line are associated with key components of the SWI/SNF complex, including BAF170, BAF180, and INI1. These protein-protein interactions are detected only in cells cultured without tetracycline (Fig. 4B, lanes 3 and 4) and are independent of treatment with vitamin D₃ for 4 h (Fig. 4B, compare lanes 3 and 4). Taken together, these results indicate that the FLAG-tagged BRG1 mutant protein is inducibly expressed and is capable of forming complexes with components normally found in SWI/SNF complexes.

We next determined how the presence of inactive SWI/SNF complexes affected the expression of the endogenous OC gene in the ROS17/2.8 cell lines. As shown in Fig. 5, in the presence of tetracycline, OC mRNA is expressed in the ROSBRG1-TA cells as detected by reverse transcription-PCR using specific primers. These mRNA levels were significantly increased following treatment of the cells with vitamin D₃ (Fig. 5, compare lanes 1 and 2). However, when the cells were cultured without tetracycline, OC mRNA was detected neither in the absence nor in the presence of vitamin D₃ (Fig. 5, lanes 3 and 4), indicating that formation of inactive SWI/SNF complexes results in inhibition of endogenous OC gene transcription. This
inhibition is not a general effect on cellular transcription, since \( \beta \)-actin and genes encoding early bone phenotypic markers such as the VDR, alkaline phosphatase, and Runx2 were not affected (Fig. 5). Since the ROS17/2.8 cells exhibit a terminally differentiated osteoblast phenotype (21), this result suggests that expression of late phenotypic markers such as OC are tightly regulated in a SWI/SNF-dependent manner in these cells.

By ChIP analyses, we then evaluated whether the inactive SWI/SNF complexes were bound to the OC promoter in the ROSBRG1-TA cells. Using specific antibodies against the FLAG epitope, we found that mutant BRG1 is associated with the OC promoter (proximal and distal regions) in cells cultured without tetracycline (Fig. 6, lanes 11 and 12). The mutant protein was also detected at the OC promoter in cells grown with tetracycline (Fig. 6, lanes 9 and 10), although exhibiting a significantly reduced signal. This indicates that reduced levels of mutant BRG1 proteins are present in the ROSBRG1TA cell lines grown with tetracycline (see Fig. 4A) and that they are sufficient to interact with the OC promoter. However, this interaction does not affect significantly basal and vitamin D\(_3\)-enhanced OC gene transcription (see Fig. 5).
Similarly, other SWI/SNF subunits, such as BAF57 and INI1, were found associated with the OC promoter, confirming that the FLAG-tagged BRG1 mutant protein forms inactive SWI/SNF complexes that are bound to this promoter (Fig. 6). As we found for the endogenous wild type SWI/SNF complexes (Figs. 3 and 4), interaction of these inactive complexes with the OC promoter is not affected by treatment of the cells with vitamin D₃ for 4 h (Fig. 6, compare lanes 11 and 12). We also find that these inactive SWI/SNF complexes interact with the distal (primers −773 to −433) and proximal (primers −198 to −28) OC promoter regions (Fig. 6). To control for an efficient sonication that results in chromatin fragments with an average size of 500 bp or less, we analyzed the samples with a set of primers that can amplify the OC promoter only when the DNA segments are larger that 700 bp (Fig. 6, see primers −773 to −28). Similar to our analyses in wild type ROS17/2.8 cells, we further controlled these ChIP experiments by demonstrating that two different unrelated DNA sequences were not amplified from the immunoprecipitates (see supplemental Fig. 2S).

Taken together, these results indicate that in the cell lines expressing FLAG-tagged mutant proteins, inactive SWI/SNF complexes are formed and bound to the OC promoter. These results also suggest that this interaction is responsible for the inhibition of OC gene transcription detected in these cells.

**Binding of Inactive SWI/SNF Complexes Results in Inhibition of the Chromatin-remodeling Events That Accompany Transcription of the Osteocalcin Gene**—We assessed whether interaction of inactive SWI/SNF complexes with OC promoter regions affected the chromatin-remodeling events that accompany transcriptional activity of the OC gene in osteoblastic cells (10). Previous studies have established that changes in chromatin organization at the OC gene promoter can be monitored by restriction endonuclease accessibility in intact nuclei (10, 24). We then compared sensitivity to DraIII, BglII, and PstI enzymes, which cut in the promoter at functionally strategic positions (see Fig. 7A), exhibit enzymatic activities that are not affected by DNA methylation and share similar optimal digestion buffer conditions.

In agreement with previous reports (10, 24), we found that all three cleavage sites were accessible in wild type ROS17/2.8 cells and that this accessibility was enhanced upon vitamin D₃ treatment for 4 h (Fig. 7B, see percentage of digestion below lanes 3 and 4). In ROSBRG1TA cells grown with tetracycline, the restriction sites PstI and BglII, which are located at the proximal and intermediate promoter regions of the OC promoter, respectively, show similar accessibility as in wild type cells. The distal DraIII site, however, shows reduced cleavage (Fig. 7B, top, lanes 5 and 6), indicating that the chromatin conformation of this region of the promoter may be slightly affected by the low levels of BRG1 mutant protein present in the ROSBRG1TA cells grown in tetracycline (leaking expression; see Fig. 4A). However, this reduced accessibility does not affect OC transcription (see Fig. 5, lanes 1 and 2).

When the ROSBRG1TA cells grown without tetracycline were analyzed, it was found that all the restriction sites exhibited decreased accessibility, indicating that the entire OC promoter adopted a closed chromatin conformation (Fig. 7B, lane 7). Treatment with vitamin D₃ for 4 h did not reverse this closed chromatin conformation (Fig. 7B, lane 8). Together, these results indicate that in ROSBRG1TA cells cultured in the absence of tetracycline, binding of inactive SWI/SNF complexes to the OC promoter blocks the chromatin-remodeling
events that accompany basal and vitamin D₃-enhanced OC transcriptional activity in osteoblastic cells.

We next determined whether the reduced accessibility at the OC gene promoter prevents the binding of key transcription factors to its cognate regulatory elements. This analysis was carried out by ChIP assays on chromatin samples isolated from ROSBRG1TA cells, which were cultured with or without tetracycline and in the presence or absence of vitamin D₃ (Fig. 8, see explanation below each panel). As shown in Fig. 8A, the transcription factors Runx2 and YY1 bind to the distal OC promoter independently of the condition in which the ROSBRG1TA cells have been grown. This result indicates that although these cells exhibit reduced accessibility in this region of the promoter (Fig. 7B), binding of these two key regulators is not significantly impaired. On the other hand, the VDR/RXR heterodimer binds to the VDRE within the distal OC promoter only in ROSBRG1TA cells incubated with vitamin D₃ and cultured with tetracycline (Fig. 8, A and B). As VDR/RXR is unable to interact with the VDRE in ROSBRG1TA cells expressing mutant BRG1 (Fig. 8A), these results confirm previous reports indicating that a closed chromatin conformation in the OC promoter prevents interaction of the VDR complex upon vitamin D₃ stimulation (24, 27).

It was also determined that the Runx2 and C/EBPβ transcription factors are bound to the proximal OC promoter independently of the condition in which the ROSBRG1TA cells are grown (Fig. 8, C and D). These two factors have been reported as the two key regulators of basal tissue-specific transcription of the OC gene in osteoblasts (12, 14, 17). Therefore, our results indicate that binding of Runx2 and C/EBPβ occurs prior to completion of the chromatin remodeling and may represent one of the initial events leading to chromatin remodeling and transcriptional activation.

Previous reports have demonstrated that in the absence of SWI/SNF activity loading of the RNA polymerase II basal transcription machinery onto a promoter is significantly reduced (7, 28). Similarly, we found that in ROSBRG1TA cells grown in the absence of tetracycline RNA polymerase II and TFIIB, both key components of the RNA
polymerase II holoenzyme are lost from the proximal OC promoter (Fig. 8, E and F). As described earlier, the specificity of these protein-DNA interactions was further evaluated by demonstrating that two unrelated DNA sequences are not amplified from our precipitated chromatin samples (Fig. 8G; see supplemental Fig. 3S). Together, our results indicate that the stable association of the RNA polymerase II transcription machinery with the OC gene promoter requires a SWI/SNF-mediated chromatin-remodeling process.

C/EBPβ Recruits SWI/SNF Complexes to the Proximal Osteocalcin Gene Promoter—C/EBPβ is a principal regulator of the OC gene as it binds to and synergizes with Runx2 to enhance basal and tissue-specific OC gene transcription (17). C/EBPβ has been shown to interact with chromatin-remodeling complexes, such as those including HAT activity (p300-p/CAF) or ATP-dependent activities (SWI/SNF) (18, 19). Hence, C/EBPβ has been proposed to recruit these chromatin-remodeling complexes to tissue-specific gene promoters, leading to transcriptional activation during cell differentiation.

We determined whether binding of C/EBPβ to the proximal OC promoter region contributes to BRG1-dependent enhancement of the OC promoter activity by assessing the effect of forced expression of BRG1 on the activity of a proximal (−208 bp) OC promoter-luciferase reporter gene construct (p208OC-LUC) in ROS17/2.8 cells. This OC promoter construct includes binding sites that are recognized by C/EBPβ (−106 to −99; see Fig. 1) and Runx2 (site C, −138 to −130; see Fig. 1) and has been shown to be sufficient to drive bone-specific expression (17). Our results indicate that BRG1 enhances the OC promoter activity in a dose-dependent manner (Fig. 9A). In agreement with previous reports (15), we also found that the overexpression of either C/EBPβ or Runx2 stimulates the proximal OC promoter activity (Fig. 9A). Interestingly, BRG1-mediated enhancement of the proximal OC promoter requires an intact C/EBP site. Thus, an equivalent OC promoter construct including a mutated C/EBP site is not stimulated by BRG1 overexpression (Fig. 9B). In addition, Runx2-mediated enhancement of the OC proximal promoter is prevented when the C/EBP site is mutated, a result that is in agreement with previous reports (17). Together, these results indicate that binding of C/EBPβ to the proximal OC promoter is required for the stimulatory effect of SWI/SNF.

By combining co-immunoprecipitation and Western blot analyses, we next determined whether in ROSBRG1TA cells grown without tetracycline for 4 days and then exposed to vehicle (−) or 10−8 M vitamin D3 (+) for 4 h (see below the blots for an explanation), A, nuclear extracts were precipitated with anti-FLAG antibodies (lanes 4 and 5) or with nonspecific mouse IgG (lanes 2 and 3). Co-immunoprecipitated proteins were analyzed with anti-C/EBPβ or anti-Runx2 polyclonal antibodies. B, nuclear extracts were precipitated with anti-C/EBPβ (lanes 4 and 5) or nonspecific rabbit IgG (lanes 2 and 3), and the co-precipitated FLAG-tagged BRG1 protein was detected using an anti-FLAG antibody. The antibodies used to reveal the proteins in the immunoprecipitate are indicated at the right of each blot. The antibodies used to immunoprecipitate are marked at the top of each blot.

We determined whether binding of C/EBPβ to the proximal OC promoter region contributes to BRG1-dependent enhancement of the OC promoter activity by assessing the effect of forced expression of BRG1 on the activity of a proximal (−208 bp) OC promoter-luciferase reporter gene construct (p208OC-LUC) in ROS17/2.8 cells. This OC promoter construct includes binding sites that are recognized by C/EBPβ (−106 to −99; see Fig. 1) and Runx2 (site C, −138 to −130; see Fig. 1) and has been shown to be sufficient to drive bone-specific expression (17). Our results indicate that BRG1 enhances the OC promoter activity in a dose-dependent manner (Fig. 9A). In agreement with previous reports (15), we also found that the overexpression of either C/EBPβ or Runx2 stimulates the proximal OC promoter activity (Fig. 9A). Interestingly, BRG1-mediated enhancement of the proximal OC promoter requires an intact C/EBP site. Thus, an equivalent OC promoter construct including a mutated C/EBP site is not stimulated by BRG1 overexpression (Fig. 9B). In addition, Runx2-mediated enhancement of the OC proximal promoter is prevented when the C/EBP site is mutated, a result that is in agreement with previous reports (17). Together, these results indicate that binding of C/EBPβ to the proximal OC promoter is required for the stimulatory effect of SWI/SNF.

By combining co-immunoprecipitation and Western blot analyses, we next determined whether in ROSBRG1TA cells grown without tetracycline, both BRG1 and C/EBPβ are components of the same nuclear complexes. Co-immunoprecipitation analyses were performed using nuclear extracts isolated from ROSBRG1TA cells grown without tetracycline for 4 days and then exposed to vehicle (−) or 10−8 M vitamin D3 (+) for 4 h (see below the blots for an explanation). A, nuclear extracts were precipitated with anti-FLAG antibodies (lanes 4 and 5) or with nonspecific mouse IgG (lanes 2 and 3). Co-immunoprecipitated proteins were analyzed with anti-C/EBPβ or anti-Runx2 polyclonal antibodies. B, nuclear extracts were precipitated with anti-C/EBPβ (lanes 4 and 5) or nonspecific rabbit IgG (lanes 2 and 3), and the co-precipitated FLAG-tagged BRG1 protein was detected using an anti-FLAG antibody. The antibodies used to reveal the proteins in the immunoprecipitate are indicated at the right of each blot. The antibodies used to immunoprecipitate are marked at the top of each blot.
This immunoprecipitation was specific, since FLAG-tagged BRG1 is not detected when a nonspecific mouse IgG is used in the reaction (Fig. 10A, top, lanes 2 and 3). More interestingly, C/EBPβ is present in the immunoprecipitated material (Fig. 10A, middle, lanes 4 and 5), strongly indicating that in these osteoblastic cells BRG1 and C/EBPβ are components of the same nuclear complex. In contrast, the bone-specific transcription factor Runx2 was not detected in the immunoprecipitate (Fig. 10A, bottom, lanes 4 and 5). As a necessary control, we repeated the co-immunoprecipitation but changed the order of the antibodies. Fig. 10B shows that when we specifically immunoprecipitated C/EBPβ from the nuclear extracts (Fig. 10B, top, lanes 4 and 5) FLAG-tagged mutant BRG1 is detected in the precipitate (Fig. 10B, bottom, lanes 4 and 5), further demonstrating the interaction of these two proteins within the nuclei of osteoblastic cells.

It was important to directly demonstrate that recruitment of BRG1-containing SWI/SNF activity to the OC promoter requires binding of the C/EBPβ to this promoter. Therefore, we transiently transfected the p208OC-LUC and p208OC-LUC-C/EBPm constructs (previously analyzed in Fig. 9) into wild type ROS17/2.8 cells and determined the binding of Runx2, C/EBPβ, and BRG1 by ChIP assays (Fig. 11). As expected, all three proteins interacted with the wild type proximal OC promoter region (Fig. 11, B and C) that contained intact binding sites for both Runx2 and C/EBPβ (p208OC-LUC; Fig. 11A). However, neither C/EBPβ nor BRG1 were associated with this promoter region (Fig. 11, B and C) when the C/EBPβ site was mutated (p208OC-LUC-C/EBPm; Fig. 11A), indicating that interaction of BRG1 with the proximal OC promoter necessitates binding of C/EBPβ. Interestingly, Runx2 was found associated with both wild type and mutant OC promoter constructs exhibiting similar levels of binding (Fig. 11, B and C). This result indicates that Runx2 binds to the OC promoter independent of C/EBPβ, although it requires bound C/EBPβ to stimulate basal OC promoter activity (Fig. 9B).

We next evaluated by ChIP analyses whether BRG1 and C/EBPβ proteins are bound simultaneously to the proximal OC promoter in intact wild type osteoblastic cells expressing the gene. We performed two sequential immunoprecipitations of the cross-linked ROS17/2.8 sonicated cell extracts with anti-BRG1 and anti-C/EBPβ antibodies (Fig. 11A, lane 4) and the collected samples were subsequently re-precipitated with anti BRG1 antibodies (Fig. 12A, lane 8), we found that the proximal OC promoter was present in the precipitate. This result indicates that both proteins are bound simultaneously to this region of the promoter. A similar result was obtained when the cell extracts were first immunoprecipitated with anti BRG1 antibodies (Fig. 12A, lane 5) and then reprecipitated with anti C/EBPβ antibodies (Fig. 12A, lane 9). Interestingly, when the chromatin extracts were sequentially immunoprecipitated with anti-Runx2 and anti-BRG1 antibodies (Fig. 12A, lane 7), the OC promoter was also detected in the collected material. This result indicates that although Runx2 and BRG1 do not form stable complexes that can be detected by co-immunoprecipitation in nuclear extracts of ROS17/2.8 cells (Fig. 10A, bottom) both proteins (and associated co-factors) are bound to the proximal OC promoter simultaneously. These interactions however, only occur within the context of the proximal OC promoter, since the distal OC promoter region that is also recognized by Runx2 was not detected in this precipitate (not shown). Whether Runx2 and BRG1 proteins, whereas bound to the OC promoter, are part of a common multisubunit complex or are components of distinct complexes remains to be established.

FIGURE 11. Recruitment of BRG1 to the proximal OC promoter requires binding of C/EBPβ. A, schematic representation of the p208OC-Luc or p208OC-Luc-C/EBPm constructs containing the proximal OC promoter. The position of the Runx2, C/EBPβ, and TATA elements is indicated. The set of primers utilized in the PCR amplifications is shown below. The mutated C/EBPβ site is marked with an X. B, ROS17/2.8 cells were transfected with either the p208OC-Luc or the p208OC-Luc-C/EBPm construct (indicated below), and the binding of Runx2, C/EBPβ, and BRG1 (indicated at the top) was analyzed by ChIP (upper panel). IgG corresponds to nonspecific immunoglobulin G. The PCR amplifications were carried out using an OC promoter-specific primer (−198) and a primer directed against the luciferase reporter gene (+99). An input sample obtained from untransfected ROS17/2.8 cells was also included as negative control for specific amplification with this set of primers. As a required negative control, each immunoprecipitate was also amplified with a set of primers directed against a sequence upstream (−5,972 to −5,666) of the OC promoter (lower panel). C, quantification of the ChIP using a molecular imager.
SWI/SNF Activity in Osteoblast-specific Transcription

FIGURE 12. BRG1 and C/EBPβ proteins interact within the context of the OC promoter in osteoblastic cells expressing OC. A, double ChIP (re-ChIP) experiments were carried out as described previously (15). Lane 1, input; lane 2, ChIP with nonspecific IgG; lane 3, ChIP with anti-Runx2; lane 4, ChIP with anti-C/EBPβ; lane 5, ChIP with anti-BRG1; lane 6, ChIP and re-ChIP with a nonspecific IgG, lane 7, ChIP with anti-Runx2 and re-ChIP against BRG1; lane 8, ChIP with anti-C/EBPβ and re-ChIP against BRG1; lane 9, ChIP with anti-BRG1 and re-ChIP against C/EBPβ. B, quantification of the ChIP analyses shown in A (lanes 1–5). C, quantification of the re-ChIP shown in A (lanes 6–9). Here, the band intensities were normalized to the corresponding input sample. As a required negative control, each immunoprecipitate was also amplified with a set of primers directed against an upstream sequence (−5,972 to −5,666; see Fig. 1) of the OC promoter (A, lower panel). Additional controls for these experiments are included as supplemental data (Fig. 45).

Similar to our previous ChIP experiments, the specificity of these protein-DNA interactions was further controlled by demonstrating that these factors do not interact with either a region far upstream of the OC gene promoter (Fig. 12A, bottom) or with a sequence within a histone H4 gene promoter (see supplemental Fig. 45).

Taken together, our results indicate that binding C/EBPβ to its cognate element in the proximal OC promoter region is required for the subsequent recruitment of SWI/SNF activity. The association of SWI/SNF with this promoter results in chromatin remodeling and transcriptional activation of the OC gene.

DISCUSSION

Here, we show that SWI/SNF-mediated chromatin-remodeling activity is required for the changes in chromatin structure that accompany transcription of the bone-specific OC gene in osteoblastic cells. We find that the SWI/SNF complex is associated with the proximal OC promoter region and up-regulates OC promoter activity. In addition, we generated stably transfected osteoblastic cell lines that inducibly express FLAG-tagged BRG1 mutant proteins that form inactive SWI/SNF complexes. These inactive complexes bind to the OC gene promoter and inhibit basal and vitamin D₃-enhanced OC transcription by preventing the chromatin-remodeling events that are required for OC expression in differentiated osteoblastic cells. Transient overexpression experiments as well as double chromatin immunoprecipitation analyses (re-ChIP), demonstrate that in osteoblastic cells the SWI/SNF complex is recruited to the OC promoter by the transcription factor C/EBPβ.

SWI/SNF components have been shown to interact with several transcriptional activators, including nuclear steroid receptors (25, 29, 30, 31), human heat shock factor 1 (32), EKLF (33), c-Myc (34), C/EBPβ (19), and C/EBPα (35). However, only few transcription factors have been demonstrated to target the SWI/SNF complex to specific cellular promoters (2, 3).

We have previously demonstrated that the proximal OC promoter region contains the regulatory elements that allow basal tissue-specific transcription of this gene and the formation of the proximal nuclease-hypersensitive site (17, 24, 36). C/EBPβ binds to this proximal OC promoter region and up-regulates OC expression (17). Therefore, while associated with the SWI/SNF complex, C/EBPβ is also facilitating the changes in chromatin structure that are required for OC transcription. C/EBPβ has been also demonstrated to functionally interact with Runx2 (17), which in turn binds to another chromatin-remodeling complex, the HAT complex p300-CAF that also is recruited to the OC promoter (16). Recently, we have shown that transcriptional activation of the OC gene during normal diploid osteoblast differentiation involves increased acetylation of core histones bound to the OC promoter (37). Together these results indicate that HAT activity may also have a principal role in the chromatin-remodeling events associated with transcription of the OC gene, although in light of the results shown here, it appears as insufficient to bring the remodeling process to completion. Therefore, we hypothesize that binding of C/EBPβ and Runx2 to the proximal promoter region of the OC gene in differentiated osteoblastic cells allows the recruitment of both SWI/SNF and HAT-containing chromatin-remodeling activities and that together these activities catalyze the changes in chromatin structure that facilitate transcription. In support of this proposition, other groups have previously reported that both SWI/SNF and p300-containing chromatin-remodeling complexes collaborate in mediating the changes in chromatin structure that lead to transcriptional activation in mammalian cells (29, 38).

Interestingly, we found that both C/EBPβ and Runx2 are bound to the OC promoter in osteoblastic cells that are not expressing the OC gene in the absence of SWI/SNF-mediated chromatin remodeling. This indicates that interaction of both factors with the OC promoter is independent of SWI/SNF activity and therefore prior to the formation of the proximal DNase I-hypersensitive site. In support of this conclusion, we have previously demonstrated that Runx factors are capable of recognizing their cognate elements within the context of a nucleosomal organization (27, 39) and independent of the level of core histone acetylation. Together these results indicate that binding of Runx2 and C/EBPβ is one of the early events

4 J. Gutierrez, R. Paredes, J. Olate, A. J. van Wijnen, G. S. Stein, J. B. Lian, J. L. Stein, A. N. Imbalzano, and M. Montecino, unpublished results.
SWI/SNF Activity in Osteoblast-specific Transcription

during chromatin remodeling and transcriptional activation of the OC gene.

It has been established that transcription in eukaryotic cells requires SWI/SNF activity (2). Recent reports indicate that SWI/SNF-mediated chromatin remodeling is necessary for the loading of the RNA polymerase II holoenzyme onto the interferon-β (28) and peroxisome proliferator-activated receptor γ (7) promoters. On the other hand, the SWI/SNF activity is required for transcriptional initiation at the α1-anti-trypsin gene. Hence, in this particular promoter, chromatin remodeling is a defining step of the transcription initiation process, acting after the assembly of the polymerase II machinery (40). We find that recruitment of functional SWI/SNF at the proximal OC promoter region is required for a stable interaction of the RNA polymerase II complex. Therefore, the lack of basal OC gene transcription in osteoblastic cells expressing inactive SWI/SNF can be due to the inability of the RNA polymerase II complex to bind to the OC promoter and initiate transcription.

In summary, our results show for the first time that transcription of a bone-specific gene that is expressed at late stages of osteoblast differentiation involves an active chromatin-remodeling process mediated by ATP-dependent SWI/SNF activity that is specifically recruited to the proximal promoter region by C/EBPβ.

Acknowledgments—We especially thank Dr. Arnold Berk and his group for the technical support in pursuing the ChIP experiments. We also thank Dr. Borja Belandia for the generous gift of the anti-BAF57 antibody and Dr. Weidong Wang for kindly providing the anti-BAF180 antibody.

REFERENCES

1. Becker, P., and W. Hörz. (2002) Annu. Rev. Biochem. 71, 247–273
2. Narlikar, G., Fan, H.-Y., and Kingston, R. (2002) Cell 108, 475–487
3. Peterson, C., and Workman, J. (2000) Curr. Opin. Genet. Dev. 10, 187–192
4. De la Serna, I., Carlson, K., and Imbalzano, A. N. (2001) Nat. Genet. 27, 187–190
5. De la Serna, I., Carlson, K., Hill, D., Guido, C., Stephenson, R., Sif, S., Kingston, R., and Imbalzano, A. N. (2000) Mol. Cell. Biol. 20, 2839–2851
6. De la Serna, I., Roy, K., Carlson, K., and Imbalzano, A. N. (2001) J. Biol. Chem. 276, 41486–41491
7. Salma, N., Xiao, H., Mueller, E., Imbalzano, A. N. (2004) Mol. Cell. Biol. 24, 4651–4663
8. Young, D., Pratrap, J., Javed, A., Weiner, B., Okhawa, Y., van Wijnen, A., Montecino, M., Stein, G., Lian, J., and Stein, J. (2005) J. Cell. Biochem. 94, 720–730
9. Lian, J., Stein, J., Stein, G., Montecino, M., van Wijnen, A., Javed, A., and Gutierrez, S. (2001) Steroids 66, 159–170
10. Montecino, M., Lian, J., Stein, G., and Stein, J. (1996) Biochemistry 35, 5093–5102
11. Banerjee, C., McCabe, L., Choi, J., Hiebert, S., Stein, J., Stein, G., and Lian, I. (1997) J. Cell. Biochem. 66, 1–8
12. Ducy, P., Zhang, R., Geoffroy, V., Ridall, A., and Karsenty, G. (1997) Cell 89, 747–754
13. Gutierrez, S., Liu, J., Javed, A., Montecino, M., Stein, G., Lian, J., and Stein, J. (2004) J. Biol. Chem. 279, 43581–43588
14. Javed, A., Gutierrez, S., Montecino, M., van Wijnen, A., Stein, J., Stein, G., and Lian, J. (1999) Mol. Cell. Biol. 19, 7491–7500
15. Paredes, R., Arriagada, G., Cruzat, F., Villagra, A., Olate, J., Zaidi, K., van Wijnen, A., Lian, J., Stein, G., Stein, J., and Montecino, M. (2004) Mol. Cell. Biol. 24, 8847–8861
16. Sierra, J., Villagra, A., Paredes, R., Cruzat, F., Gutierrez, S., Javed, A., Arriagada, G., Olate, J., Imschenetzky, M., van Wijnen, A., Lian, J., Stein, G., Stein, J., and Montecino, M. (2005) Mol. Cell. Biol. 25, 3339–3351
17. Gutierrez, S., Javed, A., Tennant, D., van Rees, M., Montecino, M., Stein, G., Stein, J., and Lian, J. (2002) J. Biol. Chem. 277, 1316–1323
18. Kitabayashi, I., Yokoyama, A., Shimizu, K., and Ohki, M. (1998) EMBO J. 17, 2994–3004
19. Kowenz-Leutz, E., and Leutz, A. (1999) Mol. Cell 4, 735–743
20. Khavari, P., Peterson, C., Tamkun, J., and Crabtree, G. (1993) Nature 366, 170–174
21. Majeska, R., Rodan, S., and Rodan, G. (1980) Endocrinology 107, 1494–1503
22. Gossen, M., and Bujard, H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5547–5551
23. Dignam, J., Lebovitz, R., and Roeder, R. (1983) Nucleic Acids Res. 11, 1475–1489
24. Montecino, M., Frenkel, B., van Wijnen, A., Lian, J., Stein, G., and Stein, J. (1999) Biochemistry 38, 1338–1345
25. Muchardt, C., and Yaniv, M. (1993) EMBO J. 12, 4279–4290
26. Shockett, P., Difilippantonio, M., Hellman, N., and Schatz, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6522–6526
27. Paredes, R., Gutierrez, J., Gutierrez, S., Allison, L., Puchi, M., Imschenetzky, M., van Wijnen, A., Lian, J., Stein, G., Stein, J., and Montecino, M. (2002) Biochem. J. 363, 667–676
28. Lomvardas, S., and Thanos, D. (2001) Cell 106, 685–696
29. Fryer, C., and Archer, T. (1998) Nature 393, 88–91
30. Ichinose, H., Garnier, J., Chambon, P., and Losson, R. (1997) Gene (Amst.) 188, 95–100
31. Ostlund-Farrants, A., Bromquist, P., Kwon, H., and Wrangle, O. (1997) Mol. Cell. Biol. 17, 895–905
32. Sullivan, E., Weirich, C., Guyon, J., Sif, S., and Kingston, R. (2001) Mol. Cell. Biol. 21, 5826–5837
33. Armstrong, J., Bieker, J., and Emerson, B. (1998) Cell 95, 93–104
34. Cheng, S., Davis, K., Belaran, R., Yu, J., and Kalpana, G. (1999) Nat. Genet. 22, 102–105
35. Pedersen, T., Kowenz-Leutz, E., Leutz, A., and Merlov, C. (2001) Genes Dev. 15, 208–216
36. Montecino, M., Frenkel, B., Stein, J., and Stein, G. (1996) J. Cell. Biol. 63, 221–228
37. Shen, J., Hovhannisyan, H., Lian, J., Montecino, M., Stein, G., Stein, I., and van Wijnen, A. (2003) Mol. Endocrinol. 17, 743–756
38. Dilworth, F., Fromental-Ramain, C., Yamamoto, K., and Chambon, P. (2000) Mol. Cell 6, 1049–1058
39. Gutierrez, J., Sierra, J., Medina, R., Puchi, M., Imschenetzky, M., van Wijnen, A., Lian, J., Stein, G., Stein, J., and Montecino, M. (2000) Biochemistry 39, 13565–13574
40. Soutoglou, E., and Talianidis, I. (2002) Science 295, 1901–1904