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# SWI/SNF-INDEPENDENT NUCLEASE HYPERSENSITIVITY AND INCREASED HISTONE ACETYLATION AT THE P1 PROMOTER ACCOMPANY ACTIVE TRANSCRIPTION OF THE BONE MASTER GENE RUNX2

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## Abstract

The Runx2 transcription factor is essential for skeletal development as it regulates expression of several key bone-related genes. Multiple lines of evidence indicate that expression of the Runx2/p57 isoform in osteoblasts is controlled by the distal P1 promoter. Alterations of chromatin structure are often associated with transcription and can be mediated by members of the SWI/SNF family of chromatin remodeling complexes, or by transcriptional co-activators that possess enzymatic activities that covalently modify structural components of the chromatin. Here, we report that a specific chromatin remodeling process at the proximal region (−400 to +35) of the Runx2 gene P1 promoter accompanies transcriptional activity in osteoblasts. This altered chromatin organization is reflected by the presence of two DNase I hypersensitive sites that span key regulatory elements for Runx2/p57 transcription. Chromatin remodeling and transcription of the Runx2 gene are associated with elevated levels of histone acetylation at the P1 promoter region and binding of active RNA polymerase II, and are independent of the activity of the SWI/SNF chromatin remodeling complex. Changes in chromatin organization at the P1 promoter are stimulated during differentiation of C2C12 mesenchymal cells to the osteoblastic lineage by treatment with BMP2. Together, our results support a model in which changes in chromatin organization occur at very early stages of mesenchymal differentiation to facilitate subsequent expression of the Runx2/p57 isoform in osteoblastic cells.

The Runx2 transcription factor is essential for skeletal formation as it regulates the expression of numerous key bone-related genes (1,2). Elimination of the Runx2 gene causes developmental defects in osteogenesis (3), and hereditary mutations in this gene in humans are linked to specific ossification defects, as observed in Cleidocranial Dysplasia (4). The Runx2 proteins are expressed in early mesenchyme of developing skeletal tissues (embryonic age E9.5) (5,6). Expression of the bone-related Runx2/p57 protein is controlled by the P1 upstream promoter, which contains regulatory elements that are recognized by several transcription factors to either activate or repress expression (see Figure 1). Among these factors are the homeodomain factors Msx2, CDP/cut, Dlx3 and Dlx5 (7,8), β-catenin/TCF (9), Hoxa10 (10), AP-1 (11), Nkx3.2 (5), and Runx2 (12).

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Also essential for commitment and differentiation of mesenchymal cells to the osteoblast lineage during bone formation are the BMP2/4/7 members of the TGFβ superfamily of signaling factors (13,14). BMP2 can block differentiation of mesenchymal cells into mature muscle cells by suppressing the master control genes for myoblast differentiation (15). At the same time, the expression of bone-phenotypic genes, including Runx2, alkaline phosphatase and osteocalcin, is induced by treatment with BMP2 (13,16). The BMP signal is transduced through binding to the heterodimeric type I and type II receptors and leads to the formation of activated Smad complexes that are translocated to the nucleus to regulate target genes (17).

It is well established that gene expression is usually accompanied by alterations in chromatin organization, as evidenced by increased nuclease hypersensitivity at specific promoter and enhancer elements (18,19,20). In the last decade a number of nuclear complexes with the ability to remodel chromatin and facilitate gene transcription have been described (19). Among them is the SWI/SNF protein complex that promotes transcription by altering chromatin structure in an ATP-dependent manner (18,21,22,23). SWI/SNF is composed of several sub-units and has been implicated in a wide range of cellular events, including gene regulation, cell cycle control, development and differentiation (19,21,22,23). The mammalian SWI/SNF complexes contain a catalytic subunit that can be either BRG1 or BRM, each of 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 (24,25,26). 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 (24) and to differentiate into muscle or adipocytic cells (24,25,26,27). 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 (28). Similarly, expression of mutant BRG1 in osteoblastic cells exhibiting a differentiated phenotype inhibits the expression of genes associated with this terminally differentiated stage (29).

Post-translational covalent modifications of histones play a major role in regulating chromatin structure and gene transcription (19,30,31,32). These modifications may alter chromatin organization by modulating intranucleosomal and/or internucleosomal histone-DNA interactions. In addition, these posttranslational modifications can provide specific docking domains on the nucleosomal surface that can be recognized by proteins that both further modify chromatin structure and regulate transcription (19,30,31,32). In particular, increased histone H3 and H4 acetylation has been found to parallel transcriptional activation of bone-specific genes during osteoblast differentiation (33,34). Similarly, suppression of histone deacetylase (HDAC) activity during osteoblast differentiation by either HDAC inhibitors or knock down of HDAC1 via siRNA, stimulated osteoblast differentiation with osteogenic gene expression and induced cell cycle arrest (34). During osteogenesis ex vivo, total HDAC activity is decreased together with significant reduction in HDAC1 expression. Consistently, recruitment of HDAC1 to promoters of osteoblast-related genes, including osteocalcin and osterix, was down-regulated, whereas H3 and H4 acetylation was increased (34).

Because of the key regulatory role of Runx2 during bone-tissue formation, there is a necessity to understand the mechanisms that control chromatin remodeling and transcriptional activation of the Runx2 gene. Here, we report that a specific chromatin remodeling process at the P1 promoter region of the Runx2 gene accompanies transcriptional activity of this gene in osteoblastic cells. This chromatin reorganization involves increased histone H3 and H4 acetylation and is independent of SWI/SNF activity.

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# EXPERIMENTAL PROCEDURES

## Expression constructs

The construct pTSCeBRG1K-R coding for flag-tagged BRG1 carrying a mutation in the ATP-binding site under the control of the tetracycline-inducible promoter system was reported previously (29).

## Cell culture

ROS 17/2.8 osteoblastic cells were cultured as described previously (35). C2C12 cells were grown in Dulbecco's modified Eagle medium with 10% fetal calf serum as described elsewhere (16). Treatments of proliferating C2C12 cells with 300 ng/ml BMP2 (R&D BioSystem, Minneapolis, MN) were carried out for 48 hrs. The ROSBRG1TA cell lines were generated and characterized previously (29). The cells were maintained in 50 μg/ml hygromycin, 100 μg/ml Geneticin, and 10 μg/ml tetracycline. ROSBRG1TA cells were evaluated for their ability to express Flag-tagged mutant BRG1 proteins by Western blot using anti-Flag antibody (Sigma). Expression of the endogenous genes osteocalcin, Runx2, MyoD and β-actin was determined by reverse transcription-PCR using the following specific primers: Osteocalcin (forward, 5′-CAGACCTAGCAGACACCATAGG-3′; reverse, 5′-CGTCCATACTTTCGAGGCAG-3′), Runx2 (forward, 5′-CCGTGTCAGCAAAGCTTCTT-3′; reverse 5′-ACAGGAAGTTGGGACTGTCG-3′), MyoD (forward, 5′-GCAGGCTCTGCTGCGCGACC-3′; reverse, 5′-TGCAGTCGATCT CTCTCAAAGCA-3′), β-actin (forward, 5′-GCTCGTCGTCGACAACGGCTC-3′; reverse, 5′-CAAACATGATCTGGGTCATCTTCT-3′).

## Western blotting

Nuclear extracts were prepared as reported earlier (36) from wild type ROS17/2.8 or ROSBRG1TA cells lines cultured with or without tetracycline and from C2C12 cells cultured in the presence or absence of 300 ng/ml BMP2 for 48 h. Proteins were detected by Western blot using specific antibodies. Anti-Runx2 and TFIIB antibodies were purchased from Santa Cruz Biotechnology.

## DNase I hypersensitive assays

DNase I hypersensitivity analyses were performed as described before (37). Nuclei isolated from wild type ROS17/2.8 cells, ROSBRG1TA and C2C12 cells, were incubated with increasing concentrations of DNase I, for 10 min at 37 °C. The genomic DNA was then purified and completely cleaved with either *EcoRI* (for samples from ROS 17/2.8 and ROSBRG1TA cells) or *Bgl II* (for samples from C2C12 cells). The samples were then electrophoresed in 1.2 or 2% (w/v) agarose gels, blotted, and hybridized with specific probes generated by PCR.

## Chromatin immunoprecipitation (ChIP)

ChIP studies were performed as described earlier (29,38) with modifications. Wild type ROS17/2.8, ROSBRG1TA and C2C12 cell cultures (100-mm diameter plates) were incubated for 10 min with 1% formaldehyde and gentle agitation at room temperature. The cross-linking was stopped by the addition of 0.125 M glycine for 5 min. The following experimental steps were performed on ice or at 4 °C. The cells were 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 $MgCl_2$, 0.1% Nonidet P-40, and a mixture of proteinase inhibitors) 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

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10 min on ice. To reduce the length of the chromatin fragments to 500 bp or smaller (confirmed by electrophoretic analysis and PCR amplification; see 29), the extract was sonicated with a Misonix sonicator (model 3000), using 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 anti-RNA polymerase II polyclonal antibody (Santa Cruz Biotechnology), anti-RNA polymerase II phospho 5 polyclonal antibody (Abcam), anti-acetyl-Histone H3 polyclonal antibody (Upstate Biotechnology) or anti-acetyl-Histone H4 polyclonal antibody (Upstate Biotechnology). 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 500mM 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 (2mM EDTA and 50 mM Tris-HCl, pH 8.0), washing each time for 5 min at 4 °C. The protein-DNA complexes were then eluted by incubation with 100 μl of elution buffer (50 mM $NaHCO_3$ 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 42 °C. NaCl was then added to the mixture to a final concentration of 200 mM and incubated at 65 °C to reverse the cross-linking. The proteins were digested with 200 μg/ml of proteinase K for 2 h at 50 °C and the DNA recovered by phenol/chloroform extraction and ethanol precipitation using glycogen (20 μg/ml) as a precipitation carrier. The PCR primers used to evaluate the mouse Runx2 P1 promoter region were: forward, 5′-GGCTCC TTCAGCATTTGTGT-3′; reverse, 5′-TAAAGTGGGACTGCCTACCA-3′. The PCR primers used to evaluate the rat RUNX2 P1 promoter region were: forward, 5-TGCTCTCCAAGTGCTTAACCT-3′; reverse, 5′-TAAAGTGGGACTGCCTACCA-3′.

## RESULTS

### Nuclease hypersensitivity at the P1 promoter accompanies transcriptional activation of the Runx2 gene

Previous reports have established that expression of the bone-related Runx2/p57 protein is controlled by the distal P1 promoter (see Figure 1; 10,11,12). Similarly, it has been described that in the presence of BMP2, the mesenchymal C2C12 cells differentiate into the osteoblastic lineage, characterized by the expression of early and late bone-related markers (13,16).

As shown in figure 2, incubation of proliferating C2C12 cells with 300 ng/ml BMP2 for 48 h results in increased osteocalcin (OC) and Runx2/p57 gene expression as measured by mRNA levels (Figure 2A). Also, this treatment produces a significant reduction in the mRNA levels of MyoD (Figure 2A), a phenotypic marker of muscle differentiation (39). Increased expression of Runx2/p57 is also shown by Western blot analysis of nuclear extracts isolated from BMP2-treated C2C12 cells, indicating that both mRNA and protein levels are elevated after incubation of these cells with BMP2 (Figure 2B).

We evaluated whether BMP2-mediated transcriptional activation of the Runx2/p57 gene involves chromatin remodeling at the P1 promoter. The alterations in chromatin organization were assessed by determining changes in DNase I hypersensitivity using the indirect end-labeling method as described in Materials and Methods. Proliferating C2C12 cells were cultured under control conditions or in the presence of BMP2 for 48 h. Nuclei were then isolated and incubated with increasing concentrations of DNase I for 10 minutes. Partially digested

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genomic DNA was purified, cleaved to completion with restriction enzymes and analyzed by Southern blot (Figure 3A and 3B).

As shown in Figure 3B, C2C12 cells cultured under control conditions exhibit two domains within the P1 promoter region that are slightly hypersensitive to DNase I activity. These hypersensitive sites (DHS) represent bona-fide remodeled domains of chromatin and not sequence-specific recognition sites for DNase I, as defined DNA sub-bands are not generated when naked genomic DNA is incubated with this nuclease (Figure 3B, right panel). These two DHS span key regulatory elements of the P1 promoter sequence as they are centered at positions −320 (DHS II) and −20 (DHS I). Interestingly, DNase I hypersensitivity in the P1 promoter region is significantly enhanced in C2C12 cells incubated with BMP2 (Figure 3B), indicating that BMP2- mediated increased transcription of the Runx2/p57 gene involves chromatin remodeling events in the P1 promoter. Complementary studies using similar approaches confirmed that there are not additional changes in chromatin accessibility occurring within a 3 kb upstream region of the P1 promoter (data not shown). Importantly, these two DNase I hypersensitive sites are also found at equivalent distance from the transcriptional start site within the Runx2 gene P1 promoter in rat-derived osteoblastic cells (ROS17/2.8) that express this gene constitutively (Figure 3C and D), as well as in rat primary normal diploid osteoblasts (ROB) differentiating in culture, which express Runx2 in a developmentally-regulated manner (not shown).

Taken together, these results indicate that modifications in the chromatin organization of the P1 promoter reflected by increased DNase I hypersensitivity, accompany the enhanced expression of the Runx2/p57 isoform.

## Transcriptional activation of the Runx2/p57 gene involves increased histone acetylation at the P1 promoter region and recruitment of active RNA polymerase II

The proximal DNase I hypersensitive site (pDHS I) of the Runx2 P1 promoter spans the TATA box and other basal regulatory elements (see Figure 1). Hence it was important to determine whether the BMP2-mediated enhancement of hypersensitivity in C2C12 cells is accompanied by changes in the binding of total and active RNA polymerase II (RNA pol II) to the P1 promoter region of the Runx2/p57 gene. Therefore, we used specific antibodies that recognize either total or CTD-phosphorylated RNA pol II and chromatin immunoprecipitation (ChIP) analyses to evaluate binding of this enzyme to the P1 promoter. As shown in Figure 4A, interaction of total RNA pol II with this promoter is significantly elevated upon BMP2 treatment of C2C12 cells. Importantly, we found increased association of RNA pol II phosphorylated in the CTD region (RNA pol II-p), further indicating that BMP2-induced expression of Runx2/p57 involves transcriptional activation of the P1 promoter. This increased recruitment of total and active RNA pol II in BMP2-treated C2C12 cells is equivalent to the levels of bound RNA pol II (total and phosphorylated) detected in ROS 17/2.8 cells that are expressing the Runx2/p57 gene constitutively (Figure 4, compare A and B). These results indicate that under our experimental conditions, treatment with BMP2 leads to transcriptional activation of the P1 promoter in C2C12 cells.

It has been established that in eukaryotes chromatin at actively transcribed genes is usually enriched in acetylated histones H3 and H4, especially in promoter and enhancer regions (19, 30,31,32). This increased acetylation promotes a more accessible chromatin conformation, thereby facilitating binding of specific and general transcription factors (18,19,31,32). Therefore we used specific antibodies that recognize and precipitate acetylated histone H3 and acetylated histone H4 to determine whether changes in the histone acetylation status at the P1 promoter region accompany Runx2/p57 gene transcription.

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As shown in figure 4C, BMP2-mediated transcriptional activation of the Runx2/p57 gene in C2C12 cells is accompanied by an increase in histone H3 and histone H4 acetylation. In agreement with these results acetylation of histones H3 and H4 associated with the P1 promoter region was also elevated in ROS 17/2.8 cells (Figure 4D). Together, these results indicate that transcriptional activity of the Runx2/p57 gene involves increased core histone acetylation at the P1 promoter region.

## Chromatin remodeling and transcriptional activity of the Runx2/p57 gene is independent of SWI/SNF activity

SWI/SNF complexes have been shown to remodel chromatin in an ATP-dependent manner, therefore contributing to tissue-specific and hormone-dependent regulation of transcription (23). Hence, we next determined whether transcription of the Runx2/p57 gene in osteoblastic cells requires SWI/SNF activity. We have previously reported the generation of ROS17/2.8 cell lines (ROSBRG1TA) that inducibly express (Tetracycline-inducible Tet-off system) flag-tagged BRG1 mutated in the ATP-binding site (Figure 5A; and also in 29). This mutant BRG1 protein is competent for assembling non-functional SWI/SNF complexes which bind to the bone-specific osteocalcin (OC) gene promoter and inhibit both chromatin remodeling and OC gene transcription (29).

As shown in Figure 5B, ROSBRG1TA cells cultured in the presence of tetracycline express high levels of mRNA coding for Runx2/p57, OC, and the control β-actin. The levels of these osteoblast-related mRNAs are comparable to those detected in wild-type ROS17/2.8 cells. As reported previously, removal of tetracycline from the cell media for 4 days results in induction of the mutant flag-tagged BRG1 (Figure 5A) and a significant reduction of OC mRNA levels (Figure 5B; and also in 29). In contrast, we find that both Runx2/p57 and β-actin mRNA levels remain unchanged in these same samples (Figure 5B). These findings have been also confirmed using real time PCR analyses (data not shown). Accordingly, we find that Runx2/p57 protein concentrations in the mutant BRG1-expressing cells are unaltered (data not shown). Together these results indicate that in contrast to the bone-phenotypic OC gene, expression of Runx2/p57 in osteoblastic cells is independent of SWI/SNF activity. This conclusion was further confirmed by using C2C12 cell lines that also express the flag-tagged mutant BRG1 in an inducible (Tet-off system) manner (see supplementary figure S1 at the “Supporting Information Available”).

We next determined whether the presence of inactive SWI/SNF complexes affects chromatin remodeling at the Runx2 P1 promoter. Nuclei isolated from ROSBRG1TA cells grown in the presence or absence of tetracycline for 4 days were incubated with DNase I and the presence of hypersensitive sites detected by the indirect end-labeling method (see Figure 3C for the restriction enzymes and specific probe used). As shown in Figure 5C, both nuclease hypersensitive domains are present within the P1 promoter region of the Runx2/p57 gene in ROSBRG1TA cells expressing inactive SWI/SNF complexes. Importantly, both hypersensitive sites span P1 promoter sequences that are the same as those found in wild type ROS 17/2.8 cells (compare Figure 3D with Figure 5C). Together, these results indicate that maintenance of a remodeled chromatin structure at the P1 promoter of the Runx2/p57 gene does not require SWI/SNF function, at least in osteoblastic ROS 17/2.8 cells that are constitutively transcribing this gene.

Using ChIP analyses it was next determined that the presence of inactive SWI/SNF complexes in ROSBRG1TA cells does not affect binding of RNA pol II (both total and CTD-phosphorylated forms) to the Runx2 gene P1 promoter (Figure 6A). Moreover, we found that BRG1-containing SWI/SNF complexes do not bind the P1 promoter region, as neither anti BRG1 nor anti flag antibody is capable of co-precipitating P1 promoter sequences (Figure 6C). Using these same chromatin samples, we determined that both wild-type and flag-tagged

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mutant Brg1 are efficiently binding to the OC gene promoter in ROSBRG1TA cells (Figure 6D), thus confirming previous reports indicating that function of the OC gene in osteoblastic cells requires SWI/SNF (29). Together, these results indicate that recruitment of the basal transcription machinery to the P1 promoter of the Runx2 gene involves a SWI/SNF-independent chromatin remodeling step.

Following the same experimental approach, we additionally determined that acetylation of histones H3 and H4 that are associated with this promoter region remains unaffected in the presence of inactive SWI/SNF complexes (Figure 6B). Moreover, ROSBRG1TA cells exhibit H3 and H4 acetylation levels at the Runx2 gene P1 promoter that are comparable to those detected in wild type ROS 17/2.8 cells (compare Figures 6B and 4D). Taken together, our results indicate that the chromatin reorganization process that accompanies transcriptional activity of the Runx2/p57 gene is independent of SWI/SNF activity and tightly associated with elevated histone acetylation.

## DISCUSSION

Changes in chromatin organization accompany transcription of eukaryotic genes (18,19). Here, we report that a specific chromatin remodeling process at the distal P1 promoter of the Runx2 gene is associated with transcriptional activity of this gene in osteoblastic cells. This alteration in chromatin structure is reflected by the presence of two DNase I hypersensitive sites that span key regulatory elements within the first 400 bp of the Runx2 P1 promoter in ROS17/2.8 osteoblastic cells that constitutively express Runx2. Importantly, strong hypersensitive sites are found in similar positions at the Runx2 P1 promoter in C2C12 mouse mesenchymal cells that have been induced to differentiate into the osteoblastic lineage by treatment with BMP2 and which express high levels of Runx2/p57. Interestingly, weaker hypersensitivity is also detectable when the C2C12 cells are cultured under control conditions, where only minimal expression of Runx2/p57 can be detected. We find that this process of chromatin reorganization involves increased histone H3 and H4 acetylation and is independent of SWI/SNF-mediated chromatin remodeling activity. DNase I hypersensitivity at the Runx2 P1 promoter is also concomitant with an increased binding of RNA polymerase II, further demonstrating the tight relationship between chromatin remodeling and active transcription of the Runx2 gene.

BMP2 signaling has been shown to be essential for osteoblast differentiation, as it up-regulates many key bone-phenotypic genes, including Runx2 (13,16). Although the precise molecular mechanisms involved in the BMP2-mediated Runx2 gene induction have not been established, recent reports have shed light on some of the transcription factors that control Runx2 expression is osteoblastic cells. Thus, it has been found that the homeodomain factor Dlx-5 is a target of BMP2 signaling that up-regulates Runx2 gene expression (40). Moreover, Runx2 expression is specifically stimulated by Dlx-5 overexpression in osteoblasts. This action of Dlx-5 is direct on the Runx2 gene P1 promoter and is antagonized by Msx-2 (7). A detailed recent analysis concluded that two homeodomain proteins, Msx-2 and CDP/cut function as repressors of the Runx2 gene expression and Runx2 P1 promoter activity while another two homeodomain factors, Dlx-3 and Dlx-5, function as activators (8). Interestingly, these homeodomain proteins exhibit distinct temporal expression profiles during osteoblast differentiation ex vivo, as well as selective association with the Runx2 gene P1 promoter, that is related to Runx2 transcriptional activity (8). Msx-2 binds to the Runx2 P1 promoter when transcription is minimal. Hence, it is tempting to speculate that this protein-DNA interaction may be directly associated with the weak DNase I hypersensitive sites that we detect at this promoter in C2C12 cells cultured in the absence of BMP2. Whether Msx-2 may also be recruiting inhibitory complexes including histone deacetylases (HDACs) that maintain low histone acetylation levels at the Runx2/p57 P1 promoter (see below) is currently investigated. The activating Dlx-3 factor is recruited transiently to the Runx2 gene in post-proliferative osteoprogenitors, while

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Dlx-5 remains associated with this gene in mature osteoblasts. Hence, the commitment to the osteogenic lineage may be operating through BMP2 induction of the transcription factors Dlx-3 and Dlx-5 which in turn activate expression of the Runx2 master gene. Interestingly, it has been reported that BMP2 treatment also activates the canonical Wnt signaling pathway in osteoblastic cells (41) and that canonical Wnt signaling up-regulates Runx2 gene transcription by targeting the P1 promoter sequence (9). Hence it is tempting to speculate that BMP2-dependent increase of Runx2 gene transcription may be also mediated through the Wnt-target sequences present in the proximal P1 promoter.

Here, we find increased histone H3 and H4 acetylation at the P1 promoter region of C2C12 cells in response to BMP2. In addition, we have recently shown that the Hoxa10 factor activates Runx2 gene transcription in mesenchymal cells (10). Hoxa10 is recruited early to the Runx2 P1 promoter and this interaction is associated with enhanced histone acetylation. Moreover, knock down of Hoxa10 in osteoblastic cells using siRNA results in reduced Runx2 gene transcription and decreased histone acetylation at the P1 promoter region.

On the other hand, it is well-established that BMP2 signaling is normally mediated by down-stream proteins, the Smad factors, which may recognize GC-rich binding elements in target promoters (17). To date, these elements have been defined in the promoter regions of only a few genes, including the collagen X and Smad6 genes (42,43). Moreover, Smad factors exhibit relatively low DNA binding affinity, leading to the general belief that they also require interaction with other sequence-specific proteins to form stable DNA-bound complexes that activate transcription (17,44). Recently, it was reported that Smad1-mediated BMP2 signaling on the Smad6 gene occurs through direct binding of Smad1 with the Runx2 transcription factor, which then recruits the complex to a Runx2 site on the Smad6 promoter (45). Whether the HAT activities that modify the Runx2 P1 promoter region and contribute to transcriptional activation of the Runx2 gene are recruited in response to BMP2 by forming complexes with the transcription factors Dlx-3, Dlx-5, Hoxa10, Wnt/β-catenin/TCF or another still unknown factor, remains under investigation.

We have recently shown that the SWI/SNF complex is recruited to the bone-specific osteocalcin (OC) gene promoter where it is required for both nuclease hypersensitivity and transcriptional activity of this gene (29). Interestingly, OC expression also involves enhanced histone H3 and H4 acetylation (Shen et al. 2002; 2003), although this modification is not sufficient to maintain active OC gene transcription (29). Therefore, even though both Runx2 and OC are expressed constitutively in ROS17/2.8 osteoblastic cells, distinct molecular mechanisms control chromatin remodeling at the promoter of each gene, perhaps reflecting the temporally-different expression pattern of the Runx2 and OC genes during bone development in vivo (1). Transcription of the Runx2/p57 gene initiates at very early stages of the osteoblast differentiation process, as Runx2/p57 functions as a master regulator of several key bone-related genes to promote the osteoblastic phenotype (1). In contrast, OC is expressed at late stages of osteoblast differentiation and its transcription is controlled, at least in part, by Runx2 (1). Our results indicate that SWI/SNF activity also is not required for Runx2 expression in C2C12 cells induced to differentiate into the osteoblastic lineage by treatment with BMP2 (Supplementary Figure S1 at the “Supporting Information Available”). In addition, we have recently reported that expression of Runx2-dependent osteoblastic genes, but not the Runx2 gene itself, is inhibited in NIH3T3 cells that inducibly express a mutant BRG1 protein and that are forced to differentiate into the osteoblastic lineage by BMP2 treatment (28). Together, these results indicate that both chromatin remodeling and transcriptional activity of the Runx2 gene are associated with SWI/SNF-independent epigenetic mechanisms that involve core histone acetylation.

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In support of our results, it has been previously found that during myeloid differentiation the activity of SWI/SNF complexes containing the catalytic subunit BRM is not universally required for tissue-specific gene expression (47). Also, it has been shown that not all the muscle-specific genes are strictly dependent on SWI/SNF activity for expression (48). Interestingly, in these latter studies it was demonstrated that forced expression of MyoD in mesenchymal cells results in histone hyperacetylation at the myogenin promoter, which occurs prior to and independently of SWI/SNF activity. Nevertheless, and in contrast to our studies in the Runx2 gene, subsequent binding of the SWI/SNF complex to the promoter is needed for myogenin expression during muscle differentiation.

In agreement with our results, it has been recently reported that there is a close relationship between histone acetylation and osteoblast differentiation. It was found that suppression of HDAC activity during osteoblast differentiation by either HDAC inhibitors or knock down of HDAC1 expression via siRNA stimulates osteogenic gene expression, thereby promoting differentiation (34). Among the genes that exhibited elevated mRNA levels in response to treatment with HDAC inhibitors is Runx2, together with well-known Runx2 downstream targets including OC, osteopontin, and alkaline phosphatase. Moreover, during osteogenesis ex vivo, recruitment of HDAC1 to promoters of osteogenic genes including OC and osterix was down-regulated, concomitant with an increase of histone H3 and H4 acetylation (34). Taken together these results indicate that enhanced chromatin acetylation represents an epigenetic landmark of the Runx2 gene activation process during osteoblast differentiation, therefore providing a new target for modulating the expression of this key regulatory gene.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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We thank Dr. Brigitte van Zundert for helpful discussions and critical reading of the manuscript. “Supporting Information Available” includes Figure S1 and Figure Legend S1, which demonstrate that in BMP2-induced C2C12 cells Runx2/p57 expression is also independent of SWI/SNF activity. This material is available free of charge via the internet at http://pubs.acs.org. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

## Abbreviations

**BMP2**
: Bone Morphogenetic Protein 2

**HDAC**
: histone deacetylase

**TGFβ**
: Transforming growth factor beta

**DHS**
: DNase I hypersensitive site

**OC**
: Osteocalcin

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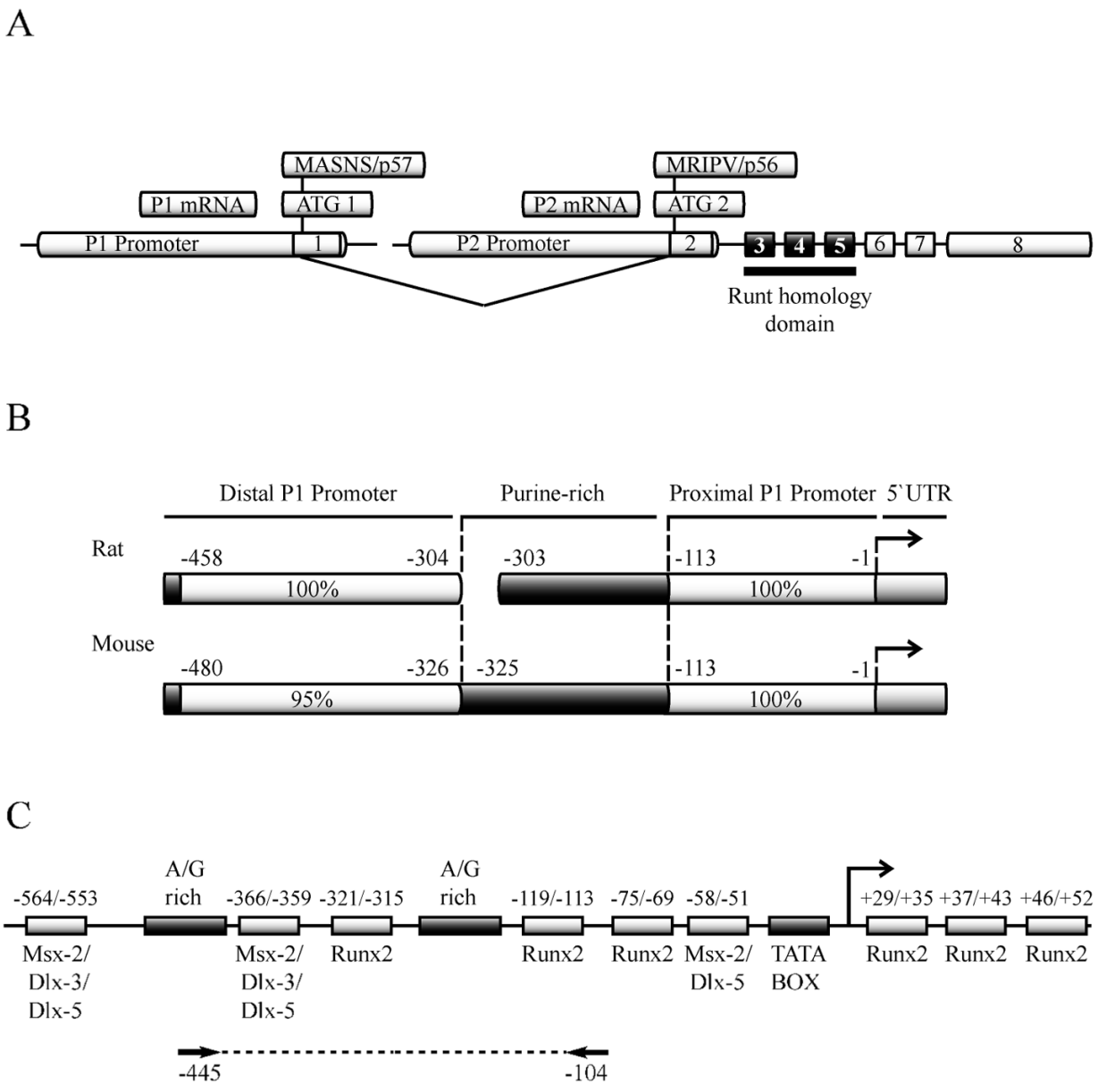


**Figure 1. Organization of the 5′ regulatory region of the Runx2 gene**

A) The diagram shows the overall organization of the Runx2 gene including the upstream (P1) and downstream (P2) promoters. The two promoters regulate expression of two distinct mRNAs encoding two isoforms with different N-termini, MASNS/p57 and MRIPV/p56. B) Comparison of the rat and mouse Runx2 P1 promoters. Both sequences share conserved proximal and distal promoter regions (open boxes) that are flanked by purine-rich segments of variable length (filled boxes). C) Schematic representation of the regulatory elements for cognate transcription factors in the P1 promoter. The diagram shows the positions of the different binding sites with respect to the transcription start site, as well as the position of the primer pair used in ChIP experiments.

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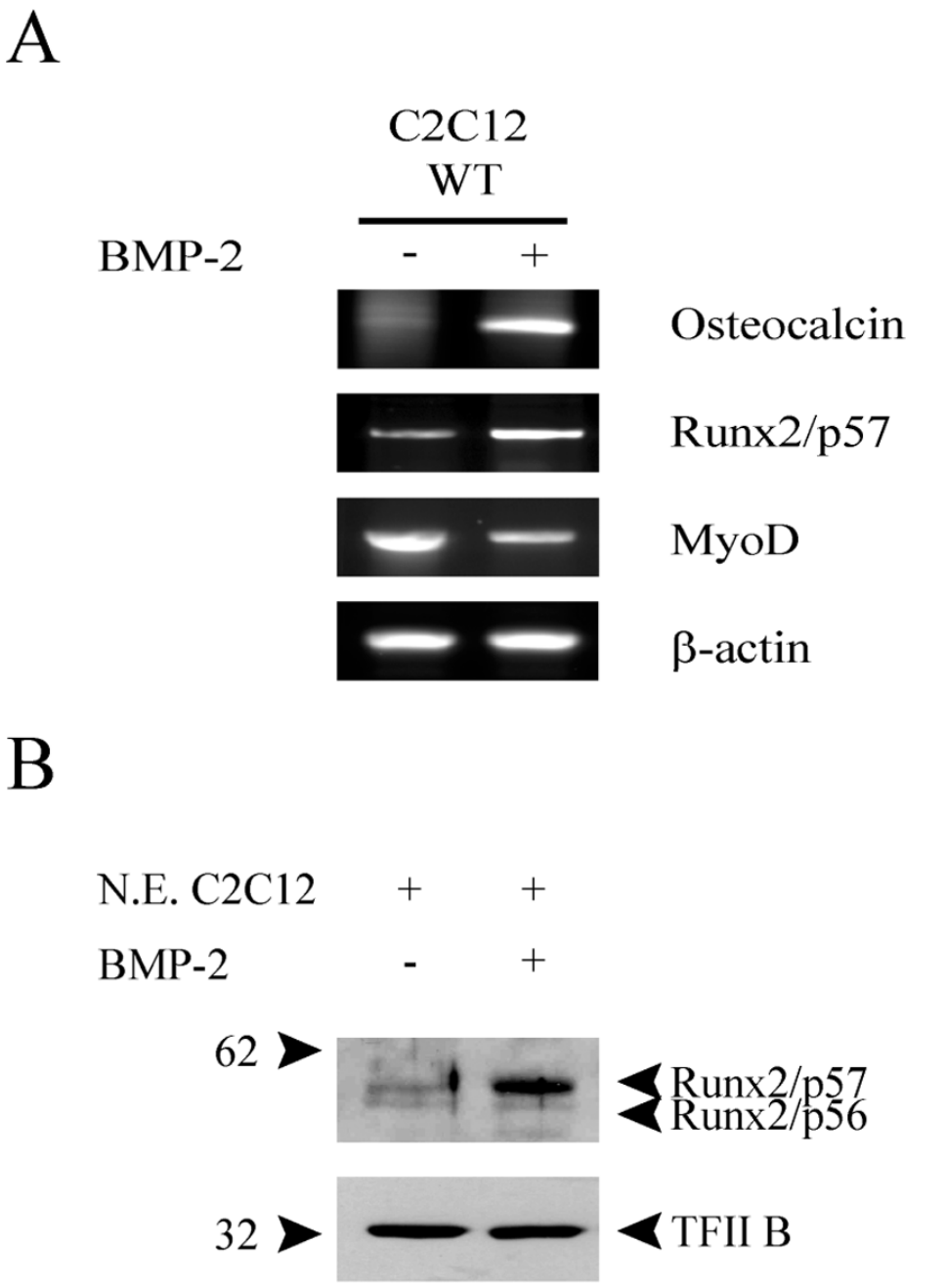


**Figure 2. BMP2-dependent induction of the Runx2/p57 isoform in C2C12 cells**

A) Total RNA samples isolated from C2C12 cells grown in the presence (+) or absence (−) of 300 ng/mL BMP2 for 48 h were reverse-transcribed and PCR-amplified using specific primers against osteocalcin (upper panel), Runx2/p57 (second panel), MyoD (third panel), and β-actin (lower panel) mRNAs. B) Nuclear extracts isolated from C2C12 cells grown in the presence (+) or absence (−) of 300 ng/mL BMP2 for 48 h were analyzed by Western blot using an anti Runx2 specific antibody (upper panel). To control for equal protein loading we detected TFIIB in each sample (lower panel).

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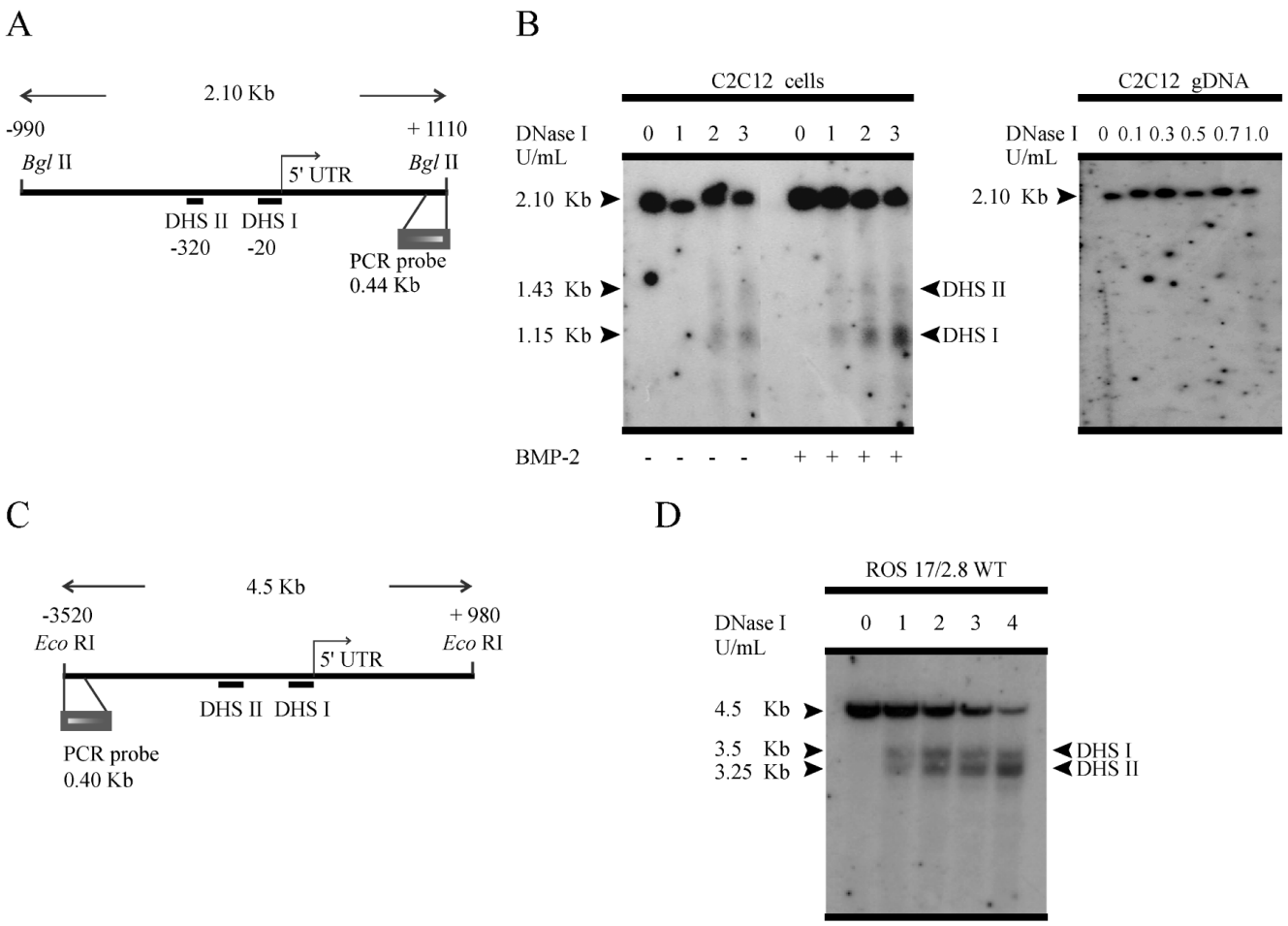


**Figure 3. BMP2-dependent stimulation of Runx2/p57 transcription in C2C12 cells is accompanied by enhanced DNase I hypersensitivity at the P1 promoter region**

A) Schematic representation of the restriction endonuclease *Bgl* II cleavage map of the mouse Runx2 gene (including exons and introns). The transcription initiation site is indicated with an arrow. The diagram also shows the PCR-generated hybridization probe (0.44 Kb) used in the indirect end-labeling analysis (see materials and methods). In addition, the positions of two DNase I hypersensitive sites (DHS II, centered at −320 and DHS I, centered at −20) are indicated. B) Nuclei isolated from mesenchymal C2C12 cells, grown in the presence (+) or absence (−) of 300 ng/mL BMP2 for 48 h, were incubated with increasing concentrations of DNase I (indicated at the top) and the purified genomic DNA was then analyzed by the indirect end-labeling method using the gene-specific probe shown in A. The positions of the DNase I-digested DNA sub-fragments representing both nuclease hypersensitive sites are indicated on the right of the blot. In the right panel, genomic DNA isolated from C2C12 cells was incubated with increasing concentrations of DNase I (indicated at the top) and the purified digestion products were also analyzed by the indirect end-labeling method. C) Schematic representation of the restriction endonuclease *EcoRI* cleavage map of the rat Runx2 gene. See A for an explanation of the symbols. D) Nuclei isolated from ROS 17/2.8 cells were incubated with increasing concentrations of DNase I (indicated at the top) and the purified genomic DNA analyzed by the indirect end-labeling method. See B for an explanation of the symbols.

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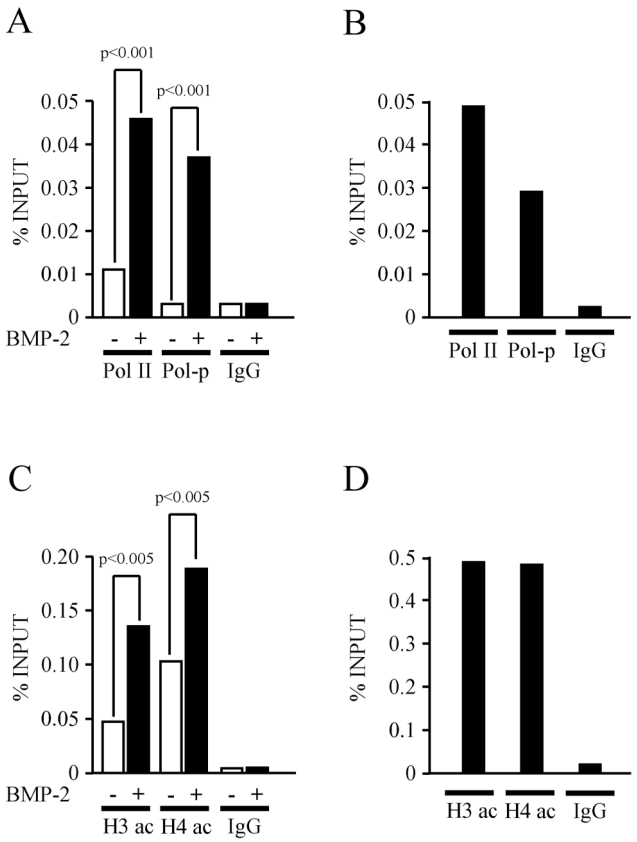


**Figure 4. BMP2 treatment induces recruitment of RNA pol II and increased histone H3 and H4 acetylation at the Runx2 gene P1 promoter**

Binding of total (Pol II) and CTD-phosphorylated (Pol-P) RNA Polymerase II and acetylated histones H3 and H4 to the Runx2 gene P1 promoter in C2C12 cells grown in the presence (+) or absence (−) of 300 ng/mL BMP2 for 48 h (A and C) or in ROS17/2.8 cells (B and D), was assessed by chromatin immunoprecipitation (ChIP). Precipitated DNA fragments were quantified in a molecular imager or by quantitative PCR using specific primers against the Runx2 P1 promoter and normalized to input material. IgG, non-specific immunoglobulin G. These results are representative of at least three independent experiments. $p<0.001$ and $p<0.005$ were determined by ANOVA test.

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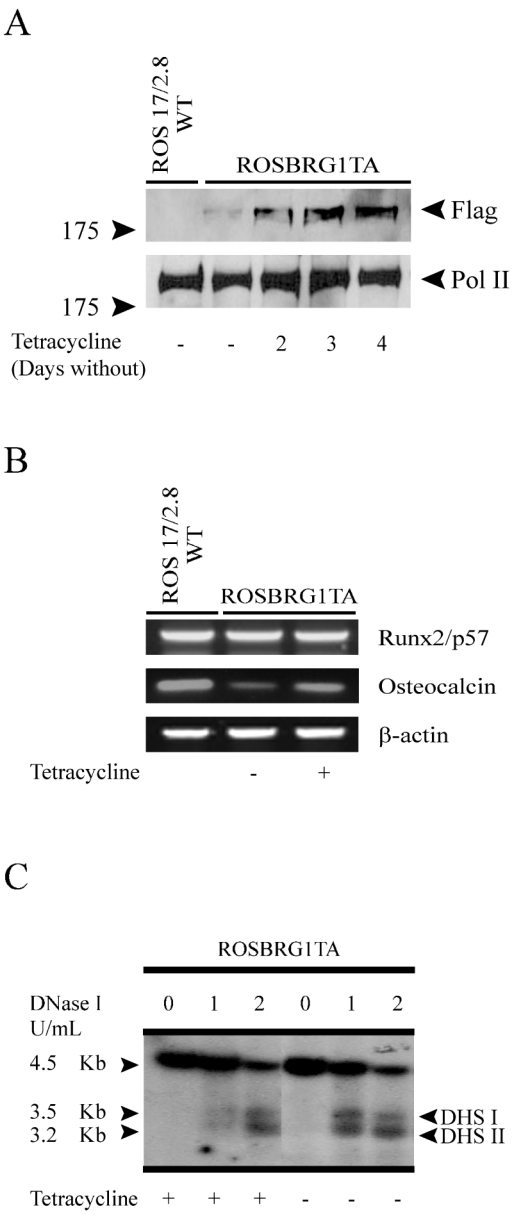


**Figure 5. Runx2/p57 expression and chromatin remodeling at the Runx2 P1 promoter occur in a SWI/SNF-independent manner**

A) Nuclear extracts isolated from wild-type ROS 17/2.8 cells and ROS17/2.8 cells expressing an inducible dominant-negative (flag-tagged) form of BRG1 (ROSBRG1TA) were analyzed by Western blot using an anti-flag antibody (upper panel). The cells were cultured in the absence (−) of Tetracycline for 2, 3, and 4 days as indicated below the blots, to induce dnBRG1 expression. To control for equal protein loading the presence of RNA pol II was detected in each sample (lower panel). The position of a molecular size marker (175 Kda) in the gel is indicated at the left of the blot. B) Total RNA isolated from ROS 17/2.8 and ROSBRG1TA cells was reverse transcribed and PCR-amplified using specific primers against the Runx2/p57, osteocalcin, and β-actin mRNAs (see materials and methods). The ROSBRG1TA cells were grown in the presence (+) or absence (−) of 10 μg/mL Tetracycline for 4 days. C) Nuclei isolated from ROSBRG1TA cells cultured in the presence (+) or absence (−) of 10 μg/mL Tetracycline

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for 4 days were incubated with increasing concentrations of DNase I (indicated at the top) and the purified genomic DNA was then analyzed by the indirect end-labeling method (see Figure 3C for probe information).

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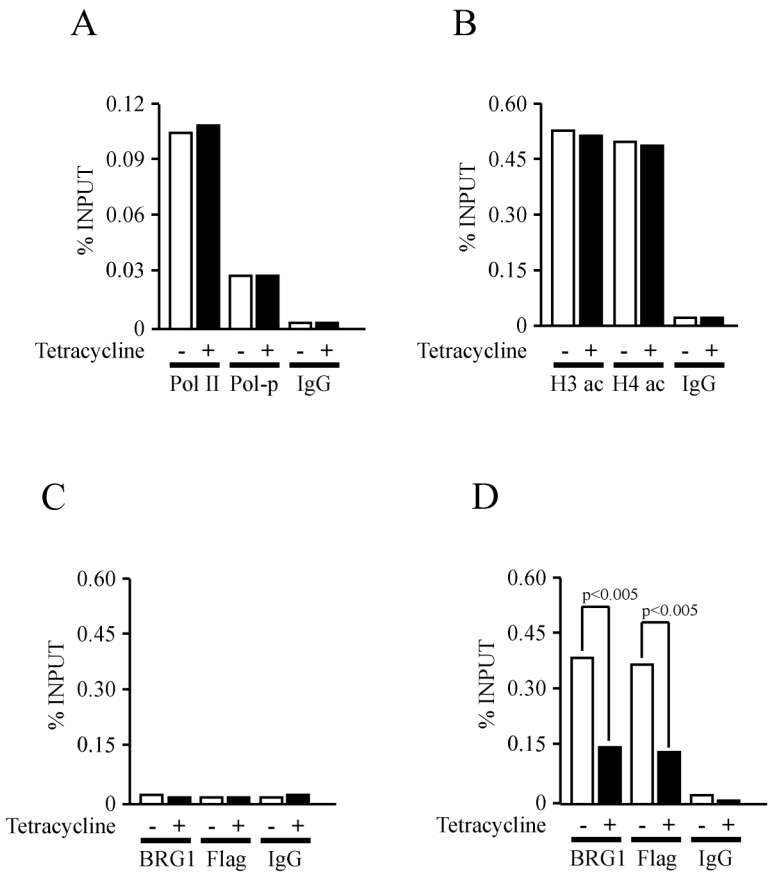


**Figure 6. Increased histone acetylation and recruitment of RNA pol II at the Runx2 P1 promoter are independent of ongoing SWI/SNF activity**

The association of RNA pol II (A), acetylated histones H3 and H4 (B) as well as endogenous (C) and flag-tagged (D) BRG1 proteins with either the Runx2 P1 (A, B, and C) or osteocalcin (D) promoters was determined by ChIP analyses using specific antibodies and primers pairs (see materials and methods). The precipitated DNA fragments were electrophoresed and then quantified in a molecular imager or directly quantified by real-time PCR. IgG represents the material precipitated when using an unrelated IgG fraction. The results are representative of at least three independent experiments. $p<0.005$ was determined by ANOVA test.