Cooperative Interactions between RUNX2 and Homeodomain Protein-binding Sites Are Critical for the Osteoblast-specific Expression of the Bone Sialoprotein Gene*

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The bone sialoprotein (Bsp) gene provides an excellent model for studying mechanisms controlling osteoblast-specific gene expression. Although the RUNX2 transcription factor directly regulates many osteoblast-related genes, its function in Bsp expression remains uncertain. By using chromatin immunoprecipitation (ChIP) analysis in MC3T3-E1 (clone MC-4) preosteoblast cells, RUNX2 was shown to bind a chromatin fragment containing the proximal Bsp promoter. Two putative RUNX2-binding sites (R1 and R2) were identified within this region of the mouse, rat, and human genes and were shown to bind RUNX2 in vitro and in vivo (by ChIP assay). Site-specific mutagenesis established that both sites act as osteoblast-specific transcriptional enhancers and together account for nearly two-thirds of the total promoter activity. In addition, functional cooperativity was observed between the R2 site and an adjacent homeodomain protein-binding site previously characterized by this laboratory (the C site). All three sites (R1, R2, and C) are necessary for maximal promoter activity in osteoblasts. DLX5 in MC-4 cell nuclear extracts binds to the C site in vitro. Furthermore, ChIP assays revealed that DLX5 is selectively associated with chromatin in the vicinity of the C site only when Bsp is transcriptionally active. Finally, co-immunoprecipitation assays detected a physical complex containing DLX5 and RUNX2. Taken together, our data show that RUNX2 is a direct regulator of Bsp in osteoblasts and that it functions in cooperation with DLX5 or a related factor to activate osteoblast-specific gene expression.

Osteoblast differentiation is a crucial event for skeletal tissue formation, repair, and maintenance. Osteoblasts secrete a complex extracellular matrix (ECM) containing collagenous and noncollagenous proteins. This ECM interacts with cells by binding to β1 subunit-containing integrins that signal to the cell nucleus to regulate gene expression (1–3). Bone sialoprotein (BSP) is one of the major noncollagenous components of the bone ECM, and its expression is primarily restricted to hypertrophic chondrocytes and osteoblasts (4). During osteoblast differentiation, BSP is up-regulated as the ECM mineralizes (5). Thus, BSP is one of the primary markers of the differentiated osteoblast, and knowledge concerning the mechanisms controlling its expression will be of critical importance to understand the regulation of bone formation and osteoblast differentiation.

Regulatory regions of all eukaryotic genes contain general and tissue-specific enhancer sequences that, on interaction with the appropriate nuclear proteins, specify the overall pattern of gene expression. Viewing transcription factors as functioning in complexes and interacting with each other provides a model that can explain how a cell lineage is defined during differentiation (6). In the case of osteoblast differentiation, gene deletion studies have so far definitively identified three transcription factors as being required for development of this cell type. The first factor, RUNX2, the bone-related product of the Cbfa1 gene, is first expressed at embryonic day 12.5 in mice and is required for early commitment of mesenchymal cells to chondro/osteoblastic lineages (7–9). Cbfa1−/− mice fail to form hypertrophic chondrocytes or osteoblasts and produce a cartilaginous skeleton that is completely devoid of mineralized matrix. The other two factors, OSX and ATF4, function downstream to further restrict chondro/osteoblastic precursors to the osteoblast phenotype. Osx−/− mice are able to form hypertrophic chondrocytes but fail to form osteoblasts (10), whereas deletion of Atf4 results in mice with stunted skeletal growth and reduced osteoblast activity (11). Members of the DLX subfamily of homeodomain-containing transcription factors have also been implicated in osteoblast differentiation. DLX5 is selectively expressed in bone where it regulates osteocalcin (Ocn), collagen 1, and alkaline phosphatase (Alp) genes (12–16). Furthermore, osteoblast activity is moderately reduced in Dlx5−/− mice (17), whereas combined deletion of Dlx5 and Dlx6 leads to defects in the transition from prehypertrophic to hypertrophic chondrocytes, severe disruptions in osteoblast gene expression, and delayed mineralization (17).

Our group has been interested in defining mechanisms controlling bone-specific gene expression by using Bsp as a model. Previous studies identified a 2.5-kb promoter region of the murine gene that exhibits osteoblast-selective activity in vitro and in vivo (18, 19). This promoter contains two putative RUNX2-binding sites having the consensus sequence, RC-CRC(AT), that is known to bind members of the Runt family of
transcription factors (20). These sites are located –61 and –1335 bp from the transcription start site. Most surprisingly, neither site was found to contribute to the osteoblast-specific activity of this promoter (18). In contrast, a homeodomain factor-binding site designated site C or osteoblast-specific homeodomain element 1 (OSHE1) was identified in the proximal Bsp promoter and shown to partially explain the tissue-specific activity of this gene in vitro and in transgenic mice (19, 21). We also discovered that DLX5 could bind to the C site in vitro and regulate its enhancer activity (21).

In contrast to our early negative results regarding the involvement of RUNX2 in Bsp expression, considerable evidence exists suggesting direct or indirect involvement of this transcription factor in the bone-specific expression of Bsp. Thus, Bsp is greatly reduced in the bones of Cbfa1–/– animals (22). Furthermore, overexpression of a dominant-negative RUNX2 can block Bsp transcription in vivo (23). More recent studies with breast cancer cells also showed that RUNX2, normally expressed only in bone-derived cells, is ectopically overexpressed in these cells and can act as a positive regulator of Bsp promoter activity (24).

In view of these findings, we considered it important to examine further the possible involvement of RUNX2 in Bsp expression by conducting a detailed analysis of the promoter structure for cryptic RUNX2-responsive regions. As will be shown, the proximal Bsp promoter contains two such sites (R1 and R2) having a sequence that slightly diverges from the consensus runt domain protein-binding site. These elements together with the C site account for most of the osteoblast-specific activity of the proximal Bsp promoter. We will also show that one of these sites (R2) functionally interacts with the C site and, furthermore, that part of this functionality can be explained by physical interactions between DLX5 bound to the C site and RUNX2 bound to R2.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The isolation of preosteoblastic clone 4 cells (MC-4) from the parent MC3T3-E1 line was described previously (5, 25). These cells were maintained in ascorbic acid-free minimal essential medium (Invitrogen), 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), and 1% penicillin/streptomycin. MC-4 cells were grown under the same conditions as MC3T3-E1 cells. The murine pluripotent mesenchymal cell line, C3H10T1/2, was obtained from American Type Culture Collection (Manassas, VA). C3H10T1/2 cells were grown under the same conditions as MC-4 cells.

**DNA Constructs**—Construction of recombinant adenovirus expressing RUNX2 (AdCMV-Runx2) was described previously (29). Control adenovirus expressing β-galactosidase (AdCMV-LacZ) was purchased from the American Type Culture Collection (Manassas, VA). AdCMV-Runx2 is a recombinant adenovirus expressing RUNX2 (AdCMV-Runx2) with an N-terminal FLAG tag, was the generous gift of Dr. Dwight M. Towler (Washington University, St. Louis, MO) (12).

**Transfection**—All transfections were performed using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. When the transfections were performed in MC-4 cells for ChIP analysis, cells were plated in 35-mm dishes at a density of 2.5 × 10^5 cell/cm^2, grown for 12 h, and transfected with 600 ng of Bsp-luciferase reporter construct plus 60 ng of pRL-SV40 Renilla luciferase vector (Promega) for normalization. After transfection, cells were switched to the appropriate differentiation media and grown for 6 days. In the case of C3H10T1/2 or COS7 cells, they were also transfected using 400 ng of pCMV-Luc-LacZ vector (Promega) plus 40 ng of Renilla luciferase vector. The cells were harvested 48 h after transfection. Luciferase expression in cell lysates was assayed using the dual luciferase reporter kit (Promega) and a Monolight 1010 luminoimeter (Pharmingen). All transfection experiments were performed in triplicate and repeated at least three times.

**Quantitative Real-Time PCR**—Cells were infected with an adenovirus containing Runx2 cDNA (AdCMV-Runx2) or LacZ (AdCMV-LacZ) at 100 plaque-forming units/cell. The infected cells were treated with ascorbic acid (50 μg/ml) every other day and harvested in TRIzol reagent (Invitrogen) on day 6. Total RNA was prepared as described by Chomczynski and Sacchi (31) and then cleaned with an RNasey mini kit (Qiagen, catalog number 74104). RNA (2 μg) was reverse-transcribed using Taqman reverse transcription reagents for first-strand cDNA synthesis (Applied Biosystems, catalog number N808-0234). Real-time PCRs were performed with the first-strand cDNA corresponding to 20 ng of total RNA and the Taqman universal PCR master mix (Applied Biosystems, Foster City, CA) as well as one of the following Taqman predeveloped assay reagents for mouse: 1) Bsp (FAM/MGB probe; Applied Biosystems, catalog number Mm00492555_m1); 2) Runx2 (FAM/MGB probe; Applied Biosystems, catalog number Mm00481342_m1); 3) Dlx5 (FAM/MGB probe; Applied Biosystems, catalog number Mm00438430_m1); and 4) Gapdh (Applied Biosystems, catalog number Mm99999915_g1). Universal mouse reference RNA (Stratagene, catalog number 740100-41) was used to generate a relative standard curve. Real-time detection of PCR products was performed on an ABI PRISM 7700 sequence detector (Applied Biosystems) in the School of Dentistry Molecular Biology Core. Levels of mRNA were normalized with Gapdh. All real-time PCR results were calculated from triplicate RNA samples.

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSA)**—Nuclei from 5-day AA-treated MC-4 cells were isolated by sucrose gradient centrifugation as described previously (25) and were used for preparation of nuclear extracts according to the method of Dinagn et al. (32). Double-stranded oligonucleotides (see Table 1) were end-labeled by filling with Klenow (Invitrogen) in a reaction containing 2 pmol of oligonucleotide, cold G, A, and T nucleotides, and 80 μCi of [α-32P]dCTP. The labeled oligonucleotide (probe) was then purified using ProbeQuant™ G-50 Micro Columns (Amer sham Biosciences) according to the manufacturer's instructions. For supershift experiments, 2 μg of nuclear extract were preincubated for 30 min at 4 °C with 200 ng of the indicated antibodies in binding buffer containing 12% glycerol, 20 mM Tris-HCl (pH 7.9), 100 mM KCl, 1 mM MgCl2, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, and 1 μg of poly(dI-dC). Nuclear extract-antibody complexes were mixed with ~10 fmol of the labeled probe in a 15-μl reaction, following 10 min of incubation at room temperature. Reactions were then electrophoresed on 5% acrylamide gels in 1× TBE (Inviron) for 120 min at 180 V at 4 °C, and gels were dried and autoradiographed. The following antibodies were used: RUNX2 (M 70), Dlx5 (Y20), Dlx6 (G20), or rabbit IgG purchased from Santa Cruz Biotechnology.
**RUNX2 and Homeodomain Protein Regulation of Bsp**

**Immunoprecipitation**—Approximately 500 μg of nuclear extract was prepared from MC-4 or COS7 cells transfected with DLX5 and RUNX2 expression plasmids. Extracts were diluted to 800 μl in co-immunoprecipitation (co-IP) buffer (50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 0.1% (v/v) Triton X-100, 1 mM dithiothreitol, 1% (v/v) protease inhibitor mixture (Sigma), and 1 mM phenylmethylsulfonyl fluoride) and pre-cleared for 1 h at 4 °C with 60 μl of protein A/plus agarose (Santa Cruz Biotechnology). Antibodies were added to the precleared nuclear extracts following incubation at 4 °C overnight. To precipitate immunocomplexes, 50 μl of protein A/plus agarose beads were added and further incubated at 4 °C for 1 h. Beads were washed three times with co-IP buffer, one time with 1× phosphate-buffered saline (containing 1 mM phenylmethylsulfonyl fluoride), suspended in 30 μl of 2× SDS sample buffer, and analyzed by Western blotting with RUNX2 (M70) antibody (1:1000 dilution) or anti-FLAG M2 monoclonal antibody (1:2000 dilution).

**Chromatin Immunoprecipitation (ChiP) Assays**—ChiP assays were performed using a protocol kindly provided by Dr. Dwight Towler (Washington University) (33). After sonication, the amount of chromatin was quantified using the PicoGreen double-stranded DNA Quantitation Assay (Molecular Probes) according to the manufacturer’s instructions. The equivalent of 10 μg of DNA was used as starting material (input) in each ChiP reaction with 2 μg of the appropriate antibody (anti-RUNX2, AdCMV-Runx2, or control rabbit or goat IgG). Fractons of the purified ChiP DNA (5%) or inputs (0.05–0.05%) were used for PCR analysis. The reaction was performed with AmpliTaq Gold DNA Polymerase (Applied Biosystems) for 30 cycles of 15 s at 95 °C, 30 s at 60 °C, and 15 s at 72 °C. PCR primer pairs were generated to detect DNA segments located near the putative RUNX2-binding site at −1335 (primers P3 and P4) or in the proximal promoter region (primers P1 and P2; see Fig. 2A and Table I). The PCR products were separated on 3% agarose gels and visualized with ultraviolet light. All ChiP assays were repeated at least two times and with duplicate samples for each antibody used in the immunoprecipitation reactions.

When the ChiP assay was performed in MC-4 cells transfected with Bsp promoter-luciferase reporter constructs, PCR primers were designed to amplify a 62-bp fragment of the Bsp promoter-luciferase chimeric gene. One primer (PBL1) was selected from the last 18 bp of Bsp promoter fragment −2472 to +41 cloned in pGL3Basic, and the second primer (PBL2) includes 21 bp of the specific primed sequence located just 8 bp before the start of luciferase coding region (see Fig. 5A and Table I). In this case the PCR proceeded for 23 cycles of amplification. Quantitative PCR analysis was also performed before immunoprecipitation to ensure equal transcription efficiencies.

**Statistical Analysis**—Unless stated otherwise, all results are expressed as means ± S.D. of triplicate independent measurements with all experiments being repeated at least three times. Statistical significance was assessed using a Tukey-Kramer multiple comparisons test with an Instat 4.0 software package.

**RESULTS**

**RUNX2-responsive Regions in the Bsp Promoter**—Gene deletion and dominant-negative approaches clearly demonstrated that RUNX2 is necessary for expression of Bsp in osteoblasts (23). However, it is not known whether the effects of this transcription factor are direct (e.g. involve binding of RUNX2 to enhancer sequences in the Bsp promoter) or require induction of secondary factors. To begin exploring the mechanism used by RUNX2 to regulate Bsp, the activity of this gene was examined in the murine C3H10T1/2 mesenchymal cell line. We showed previously (29) that these cells undergo osteoblast differentiation and form a bone-like mineralized matrix after transduction with an adenovirus expressing RUNX2 in vitro and in vivo. Fig. 1A shows that RUNX2-overexpressing cells also stimulates Bsp mRNA. For this experiment, cells were transduced with recombinant adenovirus expressing RUNX2 (AdCMV-Runx2) or control adenovirus (AdCMV-LacZ). Fig. 1A, upper panel, shows that C3H10T1/2 cells express minimal RUNX2 protein when transduced with control virus and high levels after AdCMV-Runx2 transduction. Real-time PCR analysis revealed that RUNX2 stimulated Bsp mRNA levels by more than 20-fold (Fig. 1A, lower panel).

To examine effects of RUNX2 on Bsp gene transcription, we next examined a series of previously developed 5′ deletions in the 2.5-kb murine Bsp promoter that were fused to a luciferase reporter (Fig. 1B). This promoter region contains sufficient information to direct osteoblast-selective expression of luciferase and lacZ reporter genes in vitro (19, 21). The indicated promoter constructs (p2472, p705, p338, p178, and p49) were transfected into C3H10T1/2 cells together with a Renilla luciferase normalization vector and either a control (pCMV-LacZ) or RUNX2 expression plasmid (pCMV5-Runx2). All values were corrected for transfection efficiency using a Renilla plasmid and are expressed relative to the activity of the full-length construct co-transfected with RUNX2 expression plasmid.

**FIG. 1.** RUNX2 induction of Bsp expression and promoter activity in C3H10T1/2 mesenchymal cells. A, induction of Bsp mRNA. Cells were transfected with control adenovirus (AdCMV-LacZ) or virus encoding RUNX2 (AdCMV-Runx2) and harvested for analysis of RNA and protein after 5 days in AA-containing medium. RUNX2 protein levels were measured by Western blotting (WB) (upper panel), and Bsp mRNA expression (lower panel) was determined by quantitative reverse transcription-PCR. The results are based on relative standard curves and normalized to Gapdh. Values are means ± S.D. from triplicate RNA samples. B, functional analysis of 5′ deletions of the Bsp promoter. Transient transfection assays were performed in C3H10T1/2 cells using full-length (p2472) or deletion constructs (p705, p338, p178, and p49) of the Bsp promoter fused to luciferase and either a RUNX2 expression plasmid (pCMV5-Runx2) or control plasmid expressing β-galactosidase (pCMV5-LacZ). All values were corrected for transfection efficiency using a Renilla plasmid and are expressed relative to the activity of the full-length construct co-transfected with RUNX2 expression plasmid.
As noted in the Introduction, previous work in our laboratory investigated the role of a putative RUNX2-binding site located at −1335 in the *Bsp* promoter. This site (named R3 in Fig. 2A) has the same sequence in inverted orientation contained in OSE2, the RUNX2-responsive element in the osteocalcin promoter (7). Furthermore, this site is able to specifically bind RUNX2 in vitro (18). However, luciferase reporter assays using promoter constructs containing deletions in this region revealed that it does not contribute to the activity of the 2.5-kb *Bsp* promoter in osteoblasts (18). On the other hand, functional analysis with *Bsp* promoter deletions shown in Fig. 1B suggested that RUNX2-responsive elements are present in the proximal promoter between −49 and −178 bp. To clarify this issue further, we used ChIP assays to examine the in vivo association of RUNX2 with proximal (178 to −49 bp) and distal (−1335 bp) promoter regions in osteoblast-like MC-4 cells (Fig. 2). These cells express RUNX2 and undergo osteoblast differentiation after growth in AA-containing medium (5). For this experiment, cells were treated with AA for 5 days to induce *Bsp* expression and cross-linked with formaldehyde. After shearing, soluble chromatin was immunoprecipitated with either an antibody against RUNX2 or control IgG. Two independent IP reactions were performed for each antibody. The positions and sequence of primers selected for PCR analysis of ChIP DNAs are shown in Fig. 2A and Table I, respectively. The PCR bands amplified with primers P1 and P2 and corresponding to ChIP DNAs immunoprecipitated with RUNX2 antibody (Fig. 2C, lanes 1 and 2) revealed that RUNX2 specifically interacts with a chromatin fragment containing the proximal *Bsp* promoter.

![Diagram of the *Bsp* promoter with putative RUNX2-binding sites R1, R2, and R3, and regions of interest for ChIP analysis.](image)

**TABLE I**

| Oligonucleotides used in this study | Bars are above mutated nucleotides. |
|------------------------------------|-----------------------------------|
| P1 | GCCCTGTTTGAAATACCACGTGAA |
| P2 | TCTGGACCCCTTCAATTAAATCCCAACA |
| P3 | GACCACTAAAGAAGGTTGAGGTAAT |
| P4 | AACCCTCTTTTGGCTGAGAAGACGT |
| PBL1 | TACCAACCTGCTAGGTGATCC |
| PBL2 | ATCCATGCCATCAGCTGG |
| oR1 | GAATCTCCTGGTGAAGACCCACGCTG |
| oR2 | GGTTAATCCCACATCAGCCTG |
| omR1 | TAAAGAACATCCTGTCGAGAGCGC |
| omR2 | ATTTAGCTCCTTGGATGGACTGAGCGC |
| oCR2 | GAGCCTTACCTACATTAAATCCCAACAGGA |
| oCmR2 | TTGGAACTTATAATGATGGAGAGC |

**Fig. 2.** ChIP analysis of RUNX2 binding to the *Bsp* promoter in differentiated MC-4 cells. A, schematic representation of relevant regions of the murine *Bsp* promoter. Putative RUNX2-binding sites, R1 and R2, the previously described R3 site (at −1335), and the C site are shown along with positions relative to the transcription start site. P1, P2, P3, and P4 indicate PCR primers used to analyze ChIP DNA. The positions of these primers and the size of the fragments they amplify are indicated at the bottom of the figure. B, sequence comparison of the *Bsp* promoter regions surrounding the putative RUNX2-binding sites (R1 and R2) in mouse, rat, and human promoters. R1, R2, and C sites are located within a 343-bp promoter region that shares 75% homology across these three species. In the selected regions, mouse and rat sequences show 100% homology, whereas mismatches are shown for the human sequence. Note that the C site is perfectly conserved across species and that mismatches within R1 and R2 sites are conserved for purine or pyrimidine bases. C, ChIP analysis. Differentiated MC-4 cells were prepared by growth for 6 days in AA-containing medium before cells were cross-linked with formaldehyde for ChIP analysis. Two independent IP reactions were carried out with RUNX2 antibody (Ab) (lanes 1 and 2) and two IP control reactions with normal rabbit IgG (lanes 3 and 4). The gels are ethidium bromide-stained agarose gels of the PCR products obtained using the *Bsp* promoter primers P1–P2 (top) and P3–P4 (bottom). Purified input chromatin was also used to perform parallel PCRs (lane 5) with both pair of primers. The experiment was repeated twice with similar results.
The ability of RUNX2 to bind to R1 and R2 sites in vivo. MC-4 nuclear extracts were preincubated with RUNX2 antibody (Ab) or normal rabbit IgG prior to addition of wild type (oR1 and oR2) or mutant (omR1 and omR2) 32P-labeled oligonucleotides followed by analysis of protein-DNA complexes by EMSA. Oligonucleotides oR1 and oR2 contain R1 and R2 sites, respectively, whereas the mutant oligonucleotides harbor 2-bp mutations in these sites. Supershifted complexes were observed for both wild type oligonucleotides in the presence of specific RUNX2 antibody (lanes 2 and 6), whereas the nonspecific control IgG had no effect (lanes 1 and 5). In contrast, when the oligonucleotides containing mutations (omR1 and omR2) were used as probes, little binding of nuclear proteins to DNA was detected, and no supershifted complexes were observed with RUNX2 antibody (Fig. 3, lanes 3, 4, 7, and 8). These results demonstrate that the protein-DNA complexes formed upon incubation of the labeled oR1 and oR2 with MC-4 nuclear extract contain RUNX2 and are consistent with ChIP assay results showing in vivo RUNX2 binding to the chromatin region containing R1 and R2.

**R1 and R2 Sites Act as Osteoblast-specific Transcriptional Enhancers**—The transcriptional activity of R1 and R2 sites was next examined by comparing the activity of the wild type 2.5-kb Bsp promoter with constructs containing the above described mutations in these two sites. The first set of studies (Fig. 4A) examined RUNX2-dependent activation of promoter activity in C3H10T1/2 cells as was done in Fig. 1B. Mutation of R1 (pMR1) reduced luciferase activity by 50%, whereas mutation in R2 (pMR2) caused only a small drop (~15%) in the activity relative to the wild type promoter. Furthermore, the double mutation of R1 and R2 did not inhibit promoter activity beyond that seen with the single mutation in R1. Also, these mutations did not affect basal promoter activity in cells transfected with control plasmid (pCMV5-LaeZ), indicating that mutations only affect RUNX2-dependent transcriptional activity. These results demonstrate that the R1 site plays an important role in the RUNX2-dependent transcriptional activation of the Bsp promoter and suggest that the R1 site might be functionally more important than R2. However, the functionality of R2 site could be dependent not only on RUNX2 itself but also on other osteoblast-related transcription factors not expressed in C3H10T1/2 mesenchymal cells. For this reason, we considered it important to also examine R1 and R2 mutants in an osteoblast context. To accomplish this, we compared the activity of wild type and mutant constructs in osteoblastic MC-4 cells as well as several non-osteoblast cell lines (C3H10T1/2, C2C12, and COS7). For each cell line, the promoter activity is expressed as a percent of the basal promoter activity in cells transfected with control plasmid (pMR2) caused only a small drop (~15%) in the activity relative to the wild type promoter. In contrast, the double mutant further reduced promoter activity to only 35% of wild type. In contrast, none of these mutations affected transcriptional activity in the non-osteoblast cell lines that do not contain RUNX2. Thus, both R1 and R2 sites function as transcriptional enhancers of the Bsp promoter in osteoblast-like cells, and together they account for nearly two-thirds of the total Bsp promoter activity but are not active in non-osteoblast cells.

**RUNX2 Binds to Both R1 and R2 Sites in Vivo**—Although the study of the natural Bsp promoter by ChIP assay revealed that RUNX2 binds in vivo to a chromatin fragment containing R1 and R2 sites, we could not discriminate between the occupancy of each site because they are separated by only 100 bp, and the average size of the sheared chromatin is 300–400 bp (even after extended sonication). Even though ChIP assays are most often used to examine chromatin interactions in the context of chromosomal DNA, they can also be used to examine interactions with transfected plasmid DNAs. This is possible...
because transfected DNA is assembled into nucleosomes resembling the normal chromatin structure (35). These results suggested that ChIP assays with wild type and mutant Bsp promoter fragments might be used to examine in vivo interactions of RUNX2 with this gene (Fig. 5). For these studies, MC-4 cells were transfected either with the wild type Bsp promoter-luciferase plasmid or with the different mutant plasmids harboring mutations in R1, R2, or in both sites, respectively (pMR1, pMR2, and pMR12). The transfected cells were treated for 6 days with ascorbic acid to induce differentiation. Two independent IP reactions were performed with either an antibody against RUNX2 or control nonspecific antibody (rabbit IgG). The primers used in the PCR analysis (PBL1 and PBL2) amplify a unique 62-bp fragment of the reporter plasmid without the interference from the endogenous Bsp gene. The position of this fragment relative to R1 and R2 sites are shown in Fig. 5A. PCRs with input DNAs were included as controls to demonstrate that similar inputs were used in the immunoprecipitation reactions for all transfections (Fig. 5B, lanes 5 and 6). As shown in Fig. 5B, positive ChIP assays with RUNX2 antibody were observed for reporter constructs that had at least one of the two sites intact, R1 or R2. However, when both sites were mutated, the antibody against RUNX2 failed to immunoprecipitate specific DNA fragments. These results demonstrate that RUNX2 binds to both R1 and R2 sites and that RUNX2-DNA complex was observed with probes harboring the intact C site (oCR2 and oCmR2; Fig. 6, lanes 3 and 7) but not with the oR2 probe (lane 11) that has a truncated C site. These supershifted species were not seen when IgG, DLX5, DLX6, or RUNX2 antibodies were mixed with the nuclear extract. On the other hand, as expected, the supershifted RUNX2-DNA complex was observed with probes harboring the intact R2 site (oR2 and oCR2; Fig. 6, lanes 8 and 12) but not with the mutated R2 site (oCmR2; lane 4). Taken together, these results demonstrate that endogenous DLX5 in osteoblastic MC-4 cells is able to bind to the C site in vitro.

To further investigate the functional role of endogenous DLX5 in Bsp expression, we used ChIP assays to assess DLX5 promoter occupancy in the vicinity of the C site in MC-4 cell chromatin. For this experiment, undifferentiated and differentiated (i.e. AA-treated) cells were compared. As pointed out because transfected DNA is assembled into nucleosomes resembling the normal chromatin structure (35). These results suggested that ChIP assays with wild type and mutant Bsp promoter fragments might be used to examine in vivo interactions of RUNX2 with this gene (Fig. 5). For these studies, MC-4 cells were transfected either with the wild type Bsp promoter-luciferase plasmid or with the different mutant plasmids harboring mutations in R1, R2, or in both sites, respectively (pMR1, pMR2, and pMR12). The transfected cells were treated for 6 days with ascorbic acid to induce differentiation. Two independent IP reactions were performed with either an antibody against RUNX2 or control nonspecific antibody (rabbit IgG). The primers used in the PCR analysis (PBL1 and PBL2) amplify a unique 62-bp fragment of the reporter plasmid without the interference from the endogenous Bsp gene. The position of this fragment relative to R1 and R2 sites are shown in Fig. 5A. PCRs with input DNAs were included as controls to demonstrate that similar inputs were used in the immunoprecipitation reactions for all transfections (Fig. 5B, lanes 5 and 6). As shown in Fig. 5B, positive ChIP assays with RUNX2 antibody were observed for reporter constructs that had at least one of the two sites intact, R1 or R2. However, when both sites were mutated, the antibody against RUNX2 failed to immunoprecipitate specific DNA fragments. These results demonstrate that RUNX2 binds to both R1 and R2 sites and that RUNX2-DNA complex was observed with probes harboring the intact C site (oCR2 and oCmR2; Fig. 6, lanes 3 and 7) but not with the oR2 probe (lane 11) that has a truncated C site. These supershifted species were not seen when IgG, DLX5, DLX6, or RUNX2 antibodies were mixed with the nuclear extract. On the other hand, as expected, the supershifted RUNX2-DNA complex was observed with probes harboring the intact R2 site (oR2 and oCR2; Fig. 6, lanes 8 and 12) but not with the mutated R2 site (oCmR2; lane 4). Taken together, these results demonstrate that endogenous DLX5 in osteoblastic MC-4 cells is able to bind to the C site in vitro.

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Fig. 5. Binding of RUNX2 to R1 and R2 sites in vivo. A, schematic representation of Bsp promoter-luciferase reporter construct used in transfection experiments and ChIP analysis. The relative positions of PBL1 and PBL2 primers, used in ChIP analysis, are indicated by single arrows. These primers were selected to amplify a fragment from the promoter region of the reporter plasmid not present in the endogenous Bsp gene. Double arrows indicate the distance from the 3’ primer (PBL1) to the 5’ primer (PBL2) or to R1 and R2 sites. B, modified ChIP assay. MC-4 cells were transfected with 4 μg of either wild type Bsp promoter-reporter construct (p2472) or promoter containing the indicated mutations (pMR1, pMR2, or pMR12). Transfected cells were treated with ascorbic acid for 6 days to induce differentiation. Cell lysates were then cross-linked with formaldehyde for ChIP analysis using RUNX2 antibody (Ab) (lanes 1 and 2) or rabbit IgG (lanes 3 and 4). The gels are ethidium bromide-stained agarose gels of the PCR products obtained with ChIP-DNAs after 23 amplification cycles using the primers PBL1 and PBL2 (represented in A). Purified input chromatin DNA is also included in the PCRs to compare the transfection efficiencies of the different constructs (lanes 5 and 6, respectively).

above, growth of MC-4 cells in AA-containing medium induces matrix synthesis and, after 5 days, active transcription of osteoblast-related genes such as Ocn and Bsp. In contrast, minimal expression of these genes is observed in cells grown without AA (25). ChIP analysis was performed with primers P1 and P2 located in the vicinity of the C site (see Fig. 2A). Two independent IP reactions were carried out using DLX5 and control IgG (normal goat) antibodies. ChIP assays with RUNX2 antibody were also included as internal controls. The results presented in Fig. 6B show that DLX5 is associated with chromatin near the C site only in differentiated cells (lanes 1 and 2). In contrast, RUNX2 remains chromatin-bound in both differentiated and undifferentiated cells (lane 5). This result could not be explained by changes in DLX5 protein or mRNA levels that were only slightly increased in AA-treated versus control cultures (Fig. 6, C and D). Therefore, the difference in binding seen between differentiated and undifferentiated cells is explained by changes in the chromatin structure making the C site (and possible other homeodomain sites) more accessible to DLX5 and/or changes in the affinity of DLX5 for chromatin. In summary, these results indicate that DLX5 is associated with chromatin in the vicinity of the C site in vivo only in cells actively expressing Bsp.

Cooperative Interactions between R2 and C Sites—A final series of studies examined functional and physical interactions between RUNX2 and homeodomain protein-binding sites. As shown in Fig. 2B, R1, R2, and C sites are all within an ~120-bp region of the proximal Bsp promoter (from −83 to −200). The observation that each of these sites contributes to the osteoblast-selective expression of Bsp compounded with the fact that they are in close proximity suggests that they may also cooperatively interact. To address this issue, transfection experiments were performed in MC-4 cells with the wild type Bsp promoter-luciferase reporter construct and all possible mutant combinations of R1, R2, and the C site. A 2-bp mutation in the core sequence of the homeodomain site was described previously (21). After transfection, cells were treated for 6 days with AA to induce differentiation. Results are expressed relative to wild type activity for the different constructs (Fig. 7). Statistical analysis was also performed to compare groups. Samples can be divided in two sets with similar means: group 1 (pMR1, pMR2, pMC, and pMR2C) and group 2 (pMR12, pMR1C, and pMR12C). All group 1 members were significantly different from group 2 members (p < 0.01). Taken together, these results suggest that R2 and C functionally interact, as the double mutant in these sites (pMR2C) did not cause any further decrease in the promoter activity beyond that seen with individual mutations in R2 (pMR2) or C (pMC). In contrast, the R1 site appears to be independent of R2 and C sites in that combination of an R1 mutation with mutations in R2 (pMR12), C (pMR1C), or the R2C double mutant (pMR12C) always led to an additional ~15% drop in promoter activity. These results are also consistent with those of Fig. 4A showing that R2 (unlike R1) exhibits poor functionality in mesenchymal C3H10T1/2 cells after forced expression of RUNX2. Specifically, they demonstrate that additional C site interacting factors such as DLX5 that are not present in C3H10T1/2 cells are necessary for the R2 site to be functional.

The close proximity and functional interdependence of R2 and C suggests that the factors interacting with these elements (i.e. RUNX2 and DLX5) physically interact. To test this hypothesis, we conducted the co-immunoprecipitation experiments shown in Fig. 8. Two approaches were used. In the first, protein-protein interactions were examined in nuclear extracts from osteoblastic MC-4 cells (Fig. 8A). Extracts were immunoprecipitated with either anti-DLX5 or anti-RUNX2 antibodies, and the immunoprecipitates were probed on Western blots for the presence of RUNX2 or DLX5, respectively. Strong evidence for association of RUNX2 and DLX5 was obtained. The second approach involved transfection of COS7 cells with RUNX2 and FLAG-DLX5 expression plasmids followed by immunoprecipitation and Western blotting. Both FLAG and RUNX2 antibodies were able to immunoprecipitate RUNX2 or DLX5 proteins, respectively (Fig. 8B). Together, these studies provide strong support for the concept that RUNX2 and DLX5 physically and functionally interact. This interaction could be of vital importance not only in relation to the C site but also as a general mechanism for coordinating signals between RUNX2 and homeodomain-containing transcription factors.

DISCUSSION

This study explores functional interrelationships between a previously described homeodomain protein-binding site in the murine Bsp promoter and two newly identified cryptic RUNX2-binding sites. This study relates these enhancer regions and their associated nuclear proteins to the osteoblast-specific expression of Bsp.

We characterized previously a 2472-bp (2.5kb) region of the mouse Bsp promoter (18, 19, 21). Like a similar fragment from the rat Bsp gene, this promoter region contains sufficient information to direct the osteoblast-selective expression of luciferase and lacZ reporter genes in transgenic mice (19, 38). Cell culture studies with related promoter fragments from murine, avian, and human genes also support the concept that tissue-specific enhancers reside in the proximal Bsp promoter (18, 39,
**FIG. 6. Interaction of DLX5 with the C site in differentiated MC-4 cells in vitro and in vivo.**

**A**, EMSA of DLX5 and RUNX2 binding to C site and R2-containing oligonucleotides. MC-4 nuclear extracts (2 μg) were preincubated with the following antibodies (Ab): rabbit IgG, DLX6(G20), DLX5(Y20), and RUNX2(M70). Nuclear extracts were then mixed with the indicated 32P-labeled double-stranded oligonucleotides (oCmR2, oCR2, and oR2; see Table I). Supershifted species with DLX5 and RUNX2 antibodies are indicated by filled circles or asterisks, respectively.

**B**, DLX5 ChIP analysis of control or differentiated MC-4 cells. MC-4 cells were cultured with or without ascorbic acid for 5 days. ChIP analysis was conducted with antibodies against DLX5 (lanes 1 and 2), RUNX2 (lane 5), or preimmune goat IgG (lanes 3 and 4). The gels are ethidium bromide-stained agarose gels of the PCR products obtained with ChIP DNAs using the Bsp promoter-primers P1–P2 (Fig. 1). Input chromatin is also shown (lane 6).

**C**, measurement of DLX5 protein levels by Western blotting (WB). Cell extracts were prepared from control or AA-treated cells and probed for DLX5 using Y20 antibody. Samples represent the equivalent of 5% of the input chromatin solution used in ChIP assays.

**D**, Dlx5 mRNA levels. Total RNA was isolated from control and AA-treated cells and Dlx5 mRNA levels measured by quantitative real time PCR. Results are expressed as fold increase relative to the −AA control. Values are means ± S.D. of triplicate independent RNA samples.
RUNX2 and Homeodomain Protein Regulation of Bsp

Fig. 7. Functional interaction between R2 and C sites. Luciferase (Luc.) reporter constructs containing wild type 2.5-kb Bsp promoter (p2472) or promoter constructs containing all possible combinations of R1, R2, and C-element mutations (as indicated in the left panel) were transfected into osteoblastic MC-4 cells. After transfection, cells were grown for 6 days in AA-containing medium to induce differentiation. Normalized luciferase activities are shown as a percentage of the wild type promoter construct (p2472). Values are the average ± S.D. of three independent experiments each one performed in triplicate samples. Asterisk indicates significantly different values at p < 0.01.

(A) MC-4 cells

WB: anti-RUNX2
IP-Ab

WB: anti-DLX5

RUNX2

DBX2

(B) COS7 cells

WB: anti-RUNX2
IP-Ab

WB: anti-FLAG

FLAG-DLX5

Fig. 8. Protein-protein interactions between DLX5 and RUNX2. A RUNX2-DLX5 interactions in MC-4 cells. Nuclear extracts from MC-4 cells were used to perform IP reactions with anti-DLX5 (Y20) antibody or normal goat IgG (upper panel) or anti-RUNX2 (M70) or normal rabbit IgG (lower panel). Immunocomplexes were analyzed by Western blotting (WB) using either RUNX2 (1:1000) or DLX5 (1:250) antibodies (Ab). B RUNX2-DLX5 interactions in COS7 cells. COS7 cells were co-transfected with RUNX2 and FLAG-DLX5 expression plasmids. After 48 h, nuclear extracts were prepared and used in IP reactions with anti-FLAG (M2) antibody or control mouse IgG (upper panel) or anti-RUNX2 antibody (M70) or normal rabbit IgG (lower panel). Immunoprecipitates were probed on Western blots using either anti-RUNX2 or anti-FLAG antibodies as indicated.

Although additional osteoblast-specific regulatory regions may reside in other parts of the Bsp gene, the strong sequence conservation within the proximal promoter suggests that it is critical for tissue-specific gene expression. Cross-species sequence conservation is particularly striking in the first 343 bp upstream from the transcription start site, which shares a 75% sequence homology across mouse, rat, and human genes (18). We conducted a systematic deletion analysis of the 2.5-kb mouse promoter, and we identified a region between −338 and −178 as having the most dramatic effect on osteoblast-selective activity (21). Further deletion to −49 bp eliminated all residual activity in osteoblasts. DNase I footprinting and functional analysis of the −338 to −178 region identified a homeodomain protein-binding site as being particularly important for promoter activity in osteoblasts. The importance of this site to osteoblast-specific expression was subsequently confirmed in transgenic mice (19). Homologous sites were also identified in human and avian Bsp genes and were shown to be critical for osteoblast-selective expression in cell culture (39, 40).

The present study shows that the −338 to −49 bp region is more complex than originally thought. In addition to the homeodomain protein-binding site (C site), it contains two cryptic RUNX2-binding sites (R1 and R2) whose sequence slightly diverges from the runt domain protein consensus binding sequence (RCCRC(A/T)) we originally used to screen for putative RUNX2-responsive regions (18). Most interestingly, a recent study by Barnes et al. (24) showed that a sequence homologous to R1 in the human Bsp promoter bound RUNX2 present in nuclear extracts from metastatic breast cancer cells expressing Bsp. However, the functionality of this site was not examined in osteoblasts.

On the basis of the present work, it is now clear that R1 and R2 both play major roles in directing the expression of Bsp in osteoblasts. However, their relative activity was found to vary with the experimental system used. For example, in C3H10T1/2 cells transfected with a RUNX2 expression vector, the R1 site predominated. This was observed in the deletion study shown in Fig. 1B as well as the mutational analysis in Fig. 4A. In the deletion study, the major drop in RUNX2-dependent promoter activity occurred between −178 and −49 bp, the region containing R1. In contrast, deletion from −338 bp to −178 bp, the region containing R2, did not affect inducible (or basal) activity. Similarly, mutation of R2 in the context of the 2.5-kb promoter only reduced activity in RUNX2-expressing cells by 15%, whereas the R1 or combined R1/R2 mutations caused a 50% drop. A dramatically different result was obtained when these sites were examined in osteoblast-like MC-4 cells. In this case, mutation of either R1 or R2 reduced promoter activity by 50%, and the double mutation caused a further drop to 35% of the wild type promoter. In contrast, neither mutation affected activity in non-osteoblast cells. In our earlier work, deletion from −338 to −178 bp dramatically reduced Bsp promoter activity only in osteoblast-like cells with residual osteoblast-related activity completely lost with further deletion to −49 bp (21). Although we attributed the loss in activity between −338 and −178 bp to deletion of the homeodomain-binding site or C site (see below), it is now clear that R2 is also contained within this region and contributes to the loss in activity. The increased functionality of R2 in osteoblast-like cells cannot be simply explained by the fact that they have high endogenous RUNX2 levels because this is also the case for C3H10T1/2 cells transfected with Runx2. Instead, these results imply that a second osteoblast-related factor or factors interact.
with R2 in bone cells (see discussion on homeodomain proteins below).

In addition to providing strong evidence for the functionality of R1 and R2, we showed that both sites physically interact with RUNX2 in vitro by EMSA (Fig. 3) and in vivo using ChIP assays (Figs. 2 and 5). This latter approach was particularly instructive because it detects physiological interactions between chromatin and nuclear proteins that are preserved by formaldehyde cross-linking of living cells. The specificity of ChIP analysis was established by the experiment shown in Fig. 2. In this study, ChIP assays clearly detected binding of RUNX2 to a chromatin fragment containing R1 and R2 sites. In contrast, a previously characterized upstream RUNX2 consensus binding site at −1335 bp that binds RUNX2 on EMSA, but has no transcriptional activity (18), gave a negative result when examined by ChIP. In addition, we used a modified ChIP assay that measured the association of RUNX2 with plasmids containing either wild type promoter or promoter with deletions in R1, R2, or both sites to show that R1 and R2 are both occupied in intact cells (Fig. 5). The observation that positive ChIP PCR signals were still obtained with individual R1 or R2 mutations, but were abolished with the double mutation, provides strong evidence that both sites are occupied in vivo. Although ChIP assays are most often used to detect interactions between specific nuclear components and native chromatin, they can also be used to measure interactions with plasmid DNAs that assume a chromatin-like structure after transfection (35). Even though we cannot exclude the possibility that the structure of a plasmid nucleosome may be different from the native chromatin, our ChIP analysis correlates with the functional assays in MC-4 cells and represents a close approximation in order to resolve the Runx2 interactions with R1 and R2 sites.

The present study also clarifies the role of a homeodomain protein-binding site previously characterized by this laboratory. This site, designated the C site or OSHE1, was initially identified by deletion/mutation analysis as being necessary for the osteoblast-selective expression of Bsp in cell culture and in vivo. DLX5 overexpression in COS7 cells was also shown to stimulate C site-mediated gene expression (21). However, because DLX5-specific antibodies were not available at the time, we were not able to detect binding of endogenous DLX5 present in osteoblast nuclear extracts to the C site. Instead, nuclear extracts from COS7 cells transfected with a FLAG-tagged DLX5 expression vector were shown to form a specific complex with a C site-containing oligonucleotide. In the present study, we extend this work using DLX5-specific antibodies and supershift EMSAs to directly demonstrate that DLX5 present in osteoblast nuclear extracts can bind to a C site oligonucleotide (Fig. 6A). Of greater significance, ChIP assays revealed that DLX5 associates with C site containing chromatin only in differentiated osteoblasts actively synthesizing Bsp (Fig. 6, B–D). In contrast, DLX5 protein or mRNA levels were only slightly increased with differentiation. Possible explanations for this result include post-translational modifications in the DLX5 protein, the induction of accessory factors to facilitate DLX5-C site binding or more global changes in chromatin structure that make the C site accessible to DLX5. In contrast, RUNX2 remained bound to R1 and R2 sites regardless of the differentiation state of cells. Thus, RUNX2 remains associated with Bsp promoter-containing chromatin regardless of transcriptional state, whereas the binding of DLX5 is positively correlated with promoter-containing chromatin regardless of transcriptional state of cells. Thus, RUNX2 remains associated with R1 and R2 sites in vivo.

The close physical and functional association of the C site with R2 (Figs. 2 and 7) and the observation that RUNX2 and DLX5 proteins physically interact (Fig. 8) may explain why R2 has activity comparable with R1 in osteoblast-like cells, while having little activity in RUNX2 and DLX5-negative C3H10T1/2 mesenchymal cells even after transfection with a RUNX2 expression vector (Fig. 4). It is unlikely that the R2 sequence functions only as a flanking sequence that is necessary for DLX5 binding to the C site because a 2-bp mutation in R2 did not affect DLX5 binding on gel shift assays (Fig. 6). In contrast, this same mutation totally abolished RUNX2 binding. Because only a few bases separate R2 and C sites, RUNX2 and DLX5 should be able to readily interact on the promoter surface.

DLX5 is expressed by osteoblasts and associated with induction of differentiation (13–16, 36). Consistent with our results, Lee et al. (41) showed that DLX5 is specifically induced by BMP2 in mesenchymal C2C12 cells suggesting that DLX5 is an indispensable regulator of osteoblast differentiation. We demonstrated previously that transduction of C3H10T1/2 cells with a BMP2 expression vector similarly induces osteoblast differentiation by these cells (29). In unpublished work, we observed that BMP2 treatment of C3H10T1/2 cells also induces DLX5.

Although our studies provide evidence that DLX5 is a physiologically significant C site-binding factor, we cannot exclude the involvement of other homeodomain proteins, including other members of the Dlx family in this regulation. This more complex regulation was proposed previously for the osteocalcin promoter, which contains a related homeodomain protein-binding site (15).

In summary, this study shows that osteoblast-selective expression of Bsp can be explained by interactions between two classes of enhancers residing in the first 200 bp of the promoter sequence, a homeodomain protein-binding site (the C site), and two RUNX2-binding sites, R1 and R2. All three of these sites are required for full promoter activity. The C site binds DLX5 present in osteoblasts, although we cannot exclude the possibility that other homeodomain proteins interact with this site as well. RUNX2 occupancy of both R1 and R2 was established in vivo using ChIP assays. Our studies show that full activity of R2 requires a functional C site and interaction of osteoblast-related factors such as DLX5 with this site. The finding that osteoblast-specific Bsp expression requires RUNX2 and its interaction with a homeodomain protein provides new insights into mechanisms controlling osteoblast-specific gene expression that are of crucial importance in the process of bone formation. Our ongoing studies are aimed at investigating RUNX2-DLX5 interactions as well as interactions with other homeodomain proteins and the role of these interactions in the control of the osteoblast phenotype.

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Cooperative Interactions between RUNX2 and Homeodomain Protein-binding Sites Are Critical for the Osteoblast-specific Expression of the Bone Sialoprotein Gene
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