

Dlx5 Specifically Regulates Runx2 Type II Expression by Binding to Homeodomain-response Elements in the *Runx2* Distal Promoter*

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Two major isoforms of the *Runx2* gene are expressed by alternative promoter usage: Runx2 type I (Runx2-I) is derived from the proximal promoter (P2), and Runx2 type II (Runx2-II) is produced by the distal promoter (P1). Our previous results indicate that Dlx5 mediates BMP-2-induced Runx2 expression and osteoblast differentiation (Lee, M.-H., Kim, Y.-J., Kim, H.-J., Park, H.-D., Kang, A.-R., Kyung, H.-M., Sung, J.-H., Wozney, J. M., Kim, H.-J., and Ryou, H.-M. (2003) *J. Biol. Chem.* 278, 34387–34394). However, little is known of the molecular mechanisms by which Dlx5 up-regulates Runx2 expression in BMP-2 signaling. Here, Runx2-II expression was found to be specifically stimulated by BMP-2 treatment or by Dlx5 overexpression. In addition, BMP-2, Dlx5, and Runx2-II were found to be expressed in osteogenic fronts and parietal bones of the developing cranial vault and Runx2-I and Msx2 in the sutural mesenchyme. Furthermore, *Runx2* P1 promoter activity was strongly stimulated by Dlx5 overexpression, whereas *Runx2* P2 promoter activity was not. *Runx2* P1 promoter deletion analysis indicated that the Dlx5-specific response is due to sequences between –756 and –342 bp of the P1 promoter, where three Dlx5-response elements are located. Dlx5 responsiveness to these elements was confirmed by gel mobility shift assay and site-directed mutagenesis. Moreover, Msx2 specifically suppressed the *Runx2* P1 promoter, and the responsible region overlaps with that recognized by Dlx5. In summary, Dlx5 specifically transactivates the *Runx2* P1 promoter, and its action on the P1 promoter is antagonized by Msx2.

The Runx2-related transcription factor Runx2 plays an essential role in osteoblast differentiation and bone mineralization (1, 2). Two major isoforms are expressed from the mouse *Runx2* locus, and these isoforms are generated by different promoter usage. Runx2 type I (Runx2-I)² referred to as the Cbfa1/p56 isoform or PEBP2 α A, is a 513-amino acid

protein that starts with the amino acid sequence MRIPV (3) and is derived from the proximal P2 promoter of the gene (4). More recently, upstream exons of the *Runx2* gene that potentially encode the N termini of Runx2 isoforms expressed in osteoblasts have been identified (5, 6). These upstream exons contain a 5'-untranslated region and encode the N-terminal 19 amino acids of Runx2 type II (Runx2-II; also referred to as Cbfa1/p57 and OSF2), which starts with the sequence MASNSL (7). This isoform is expressed from the P1 or "bone-related" upstream promoter (8), and its expression is predominant in osteoblasts (9). The alternative promoter usage strongly implies that the expression pattern of each isoform differs temporally and/or spatially. Indeed, they exhibit distinct expression patterns during bone development (10, 11). Thus, it is natural to assume that these two promoters differently respond to different extracellular signals or their downstream transcription factors because these promoters have distinct transcription factor-binding sites.

Runx2 plays a central role in the BMP-2-induced *trans*-differentiation of C2C12 cells at an early restriction point by diverting them from the myogenic pathway to the osteogenic pathway (12, 13). We found that the homeobox gene Dlx5 is an upstream target of BMP-2 signaling and that it plays a pivotal role in stimulating the downstream osteogenic master transcription factor Runx2. In turn, Runx2 acts simultaneously or sequentially to induce the expression of bone-specific genes that represent BMP-2-induced osteogenic *trans*-differentiation. In addition, it has also been suggested that Dlx5 is a critical target of the inhibitory action of transforming growth factor- β 1 (TGF- β 1) on BMP-2-induced osteoblast *trans*-differentiation (14).

Several lines of *in vivo* evidence indicate that the Dlx and Msx families of homeodomain proteins include regulatory factors that preferentially support skeletal tissue differentiation. Among them, Dlx5 is a bone-inducing transcription factor that is expressed in the later stages of osteoblast differentiation (15). Forced expression of Dlx5 in cultured cells leads to osteocalcin expression and a fully mineralized matrix (14, 16, 17). These results strongly suggest that Dlx5 plays important roles in the development of mineralized tissues. Another homeodomain protein that plays critical roles in bone formation and osteoblast differentiation (Msx2) is also induced by bone morphogenetic proteins (BMPs) (18). Several *in vitro* studies have shown that Msx2 negatively regulates the transcription of osteoblast-specific genes such as osteocalcin (15, 19). This was further supported by findings that the Dlx5 and Msx2 proteins bind the promoters of osteoblast markers and appear to have opposing transcriptional properties because Msx2 functions as a transcriptional

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² The abbreviations used are: Runx2-I, Runx2 type I; Runx2-II, Runx2 type II; TGF- β 1, transforming growth factor- β 1; BMPs, bone morphogenetic proteins; AS, antisense; HDREs, homeodomain-response elements; EMSA, electrophoretic mobility shift assay.

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TABLE ONE

Primer sequences for construction of Runx2 promoter deletion mutants and site-directed mutagenesis

The lowercase letters correspond to restriction enzyme sites for KpnI (forward primers) or StuI (reverse primer). The lowercase italic boldface letters designate the substitution of nucleotide for site-directed mutagenesis. The sequence location was calculated from the transcription start site of the *Runx2* P1 promoter.

Name	Oligonucleotide sequence	Sequence location
		<i>bp</i>
P1-610	5'-GCggtaccGCCACACACTCAGTTGAGAC-3' (forward)	-610 to -591
P1-458	5'-CAggtaccTGCTCTCCAGAGGCTTAACC-3' (forward)	-458 to -439
Reverse	5'-CTTGTGGTAaggcctTCCTG-3'	-331 to -350
MTD1	5'-GATACAATCCCAAGATGCGAgcgggTGCAAAGCAGCACTGTTGCTC-3' (forward)	-661 to -616
MTD2	5'-CAATTTTGCTCACTTTTCCATAGACaccgtcgcAAGGAAAGGGAGGAGGGGTAG-3' (forward)	-591 to -537
MTD3	5'-CAAATCCTCATGAGTCACAAAagcggcAAAGCTATAACCTTCTGAATG-3' (forward)	-399 to -352
GL2	5'-CTTTATGTTTTTGGCGTCTTCCA-3' (reverse)	
RV3	5'-CTAGCAAATAGGCTGTCCC-3' (forward)	

repressor (20–22), whereas Dlx5 is an activator of osteoblast marker gene expression (15, 23–25).

Dlx5 is the key mediator of BMP-induced Runx2 expression (14), but little is known about how it regulates Runx2 expression in response to BMP-2 signaling. Therefore, the first aim of this study was to assess which promoter responds to BMP signaling because Runx2 has two major isoforms whose expression is regulated by alternative promoters. In addition, as Dlx5 acts downstream of BMP-2, we undertook to define *cis*-acting elements in the BMP-2-responsive *Runx2* promoter that interact with Dlx5. Finally, as *Msx2* is thought to inhibit the Dlx5-induced expression of osteoblast marker genes (20, 21), we also explored the relationship of these two transcription factors with respect to the *Runx2* promoter. Here, we demonstrate that Dlx5 up-regulates Runx2 (more specifically, Runx2-II) expression and that the effect of Dlx5 on the *Runx2* P1 promoter is antagonized by *Msx2*.

EXPERIMENTAL PROCEDURES

Materials—Bioactive recombinant human BMP-2 was from Wyeth Pharmaceuticals (Cambridge, MA). Recombinant human TGF- β 1 was purchased from R&D Systems (Minneapolis, MN). Dulbecco's modified Eagle's medium, α -minimal essential medium, fetal bovine serum, and Lipofectamine PLUSTM reagent were from Invitrogen. Tissue culture plasticware was from Corning (Corning, NY); the Megaprime DNA labeling system was from Amersham Biosciences; DNA midi-prep kits were from Qiagen Inc.; and ExpressHyb hybridization solution was from Clontech. The Zeta-Probe blotting membrane was from Bio-Rad, and the Dual-Luciferase reporter assay kit, *Taq* polymerase, *Pfu* polymerase, dNTP mixture, pGL3-Basic vector, and TNT coupled reticulocyte lysate system were from Promega Corp. Anti-FLAG monoclonal antibody M2, HEPES, Nonidet P-40, EDTA, dithiothreitol, phenylmethylsulfonyl fluoride, glycerol, and poly(dI-dC) were purchased from Sigma.

Cell Culture and Northern Blot Analysis—Mouse myogenic C2C12 cells, osteoblast-like MC3T3-E1 cells, and the rat osteosarcoma cell line ROS17/2.8 were maintained as described previously (12). C2C12 cells stably transfected with *Dlx5*, antisense (AS) *Dlx5* (*Dlx5*-AS), *Msx2*, and antisense *Msx2* (*Msx2*-AS) constructs were established and maintained as described previously (14). The Runx2^{-/-} calvarial cell line H1-127-21-2 was maintained as described previously (13). Chinese hamster ovary cells was maintained in α -minimal essential medium in the presence of 10% fetal bovine serum. C2C12 cells were plated at a density of 1×10^6 cells/100-mm culture dish. To examine the effects of BMP-2 or TGF- β 1 on cell differentiation, the cells were cultured for the indicated periods with or without the indicated factors in medium with 5% fetal bovine serum. All cells were harvested with phosphate-buffered saline by scraping with a rubber policeman at 4 °C. Total RNA was extracted

from the cells, and Northern blot analysis for Runx2 was performed as described previously (14).

Reverse Transcription-PCR and Quantitative Real-time PCR—Conventional reverse transcription-PCR for murine Runx2 was performed with the primers described previously (26). Quantitative real-time PCR was carried out using SYBR Green fluorescence dye on a LightCycler machine (Roche Applied Science, Mannheim, Germany) as described previously (27). PCR primers for mouse Runx2 (forward, 5'-CCA-GAATGATGGTGTGACG-3'; and reverse, 5'-GGTTGCAAGAT-CATGACTAGGG-3') and mouse glyceraldehyde-3-phosphate dehydrogenase (26) were synthesized by TaKaRa Korea (Seoul, Korea). All samples were run in triplicate, and the relative levels of *Runx2* mRNA were normalized to those of glyceraldehyde-3-phosphate dehydrogenase.

DNA Constructs—The construction of the *Dlx5*, *Dlx5*-AS, *Msx2*, and *Msx2*-AS expression vectors and the establishment of each stable cell line have been described previously (14, 25). The mouse *Runx2* P1 promoter (-2782 to +112 bp) and the *Runx2* P2 promoter (-4056 to +246 bp) were generated based on GenBankTM accession numbers AF155360 (from 1784 to 4677 bp) and AF155361 (from 1 to 4303 bp), respectively. The *Runx2* P1 and *Runx2* P2 promoter deletion constructs were generated by serial deletion from the 5'-end of the promoter with mung bean nuclease, and the fragments were ligated into the KpnI/BglII and SmaI sites, respectively, of the pGL3-Basic vector. The *Runx2* P1 promoter deletion constructs P1-342 (-342 to +112 bp), P1-756 (-756 to +112 bp), P1-1664 (-1664 to +112 bp), and P1-2782 (-2782 to +112 bp) were cloned into the pGL3-Basic vector, as were the *Runx2* P2 promoter deletion constructs P2-857 (-857 to +246 bp), P2-1843 (-1843 to +246 bp), P2-2648 (-2648 to +246 bp), and P2-4056 (-4056 to +246 bp). The *Runx2* P1 promoter deletion constructs P1-458 (-458 to +112 bp; containing the Dlx5 D3-binding site) and P1-610 (-610 to +112 bp; containing the Dlx5 D2/D3-binding sites) were constructed using P1-756 as a template. The forward primers (bearing a KpnI restriction site for the P1-458 and P1-610 constructs) and the reverse primer (bearing a StuI restriction site) are listed in TABLE ONE. Each PCR product was digested with KpnI and StuI and subcloned into the KpnI/StuI-digested *Runx2* P1-756 vector. Myc-tagged *Dlx5* and FLAG-tagged *Msx2* were subcloned into pcDNA3.1.

Site-directed Mutagenesis of Dlx5-binding Sites—To produce constructs that bear mutations of each binding site alone, two sites, or all three sites in the three putative homeodomain-response elements (HDREs) of the *Runx2* P1-756 construct, a site-directed mutagenic two-step PCR strategy was followed. For the first set of PCRs, three mutant primers designated MTD1 (-661 to -616 bp), MTD2 (-591 to -537 bp), and MTD3 (-399 to -352 bp) (TABLE ONE) were used as

the forward primers, and the GL2 primer (Promega Corp.) was used as the reverse primer to create Runx2-MTD1, Runx2-MTD2, and Runx2-MTD3, respectively, using the *Runx2* P1-756 construct as a template. In the second round of PCR, the RV3 primer (Promega Corp.) was used as the forward primer, and PCR products from the first round were used as the reverse primers with P1-756 as a template. The cycling parameters for PCR were as follows: 94 °C for 60 s, 48 °C for 1 min (first round) or 44 °C for 1 min (second round), and 72 °C for 2 min for 35 cycles, followed by 72 °C for 10 min. PCR products with mutated binding sites were digested with KpnI/PstI, and the resulting 868-bp fragments were ligated to pGL3-Basic. Derivatives with two or three mutated sites were constructed using combinations of each mutant construct described above. All constructs were confirmed by sequencing (Macrogen, Seoul).

In Situ Hybridization—Probe preparation (BMP-2, *Dlx5*, *Msx2*, *Runx2-I*, and *Runx2-II*), tissue preparation, and *in situ* hybridization procedures were as described previously (10). Calvariae of ICR mice (embryonic day 16) were prepared as described previously (28). Sections were stained with hematoxylin and eosin to assess the developing calvaria. The 240-bp murine BMP-2 fragment in pGEM3 (Promega Corp.) was digested with HindIII or EcoRI. The 800-bp rat *Dlx5* fragment in pCR2 (Invitrogen) was digested with BamHI or XbaI. The 850-bp murine *Msx2* fragment in pSP72 (Invitrogen) was digested with HindIII or BglII. In all three cases, antisense and sense riboprobes were produced by T7 and SP6 RNA polymerases, respectively. *Runx2-I*- and *Runx2-II*-specific riboprobe preparation and *in situ* hybridization were as described previously (10, 28).

Transfection and Luciferase Assay—C2C12, ROS17/2.8, and *Runx2*^{-/-} cells were plated in 6-well plates at a density of 1×10^5 cells/well. After overnight culture, cells were transfected with Lipofectamine PLUSTM according to the manufacturer's instructions. Each transfection assay was performed with 0.5 μ g of the *Dlx5* or *Msx2* expression vector or pcDNA3 and 0.5 μ g of the *Runx2* promoter-luciferase reporter vector. All plasmid DNA was prepared using a DNA midi-prep kit. Three hours after transfection, the medium was changed, and the cells were cultured for the indicated periods. The cells were then harvested, and luciferase activity was determined with a Dual-Luciferase reporter assay kit. The results presented are representative data from at least three independent experiments with triplicate samples.

Electrophoretic Mobility Shift Assay (EMSA)—The oligonucleotide sequences of three putative homeodomain-binding sites between -342 and -756 bp from the transcription start site of the *Runx2* P1 promoter were synthesized (Takara Bio Inc., Shiga, Japan) (see Fig. 4D). The mutant D3 oligonucleotides were synthesized by substituting the ATTA sequence with the underlined sequences in Fig. 4D. These double-stranded DNA probes were end-labeled with [α -³²P]dCTP using Klenow enzyme. The *Dlx5* and *Msx2* proteins were produced by *in vitro* transcription and translation using the TNT coupled reticulocyte lysate system. Nuclear extracts from C2C12 or Chinese hamster ovary cells that were transiently transfected with *Dlx5* for 24 h were prepared as described previously (29). The *Dlx5* or *Msx2* protein was incubated with labeled double-stranded DNA probes in the absence or presence of a 50-, 100-, or 500-fold molar excess of unlabeled competitor for 20 min at room temperature. For the supershift assay, *Dlx5* or *Msx2* was preincubated with anti-*Dlx5* rabbit polyclonal antibody (14) or anti-FLAG monoclonal antibody M2, respectively, for 20 min at room temperature before incubation with the labeled probe. Protein-DNA complexes were separated at 4 °C on a 6% polyacrylamide gel containing 0.5 \times Tris borate/EDTA.

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation assays were performed as described in detail previously (30,

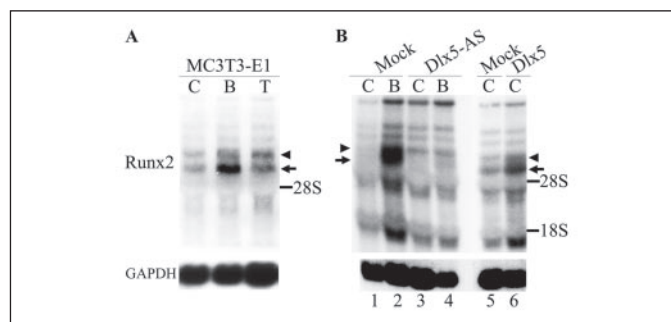


FIGURE 1. **Runx2-II is specifically stimulated by BMP-2.** A, MC3T3-E1 cells were untreated (control (C)) or treated with BMP-2 (B; 300 ng/ml) or TGF- β 1 (T; 5 ng/ml) for 72 h. B, mock-transfected C2C12 cells or C2C12 cells stably transfected with *Dlx5* or *Dlx5-AS* (14) were untreated or treated with 300 ng/ml of BMP for 72 h. The *Dlx5-AS*-expressing cell lines did not express *Dlx5* protein in the treatment with BMP-2 in our previous study (14). Total RNA was analyzed by Northern blot hybridization with a full-length *Runx2* cDNA probe with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a loading control. The two major *Runx2* transcripts, *Runx2-I* (~6.0 kb; arrowheads) and *Runx2-II* (~5.4 kb; arrows), and 18 S and 28 S rRNAs are designated.

31). C2C12 cells were transiently transfected with the Myc-*Dlx5* or FLAG-*Msx2* construct for 24 h. PCR primer pairs were generated to detect DNA segments located between nucleotides -715 and -431 of the mouse *Runx2* distal promoter (nucleotide +1 is the mRNA cap site): forward, 5'-AACAGAAGGAAGCAGCCACC; and reverse, 5'-CCA-CACTCCTGTAAGGTTAAGC.

RESULTS

Runx2-II Is Specifically Stimulated by BMP-2 and *Dlx5*—Our previous study showed that both BMP-2 and TGF- β 1 stimulate *Runx2* expression in C2C12 cells (12). In this study, we found that BMP-2 treatment stimulated the expression of *Runx2-II* (5.4 kb) (Fig. 1A, arrow) and *Runx2-I* (6.0 kb) (arrowhead) transcripts, whereas TGF- β 1 treatment stimulated mainly *Runx2-I* expression. *Runx2-II* expression was more strongly stimulated by BMP-2 treatment compared with *Runx2-I* expression. In addition, overexpression of *Dlx5*, which mediates *Runx2* expression in response to BMP-2 signaling (14), also specifically stimulated *Runx2-II* transcription (Fig. 1B, lanes 5 and 6). Moreover, in cells stably transfected with *Dlx5-AS* (14), the antisense intervention of *Dlx5* specifically blocked the BMP-2-stimulated expression of *Runx2-II*, but not *Runx2-I* (Fig. 1B, lanes 1-4).

We previously demonstrated that these two *Runx2* isoforms are differentially expressed during intramembranous bone formation in mouse cranial and axial bone development (10, 11). As *Runx2-II* expression was specifically induced by BMP-2 treatment or by overexpression of its immediate downstream target gene *Dlx5*, we examined the expression of BMP-2, *Dlx5*, and *Runx2* in the developing mouse calvaria. The pattern of *Dlx5* expression matched those of BMP-2 and *Runx2-II* (Fig. 2, A-C). All three transcripts were strongly expressed in osteogenic fronts and parietal bones, but not in poorly differentiated sutural mesenchymal cells, whereas *Runx2-I* and *Msx2* were strongly expressed in the latter cells (Fig. 2, E and F).

***Dlx5* Specifically Enhances *Runx2* P1 Promoter Activity**—Because expression of the two major isoforms of *Runx2* is differentially regulated by two different promoters and because both BMP-2 treatment and *Dlx5* overexpression are more closely associated with *Runx2-II*, the P1 promoter product, we analyzed the responsiveness of the *Runx2* P1 and P2 promoters to *Dlx5* overexpression. For this purpose, we prepared a series of 5'-deletion constructs as illustrated in Fig. 3A. The basal promoter activity of P1 promoter deletion constructs was notably stronger than that of P2 promoter deletion constructs (Fig. 3B).

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FIGURE 2. Expression patterns of BMP-2, Dlx5, Msx2, and Runx2 isoforms in the developing mouse sagittal suture. Serial coronal tissue sections of embryonic day 16.5 mouse calvariae were hybridized with ³⁵S-UTP-labeled riboprobes *BMP-2* (A), *Dlx5* (B), *Runx2-II* (C), *Msx2* (E), *Runx2-I* (F). The intensity of the red dots indicates the expression level of each mRNA. Hematoxylin and eosin staining (H&E) of the tissue section is shown in D. D, dura mater; P, parietal bone; OF, osteogenic fronts; S, skin; SM, sutural mesenchyme.

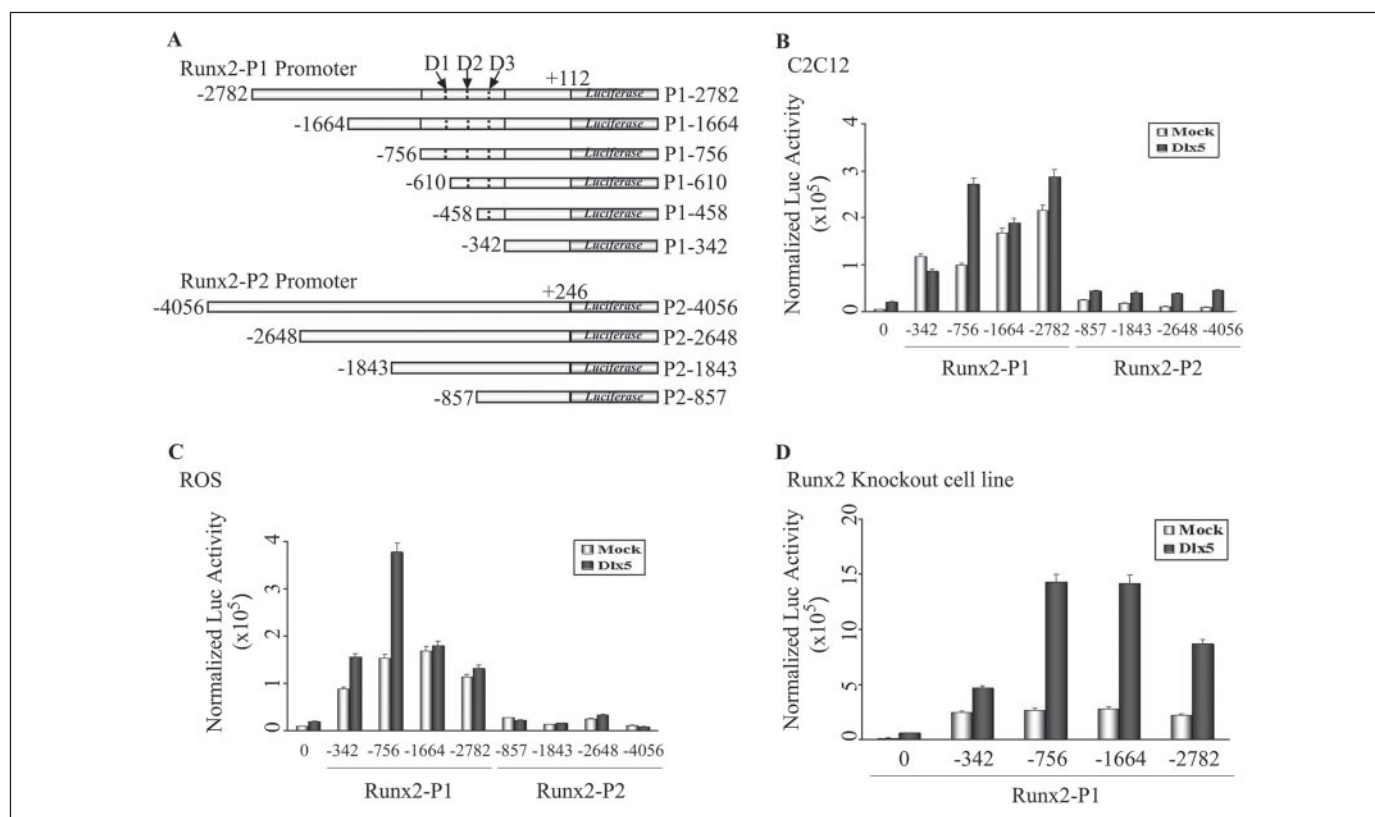
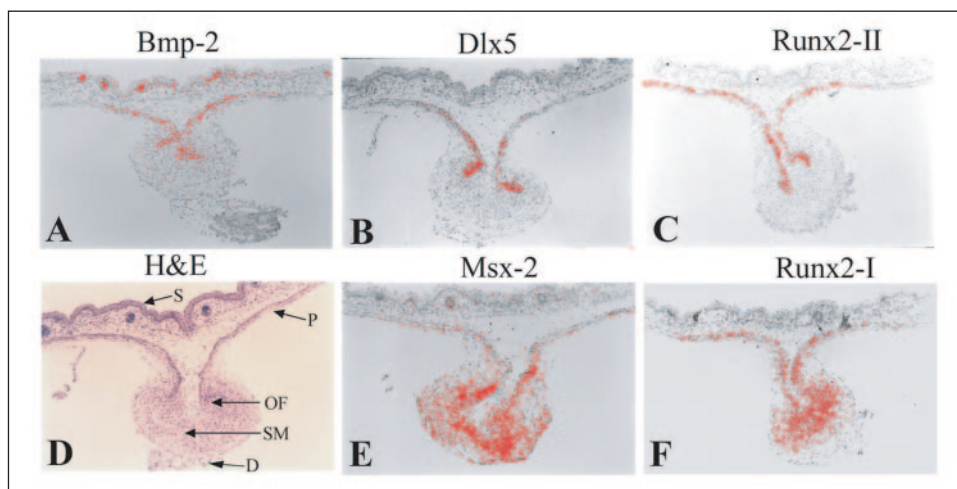


FIGURE 3. The Runx2 P1 promoter is specifically activated by Dlx5 overexpression. Serial deletion constructs of the *Runx2* P1 and P2 promoters are illustrated in A. C2C12 cells (B), ROS17/2.8 cells (C), and *Runx2*^{-/-} cells (clone H1-127-21-2) (D) were transfected with *Runx2* P1 promoter-reporter constructs (P1-342, P1-756, P1-1664, and P1-2782) or with *Runx2* P2 promoter-reporter constructs (P2-857, P2-1843, P2-2648, and P2-4056) together with a *Dlx5* expression vector or a mock vector. The luciferase (*Luc*) activity was measured and normalized to the protein concentration in the cell lysates.

P1 promoter deletion analysis indicated a strong increase in reporter activity between P1-342 and P1-756 in both non-osteogenic (C2C12) and osteogenic (ROS17/2.8) cells. Interestingly, the stimulatory activity of *Dlx5* disappeared for longer constructs in these cells (P1-1664 and P1-2782) (Fig. 3, B and C). In contrast, the stimulatory effect of *Dlx5* was determined in P1-756 and was still observed for longer constructs (P1-1664 and P1-2782) in *Runx2*^{-/-} cells (Fig. 3C). Taken together, these results suggest that the *Dlx5*-response element in the P1 promoter is located between -756 and -342 bp from the *Runx2-II* transcription start site.

Identification of Dlx5-response Elements in the Runx2 P1 Promoter—*In silico* analysis of the *Runx2* P1 promoter indicated that it contains three putative

HDREs between nucleotides -756 and -342, which have the core binding sequence ATTA. We designated these sites as D1, D2, and D3 (Fig. 4D). The D2 site includes two successive homeodomain-binding core sequences, and the D3 site is located closely to the Smad1- and AP1-binding consensus sequences. As *Runx2-II* expression is stimulated by *Dlx5*, we determined whether *Dlx5* binds to these putative HDREs by gel mobility shift assays. For this experiment, D1, D2, and D3 oligonucleotides bearing the core sequence plus surrounding nucleotides (designated WT in Fig. 4D) were synthesized, labeled, and used in EMSAs with *in vitro* translated *Dlx5* protein. The radiolabeled D1, D2, and D3 probes clearly formed a binding complex with the *Dlx5* protein (Fig. 4A, lanes 2). Furthermore, binding of *Dlx5* to each labeled probe was diminished in the presence of a molar excess of unlabeled D1 (Fig. 4A, lanes 3–5), D2 (lanes 6–8),

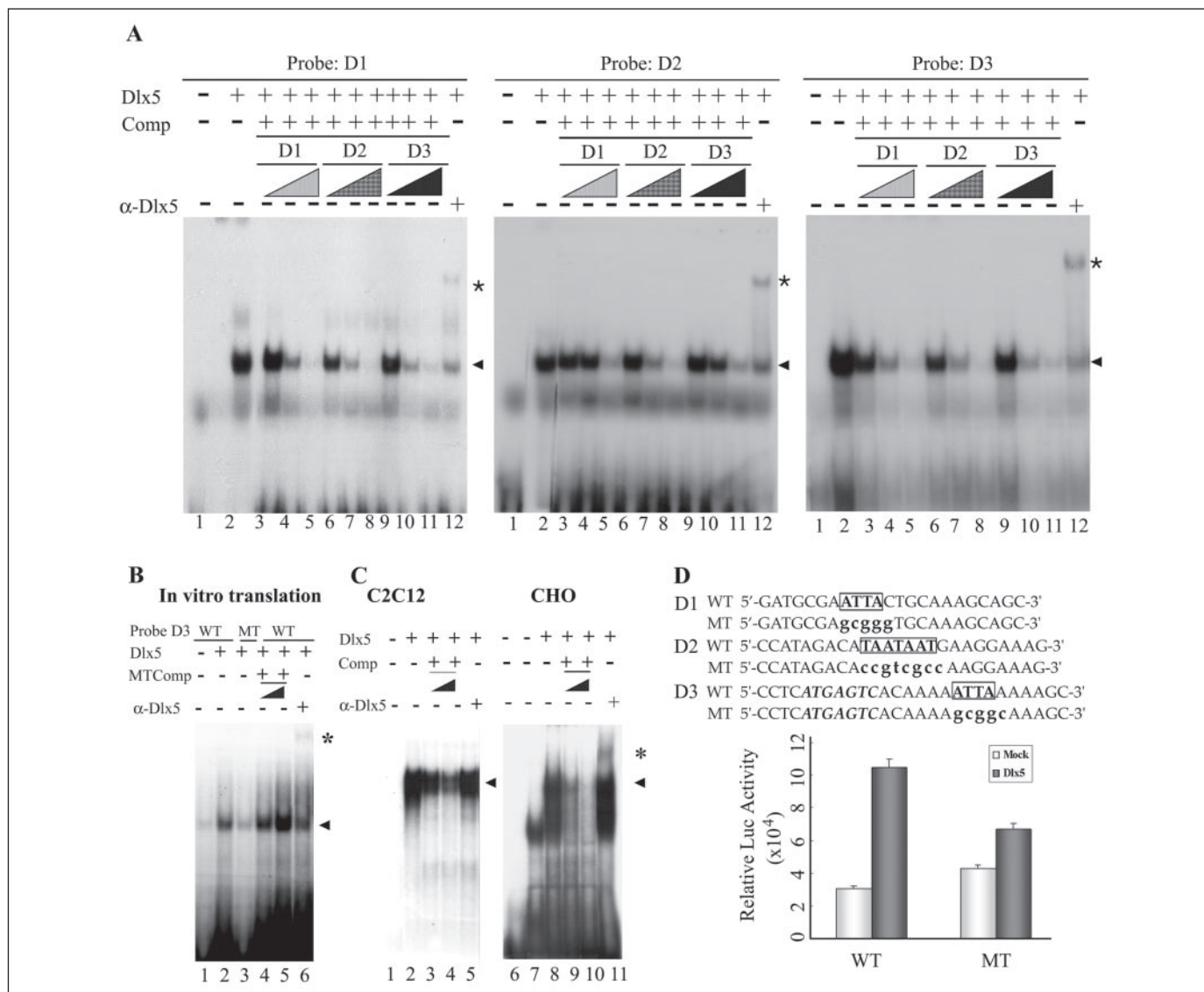


FIGURE 4. Three putative HDREs in the Runx2 P1 promoter specifically bind to Dlx5. *A*, 32 P-labeled D1 (left panel), D2 (center panel), and D3 (right panel) probes were incubated with *in vitro* translated Dlx5. Lanes 1, free probe; lanes 2–11, Dlx5 protein incubated with the labeled D1, D2, or D3 probe alone (lanes 2) or in the presence of a 50-, 100-, or 500-fold molar excess of unlabeled D1 oligonucleotides (lanes 3–5, respectively), unlabeled D2 oligonucleotides (lanes 6–8, respectively), or unlabeled D3 oligonucleotides (lanes 9–11, respectively); lane 12, Dlx5 binding confirmed by a supershift assay with anti-Dlx5 antibody. The arrowheads indicate binding of Dlx5 to each probe, and the asterisks indicate a supershift by anti-Dlx5 antibody. *B*, 32 P-labeled D3 oligonucleotides corresponding to the wild-type (WT) and mutant (MT) D3 probes were incubated with *in vitro* translated Dlx5 protein. The probe was incubated with the rabbit reticulocyte lysates that were used for *in vitro* transcription and translation (lane 1). The Dlx5 protein was incubated with the labeled wild-type D3 probe (lane 2), whose binding to Dlx5 was competed by a 50- or 100-fold molar excess of unlabeled mutant competitor (MTComp) oligonucleotides (lanes 4 and 5, respectively). The Dlx5 protein was incubated with the labeled mutant D3 probe (lane 3). Dlx5 binding was confirmed by a supershift assay (lane 6). *C*, C2C12 and Chinese hamster ovary (CHO) cells were transiently transfected with Dlx5 for 1 day. The nuclear extracts were prepared and incubated with oligonucleotides corresponding to the wild-type D3 probe. Lanes 1 and 6, free probe; lanes 2–5 and 8–11, Dlx5-transfected nuclear extracts, lane 7, mock vector-transfected nuclear extract. Binding of the D3 probe to Dlx5 was subjected to competition (Comp) from a 50-fold (lanes 3 and 9) or 100-fold (lanes 4 and 10) molar excess of unlabeled wild-type oligonucleotides. The mobility shift caused by Dlx5 binding was confirmed by a supershift assay (lane 11). The arrowheads indicate binding of Dlx5 to the probe, and the asterisk indicates a supershift by anti-Dlx5 antibody. *D*, the Runx2 P1 promoter construct bearing three putative HDREs in the region between –756 and +112 bp was subjected to site-directed mutagenesis of all three sites to substitute the ATTA sequence with the designated sequences (boxed). ROS17/2.8 cells were cotransfected the wild-type Runx2 P1–756 construct (WT bars) or the triple mutant reporter vector (MT bars) together with the Dlx5 expression vector or empty vector. Luc, luciferase.

or D3 (lanes 9–11) probe. There was no remarkable difference in Dlx5 binding affinity among these sites. Bands corresponding to each protein–DNA probe complex were supershifted when anti-Dlx5 antibody was introduced (Fig. 4A, lanes 12).

This EMSA analysis was performed with D3 site mutants (designated D3 MT in Fig. 4D). The Dlx5 binding affinity almost disappeared in the mutant D3 probe (Fig. 4B, lane 3). Furthermore, the binding between the labeled wild-type D3 probe and Dlx5 was not competed by a 50- or 100-fold molar excess of unlabeled mutant D3 probe (Fig. 4B, lanes 4 and 5). The supershift (asterisk) and weakened original shift (arrow) by

anti-Dlx5 antibody confirmed the specificity (Fig. 4B, lane 6). Dlx5 binding to the sequence was further confirmed using nuclear extracts from C2C12 and Chinese hamster ovary cells that were transiently transfected with Dlx5 (Fig. 4C, lanes 2–5 and 8–11).

To understand the functional consequence of Dlx5 binding to each HDRE in Runx2 P1 promoter activity, we performed site-directed mutagenesis on each binding site alone (D1, D2, or D3 site mutant) or on two (D1/D2, D2/D3, or D1/D3 site mutants) or three sites in the Runx2 P1–756 construct. Constructs with single or double binding site mutations were not sufficient to block P1 promoter activity to <50% of

Dlx5 Specifically Regulates Runx2-II Expression

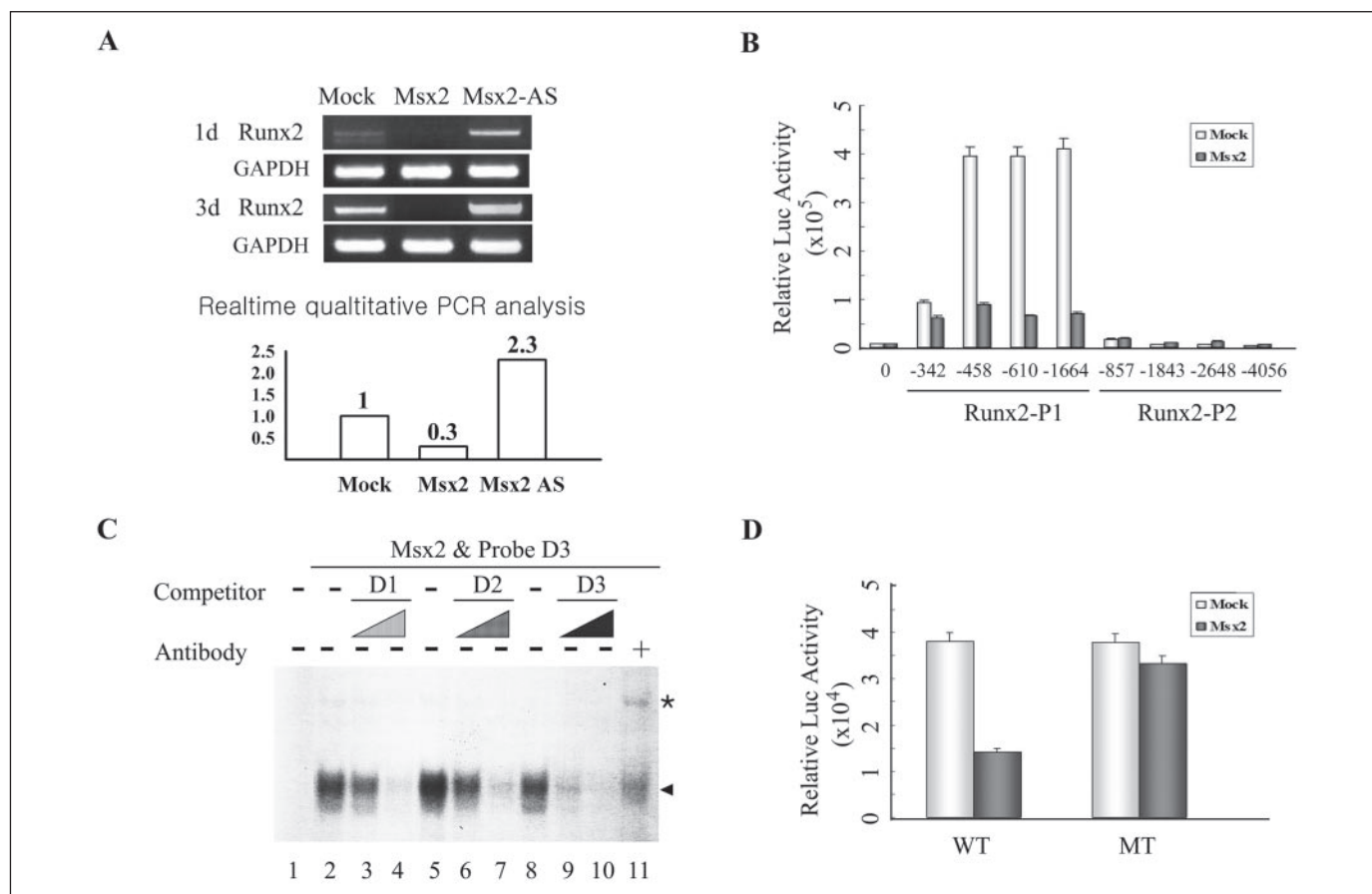


FIGURE 5. Msx2 suppresses Runx2-II expression by binding to the Runx2 P1 promoter. *A*, mock-transfected C2C12 cells or C2C12 cells stably transfected with *Msx2* or *Msx2-AS* (25) were treated with 100 ng/ml BMP-2 for 1 (*1d*) or 3 (*3d*) days after reaching visual confluence, and Runx2 expression was determined by conventional reverse transcription-PCR or quantitative real-time PCR (3 days). The relative levels of *Runx2* mRNA were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *B*, ROS17/2.8 cells were transfected with *Runx2* P1 or P2 promoter deletion constructs along with the *Msx2* expression vector or empty vector. *Luc*, luciferase. *C*, the 32 P-labeled D3 probe was incubated with *in vitro* translated FLAG-tagged Msx2. The free probe is shown in lane 1. The shift by FLAG-tagged Msx2 protein (lanes 2, 5, and 8) was competed by a 50- or 100-fold molar excess of unlabeled D1 (lanes 3 and 4, respectively), D2 (lanes 6 and 7, respectively), or D3 (lanes 9 and 10, respectively) oligonucleotides. The mobility shift caused by Msx2 binding was confirmed by a supershift assay with anti-FLAG antibody (lane 11). The arrowhead indicates a shift complex, and the asterisk indicates a supershift complex. *D*, ROS17/2.8 cells were cotransfected with the wild-type *Runx2* P1-756 construct (*WT* bars) or the triple mutant (*MT* bars) together with the *Msx2* expression vector or empty vector.

the wild-type level (data not shown). However, the mutations of all three binding sites finally blocked the response to Dlx5 in ROS17/2.8 cells, from 3.4-fold (Fig. 4D, *WT* bars) to ~1.5-fold (*MT* bars). These observations indicate that all three HDREs are required for Dlx5 binding and that they are responsible for the stimulation of Runx2-II expression by Dlx5.

Msx2 Suppresses Runx2-II Expression by Binding to the Runx2 P1 Promoter—The BMP-induced transcription of Runx2 was suppressed by stable transfection of *Msx2* and increased by stable transfection of *Msx2-AS* (Fig. 5A). The level of Runx2 expression was only 30% of the control level in *Msx2*-overexpressing cells and 2.3-fold higher in *Msx2-AS*-overexpressing cells as determined by real-time PCR analysis (day 3 sample). The real-time PCR products after 30 cycles are shown in the day 3 sample.

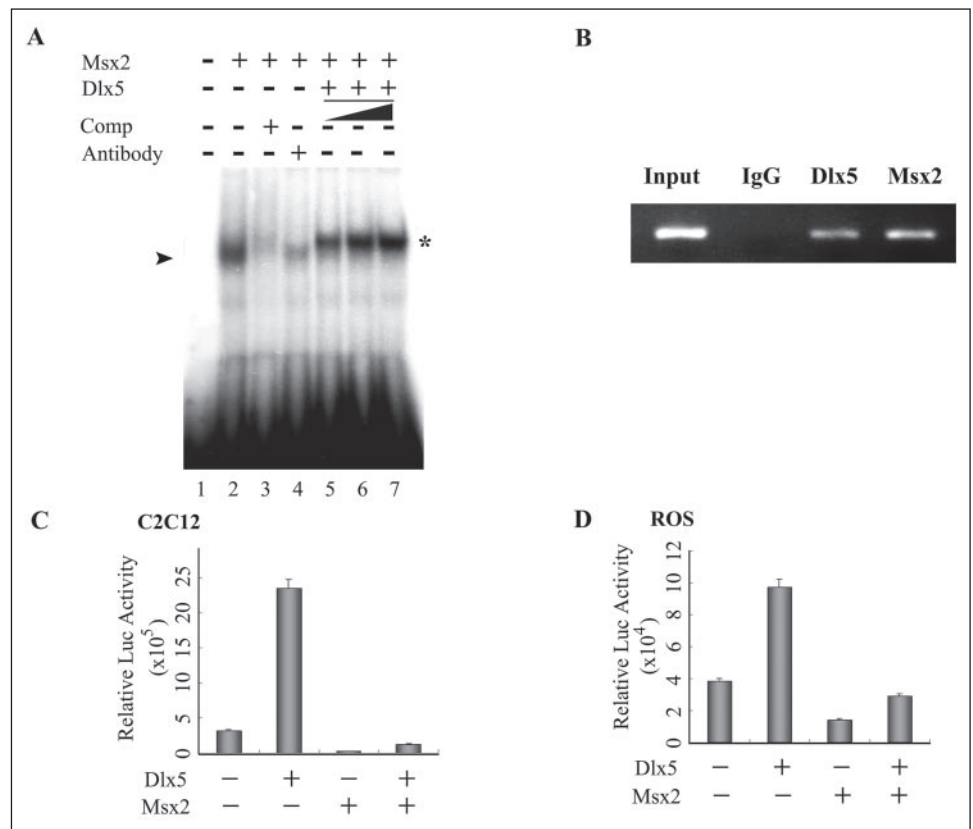
In addition, when ROS17/2.8 cells were cotransfected with *Runx2* promoter-reporter constructs together with the *Msx2* expression vector, *Msx2* strongly suppressed the reporter activity of the *Runx2* P1-1664, P1-610, and P1-458 constructs, but not of the *Runx2* P1-342 construct (Fig. 5B). P1 promoter activity was suppressed to 25% of the control level, but the P2 promoter was not inhibited by *Msx2* overexpression.

Based on these results, we speculate that *Msx2* also recognizes the response elements in the P1 promoter that are occupied by Dlx5. To test

the binding activity of *Msx2* and these elements, we performed EMSA. The labeled D3 probe effectively bound to *Msx2* (Fig. 5C, lanes 2, 5, and 8). Furthermore, binding of *Msx2* to the labeled D3 probe was reduced in the presence of a 50- or 100-fold molar excess of unlabeled D1 (Fig. 5C, lanes 3 and 4), D2 (lanes 6 and 7), or D3 (lanes 9 and 10) probe, suggesting that their binding affinity might be equivalent. The supershift confirms that the binding complex has *Msx2* (Fig. 5C, lane 11). We also found that mutation of all three binding sites in the *Runx2* P1-756 construct almost completely abrogated the suppressive ability of *Msx2* overexpression in ROS17/2.8 cells (Fig. 5D), whereas mutation of each binding site alone or two sites was not sufficient to allow P1 promoter activity to be suppressed to <50% of the wild-type level (data not shown).

Dlx5 and Msx2 Reciprocally Antagonize Each Other's Action by Competing Common Response Elements—To examine the antagonistic mechanism between Dlx5 and *Msx2* on the *Runx2* P1 promoter, we performed EMSA in which the labeled D3 probe was incubated with a constant amount of *Msx2* (Fig. 6A, arrowhead) and increasing amounts of Dlx5 (asterisk). When more Dlx5 protein was present, binding of *Msx2* to the D3 probe was decreased. The *in vivo* binding/recruitment of Dlx5 and *Msx2* to the *Runx2* P1 promoter (D1, D2, and D3 sites) was shown by chromatin immunoprecipitation (Fig. 6B).

FIGURE 6. Dlx5 and Msx2 reciprocally antagonize each other's action by competing common response elements. A, binding of the radiolabeled D3 probe to the Msx2 protein was competed by the Dlx5 protein. Each protein was translated *in vitro*. The free probe is shown in lane 1. A major binding complex with FLAG-Msx2 (lane 2) was competed by a 100-fold molar excess of unlabeled D3 competitor (Comp; lane 3) or anti-FLAG antibody (lane 4). Lanes 5–7, the binding complex with Msx2 (arrowhead) was gradually completed by the addition of increasing amounts of Dlx5 (asterisk). The binding complex with Msx2 (arrowhead) was gradually competed by the addition of increasing amounts of Dlx5 (asterisk). B, chromatin immunoprecipitation assays were performed with C2C12 cells transiently transfected with Myc-Dlx5 or FLAG-Msx2. Anti-Myc or anti-FLAG antibody mediated the precipitation of chromatin fragments PCR-amplified with *Runx2* P1 primers. C and D, C2C12 and ROS17/2.8 cells, respectively, were transiently cotransfected with the *Runx2* P1–756 construct together with the *Dlx5* and/or *Msx2* expression vector.



The antagonistic action of Dlx5 and Msx2 on Runx2 expression was investigated. C2C12 and ROS17/2.8 cells were transfected with the *Runx2* P1–756 promoter-reporter construct together with the *Dlx5* and/or *Msx2* expression vector. The stimulatory effect of Dlx5 and the inhibitory effect of Msx2 were reciprocally compromised by the other's action (Fig. 6C). Taken together, these results indicate that Msx2 antagonizes the stimulatory effect of Dlx5 on the *Runx2* P1 promoter and that all three Dlx5-response elements in the *Runx2* P1 promoter show functional consequences of Msx2 antagonism in the regulation of Runx2-II expression.

DISCUSSION

BMP Signaling Specifically Stimulates the Expression of Runx2-II—The overall genomic organization of RUNX2, including the presence of dual promoters, is remarkably conserved in other members of this family of transcription factors, including RUNX1 and RUNX3 (32, 33). According to previous reports, the dual promoters are quite distantly separated, by ~7 kb in RUNX1 and 35 kb in RUNX3. A data base analysis of human chromosome 6p12 indicated that the two *RUNX2* promoters are the most distantly separated, by ~93 kb. Similarly, the two *Runx2* promoters are also ~80 kb apart on mouse chromosome 17. We previously indicated that these alternative promoters produce two Runx2 isoforms, which are differentially expressed in intramembranous and endochondral bone-forming processes (10, 11). More recently, we showed that Dlx5 is an indispensable mediator of BMP-2-induced Runx2 expression in the osteogenic *trans*-differentiation of C2C12 myogenic cells (14).

In addition to these prior findings, in this study, we have shown that expression of Runx2-II, a bone-specific isoform, commonly co-localizes with that of BMP-2 and Dlx5 during mouse calvarial bone development (Fig. 2). These observations suggest that the *Runx2* P1 promoter is a specific target of BMP-2 signaling and Dlx5 action. As two major transcripts of Runx2 are similar in size (5.4 and 6.0 kb), it is not easy to demonstrate their differential expression by Northern blot analysis. In

this study, we used lower concentration of agarose gel (1%) and longer running times to separate these higher molecular mass transcripts more clearly. Our results indicate that Runx2-II expression is specifically regulated by BMP-2 treatment. Consistently, Runx2-II expression was clearly stimulated by Dlx5 overexpression and inhibited by Dlx5-AS treatment. As far as we have observed, the two Runx2 isoforms are not functionally different with respect to osteoblast differentiation in transgenic mice (34). Nonetheless, it is evident that the *Runx2* P1 promoter is more specifically regulated by Dlx5 compared with the *Runx2* P2 promoter. Thus, the differential regulation of the two promoters by certain signaling pathways may explain the distinct temporal/spatial expression patterns of the two isoforms during bone development (10, 11).

Runx2 Isoforms: Functional Difference Versus Differential Transcriptional Regulation—BMP-2 is very important for the normal bone-remodeling process. It is produced and secreted by osteoblasts as a propeptide and is accumulated in the bone matrix. When bone resorption occurs, enzymes secreted from osteoclasts degrade the bone matrix and release the matrix-incorporated BMP-2 in a short mature form, which in turn stimulates new bone formation in the area.

The selective *Runx2-II* deficiency in mice by targeted deletion of the *Runx2* P1 promoter and exon 1 demonstrates that Runx2-I is sufficient for early skeletogenesis and intramembranous bone formation, whereas Runx2-II is necessary for endochondral bone formation and turnover in mature bones (35, 36). These findings highly correlate with the fact that *Runx2* 3-kb P1 promoter-driven LacZ expression continues throughout chondrogenesis and is restricted to the axial skeleton (37).

In this study, we first identified three Dlx5-response elements in the *Runx2* P1 promoter. In contrast, the *Runx2* P2 promoter did not respond to Dlx5, a crucial transcription factor for BMP-2 action. Thus, it can be assumed that there was a great disturbance in BMP-2-induced endochondral bone formation as well as BMP-2-mediated normal bone remodeling in the Runx2-II-deleted mice. Meanwhile, Runx2-I expres-

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sion stimulated by other factors than the BMP-2 signal (for example, fibroblast growth factor or TGF) is sufficient for embryonic skeletogenesis and intramembranous bone formation.

Contribution of Dlx5 and Msx2 to Bone Development—The Dlx5 and Msx2 proteins appear to act in an antagonistic manner during osteoblast differentiation. Dlx5 is expressed during later stages, coincident with the expression of osteocalcin (15), and it has been found to activate the promoters of osteocalcin and other bone-specific marker genes (14, 16, 20), clearly indicating that it stimulates osteoblast differentiation. In contrast, the role played by Msx2 is negative. It has been suggested that Msx2 stimulates cell proliferation and suppresses osteogenic differentiation because its expression precedes osteocalcin expression and prevents the terminal differentiation of osteoblasts (38).

Several models can be proposed to explain the antagonistic action of Dlx5 and Msx2. First, the two proteins may interact to form a complex that is functionally inactive because DNA-binding homeodomains are obscured (39). This idea is illustrated by their interaction with the Runx2 protein: Runx2 and Msx2 interact to form a complex, and Msx2-bound Runx2 is transcriptionally inactive. The addition of Dlx5 to the Runx2-Msx2 complex releases Runx2 because Dlx5 sequesters the Msx2 protein, thus restoring the transcriptional activity of Runx2 (21). Second, the homeodomains of the Dlx5 and Msx2 proteins may compete for binding to their common response elements in bone-specific marker genes such as osteocalcin (15, 40) and alkaline phosphatase (25). Our present results strongly suggest another possibility: Dlx5 and Msx2 reciprocally regulate osteoblast differentiation through their antagonistic regulation of the expression of Runx2-II, the major Runx2 isoform in bone tissue. Furthermore, based on their expression patterns in calvarial bone development (28) and in *in vitro* osteoblast differentiation (41), it is also possible that they regulate each other at the transcriptional level (*i.e.* Msx2 suppresses *Dlx5* expression and vice versa).

Two recently reported studies proposed an alternative action of Msx2 in osteoblast differentiation. One is that BMP-2-induced Msx2 expression in vascular smooth muscle cells stimulates their osteogenic differentiation, which is consequently related to the development of an atherosclerotic lesion (42). The other is that overexpression of Msx2 in pluripotent mesenchymal progenitor cells suppresses adipogenic differentiation and stimulates osteogenic differentiation (43). These results appear incompatible with our present data, but correspond well with other *in vivo* results that a gain-of-function mutation in Msx2 (P148H) that increases its affinity for DNA (44) results in Boston-type craniosynostosis, which is characterized by premature cranial suture closure (45). However, osteoblast differentiation was determined 7 days after viral infection of *Msx2*, in contrast to our study, in which we assayed osteogenesis 24 h after transfection. Thus, it is unclear whether the stimulatory activity of Msx2 that was shown previously was due to direct action on bone marker genes or to an accumulation of secondary effects of Msx2 overexpression. In contrast, our results clearly indicate that the Msx2 action on Runx2-II regulation is direct. Nevertheless, this double-edged function of Msx2 might also be explained by temporal considerations. For example, Msx2 promotes the proliferation of osteoprogenitor cells in the early stage of cell differentiation and prevents terminal osteoblast differentiation by acting as a repressor. Supporting the proliferation of osteoprogenitor cells increases the population size of these cells; because these cells will go on to differentiate into osteoblasts, Msx2 can be seen to stimulate osteoblast differentiation.

In conclusion, we have shown that Runx2-II expression is specifically regulated by BMP-2 signaling and that this regulation is mediated by the Dlx5 protein and response elements between -756 and -342 bp of the *Runx2* P1 promoter, where three putative homeodomain-binding sites

are located. All three response elements contribute to Dlx5 binding and stimulation of promoter activity. Similar to what was found for the alkaline phosphatase promoter (25), the action of Dlx5 on the *Runx2* P1 promoter is antagonized by Msx2. Thus, for the BMP-2-induced osteogenic *trans*-differentiation of C2C12 cells, immediately induced Msx2 suppresses Runx2-II expression and permits an increase in a committed cell population. The BMP-2-induced osteogenic differentiation may continue until Msx2 is down-regulated and Dlx5 is up-regulated. Collectively, Msx2, Dlx5, and Runx2 may define a cascade of factors that contribute to initial lineage determination, proliferation, and differentiation in BMP-2-induced osteoblast differentiation, and Dlx5 appears to be a component of the combinatorial mechanism that controls the formation and differentiation of skeletal tissues.

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