Bone Morphogenetic Protein-2-induced Alkaline Phosphatase Expression Is Stimulated by Dlx5 and Repressed by Msx2*

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Bone morphogenetic protein-2 (BMP-2) is one of the most potent bone-inducing agents in osteoblast differentiation. Moreover, it induces the osteogenic transdifferentiation of fibrogenic, myogenic, and adipogenic cells both in vitro and in vivo (3–5). Several lines of evidence indicate that BMP-2 treatment increases ALP mRNA expression and ALP activity (6, 7). Thus, the osteogenic differentiation of various cells that is induced by BMP-2 treatment could be determined by measuring ALP activity or its mRNA expression. We have previously reported that Runx2 also plays an important role in the BMP-2-induced transdifferentiation of myogenic C2C12 cells into osteogenic cells (8, 9). Moreover, it was found that BMP-2 stimulates Runx2 mRNA expression in C2C12 cells and that the forced expression of Runx2 in C3H10T1/2 cells stimulates ALP promoter activity (10). Thus, it is speculated that ALP transcription is directly regulated by Runx2. However, the observation that ALP activity is still stimulated by BMP-2 in Runx2-deficient cells (11) strongly suggests that there must be other upstream mediators that regulate the BMP-2-induced expression of ALP.

We recently found that the homeobox gene Dlx5 is an indispensable upstream regulator of the expression of Runx2 that is generated by BMP signaling because of the following observations (12). First, the forced expression of Dlx5 stimulated Runx2 and ALP expression even in the absence of BMP-2 stimulation. Second, antisense blocking of Dlx5 completely abrogated BMP-2-induced stimulation of Runx2 and ALP expression. These observations suggest that Dlx5 enhances ALP expression by up-regulating Runx2 expression. In addition, we speculated a possibility that BMP-2-stimulated ALP expression may also be directly regulated by Dlx5 and this may even be the primary regulatory mechanism. In this paper, we dissected the murine ALP promoter and found that it contains a Dlx5-response element, which indicates that ALP can indeed be directly targeted by Dlx5. We also found that the binding of Dlx5 to this element is important for BMP-2-induced ALP expression. Moreover, we examined the role of Msx2,

Alkaline phosphatase (ALP) is a widely accepted bone marker. Its expression is stimulated by bone morphogenetic protein (BMP)-2 treatment, the activation of BMP receptors and R-Smads, and the expression of Dlx5 and Runx2. However, how BMP-2 induces ALP expression is not clearly understood. We dissected the murine ALP promoter and found within it a Dlx5-binding cis-acting element by electrophoretic mobility shift assays and site-directed mutagenesis of the element. Dlx5 and the product of its target gene, Runx2, stimulated ALP promoter activity in an additive manner. However, because Dlx5 continued to stimulate ALP expression in Runx2−/− cells, the ALP stimulatory activity of Dlx5 is independent of Runx2. We also found that overexpression of Msx2 suppressed the mRNA level and enzyme activity of ALP that were induced by BMP-2 stimulation, and suppressed the Dlx5-stimulated ALP promoter activity by competing with Dlx5 for the cis-acting element in the ALP promoter. Moreover, Msx2 levels are constitutively high in C2C12 myogenic cells but decrease over time after BMP-2 treatment. This may explain why BMP-2 treatment of these cells results in immediate Dlx5 expression yet ALP expression commences only 1–2 days later. In other words, Msx2 in high levels counteracts initially the transcriptional activity of Dlx5 in low levels until a threshold Dlx5:Msx2 ratio is reached to the levels that allow the ALP stimulatory activity of Dlx5 to prevail. Thus, Dlx5 transactivates ALP expression, directly by binding to its cognate response element and/or indirectly by stimulating Runx2 expression, and Msx2 counteracts the direct transactivation of Dlx5.

Alkaline phosphatase (ALP) EC 3.1.3.-: orthophosphoric-monoester phosphohydrolase, alkaline optimum) was first suggested to be important in bimolecularization by Robison in 1923 (1). Because of this discovery, the role that it plays in osteoblast differentiation and bone mineralization has been the subject of considerable research. Its function in ossification remains controversial but it is now known that the primary biological function of ALP in bone formation is associated with the calcification of the skeleton. This function is exerted either by its ability to catalyze the hydrolysis of organic phosphate esters, thereby providing inorganic phosphate, or by removing the inorganic pyrophosphate that inhibits calcification at the site of active mineralization.

The ALP family is constituted by several isoenzymes that are encoded by four genes and named after the tissues in which they are predominantly expressed, namely, the intestinal (IntALP), placental (PALP), germ cell (GALP), and tissue nonspecific or kidney/bone/liver (TNSALP) isoenzymes. Of these, TNSALP is the major isoform that is expressed in osteoblasts (2) and it is widely accepted as a marker of differentiating osteoblasts both in vivo and in vitro. The ALP protein described in the text from this point onwards refers to this isoform.

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another homeobox protein, in regulating Dlx5-induced ALP expression. Max2 is thought to reverse the Dlx5-induced expression of osteoblast marker genes like osteocalcin by binding to Dlx5 rather than by competing with it for the same element on the target gene promoter (13, 14). However, we found here that BMP-2-induced and Dlx5-mediated ALP expression is counteracted by Max2 through its ability to compete with Dlx5 for the same cis-acting element on the ALP promoter.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bioactive recombinant human BMP-2 protein was from Wyeth, Inc. (Cambridge, MA) and Dulbecco’s modified Eagle’s medium, α-minimal essential medium, and fetal bovine serum were from Invitrogen. The Superscript™ first-strand synthesis system for reverse transcription and LipofectAMINE Plus were from Invitrogen (Carlsbad, CA) and Taq polymerase, the dNTP mixture, and G418 were from Promega (Madison, WI). The Megaprome DNA labeling system kit was from Amersham Biosciences and the Express hybridization solution was from Clontech (Palo Alto, CA). The Zeta probe membrane was from Bio-Rad, whereas the cytochemical ALP staining kit was from Sigma.

**DNA Construction**—The construction of the Dlx5 expression vector and Dlx5 antisense expression vector have been described previously (12). The mouse TNSALP promoter (−1838 bp) in the pG3L3 basic vector was kindly provided by Dr. Hideyuki Harada (10). ALP promoter deletion constructs were generated by serial deletion from the 5′ end of the promoter by using restriction sites. Thus, the ALP−1838 promoter in the pG3L3 basic vector was digested with NheI, SacI, Smal, or PvuII, and HindIII, respectively. The ALP−1838 (−1450 bp), ALP−1050 (−1050 to −81 bp), ALP−553 (−553 to +81 bp), or ALP−229 (−292 to +81 bp), respectively. Each deletion construct was then ligated into the corresponding restriction sites in the multiple cloning sites of the vector. To produce a construct that bears a mutation in the putative homeodomain-binding site, a site-directed mutagenic PCR was performed with the T1 forward oligonucleotides (see Fig. 3A for the sequence) and unlabeled, mutagenic reverse PCR product. The mutagenic binding site was digested with PstI and PvuII and substituted with the counterpart of wild type ALP−553. The construct was denoted Mt ALP (Mt). The FLAG-tagged Max2 expression vector was constructed by subcloning the BamHI-XhoI fragment from pcDNA3-Flag into the corresponding sites of pcDNA3.1-Flag. To produce the Max2 antisense expression vector, antisense Max2 DNA was subcloned into HindIII and BamHI of pcDNA3.1.

**Cell Culture and Determination of mRNA Levels and ALP Activity**—Myogenic murine C2C12 cells and fibroblastic murine C3H10T1/2 cells were cultured in Dulbecco’s modified Eagle’s medium. Runx2−/− cells from the primordium of calvarial tissue of Runx2-deficient mice (9), osteoblast-like MC3T3-E1 cells, and ROS 17/2.8 cells from rat osteosarcoma were maintained in α-minimal essential medium. The cell maintenance and culture conditions used were as described (12). Total cellular RNA was extracted from the cells and its concentration was measured by spectrophotometry. RNA integrity was assessed by the ratio of 28S/18S ribosomal RNA after electrophoresis on a 1.5% agarose, 5.0% formaldehyde gels. To determine the Dlx5, Dlx5, and ALP mRNA levels, Northern blot analysis or reverse transcriptase-PCR were performed as previously described (12) and ALP-specific primers were as follows: ALP forward, 5′-CTTGAAGCTGTTAGTTACTGCTG-3′; ALP reverse, 5′-GGAGCTTATTGCTACATGGAG-3′. ALP activity was cytochemically determined by using an ALP staining kit.

**Electrophoretic Mobility Shift Assay (EMSA)**—The sequences of the wild type and mutant oligonucleotides used are listed in Fig. 3A. These double-stranded DNA probes were end-labeled with [α-32P]dCTP using Klenow enzyme. The Dlx5 and Max2 proteins were produced by in vitro transcription and translation using TNT Coupled Reticulocyte Lysate (Promega, Madison, WI). The Dlx5 and/or Max2 proteins were incubated with the labeled, double-stranded DNA probes in the presence or absence of a 100-fold molar excess of the unlabeled competitor for 20 min at room temperature. For the supershift assay, the Dlx5 protein was preincubated with an anti-Dlx5 rabbit polyclonal antibody (12) for 20 min at room temperature before it was incubated with the labeled probe. The protein-DNA complexes were then separated at 4 °C on a 6% polyacrylamide gel containing 0.5× TBE buffer.

**Transfection and Luciferase Assay**—Cells were plated in 6-well plates at a density of 1.5 × 10^5 cells/well. After overnight culture, the cells were transfected with the LipofectAMINE Plus reagent. Each transfection assay was performed with 0.5 μg of the Dlx5-Max2 expression vector or pcDNA3 and 0.5 μg of the ALP-luciferase reporter vector. Three hours after transfection, the medium was changed and the cells were cultured for an additional 24 h. The cells were then harvested and luciferase activity was determined by using a Luciferase Assay kit (Promega). To establish Dlx5 overexpressing Runx2−/− cells that are stably transfected with Dlx5, Runx2−/− cells were transfected with the Dlx5 expression vector that had been cloned into the pcDNA3.1/Zeo expression vector. Stably transfected cell clones were selected with 800 μg/ml Zeocin (Invitrogen, Groningen, The Netherlands).

**RESULTS**

**Dlx5 Overexpression Stimulates ALP Expression in a Runx2-independent Manner**—It has been shown previously that the activation of the BMP signaling pathway by BMP-2 treatment or by overexpressing constitutively active BMP receptors or BMP-stimulated R-Smads strongly stimulates ALP activity (12). We found here that BMP-2 treatment of C2C12 cells induced them to express both the ALP and Dlx5 mRNA (Fig. 1A). However, treating these cells with cycloheximide before introducing BMP-2 indicated that the expression of ALP requires de novo protein synthesis, whereas the Dlx5 gene is directly targeted by BMP signaling (Fig. 1A). Moreover, when Dlx5 was overexpressed by transfecting C2C12 cells with the Dlx5 expression vector, ALP expression was stimulated even in the absence of BMP-2 treatment (Fig. 1B). In addition, when C2C12 cells stably expressed Dlx5 antisense, BMP-2 treatment no longer stimulated ALP activity or stimulated it only very weakly (Fig. 1C). Furthermore, when Runx2−/− cells overexpressed Dlx5 because of their transfection with the Dlx5 expression vector, ALP activity was stimulated despite the lack of Runx2 (Fig. 1D). However, when Dlx5 and/or Runx2 were overexpressed in C2C12 cells along with the ALP promoter reporter construct, both transcription factors stimulated ALP promoter activity and the coexpression of both of the transcription factors had an additive effect (Fig. 1E). These observations together strongly suggest that (i) ALP is a downstream target of Dlx5, (ii) Dlx5 and Runx2 can both regulate ALP expression, and (iii) the stimulation of ALP expression by BMP-2 involves the positive regulation of these two transcription factors.

**Characterization of the Murine ALP Promoter and Identification of its Dlx5-response Element**—To identify the Dlx5-response element(s) in the murine ALP promoter, we compared the murine and rat ALP promoters. The nucleotide sequences of the two promoters are highly conserved and both contain several transcription factor-response elements in the region between the −900 nucleotide and the putative transcription start site. A series of ALP promoter deletion constructs were constructed and introduction of these into various types of murine and rat non-osteogenic (Fig. 2, A and B) and osteogenic (Fig. 2, C and D) cells indicates that the smallest deletion construct, ALP−229, still bears minimal basal promoter activity. Moreover, the segment between nucleotides −1838 and −1050 of the ALP promoter seems to bear common enhancer elements for both the non-osteogenic and osteogenic cells (Fig. 2). In contrast, the region of the promoter between nucleotides −553 and −229 contains an element(s) that stimulates ALP promoter activity in osteoblast-like cells only (Fig. 2, C and D). Significantly, cotransfection of a non-osteogenic and an osteogenic cell line with the ALP−553 or ALP−229 promoter reporter construct along with the Dlx5 expression vector showed that Dlx5 overexpression significantly stimulated ALP−553 but could not stimulate ALP−229 (Fig. 2, E and F). These results suggest that there is a Dlx5-response element in the ALP promoter between nucleotides −553 and −229. Supporting this is that data base analysis indicates that there is a putative homeodomian-response element (HDRE) between nucleotides −486 and −483 that has the sequence ATTA. As ALP expression is stimulated by Dlx5, and an ALP pro-
moter that contains the putative HDRE responded to Dlx5 overexpression, we checked whether Dlx5 can bind to the putative HDRE. To do this, an oligonucleotide bearing the ATTA sequence plus surrounding nucleotides (Fig. 3A) was synthesized, labeled, and used in an EMSA experiment with an in vitro-translated Dlx5 protein. The labeled probe clearly bound to the Dlx5 protein, and this binding was diminished by a 100 molar excess of the unlabeled probe (Fig. 3B, lanes 3 and 4). Mutant probes were also constructed in which the ATTA sequence was wholly or partly mutated (M1, M2, and M3, Fig. 3A). However, these probes could not compete with the wild type probe for binding to Dlx5 (Fig. 3B, lanes 5–7). To confirm that the binding complex includes Dlx5, an EMSA supershift assay was performed by preincubating the Dlx5 protein with an anti-Dlx5 rabbit polyclonal antibody before adding it to the wild type oligonucleotide probe. The protein-DNA probe complex was supershifted by Dlx5 antibody (Fig. 3B, lane 8). We also found that mutation of the ATTA homeodomain-binding sequence in the ALP-553 promoter construct to CGAC (which generated Mt ALP) completely abrogated the ability of Dlx5 overexpression in C2C12 and Runx2−/− cells to stimulate the ALP promoter (Fig. 3, C and D). These observations indicate that the HDRE is indeed recognized and bound by Dlx5. Notably, mutation of the HDRE in the ALP-553 promoter construct strongly stimulated the basal ALP promoter activity. This suggests that there may be a strong negative regulator that can also bind to the Dlx5 response element and basally down-regulates ALP expression. 

Msx2 Antagonizes the Stimulatory Effect of Dlx5 on ALP Expression—We have noted repeatedly in previous reports that while BMP-2 strongly stimulates ALP mRNA expression, the expression of Dlx5 is immediate, whereas ALP expression takes place only 1–2 days later (5, 9, 12). Here we found that when we transfected C2C12 cells with the ALP-1838 or ALP-553 promoter constructs, treated them with BMP-2, and then analyzed their luciferase activities 24 h later, the ALP promoter was not activated at that time point (Fig. 4A). These observations strongly support the notion that there is a strong negative regulator that inhibits Dlx5 activity early after BMP2 treatment. We speculated that it may be Msx2, because Msx2 is widely known to be a Dlx5 antagonist (13, 14, 16) whose expression is stimulated by BMP-2. To assess this, we determined Msx2, Dlx5, and ALP expression over time after BMP-2 treatment (Fig. 4B). We found that Msx2 mRNA levels were very high even before BMP-2 treatment and that these levels were maintained up to 1 day later. However, after 3 days of culture, the Msx2 mRNA disappeared almost completely. With regard to Dlx5, however, it was not expressed before BMP-2 treatment but was first expressed 3 h later (Fig. 4B). These mRNA levels gradually increased over time and were still high 3 days after BMP-2 stimulation. ALP was not stimulated in the 3 h after BMP-2 treatment (at which point the Msx2 levels were high and the Dlx5 levels were low). Rather, ALP expression was stimulated only when the Msx2 levels were decreasing and Dlx5 levels were increasing (Fig. 4B). To confirm the negative action of Msx2 in the regulation of ALP expression, we stably transfected Msx2 sense or antisense expression vectors in C2C12 cells and established cell clones. Each cell clone was treated with BMP-2 and ALP activity was determined by cytochemical staining. Overexpression of Msx2 significantly suppressed BMP-2 stimulated ALP activity. On the contrary, antisense blocking of Msx2 slightly stimulated BMP-2-stimulated ALP activity (Fig. 4C). Consistent with these results, the stable transfection of Msx2 suppressed the BMP-2-induced ALP mRNA level and antisense increased the BMP-2-induced ALP mRNA level (Fig. 4D). In addition, when C2C12 cells were cotransfected with the ALP-1838, -1488, or -229 promoter reporters together with the Msx2 expression vector, Msx2 could significantly suppress the baseline activity of ALP-1838, -1488,
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Moreover, it appears that Mxs2 may also recognize the same element containing labeled probe was incubated with a constant amount of Dlx5 protein levels can stimulate increase only 1 day after BMP-2 stimulation, at which point the (Fig. 4A). The basal luciferase activity of the wild type murine ALP promoter construct (ALP-1838) and that of the four truncated promoter constructs (−1488, −1050, −553, and −229) in osteogenic and non-osteogenic cells was determined by transfecting the cells with each construct and determining their luciferase activities. The cells tested included myogenic C2C12 cells (A), fibroblastic C3H10T1/2 cells (B), osteoblast-like MC3T3-E1 cells (C), and rat osteosarcoma ROS 17/2.8 cells (D). C2C12 cells (E) or MC3T3-E1 (F) cells were also transfected with the ALP-1853, ALP-553, or ALP-229 promoter reporter constructs along with the Dlx5 expression vector or the empty vector. The cells were harvested 24 h later and luciferase activity was measured and normalized by the protein concentrations in the cell lysates.

and -553 but it could not suppress ALP-229 activity (Fig. 4E). This suggests that Mxs2, like Dlx5, may also recognize an element lying between nucleotides −229 and −553 of the ALP promoter. Supporting this repressive effect of Mxs2 on ALP expression is that when C2C12 cells were transfected with the ALP-1838 promoter reporter together with the Dlx5 and/or Mxs2 expression vectors, the stimulatory effect of Dlx5 was suppressed by Mxs2 overexpression (Fig. 4F). To rule out the possibility that Runx2, which is secondarily induced by Dlx5 overexpression, may have an indirect effect, we examined the effect of overexpressing Dlx5 and/or Mxs2 together with the ALP-1838 promoter reporter in Runx2−/− cells. Dlx5 and/or Mxs2 overexpression had the same effect on the reporter that was observed in C2C12 cells (Fig. 4, F and G). We also found that mutating the HDRE in the ALP-553 promoter reporter abrogated the ability of Dlx5 and Mxs2 to regulate its activity (Fig. 4H). Thus, Mxs2 levels are constitutively high and decrease only 1 day after BMP-2 stimulation, at which point the increasing Dlx5 protein levels can stimulate ALP expression. Moreover, it appears that Mxs2 may also recognize the same element in the ALP promoter that is bound by Dlx5.

Mxs2 Competes with Dlx5 for Binding to the HDRE in the ALP Promoter—Because our observations to date indicate that Mxs2 inhibits the ability of Dlx5 to activate ALP transcription, the next question to be addressed was, what is the molecular mechanism by which Mxs2 antagonizes Dlx5 activity? We examined this by performing EMSA in which the HDRE-containing labeled probe was incubated with a constant amount of Dlx5 or Mxs2 and increasing levels of the counterpart protein (Fig. 5A). The binding of Dlx5 to the HDRE probe resulted in a single band (indicated by an arrowhead), whereas the binding of Mxs2 to the HDRE probe resulted in two bands (indicated by two arrows), the upper of which was located at the same level as the Dlx5-probe complex. All bands were eliminated by competition from the cold competitor (Fig. 5A, lanes 3–7). Thus, the mobility shift caused by Dlx5 binding was confirmed by a supershift assay where the in vitro-translated Dlx5 was incubated with an anti-Dlx5 antibody before being added to the wild type oligonucleotide (lane 8). The arrowhead indicates the binding of Dlx5 to the wild type oligonucleotide and the asterisk indicates a supershift because of the Dlx5-antibody complex. C and D, the binding of Dlx5 to the ATTA consensus homeodomain response element in the ALP promoter specifically binds to Dlx5. A, the sequence of an oligonucleotide whose sequence consists of the ATTA element in the ALP promoter and surrounding nucleotides and those of three mutant oligonucleotides, Mt1, Mt2, and Mt3, are illustrated. The putative Dlx5-binding site is indicated by the underlined nucleotides and the mutated nucleotides are marked by shaded boxes. B, the 32P-labeled wild type oligonucleotide was incubated with in vitro-translated Dlx5. Lane 1, free probe; lane 2, the probe was incubated with the reticulocyte lysates that were used for in vitro transcription and translation (n); lanes 3–7, the Dlx5 protein was incubated with the labeled probe (lane 3), whose binding to Dlx5 was subjected to competition (Comp) from a ×100 molar excess of the cold wild type oligonucleotide (lane 4) or Mt1, Mt2, or Mt3 (lanes 5–7, respectively). The mobility shift caused by Dlx5 binding was confirmed by a supershift assay where the in vitro-translated Dlx5 was incubated with an anti-Dlx5 antibody before being added to the wild type oligonucleotide (lane 8). The arrowhead indicates the binding of Dlx5 to the wild type oligonucleotide and the asterisk indicates a supershift because of the Dlx5-antibody complex. C and D, the binding of Dlx5 to the ATTA homeodomain-binding consensus sequence is responsible for the stimulation of ALP transcription caused by Dlx5 overexpression. The ALP promoter construct bearing the −553 to +322 region was subjected to site-directed mutagenesis to substitute the ATTA consensus homeodomain-binding consensus sequence with CGAC (Mt ALP). C2C12 cells (C) or Runx2−/− cells (D) were cotransfected with the wild type ALP-553 promoter construct or with Mt ALP together with the Dlx5 expression vector and luciferase activity was determined 24 h later.
FIG. 4. Msx2 down-regulates the activity of the ATTA response element-containing ALP promoter and counteracts the ability of Dlx5 to induce ALP expression. A, C2C12 cells were transfected with ALP-1838, ALP-553, or the empty vector (pGL3) and the next day cells were treated with or without 300 ng/ml BMP-2 for 1 day. Luciferase activity was then measured. B, Dlx5, Msx2, and ALP expression in C2C12 cells over time after BMP-2 treatment. C2C12 cells were treated with 300 ng/ml BMP2 for the indicated periods after reaching visual confluence. Dlx5 expression was determined by reverse transcriptase-PCR using GAPDH as an internal control, whereas ALP and Msx2 expression was determined by Northern blot analysis. C, cells had been stably transfected with the Msx2 expression vector (#1, #2, and #4), antisense Msx2 (Msx2 AS) or empty vector pcDNA3 (Mock), respectively, in C2C12 cells were cultured with or without 10, 30, or 50 ng/ml of BMP2 treatment for 24 h. The ALP enzyme activity was determined by cytochemical staining. D, Msx2#2, Msx2#4, Msx2 AS, or Mock clone cells were treated with 100 ng/ml BMP-2 for 24 h after reaching visual confluence and ALP expression was determined by reverse transcriptase-PCR. E, C2C12 cells were cotransfected with 0.5 μg of one of the ALP deletion constructs together with 0.5 μg of the Msx2 expression vector or the empty vector, and luciferase activity was determined. F and G, C2C12 cells (F) and Runx2−/− cells (G) were transiently cotransfected with the ALP-1838 promoter (Wt Alp) together with the Dlx5 and/or Msx2 expression vectors, and luciferase activity was determined. H, C2C12 cells were cotransfected with the mutant ALP promoter reporter vector (Mt Alp) along with the Dlx5 and/or Msx2 expression vectors and luciferase activity was determined. All luciferase activities were determined 24 h after each transfection.
In contrast, formation of the Msx2-HDRE binding complexes was decreased when increasing amounts of Dlx5 protein were added (Fig. 5A, lanes 9–11). Moreover, the levels of the supershifted Dlx5-HDRE-anti-Dlx5 antibody complex (asterisk) gradually decreased as the amount of the Msx2 protein that was added to the mixture were increased (Fig. 5B, lanes 3–6). Thus, Msx2 and Dlx5 can compete with each other for the HDRE in the ALP promoter.

**DISCUSSION**

ALP has been widely accepted to be a faithful marker of osteoblast differentiation and BMP-2-induced osteogenic transdifferentiation of myogenic cells (2, 12). However, the molecular mechanism by which BMP-2 induces ALP transcription is still not clearly understood, although it is known that various BMP-2-induced transcription factors such as Runx2 and Dlx5 can regulate the ALP promoter. Here, we analyzed the murine ALP promoter and found that it contains a Dlx5-response element that is used to regulate BMP-2-induced ALP expression.

**Dlx5 Regulates ALP Expression by Binding Directly to the ALP Promoter**—We showed previously that Dlx5 mediates BMP-2-induced osteoblast differentiation by stimulating two key master genes, namely, Runx2 and Osx (12, 17). In addition, we demonstrated that Dlx5 overexpression in Runx2−/− cells is sufficient to stimulate ALP mRNA expression. These observations together led us to propose that Dlx5 may directly regulate ALP expression. In this paper, we present evidence that strongly supports this notion because we located a Dlx5-response element in the ALP promoter and found that the binding of Dlx5 to this element is responsible for activating the ALP promoter. We also showed that blocking of Dlx5 expression abrogated BMP-2-induced ALP expression. These observations indicate that Dlx5 is a critical mediator of ALP expression.

**Dlx5 Stimulates ALP Expression in the Absence of Runx2**—Whereas our previously published observations indicated that Dlx5 plays a critical role in BMP-2-induced ALP expression, it was not clear at that point whether Dlx5 directly up-regulates ALP expression or whether it does so through its ability to stimulate the expression of Runx2 (12), which is also known to strongly activate ALP expression (9). However, here we show that Dlx5 overexpression stimulates ALP expression and activates an ALP promoter reporter in Runx2−/− cells. This indicates clearly that Dlx5 can stimulate ALP transcription without involving Runx2. This is supported by a report that shows that the BMP-2-induced increase in ALP enzymatic activity is delayed in calvarial cells from Runx2−/− mice compared with cells from wild-type mice (11). However, because it has been reported that the ALP promoter bears a Runx2-binding consensus sequence (10) that is located upstream of the Dlx5-binding site that we identified here, we suspected that these two transcription factors may collaborate in up-regulating ALP transcription. Indeed, we found evidence supporting this in the study reported here because cells overexpressing both Dlx5 and Runx2 along with the ALP promoter reporter showed that these transcription factors act in an additive manner to activate the reporter, although synergism was not observed. Thus, Dlx5 and Runx2 are independently involved in BMP-2-induced ALP expression, and Dlx5 can up-regulate ALP expression either directly by binding to the ALP promoter or indirectly by stimulating Runx2 expression.

**Msx2 Counteracts the Ability of Dlx5 to Activate the ALP Promoter**—We have previously noted that despite the fact that BMP-2 immediately induces Dlx5 expression, the expression of ALP is markedly delayed by 1–2 days (9, 12). Moreover, we could not detect ALP promoter-driven luciferase activity within the first 24 h after BMP-2 treatment. In addition, we found that disrupting the Dlx5-response element in the ALP promoter strongly stimulated its baseline activity. On the basis of these observations, we reasoned that ALP expression may normally be down-regulated by a negatively acting transcription factor in the early stage of differentiation and binds to the same
response element recognized by Dlx5, this repressed status continues for 1 day after BMP-2 treatment until the Dlx5 level exceeds those of the negatively acting factor. Msx2 is believed to potently antagonize Dlx5 activity because it represses the promoters of various common osteoblastic markers such as osteocalcin (13), bone sialoprotein (16), and α1(1) collagen (18). In addition, it has been reported that Msx1 suppresses ALP expression in primary cultured chicken chondrocytes and osteoblasts (19) and that Msx2 suppresses ALP mRNA expression in primary chick calvarial osteoblasts (20). When we examined the expression kinetics of Msx2, Dlx5, and ALP, we found that ALP expression was suppressed when Msx2 levels were high and Dlx5 levels were low, and that it was stimulated when Dlx5 levels were high and Msx2 levels were low. This suggests that the Dlx5 and Msx2 levels are reciprocally regulated and that their relative abundance determines whether the ALP promoter is repressed or activated. Supporting these observations was that we found that both ALP mRNA level and ALP promoter activity in C2C12 cells were stimulated by the overexpression of Dlx5 but suppressed by Msx2 overexpression. Thus, it appears that the high levels of Msx2 that are present in osteoblasts early after BMP-2 stimulation counteracts the stimulatory effect of the initially low levels of Dlx5 protein until these levels reach a particular threshold that overpowers the repressive activity of Msx2.

Msx2 Counteracts Dlx5 by Competing for the Same Response Element in the ALP Promoter—Two underlying mechanisms have been suggested to explain how Msx2 counteracts Dlx5. First, it may compete with Dlx5 for the same response element within the target promoter. Second, it may bind to Dlx5 and thus prevent it from binding to the target promoter. The former idea originated from the observations that Msx2 (21) and Dlx5 (22) both bind to the same response element in the rat osteocalcin promoter, as demonstrated by EMSA. The latter idea comes from the discovery that Dlx5 and Msx2 interact with each other through their homeodomains, thereby forming a heterodimer (23). Because their homeodomains were involved in this protein-protein interaction, neither transcription factors could recognize their response element thus both became transcriptionally inactive. The latter idea was also further supported by the findings that transcriptional repression was mediated by Max1 or Msx2 does not require cognate binding sites (15, 24), and that a mutation in the N-terminal arm of Msx2 that disrupts its ability to bind to DNA (T147A) has no effect on Msx2-mediated repression (25). However, in this paper we provide EMSA evidence that clearly suggests that Dlx5 and Msx2 compete with each other for the same response element in the murine ALP promoter. First, we found that the labeled oligonucleotide mimicking the Dlx5-response element in the ALP promoter bound less Msx2 when more Dlx5 protein was present. We then found that the amount of Dlx5 bound by the probe decreased when more Msx2 protein was available. Furthermore, we showed that the poor binding of Max2 to a mutated form of the response element closely matched its inability to repress ALP transcription. Thus, our data clearly indicate that binding competition is certainly involved in Msx2-mediated repression of Dlx5 activity, although we cannot entirely rule out the possibility that Msx2 may also counteract Dlx5 activity by binding to this protein.

Dlx5 and Msx2 Contribute to Bone Development—The roles that the Dlx and Max homeoproteins play in vertebrate development have been thoroughly summarized by Bendall and Abate-Shen (26). Dlx5 and Msx2 are primarily expressed in developing hard tissues, such as the skull, teeth, limbs, and the axial and appendicular skeleton (27–29). Examination of the expression patterns of these homeodomain transcription factor genes in the mesenchyme of the branchial arches and limbs has shed light on their functions during development. In terms of osteoblast differentiation and proliferation, the Dlx5 and Msx2 proteins appear to act in an antagonistic manner. Dlx5 is expressed during later stages of osteoblast differentiation, which coincides with the expression of osteocalcin (22), and it has been found to activate the promoters of osteocalcin and other bone marker genes (12, 13, 30). This indicates clearly that Dlx5 stimulates osteoblast differentiation. However, the role played by Msx2 in osteoblast differentiation is controversial. It has been suggested that Msx2 stimulates cell proliferation and suppresses osteogenic differentiation because Msx2 expression precedes osteocalcin expression and prevents the terminal differentiation of osteoblasts (31). However, it has also been proposed that Msx2 stimulates osteoblast differentiation because a gain-of-function mutation in Msx2 (P148H) that increased its affinity for DNA (32) resulted in Boston-type craniosynostosis characterized by premature cranial suture closure (33). In addition, it was found that Msx2 stimulates osteogenic differentiation and suppresses the adipogenic differentiation of pluripotent mesenchymal progenitor cells (34, 35). It was shown that Msx2 overexpression stimulates ALP mRNA expression and ALP enzymatic activity in pluripotent mesenchymal progenitor cells. Those results are apparently quite controversial considering our present result; they determined ALP activity 7 days after viral infection of Msx2 differently from our result done in 24 h after transfection. Thus, it is unclear whether this stimulatory activity of Msx2 is because of its direct binding to the ALP promoter or the accumulation of the secondary effects of Msx2 overexpression. In contrast, our present results clearly indicate that the Msx2 action on ALP regulation is a direct effect. It could be assumed that there is another transactivator such as Osx that might be secondarily induced by the primary action of Msx2 (34) and then the transactivator stimulates osteogenic differentiation by inducing ALP etc. Nevertheless, this double-faced function of Msx2 also might be explained by its time point-dependent manner. Namely Msx2 directly functions as a negative mode in the early stage and indirectly as a positive mode in the later stage.

Meanwhile, in developing long bones, Dlx5 is expressed in the hypertrophic zone and at the transition stage of proliferating chondrocytes into hypertrophic chondrocytes. Consistent with this expression pattern, forced expression of Dlx5 potentiates early and late chondrocyte differentiation and inhibits proliferation in cultured cells (26). Moreover, Dlx5 misexpression in developing skeleton markedly reduces chondrocyte proliferation concomitant with promoting hypertrophic maturation (36). In contrast, microinjection of the Max2-adenovirus inhibited BMP4-induced chondrogenesis (37) and the expression of loss-of-function Msx2 mutants accelerated chondrogenic maturation (38). Collectively, it can be assumed that the same reciprocal action of Dlx5 and Msx2 in osteoblast differentiation is commonly working in chondrocyte differentiation as well. However, the exact working mechanism of these two transcription factors in the regulation of chondrogenic marker genes remains to be clarified until the promoters of these genes are fully analyzed and a critical homeodomain response element is clearly understood.

In conclusion, we suggest that Msx2 promotes the proliferation of osteoprogenitor cells in the early stage of chondrogenesis and inhibits 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. Thus, we suggest a possibility that Dlx5 and Msx2 work as a
team in osteogenesis by cross-regulating each other in addition to the mechanism through their reciprocal ability to antagonize each other by protein-protein interaction or by the binding competition to a common response element.

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