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Differentiation and Mesoderm Induction

Katsuyoshi Miyama,* Gen Yamada,† Takamasa S. Yamamoto,‡ Chiyo Takagi,‡ Kenji Miyado,† Miho Sakai,† Naoto Ueno,‡,§ and Hiroshi Shibuya‡,¶,[1]

**New Product Research Laboratories III, Daiichi Pharmaceutical Co., Ltd., Tokyo R&D Center, Kita-Kasai, Edogawa-ku, Tokyo 134, Japan; †Center for Animal Resources and Development (CARD), Kumamoto University, Honjo 2-2-1, Kumamoto 860-0811, Japan; ‡Division of Morphogenesis, Department of Developmental Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan; §Department of Molecular Biomechanics, The Graduate University for Advanced Studies, Okazaki 444-8585, Japan; and ¶Precursory Research for Embryonic Science and Technology, Japan Science and Technology Corporation, Hikari-dai, Kyoto 619-02, Japan*

Bone morphogenetic proteins (BMPs), members of the transforming growth factor *β* superfamily, have been identified by their ability to induce cartilage and bone from nonskeletal cells and have been shown to act as a ventral morphogen in *Xenopus* mesoderm. We isolated a murine homeobox-containing gene, distal-less 5 (mDlx5), as a BMP-inducible gene in osteoblastic MC3T3-E1 cells. Stable transfectants of MC3T3-E1 that overexpress mDlx5 mRNA showed increase in various osteogenic markers, a fourfold increase in alkaline phosphatase activity, a sixfold increase in osteocalcin production, and appearance in mineralization of extracellular matrix. Furthermore, mDlx5 was induced orthotopically in mouse embryos treated with BMP-4 and in fractured bone of adult mice. Consistent with these observations, we also found that injection of mDlx5 mRNA into dorsal blastomeres enhanced the ventralization of *Xenopus* embryos. These findings suggest that mDlx5 is a target gene of the BMP signaling pathway and acts as an important regulator of both osteogenesis and dorsoventral patterning of embryonic axis.

INTRODUCTION

The bone morphogenetic proteins (BMPs) consist of at least seven separate but related genes, BMP-2 to -8 (Celeste *et al.*, 1990; Ozkaynak *et al.*, 1990; Wozney *et al.*, 1988). BMPs stimulate the differentiation from mesenchymal progenitor cells and osteoblastic cell lines into mature osteoblasts (Li *et al.*, 1996; Wang *et al.*, 1993). In addition to their ability to induce bone formation, BMPs play important roles in early development, particularly as essential components in embryogenesis (Kawakami *et al.*, 1996; Maeno *et al.*, 1994; Suzuki *et al.*, 1994; Winnier *et al.*, 1995). In *Xenopus*, BMP-2 and BMP-4 specify ventral tissue development and act as negative regulators of neuralization (Harland, 1994; Sasai *et al.*, 1995; Wilson and Hemmati, 1995). Therefore, BMPs are of fundamental importance for both therapeutic agents for fracture healing and embryonic development (Giannobile, 1996; Linkhart *et al.*, 1996).

A number of the homeobox-containing genes have been cloned in both vertebrates and invertebrates. Homeobox-containing genes have been shown to function as important regulators of developmental processes by giving cells positional and functional identities (Morasso *et al.*, 1996). The Distal-less (Dll) gene has been identified and characterized in the *Drosophila* mutant Distal-less, in which limb development is completely impaired (Cohen *et al.*, 1989). In mammals there appear to be at least seven genes (Dlx1–Dlx7) that are related to the Dll gene (Price *et al.*, 1991; Robinson and Mahon, 1994; Simeone *et al.*, 1994; Stock *et al.*, 1996). The Dlx gene family shows similar patterns of expression principally in the forebrain, limbs, and branchial

[1] To whom correspondence should be addressed at the Division of Morphogenesis, Department of Developmental Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan. Fax: 81-564-55-7571. E-mail: shibuya@nibb.ac.jp.

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arches during embryogenesis (Price *et al.*, 1991; Robinson and Mahon, 1994). However, among the Dlx gene family, Dlx5 and Dlx6 are expressed uniquely in the perichondrial region of nearly all developing fetal skeletal elements (Chen *et al.*, 1996). Several lines of evidence suggest the involvement of the Dlx family of homeodomain proteins in the regulation of osteoblast development and bone tissue-specific gene expression. Actually, Dlx5 is expressed in fetal rat calvarial cells during the mineralization stage (Ryoo *et al.*, 1997). BMPs have been shown to induce the homeobox-containing genes. Hox (homeobox) and Msx (mammalian msh-like homeobox) genes are expressed during BMP-induced ectopic bone formation (Iimura *et al.*, 1994). Perhaps, the BMP signaling pathways sequentially induce several homeobox-containing genes, possibly with tissue specificity. Several studies have suggested that Dlx5 and Msx2 are key regulators of osteocalcin gene expression (Hoffmann *et al.*, 1994; Ryoo *et al.*, 1997; Towler *et al.*, 1994). While these studies suggest a role for the Dlx-family genes involving Dlx5 gene for osteogenesis, at present there is no functional evidence for a role of Dlx5 in osteoblast differentiation and embryonic development.

In this report, we investigated potential functional roles for Dlx5, which was isolated as a BMP-inducible gene by a differential display technique. We demonstrated that stable mDlx5-transfected cells show a more differentiated osteoblast phenotype compared to parental MC3T3-E1 cells. mDlx5 was induced orthotopically in mouse embryos treated with BMP-4 and in fractured bone of adult mice, suggesting that Dlx5 functions downstream of BMP signaling. Consistent with the observations, injection of mDlx5 mRNA into dorsal blastomeres enhanced the ventralization of *Xenopus* embryos, mimicking the phenotype of BMP-overexpressing embryos. We propose that mDlx5 is a gene target of BMP signaling and an important regulator of mesoderm induction and osteoblastic differentiation through early embryogenesis to organogenesis.

MATERIALS AND METHODS

Cell Culture

The mouse osteoblast cell line, MC3T3-E1 cells, was maintained in α-minimal essential medium (α-MEM; GIBCO BRL) supplemented with 10% fetal calf serum (Sigma), 10 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL) under 5% CO_2 and 95% air until near confluence.

Differential Display of mRNA and Isolation of Novel Clones

Total RNA was extracted from untreated cells or from cells treated with 100 ng/ml hBMP-2 or 1 μM retinoic acid for 24 h. Poly(A)$^+$ RNA was isolated from each total RNA using an oligo(dT)-cellulose column (Pharmacia). Differential display was performed using RNA-Map Kits A, B, C, and D (GeneHunter) according to the manufacturer's recommendation with slight modifications. Briefly, 5 μg of poly(A)$^+$ RNA was reverse transcribed with T12MG, T12MA, T12MT, or T12MC (where M is dG, dA, or dC) as a primer, followed by PCR amplification in the presence of [α-^{33}P]dCTP using the corresponding T12MN and one of the arbitrary 10-mers (AP1–20) as primers. The cycling parameters were as follows: 94°C for 30 s, 40°C for 2 min, 72°C for 30 s for 40 cycles, followed by 72°C for 5 min. The PCR-amplified products were loaded next to each other in a 6% denaturing polyacrylamide gel for electrophoresis. The dried gel was exposed to Kodak XAR film. The reaction showing uniquely expressed products from one or more of each cell preparation was repeated (RT-PCR) to confirm the finding. Products showing reproducible unique expressions were then cut out from the dried gels and reamplified. The amplified PCR products were subcloned into the pUC18 vector and sequenced. The novelty of an isolated clone was determined by computer search and comparison against the GenBank and EMBL DNA databases.

Library Construction and Screening

A cDNA library was constructed in Lambda ZAP II (Stratagene) using 5 μg of poly(A)$^+$ RNA from cells treated with hBMP-2. After amplification of the library, 1×10^6 clones were screened with the ^{32}P-labeled PCR products to isolate full-length cDNA clones under standard hybridization conditions. Positive clones were converted into pBluescript plasmids and used for further studies. DNA sequence analysis indicated that the clone contained the complete open reading frame.

Northern Blot Hybridization Analysis and in Situ Hybridization

Total RNA (10 μg) was purified using guanidinium thiocyanate extraction procedures and fractionated on 1% (wt/vol) agarose–formaldehyde gels. After electrophoresis, samples were transferred to Hybond-N membranes (Amersham) by capillary action. The following cDNA probes were used to measure mRNA levels: mDlx5 and human β-actin. Hybridization was performed with the appropriate cDNA probe labeled with [α-^{32}P]dCTP using a random labeling kit (Amersham) at 60°C for 16 h. The membrane was washed in 20 mM sodium phosphate buffer (pH 7.0) containing 0.1% SDS at room temperature for 15 min three times and then in the same solution at 60°C for 30 min. Hybridized bands were visualized by radioautography and the radioactive bands were quantified on a Fuji-Bas 2000 imaging system. Whole-mount *in situ* hybridization was performed by standard procedures.

DNA Constructs and MC3T3-E1 Cell Transfections

The coding region of mDlx5 cDNA, lacking 5′ and 3′ untranslated sequences, was cloned into the *Eco*RI site of the pEF expression vector. The plasmids were cotransfected with the pSV2/neo vector into MC3T3-E1 cells using Tfx-50 reagent (Promega). For selection of stable cell lines, cells were plated at a density of 4×10^5 cells per 60-mm dish and transfected with 10 μg of pEF/Dlx5 and 1 μg of pSV2/neo per dish. G418 at 500 μg/ml (GIBCO BRL) was added in the medium to select appropriate colonies. G418-resistant colonies were subcultivated and selection pressure was maintained during the entire cultivation period.

Measurement of Alkaline Phosphatase Activity

The cells were plated into a 96-well plate at a density of 3×10^3 cells per well and cultured in α-MEM containing 5% FCS for 3

days. The cells were scraped into 50 μl of alkaline buffer (Sigma). Aliquots of 50 μl were assayed for enzyme activity in a 96-well plate with 50 μl of *p*-nitrophenyl phosphate substrate (Wako) in alkaline buffer. After the reaction, mixtures were incubated for 5 min at 37°C and the reaction was stopped with 100 μl of 2 M NaOH. Absorbance was measured at 405 nm with a microplate reader (Bio-Rad) with *p*-nitrophenol as a standard. The cell number was measured using a cell counting kit (Wako). The enzyme activity was expressed as picomoles of PNPP cleaved per minute per cell.

Measurement of Osteocalcin Production

Mouse osteocalcin levels in conditioned media were determined by a 2-day nonequilibrium radioimmunoassay, employing goat anti-mouse osteocalcin (Biomedical Technologies) and donkey anti-goat IgG.

Bone Nodule Assays

The cells were plated into a 12-well plate at a density of 4×10^4 cells per well and cultured in α-MEM containing 15% FCS and 2 mM β-glycerophosphate (Wako) for 27 days. The culture medium was changed every third day. After 27 days in culture, cells were fixed with 10% formalin. Alkaline phosphate activity was visualized by cellular staining with α-naphthol AS-MX phosphate (Sigma) and Fast Blue RR salt (Sigma). Bone nodule formation was monitored by von Kossa staining.

Experimental Model for Fracture Healing

The right eighth ribs of nine ICR mice (4–8 weeks) were fractured as previously described (Nakase *et al.*, 1994). Briefly, each mouse was anesthetized and the eighth ribs on the right side was exposed and cut vertically to the axis of the rib with scissors. For sham-operated controls, the right eighth ribs of nine ICR mice were similarly exposed but not fractured. After the operation, the animals were maintained in cages for 2 days with free access to food. The experimental procedures were undertaken in accordance with the guiding principles in the care and use of animals described in the *American Journal of Physiology*.

DMZ Assay

The mDlx5 cDNA was cloned into the pSP64T vector (Krieg and Melton, 1987). Capped mRNA was synthesized from linearized vectors using the mMessage Machine kit (Ambion). RNAs were then injected into the marginal zones of early stage *Xenopus* embryos. The amounts of injected *in vitro*-synthesized RNAs and sites of injection are described in the text. Dorsal marginal zone (DMZ) fragments were cut from early gastrula and allowed to grow until control sibling embryos reached either the gastrula or the tadpole stage. Total RNA was then extracted and analyzed with RT-PCR. RT-PCR assays and primer sequences were as published (Wilson and Hemmati, 1995; Wilson and Melton, 1994).

RESULTS

Isolation and Expression of Mouse Dlx5 Gene

We employed the differential display reverse transcription technique to identify target genes regulated by BMP-2 in MC3T3-E1 cells. A clone that specifically responded to BMP stimulation was subcloned into the pUC18 vector and sequenced. This clone has significant homology to the 3′ untranslated region of the rat homeoprotein (rDlx) cDNA. We isolated the full-length cDNA from a MC3T3-E1 cDNA library prepared following a 24-h pretreatment with 100 ng/ml hBMP-2. A database search revealed that the mouse homolog of rDlx was identical to mDlx5 cDNA (Stock *et al.*, 1996).

The expression pattern of mDlx5 in adult murine tissues was examined by Northern blot analysis. Northern blot analysis detected a single band of 1.5 kb. As shown in Figs. 1A and 1B, mDlx5 transcript was present only in brain and bone. Next, we examined the time course of mDlx5 mRNA induction in MC3T3-E1 cells. Transcripts of mDlx5 were detectable as early as 1 h after BMP stimulation. Maximal induction of mDlx5 mRNA by hBMP-2 occurred after 48 h (Fig. 1C). The translational inhibitor cycloheximide was employed to determine whether *de novo* protein synthesis was required for induction of mDlx5 mRNA by hBMP-2 in MC3T3-E1 cells. Pretreatment of MC3T3-E1 cells with the translational inhibitor cycloheximide did not attenuate the effect of BMP-2 on mDlx5 mRNA induction (Fig. 1D). Therefore, the induction of mDlx5 mRNA by BMP-2 in MC3T3-E1 cells does not require new protein synthesis.

Expression of mDlx5 in Early Mouse Embryo

It is known that BMP-2 and/or -4 is expressed dynamically during organogenesis. In order to get insights on whether mDlx5 gene expression pattern is correlated with BMP gene expression, we performed *in situ* analysis in the developing face. Several reports suggested the importance of epithelial–mesenchymal interactions during arch development (Wall and Hogan, 1995). Whole-mount *in situ* hybridization of mouse embryos revealed that the mDlx5 gene was expressed in midgestation mouse embryos in the branchial arches and tooth primordia. Initially, there was strong mDlx5 expression in the mesenchyme of the brachial arches (data not shown). At E10.5, mDlx5 expression remained abundant in the first and second branchial arches (Fig. 2A). Indeed, the murine BMP-4 gene was expressed in the first arch epithelium adjacent to the neighboring mDlx5-expressing mesenchyme (Fig. 2B). These data strongly suggest that mDlx5 gene expression is influenced under the control of BMP gene expression, although several other inductive mechanisms would also operate in other embryonal regions (Barlow and Francis-West, 1997).

Induction of mDlx5 after BMP Treatment

To examine the effect of BMP on mDlx5 expression, mouse embryos at day 10.5 were collected and exposed to BMP-4 *in vitro* for 6 h and mDlx5 gene expression was examined by whole-mount *in situ* hybridization. As shown in Fig. 3A, strong enhancement of mDlx5 gene expression was observed in the branchial arches and the nasal pit.

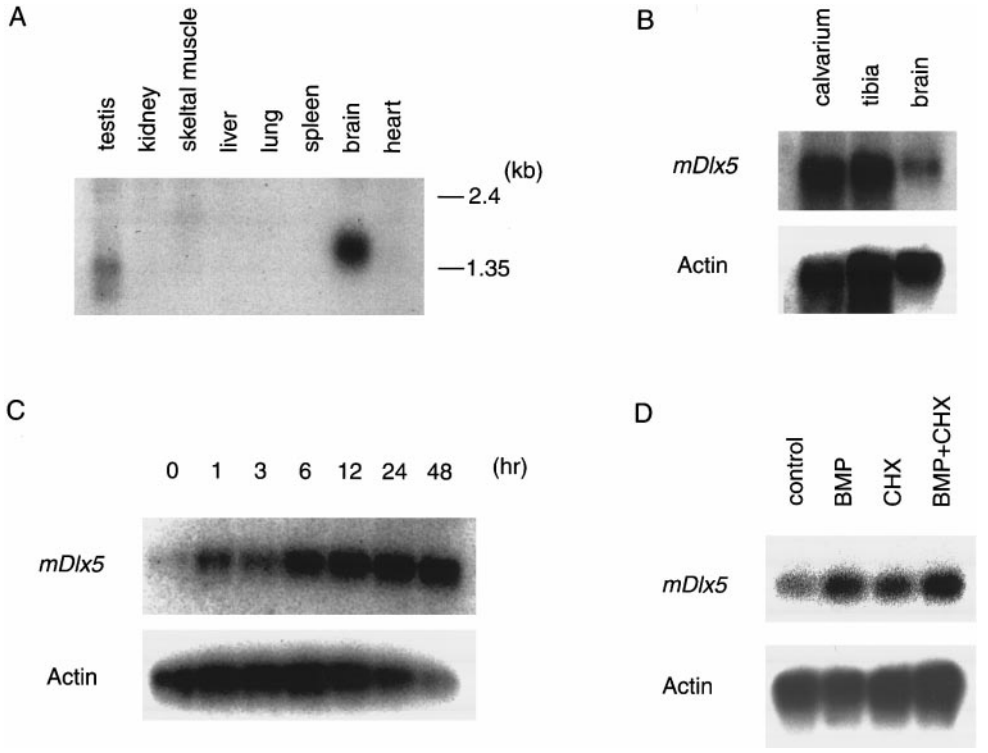


FIG. 1. Expression of mDlx5 mRNA. (A) Northern blot analysis of mDlx5 mRNA in various mouse tissues. A blot with poly(A)$^+$ RNA from mouse tissues (Clontech) was hybridized with ^{32}P-labeled mDlx5 probe (*Eco*RI–*Kpn*I fragment) as described under Materials and Methods. (B) Expression of mDlx5 mRNA in bone and brain of BALB/c mice. Total RNA was isolated from calvarium, tibia, and brain of BALB/c mice. A blot with total RNA (10 μg per lane) from each tissue was hybridized with ^{32}P-labeled mDlx5 probe (*Eco*RI–*Kpn*I fragment). The blot was rehybridized with a β-actin cDNA probe to demonstrate RNA loading. (C) Time course of mDlx5 mRNA expression in MC3T3-E1 cells stimulated by hBMP-2. MC3T3-E1 cells were seeded at 8×10^5 cells per 100-mm dish. After 24 h, they were treated with 100 ng/ml hBMP-2 for 1, 3, 6, 12, 24, or 48 h. A blot with total RNA (10 μg) from each incubation was hybridized with ^{32}P-labeled mDlx5 probe (*Eco*RI–*Kpn*I fragment) as described under Materials and Methods. Lane 1 contains total RNA from untreated cells. The β-actin signal is shown at the bottom to demonstrate RNA loading. (D) Inhibition of new protein synthesis does not block the effect of BMP-2 on mDlx5 mRNA expression. MC3T3-E1 cells were incubated for 12 h with medium (control), medium with hBMP-2 (100 ng/ml), or medium with cycloheximide (1.0 mg/ml) or preincubated with cycloheximide (1.0 mg/ml) for 30 min prior to the addition of hBMP-2 (100 ng/ml) for 12 h.

Moreover, we confirmed the induction profile of the mDlx5 mRNA upon BMP-4 stimulation in mouse embryo culture by Northern analysis (Fig. 3B). Densitometric analysis revealed eightfold mDlx5 gene induction compared with control. These observations offer evidence for the BMP inducibility of the mDlx5 gene at the tissue level. Localized induction of the mDlx5 gene was confined to the arch and the nasal pit regions, suggesting the cellular competence of these regions for BMP signaling in midgestation embryos.

mDlx5 Expression during Bone Fracture Healing

Bone fracture and healing involve multiple cellular events, such as the induction of genes which progressively trigger cell proliferations, inflammation, and differentiation including bone calcification. Since BMP-4 is induced immediately after bone fracture (Nakase *et al.*, 1994), we wondered what the roles of BMP and mDlx5 were in the process. On the basis of these observations, we hypothesized that the mDlx5 gene might be induced *in vivo* during the multiple reactions following bone fracture. Northern blot hybridization with the mDlx5 probe revealed it is strongly induced after bone fracture compared with the control unfractured mice which received sham operations (Fig. 3C). This result strongly suggests that the mDlx5 gene is induced in cartilage and/or bone formation *in vivo*.

Overexpression of Dlx5 Stimulates Osteoblast Differentiation

In order to examine the effect of mDlx5 on the MC3T3-E1 cells, stable cell lines expressing mDlx5 were established by cotransfection with a mammalian expression vector, pEF/Dlx5, which encodes mDlx5 cDNA, and with the pSV2/neo vector for G418 selection. The level of mDlx5 mRNA expression among six independently trans-

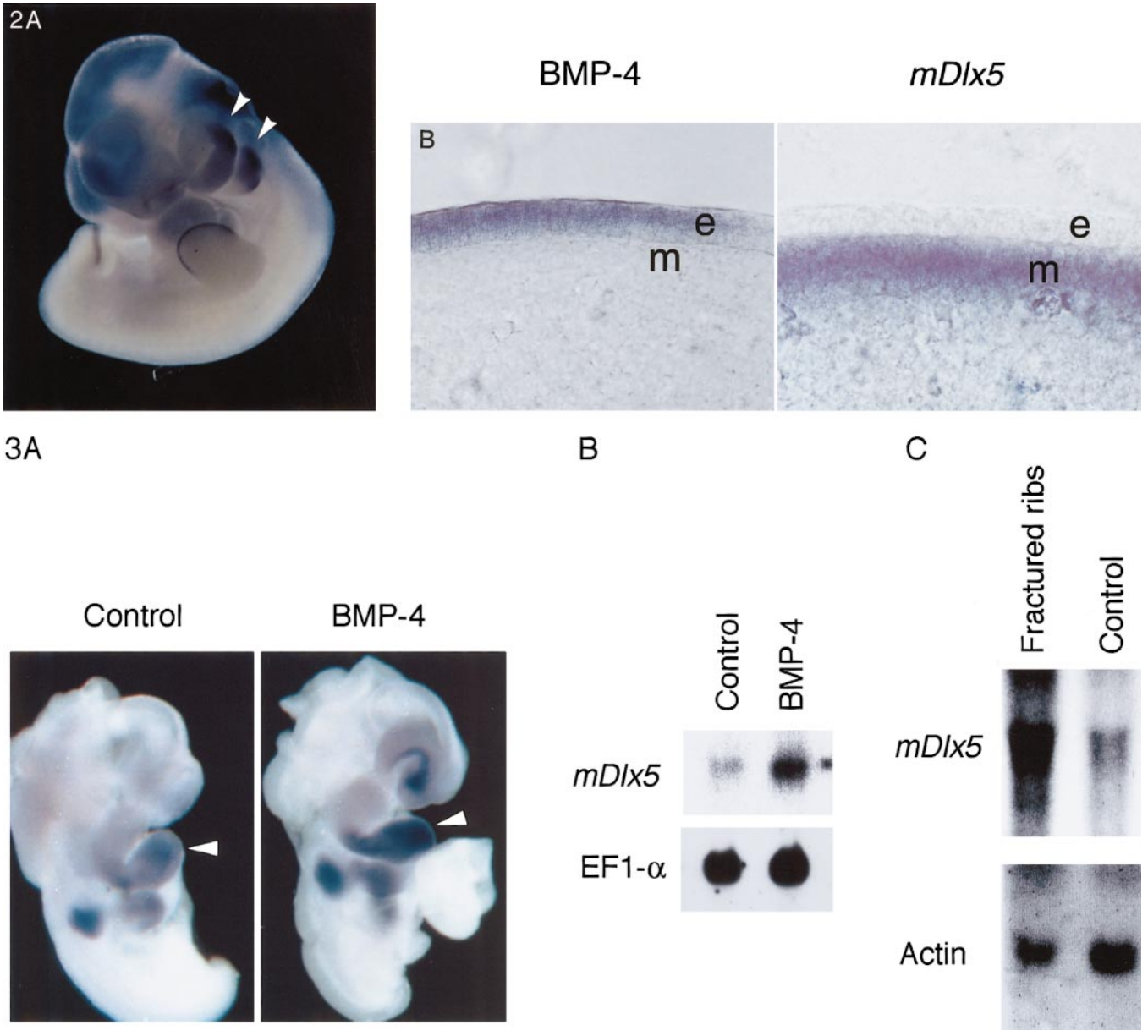


FIG. 2. Expression of mDlx5 in early mouse embryo. (A) Whole-mount *in situ* hybridization revealed the localization of the mDlx5 transcript. At E10.5, mDlx5 is expressed in the branchial arch and AER region of the forelimb. (B) Epithelial and mesenchymal expression of BMP-4 and mDlx5 genes. E10.5 embryo was analyzed for each gene expression by standard *in situ* hybridization. Stained embryos were sectioned and anterior side (oral side) of the first branchial arch region was shown. (Original magnification 100×; e, epithelium; m, mesenchyme.)

FIG. 3. mDlx5 expression during mouse development and fracture healing. (A) Induction of mDlx5 gene expression shown by a lateral view of the dissected embryo cultured in DMEM with or without BMP-4 for 6 h. Left side, DMEM; right side, DMEM containing 100 ng/ml BMP-4. (B) Induction profile of mDlx5 mRNA upon BMP-4 stimulation by mouse embryo culture. 16 embryos (E10) were treated with recombinant BMP-4 under the same conditions as the *in situ* experiment. RNA was extracted by a standard procedure and the transferred filter (each lane contains 5 μg of total RNA) was hybridized with mDlx5 probe or murine elongation factor 1-α probe. (C) mDlx5 expression during fracture healing. The experimental model is described under Materials and Methods. Left side, mouse with fractured rib; right side, control mouse. β-Actin signal is shown at the bottom.

fected lines was determined by Northern blot analysis. mDlx5 gene expression levels showed variability among the six cell lines (data not shown). While the parental MC3T3-E1 cells constitutively expressed low levels of mDlx5 mRNA (Fig. 4A),all transfected lines showed increasing levels of mDlx5 mRNA. Among them, one clonal cell line expressing high levels of mDlx5 mRNA was selected for further study. Other clones gave similar results (data not shown).

During the maturation of osteoblast progenitors into osteoblasts, various phenotypes of bone-forming cells are expressed, such as production of extracellular matrix proteins including type I collagen, osteocalcin, and mineralization, as well as high levels of alkaline phosphatase (ALP) activity. The stable mDlx5-transfected cells were compared to parental MC3T3-E1 cells and pSV2/neo-transfected cells for various differentiated osteoblast phenotypes in the absence of any treatment. Among characteristics of osteoblast differentiation processes, the level of ALP activity was found to be approximately fourfold elevated in mDlx5-transfected cells compared to parental MC3T3-E1 cells or pSV2/neo-transfected cells (Fig. 4B). Moreover, the level of ALP activity in mDlx5-transfected cells was higher than that in the control cells with treatment of BMP. The effect of mDlx5 expression on the induction of osteocalcin production was examined by radioimmunoassay of conditioned medium. Samples were analyzed on days 3, 9, 15, 21, and 27. Osteocalcin production in mDlx5-transfected cells was readily detected on day 9 and increased a maximum of sixfold compared to parental MC3T3-E1 cells by day 27 (Fig. 4C). Mineralization of extracellular matrix was also monitored by alkaline phosphatase staining and by the von Kossa staining procedure. After 9 days in culture, mDlx5-transfected cells began to grow in multilayers and the levels of alkaline phosphatase had increased markedly in mDlx5-transfected cells compared to parental MC3T3-E1 cells or pSV2/neo-transfected cells (Fig. 5A). After 27 days in culture, a higher level of mineralization of extracellular matrix was observed for mDlx5-transfected cells in regions that were densely populated and that stained positively for alkaline phosphatase. Furthermore, in mDlx5-transfected cells, uncalcified nodules were detected as multilayered cell clusters on day 10 and their number increased until day 27. Calcified nodules appeared about 4 days after nodule formation as revealed by the appearance of the sections of the calcified nodules consisting of cell layers and collagen matrices, in which some cells were embedded (Fig. 5B). Mineralization and calcified nodule were never detected in pSV2/neo-transfected control cells for time culture. These phenotypes of mDlx5-transfected cells were similar to that of the parent MC3T3-E1 cells incubated with BMP-4 (data not shown). Therefore, these results suggest that mDlx5 can induce osteoblast differentiation in MC3T3-E1.

Supernatant from mDlx5-transfected cells did not support differentiation of parental MC3T3-E1 cells or pSV2/neo-transfected cells (data not shown), indicating that mDlx5 expression did not induce endogenous growth factors such as BMP-4, at least not at levels sufficient to induce this osteoblast differentiation. Accordingly, Northern blotting analysis with a BMP-4 cDNA probe did not show any change in BMP-4 mRNA levels in mDlx5-transfected cells (data not shown). We also failed to detect the induction of Cbfa-1, which is known to function as a positive transcriptional factor of the osteocalcin gene and a positive regulator for bone formation (Ducy *et al.*, 1997; Komori *et al.*, 1997; Mundlos *et al.*, 1997; Otto *et al.*, 1997). While mDlx5-transfected cells showed no change in Cbfa-1 mRNA expression (Fig. 4A), BMP-2 induced the expression of the Cbfa-1 gene in parental MC3T3-E1 cells (data not shown). Taken together, these results suggest that overexpression of mDlx5 can induce osteoblast differentiation in the absence of BMP in MC3T3-E1 cells.

Effects of mDlx5 on BMP Signaling Pathway in Xenopus Development

Since our current results indicated the role of mDlx5 functioning downstream of BMP signaling, we wished to examine whether mDlx5 plays a similar role in early *Xenopus* embryos. When 50 pg of mDlx5 mRNA was injected into the dorsal marginal zone of a four-cell-stage embryo, the embryos were significantly ventralized [average dorsoanterior index = 2.3, $n = 30$] (Fig. 6A), mimicking the effect of BMP-2/4. On the other hand, injection of mDlx5 mRNA into the ventral side had no effect on the development of the embryos (data not shown). These results suggest that mDlx5 can ventralize embryonic axis formation.

To investigate the observed phenotypes at the molecular level, various marker gene expressions were analyzed. Injection of 50 pg of mDlx5 mRNA near the dorsal midline of four-cell embryos led to induction of ventral posterior mesoderm marker genes in dorsal marginal zone explants relative to explants isolated from uninjected embryos. As shown in Fig. 6B, ventral (Xvent-1 and Xwnt-8) and posterior (Xhox-3) mesoderm markers were induced in embryos injected with mDlx5. In contrast, injection of mDlx5 mRNA reduced a dorsal mesoderm marker (goosecoid). These results indicate that the induction of ventral mesoderm appears to be an event induced by mDlx5. As the pattern of mesoderm induction by mDlx5 is similar to that induced by BMP-4, we propose that mDlx5 functions in the BMP-4 signaling pathway.

DISCUSSION

BMPs and other members of the TGF-β superfamily interact with a family of related transmembrane serine/threonine kinase receptors, known as type I and type II (Koenig *et al.*, 1994; Nohno *et al.*, 1995; ten Dijke *et al.*, 1994). Propagation of the signal to downstream components requires the formation of a heteromeric complex of type I and type II receptors (Liu *et al.*, 1995; ten Dijke *et al.*, 1994;

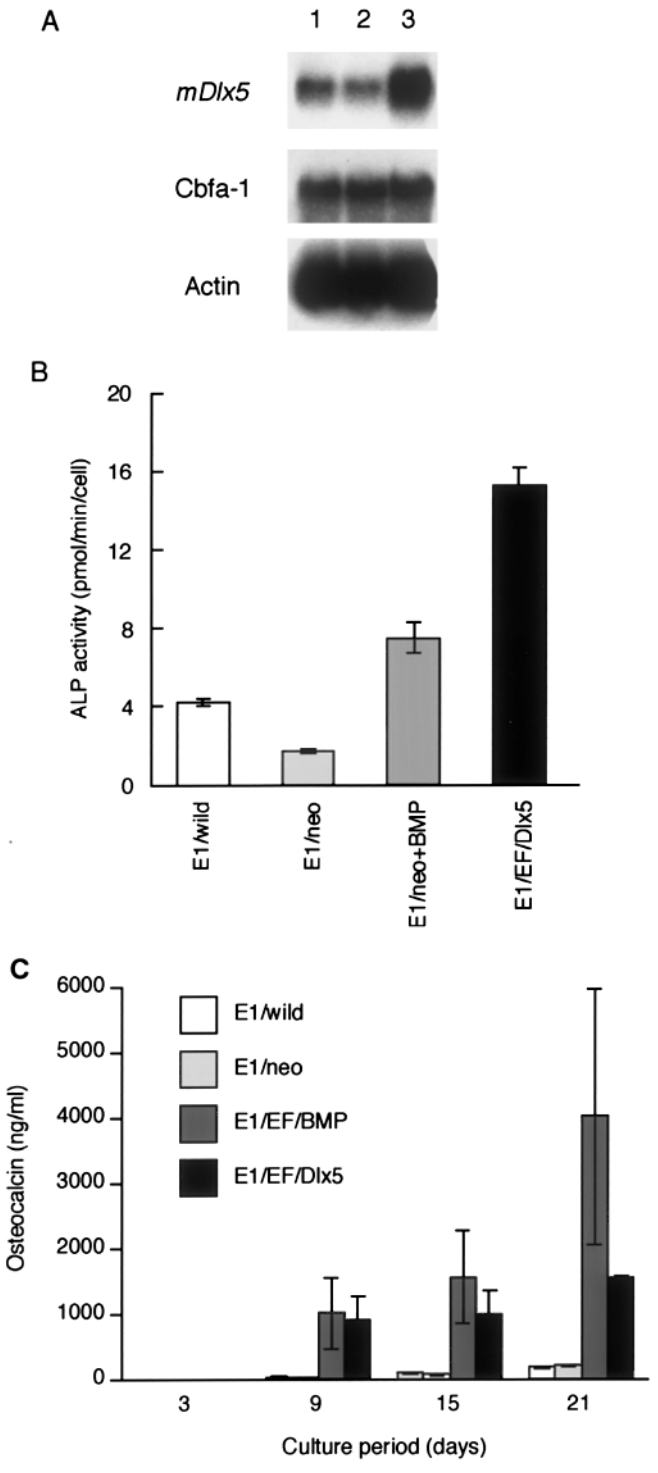
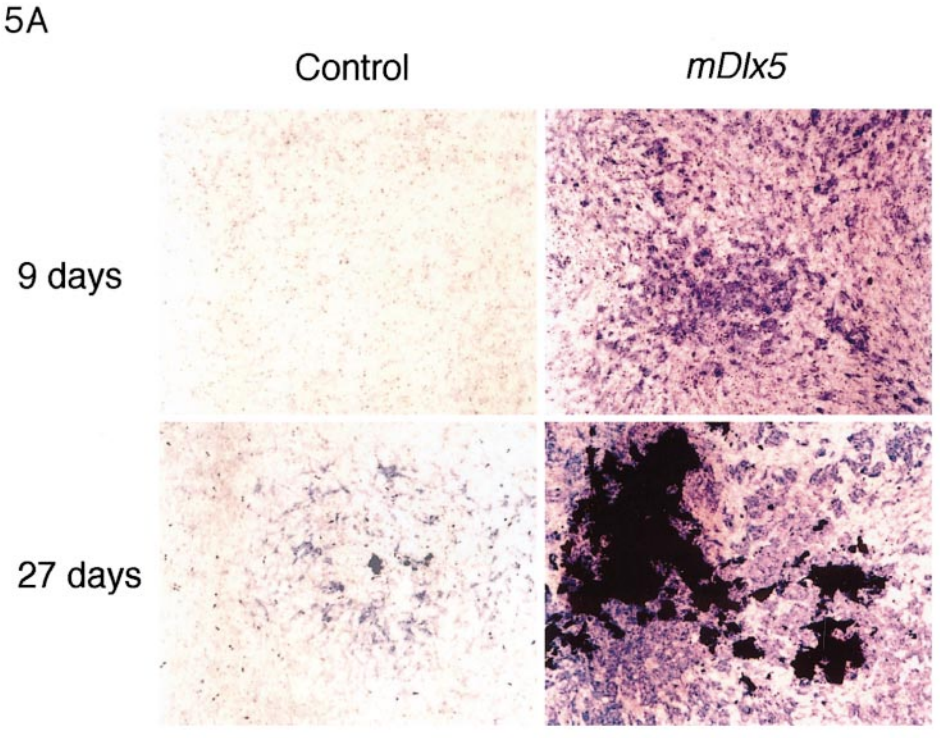
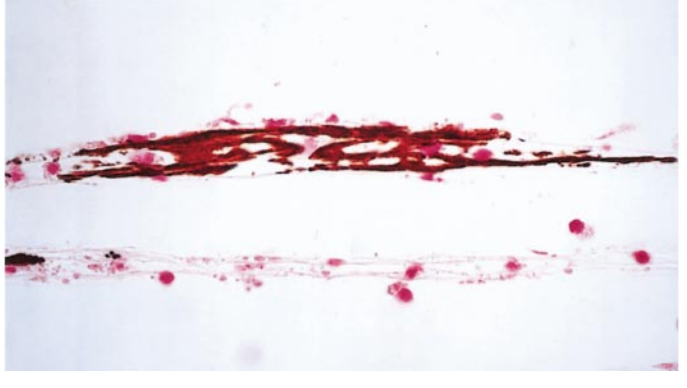


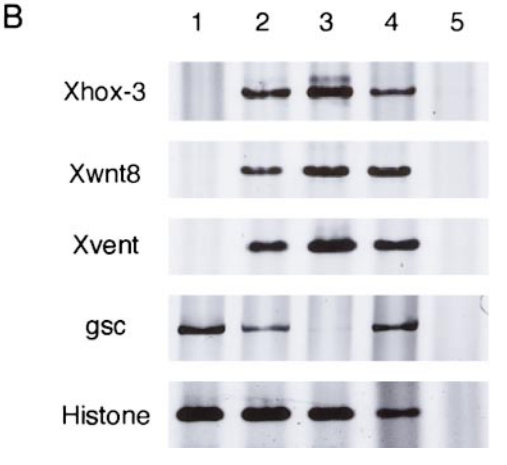
FIG. 4. Osteoblast differentiation induced by overexpression of mDlx5 in MC3T3-E1 cells. (A) Expression of mDlx5 and Cbfa-1 mRNA in stably transfected MC3T3-E1 cells. MC3T3-E1 cells were cotransfected with 10 μg of pEF/Dlx5 expression vector and 1 μg of pSV2/neo vector as described under Materials and Methods. After reaching confluence, total RNA was isolated and hybridized with a ^{32}P-labeled mDlx5 probe (*Eco*RI–*Kpn*I fragment) or mCbfa-1 probe as described under Materials and Methods. β-Actin signal is shown at the bottom to demonstrate RNA loading. Lane 1, parental MC3T3-E1 cells; lane 2, pSV2/neo vector-transfected cells; lane 3, pEF/mDlx5 and pSV2/neo-transfected cells. (B) Induction of alkaline phosphatase activity in stably transfected cells. Each value represents the mean ± SD of five individual values. Significant differences from control parental MC3T3-E1 cells were calculated by Dunnett's multiple comparisons. E1/neo + BMP, incubation with 10% conditioned medium of BMP-4 transfected CHO cells. (C) Induction of osteocalcin production in stably transfected cells. The cells were seeded at 4×10^4 cells per well in a 12-well plate and cultured in α-MEM containing 10% FCS for 27 days. The cultured medium was changed every 3 days and collected. Osteocalcin levels in the conditioned medium were determined by radioimmunoassay. Each value represents the mean ± SD of three individual values.

Wrana *et al.*, 1994). Several downstream components in BMP signaling have been identified, including a TGF-β-activated kinase (TAK1) and Smads (Hoodless *et al.*, 1996; Liu *et al.*, 1996; Thomsen, 1996; Yamaguchi *et al.*, 1995). TAK1 is a potential component of both TGF-β and BMP-4 signaling pathways. It is a member of the mitogen-activated protein kinase kinase kinase family and activates MKK4 and MKK6. We recently showed that ectopic coexpression of *Xenopus* TAB1 (xTAB1) and xTAK1 can induce ventral mesoderm formation and suppress neural differentiation (Shibuya *et al.*, 1998). Smads are mammalian homologs of mothers against dpp in *Drosophila*. They are ligand-dependent, are rapidly phosphorylated, and translocate into the nucleus. While signaling components involved in BMP signaling per se are being revealed, actual target genes that play a direct role in executing osteogenic function are not well understood. One reason for this is that the osteoblastic differentiation-related genes and differentiation-determined genes are unclear.

Using a differential display technique, we isolated a BMP-inducible target gene, mDlx5, which is a member of the homeobox-containing *Drosophila* distal-less gene family. Dlx gene products have a conserved 60-amino-acid homeodomain and are members of a large family of DNA-binding transcription factors that are believed to control the transcription of target genes to affect organogenesis for skeletal formation. Because some homeobox genes are expressed in the limb mesenchyme during precartilaginous condensation, it has been proposed for some hox genes, such as HoxA13 (Yokouchi *et al.*, 1995), that they may control chondrogenesis. Our present study suggests that osteogenesis is regulated by the homeobox gene, mDlx5. mDlx5-transfected cells showed higher alkaline phosphatase activities, osteocalcin production, and mineralization of extracellular matrix, suggesting that mDlx5 is a positive regulator of osteoblast differentiation. We propose that the mDlx5 gene product plays an important role at the later stage of differentiated osteoblast, rather than in the immature osteoblast, because the induction of mDlx5 continu-







ously occurs 48 h past BMP treatment at the time osteoblastic fate has been clearly determined. Therefore, ectopic expression of mDlx5 results in activation of various terminal markers such as increase of alkaline phosphatase activity, induction of the osteocalcin gene, and increasing mineralized nodule formation.

Recently, Cbfa-1, a runt-domain-containing transcription factor, has been shown to be a key regulator of bone formation in the developing embryo and osteoblast differentiation *in vitro* (Banerjee *et al.*, 1997; Ducy *et al.*, 1997; Komori *et al.*, 1997; Otto *et al.*, 1997). When the expression of Cbfa-1 was examined in the parental MC3T3-E1 and mDlx5-transfected cells, no change in Cbfa-1 mRNA levels was observed. The data suggest that induction of Cbfa-1 gene by mDlx5 is not required for this differentiation, and induction of mDlx5 occurs independent of the Cbfa-1 pathway. Because we did detect basal expression levels of Cbfa-1 mRNA, these factors together may contribute to the progression of osteoblast differentiation.

Moreover, we showed that mDlx5 was induced orthotopically in mouse embryos treated with BMP-4 and in fractured bone of adult mice. A previous study showed that BMP-4 mRNA was detected from 12 to 72 h after the onset of fracture, before the advent of differentiated osteoblasts and mature chondrocytes, suggesting that BMP-4 is produced by less differentiated osteoprogenitor cells (Nakase *et al.*, 1994). In contrast, it has been suggested that BMP is not produced by mature osteoblasts and that the BMP-4 gene expression is located in callus-forming tissue, such as periosteum and parifracture soft tissue (Nakase *et al.*, 1994). It is not clear at present whether mDlx5 is induced by the newly synthesized BMP during bone fracture healing or is induced by other gene products which are activated during the multiple cascades of the bone healing responses. Further studies are necessary to clarify this issue.

Overexpression of mDlx5 in dorsal cells of the early *Xenopus* embryo caused partial loss of dorsoanterior structures, which was accompanied by increased expression of ventral mesoderm marker genes and reduced expression of dorsal mesoderm marker gene. These effects of mDlx5 resemble those of overexpression of BMP-4 (Clement *et al.*, 1995; Dale *et al.*, 1992; Jones *et al.*, 1992), suggesting that mDlx5 mediates, at least in part, the effects of BMP-4 in the *Xenopus* embryo. However, the effects of mDlx5 are not as marked as those of BMP-4. There are other genes involved in the full ventralization of the *Xenopus* embryo by BMP-4. Another homeobox gene, Msx1, is also known to be a target gene whose overexpression also leads to ventralization of the *Xenopus* embryo (Maeda *et al.*, 1997; Suzuki *et al.*, 1997). It would be intriguing to examine whether mDlx5 and Msx1 induce ventralized characteristics of mesoderm in a cooperative manner.

In conclusion, we have shown that BMP-2 stimulation increases mDlx5 mRNA expression and that the overexpression of mDlx5 gene product partly mimics several aspects of the osteoblast differentiation process by inducing the expression of osteoblast-specific markers. The precise molecular mechanism for the activation of osteoblast-specific gene by mDlx5 awaits further studies.

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FIG. 5. mDlx5 gene is involved in bone formation. (A) Cellular phenotyping of stably transfected MC3T3-E1 cells. The pSV2/neo-transfected cells and mDlx5-transfected cells were cultured for 9 or 27 days in the presence of β-glycerophosphate. These cells were stained for alkaline phosphatase and mineralized nodules as described under Materials and Methods. (B) Light microscopy of representative cross-sections of mineralized nodules in mDlx5-transfected cells. Cells were cultured for 27 days in the presence of 2 mM β-glycerophosphate. The sections were stained by von Kossa's method and counterstained with hematoxylin. Nodules consisted of cell layers and mineralized matrix, in which some cells were embedded.

FIG. 6. Dorsal injection of mDlx5 mRNA caused severe ventralization in *Xenopus* embryos. (A) Synthetic mRNA encoding the mDlx5 DNA sequences was injected into the equatorial regions of two dorsal or two ventral *Xenopus* blastomeres at the four-cell stage, and phenotypes were scored at tadpole stage 36. Top, uninjected; bottom, mDlx5 (50 pg) mRNA. The average dorsoanterior index (Kao and Elinson, 1989), a measure of the degree of dorsal and anterior mesodermal patterning, was for mDlx5 2.3 ($n = 30$). (B) RT-PCR analysis of mesodermal marker gene expression in DMZ assay. Synthetic mRNAs encoding the indicated DNA sequences were injected into the two dorsal blastomeres at the four-cell stage. DMZ fragments injected with the indicated mRNAs were cultured until gastrula stage 11, and total RNA was harvested. RNA was analyzed by RT-PCR for the presence of the indicated transcripts: 1, uninjected; 2, mDlx5 (50 pg); 3, BMP-4 (200 pg); 4, whole embryo; 5, −RT. Histone, ubiquitously expressed, was used as a loading control. Xvent is a marker of ventral mesoderm. Xhox-3 is a marker of ventral and posterior mesoderm. Xwnt-8 is a marker of ventral and lateral mesoderm. Goosecoid (gsc) is marker of dorsal mesoderm.

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