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BMP2 Commitment to the Osteogenic Lineage Involves Activation of *Runx2* by DLX3 and a Homeodomain Transcriptional Network*[S]

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Mohammad Q. Hassan[‡], Rahul S. Tare[‡1], Suk Hee Lee[‡], Matthew Mandeville[‡], Maria I. Morasso[§], Amjad Javed[‡2], Andre J. van Wijnen[‡], Janet L. Stein[‡], Gary S. Stein[‡], and Jane B. Lian[‡3]

From the [‡]Department of Cell Biology and Cancer Center, University of Massachusetts Medical School, Worcester, Massachusetts 01655 and [§]Developmental Skin Biology Unit, NIAMS, National Institutes of Health, Bethesda, Maryland 20892

Several homeodomain (HD) proteins are critical for skeletal patterning and respond directly to BMP2 as an early step in bone formation. RUNX2, the earliest transcription factor proven essential for commitment to osteoblastogenesis, is also expressed in response to BMP2. However, there is a gap in our knowledge of the regulatory cascade from BMP2 signaling to the onset of osteogenesis. Here we show that BMP2 induces DLX3, a homeodomain protein that activates *Runx2* gene transcription. Small interfering RNA knockdown studies in osteoblasts validate that DLX3 is a potent regulator of *Runx2*. Furthermore in *Runx2* null cells, DLX3 forced expression suffices to induce transcription of *Runx2*, osteocalcin, and alkaline phosphatase genes, thus defining DLX3 as an osteogenic regulator independent of RUNX2. Our studies further show regulation of the *Runx2* gene by several homeodomain proteins: MSX2 and CDP/cut repress whereas DLX3 and DLX5 activate endogenous *Runx2* expression and promoter activity in non-osseous cells and osteoblasts. These HD proteins exhibit distinct temporal expression profiles during osteoblast differentiation as well as selective association with *Runx2* chromatin that is related to *Runx2* transcriptional activity and recruitment of RNA polymerase II. *Runx2* promoter mutagenesis shows that multiple HD elements control expression of *Runx2* in relation to the stages of osteoblast maturation. Our studies establish mechanisms for commitment to the osteogenic lineage directly through BMP2 induction of HD proteins DLX3 and DLX5 that activate *Runx2*, thus delineating a transcriptional regulatory pathway mediating osteoblast differentiation. We propose that the three homeodomain proteins MSX2, DLX3, and DLX5 provide a key series of molecular switches that regulate expression of *Runx2* throughout bone formation.

Skeletal development is a complex process whereby multiple signaling pathways converge to induce formation of bone structures. The BMP2/4/7 [4] members of the transforming growth factor-β superfamily of signaling polypeptides are essential for commitment and differentiation of mesenchymal cells to the osteoblast lineage for bone tissue formation. The BMP signal is transduced through the binding to their heterodimeric type I and type II serine/threonine kinase receptors with formation of activated Smad complexes that are translocated to the nucleus to regulate target genes (1). BMP2 target genes include a cohort of transcription factors with specialized activities for induction of cell type-specific lineages. Among the BMP2 target genes are transcription factors that have essential roles in skeletal development, including several homeodomain proteins, the bone-related runt homology domain factor RUNX2 (CBFA1/AML3), and an SP1 family member, OSTERIX (2–4). BMP/transforming growth factor-β intracellular Smads will also form co-regulatory complexes with transcription factors to regulate target genes. The potent osteogenic effects of BMPs are mediated in part by formation of RUNX2-SMAD complexes for phenotypic gene induction (5, 6).

RUNX2 is essential for development of the osteoblast phenotype (2, 7, 8). The protein is expressed in the early mesenchyme of developing skeletal tissues (embryonic age E9.5) (9–12), and null mouse models have clearly established that RUNX2 is required for osteoblast differentiation and bone formation at later stages of embryonic development. In undifferentiated mouse embryo fibroblasts, RUNX2 is repressed by the chondrogenic homeodomain protein NKX3.2 (13, 14) providing a mechanism for lineage switching and allowing the mesenchymal cells to enter the chondrogenic lineage under appropriate conditions. RUNX2 becomes up-regulated in hypertrophic chondrocytes and the differentiated osteoblast (15–17). A compelling question to be addressed relates to tissue-specific regulatory sequences that support *Runx2* transcription for osteogenic lineage direction upon BMP2 induction of osteogenesis. Given the early response of RUNX2 and many homeodomain proteins to the BMP osteogenic signal as identified in several microarray gene profiling studies (18, 19), a clear understand-

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[1] Present address: University Orthopaedics, Southampton General Hospital, Southampton S016 6YD, UK.

[2] Present address: Inst. of Oral Health Research, School of Dentistry, University of Alabama, Birmingham, AL 35294.

[3] To whom correspondence should be addressed: Dept. of Cell Biology, University of Massachusetts Medical School, 55 Lake Ave. N., Worcester, MA 01655. Tel.: 508-856-5625; Fax: 508-856-6800; E-mail: jane.lian@umassmed.edu.

[4] The abbreviations used are: BMP, bone morphogenetic protein; HD, homeodomain; POL, polymerase; MEM, minimal essential medium; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; qPCR, quantitative PCR; RT, real time; Col, collagen; CDP/cut, CCAAT displacement protein; DLX, distal-less homeodomain; MSX, MSH homeodomain.

ing of how the activity of multiple HD proteins and RUNX2 are coordinated for control of the onset of osteogenesis requires further study.

Homeodomain proteins play a key role in skeletal development. For example, MSX1 and MSX2 members of the *Msh* family of homeobox genes are classical repressor proteins that are expressed in neural crest cells and limb tissue to regulate skeletal patterning and the number of osteoprogenitor cells. *Msx1* and *Msx2* null double mutant phenotypes exhibit severe craniofacial abnormalities, skull ossification, and limb defects (20–23). MSX2 inhibits chondrogenesis and is critical for regulation of osteoblast differentiation (24–27). The mammalian homologues of *Drosophila* distal-less (DLX) proteins are among the homeodomain proteins that also function in specification of skeletal structures (3, 28, 29). DLX2 and DLX3 contribute to craniofacial development but with distinct temporal and spatial patterns of expression (30, 31). DLX2 also has an important role in mesenchymal condensation (32). DLX5 and DLX6 are essential for development of jaw, axial, and appendicular bone (29). Several *in vitro* studies demonstrate that DLX5, an inducer of mesoderm differentiation, is up-regulated during osteoblast differentiation (33) and is competent to induce chondrocyte and osteoblast differentiation (34, 35). Furthermore DLX5 is required for BMP2-mediated induction of osteogenesis in C2C12 myogenic cells concomitant with increased *Runx2* expression (36, 37). Although the *Dlx3* null mouse is embryonic lethal prior to skeletal development due to placental failure (38, 39), recent studies showed that DLX3 is expressed in periosteum, osteoblasts, and chondrocytes of the developing limb and in osteogenic lineage cells (40, 41). In this study we have therefore addressed a mechanism for activation and regulation of the RUNX2 transcription factor by DLX3 and in relation to other homeodomain proteins expressed during osteoblastogenesis.

Here we provide evidence for molecular switches that are operative on the bone-related P1 *Runx2* promoter in normal diploid osteoblasts. These switches are mediated by the MSX2, DLX3, and DLX5 homeodomain proteins and support *Runx2* repression and activation to regulate osteoblast differentiation and bone formation. Recent studies have identified regulatory sequences in the proximal promoter contributing to enhanced transcriptional control of endogenous *Runx2* gene expression in response to WNT-β catenin signaling (42), DLX5 (37), and AP-1 factors (43, 44) as well as autoregulation by RUNX2 itself (45). The present studies identify classical homeodomain regulatory sequences in the proximal promoter that are either activated by DLX3 and DLX5 or repressed by MSX2 and CDP/cut. Importantly these proteins are recruited to the *Runx2* promoter at different stages of osteoblastogenesis in relation to *Runx2* transcription. DLX3, DLX5, and RUNX2 are coordinately recruited with significant increases in RNA POL II (*Runx2* transcription). However, DLX3 association is early and transient, whereas the DLX5 functional associations with the *Runx2* gene promoter are sustained in mature osteoblasts. By overexpression and knockdown studies, the functional effects of MSX2, DLX3, and DLX5 on *Runx2* promoter activity and endogenous gene expression in osseous and non-osseous cells are established. We also show that DLX3 is a potent activator of *Runx2* transcription in non-osseous cells and that DLX3 and DLX5 can activate RUNX2 target genes in *Runx2* null cells. These findings support mechanisms by which BMP2 allows commitment to the osteogenic lineage through induction of both HD and RUNX2 proteins as early response genes.

MATERIALS AND METHODS

Cell Cultures—C3H10T1/2 and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA). MC3T3 cells were maintained in α-MEM supplemented with 10% fetal bovine serum. Primary rat osteoblast cells were isolated from calvaria of fetal rats at day 21 of gestation by three sequential digestions with collagenase P (2 mg/ml; Roche Applied Science) at 37 °C and 0.25% trypsin (Invitrogen) treatment as detailed previously (46). Cells from the third digestion were plated at a density of 4×10^5 cells/100-mm dish and fed every 2nd day with minimal essential medium (MEM; Invitrogen) supplemented with 10% fetal bovine serum. The same procedure was used for isolation of cells from calvaria of *Runx2* null mice (2, 47). A *Runx2*$^{-/-}$ stable cell line was derived by telomerase reverse transcriptase immortalization (48). Establishment and characterization of a Runx2$^{-/-}$ stable cell line is described previously (62). In both MC3T3 and primary cells, differentiation was induced at monolayer confluency by addition of 50 μg of ascorbic acid/ml and 10 mM β-glycerol phosphate to the medium with every feeding.

Transient Transfections and Luciferase Reporter Assays—Transient transfections were performed in 6-well plates at 50–70% confluence using 5 μl of FuGENE 6 transfection reagent (Roche Applied Science) and 0.5–1 μg of promoter reporter DNA/well in accordance with the manufacturer's protocol. For MSX2, DLX3, and DLX5 expression, a variable amount (50, 100, and 200 ng) of the respective expression clone in pcDNA plasmid backbone was transfected into each well unless otherwise noted. As a control, 100–400 ng of empty pcDNA expression vector was transfected into each well as appropriate. To monitor transfection efficiency, transfections included 25 ng of a promoterless *Renilla luciferase* reporter construct/well. For *Runx2* promoter-reporter assays, transfections included 0.5 μg of either a *Runx2* 0.6-kb promoter-luciferase construct or the deletion mutant of 0.6-kb *Runx2* promoter construct. The promoterless pGL3 luciferase construct (0.5 μg) was used as a control to examine the background luciferase activity. All results were normalized to activity of the pGL3 empty vector (Promega, Madison, WI).

Cells transfected with *Runx2* promoter-luciferase reporter constructs were harvested 24–36 h and in some cases 72 h after transfection, and each well was lysed at room temperature for 20 min in the presence of 0.3 ml of Passive Lysis Buffer (Promega). Firefly luciferase activity was quantitated in a luminometer using a 12-s measurement immediately after addition of 10–20 μl of cell lysate to 100 μl of substrate (Promega Luciferase Assay System). All results were normalized to the luciferase

TABLE 1
Primers for PCR assays

Primers for real time PCR assays	
Homeodomain primers (mouse)	
Dlx2	Forward primer, 5′-AGT TCG TCT CCG GTC AAC AA-3′
	Reverse primer, 5′-TTT CAG GCT CAA GGT CTT CC-3′
Dlx3	Forward primer, 5′-GTA CCG GGA GCA GCC TTT-3′
	Reverse primer, 5′-CTT CCG GCT CCT CTT TCA C-3′
Dlx5	Forward primer, 5′-GCC CCT ACC ACC AGT ACG-3′
	Reverse primer, 5′-TCA CCA TCC TCA CCT CTG G-3′
Msx2	Forward primer, 5′-ATA CAG GAG CCC GGC AGA TA-3′
	Reverse primer, 5′-CGG TTG GTC TTG TGT TTC CT-3′
CDP-cut	Forward primer, 5′-GAG GGT CAA AGA GGT GCT GA-3′
	Reverse primer, 5′-CCA AAA TGG TCT CCC CAA AT-3′
Bone marker primers (mouse)	
Bone Sialoprotein	Forward primer, 5′-GCA CTC CAA CTG CCC AAG A-3′
	Reverse primer, 5′-TTT TGG AGC CCT GCT TTC TG-3′
Alkaline phosphatase	Forward primer, 5′-TTG TGC CAG AGA AAG AGA GAG A-3′
	Reverse primer, 5′-GTT TCA GGG CAT TTT TCA AGG T-3′
Runx2	Forward primer, 5′-CGG CCC TCC CTG AAC TCT-3′
	Reverse primer, 5′-TGC CTG CCT GGG ATC TGT A-3′
Osteocalcin	Forward primer, 5′-CTG ACA AAG CCT TCA TGT CCA A-3′
	Reverse primer, 5′-GCG GGC GAG TCT GTT CAC TA-3′
Primers for RT-PCR assays	
Homeodomain primers (mouse)	
Dlx2	Forward primer, 5′- GGC ACC AGT TCG TCT CCG GCT-3′
	Reverse primer, 5′-CGC CGA AGT CCC AGG ATG CTG-3′
Dlx3	Forward primer, 5′-TCT GGT TCC AGA ACC GCC GCT-3′
	Reverse primer, 5′-CAG TAC ACA GCC CCA GGG TTA-3′
Dlx5	Forward primer, 5′-CCA CCG ATT CTG ACT ACT AC-3′
	Reverse primer, 5′-TAC CAT TCA CCA TCC TCA CC-3′
Msx2	Forward primer, 5′-GTC ATG GCT TCT CCG ACT AA-3′
	Reverse primer, 5′-ATT TTC CGA CTT GAC CGA GG-3′
CDP-cut	Forward primer, 5′-TCT CCG ACC TCC TTG CCC G-3′
	Reverse primer, 5′-G CCC CCA GCC ACA ACA CCA-3′
Bone marker primers (mouse)	
Alkaline phosphatase	Forward primer, 5′-TTG TGC CAG AGA AAG AGA GAG A-3′
	Reverse primer, 5′-GTT TCA GGG CAT TTT TCA AGG T-3′
Osteocalcin	Forward primer, 5′-CCA GTC CCA CAC AGC AGC CTT-3′
	Reverse primer, 5′-AAA GCC GAG CTG CCA GAG TT-3′

activity resulting from transfection of the promoterless PGL3 luciferase construct (Promega).

RNA Isolation and Q-PCR Analysis—RNA was isolated from cultures of MC3T3, NIH3T3, C3H10T1/2, and *Runx2*$^{-/-}$ cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. After purification, 5 μg of total RNA was DNase I-treated using a DNA-free RNA column purification kit (Zymo Research, Orange, CA). RNA (1 μg) was then reverse transcribed using oligo(dT) primers and the SuperScript first strand synthesis kit (Invitrogen) according to the manufacturer's protocol. Gene expression was assessed by quantitative real time PCR performed using 2× SYBR Green Master Mix (Eurogentec) and a two-step cycling protocol (anneal and elongate at 60 °C, denature at 94 °C). Specificity of primers was verified by dissociation/melting curve for the amplicons when using SYBR Green as a detector. Primers used for PCRs are listed in Table 1.

Western Blotting—For the detection of MSX2, DLX3, DLX5, RUNX2, and Actin proteins, each well of a 6-well plate was lysed in 400 μl of lysis buffer containing 2% SDS, 10 mM dithiothreitol, 10% glycerol, 12% urea, 10 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture (Roche Applied Science), 25 μM MG132 proteasome inhibitor and boiled for 5 min. Proteins were then quantified using Bradford reagent (Pierce) and taking spectrophotometric readings at 590 nm. Concentrations were estimated against a standard curve generated using bovine serum albumin. Total protein (20 μg) was subjected to electrophoresis in a denaturing 10% polyacrylamide gel containing 10% SDS. Proteins were then transferred onto Immobilon-P membranes (Millipore, Billerica, MA) using a semidry transfer apparatus. Membranes were blocked in phosphate-buffered saline, 0.1% Tween 20 containing 2% nonfat powdered milk (Bio-Rad). Proteins were detected by incubating with antibodies at a concentration of 50 ng/ml in blocking solution. Antibodies used in this study were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, except for a RUNX2 mouse monoclonal antibody that was a generous gift from Drs. Yoshi Ito and Kosei Ito, National University, Singapore (Research & Development Antibodies, Benicia, CA). Anti-Actin goat polyclonal antibody (I-19) was from Santa Cruz Biotechnology. Primary antibodies were detected with goat anti-mouse secondary antibody conjugated to horseradish peroxidase. Secondary antibodies were detected using Western Lightning chemiluminescence reagent (PerkinElmer Life Sciences).

Chromatin Immunoprecipitation (ChIP) Assays—To cross-link proteins to DNA, rat calvarial osteoblast and preosteoblast MC3T3 cells were incubated for 15 min at room temperature in 12.5 ml of serum-free media containing 1% formaldehyde. A final concentration of 0.125 M glycine was added to the 1% formaldehyde-media solution for neutralization for another 15 min. The cells were collected in phosphate-buffered saline (1×) after plates were washed twice with ice-cold phosphate-buffered saline. Cells were then resuspended in SDS lysis buffer (1% SDS, 50 mM Tris, pH 8.1, 10 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (Sigma), 25 μM MG-132 (Calbio-

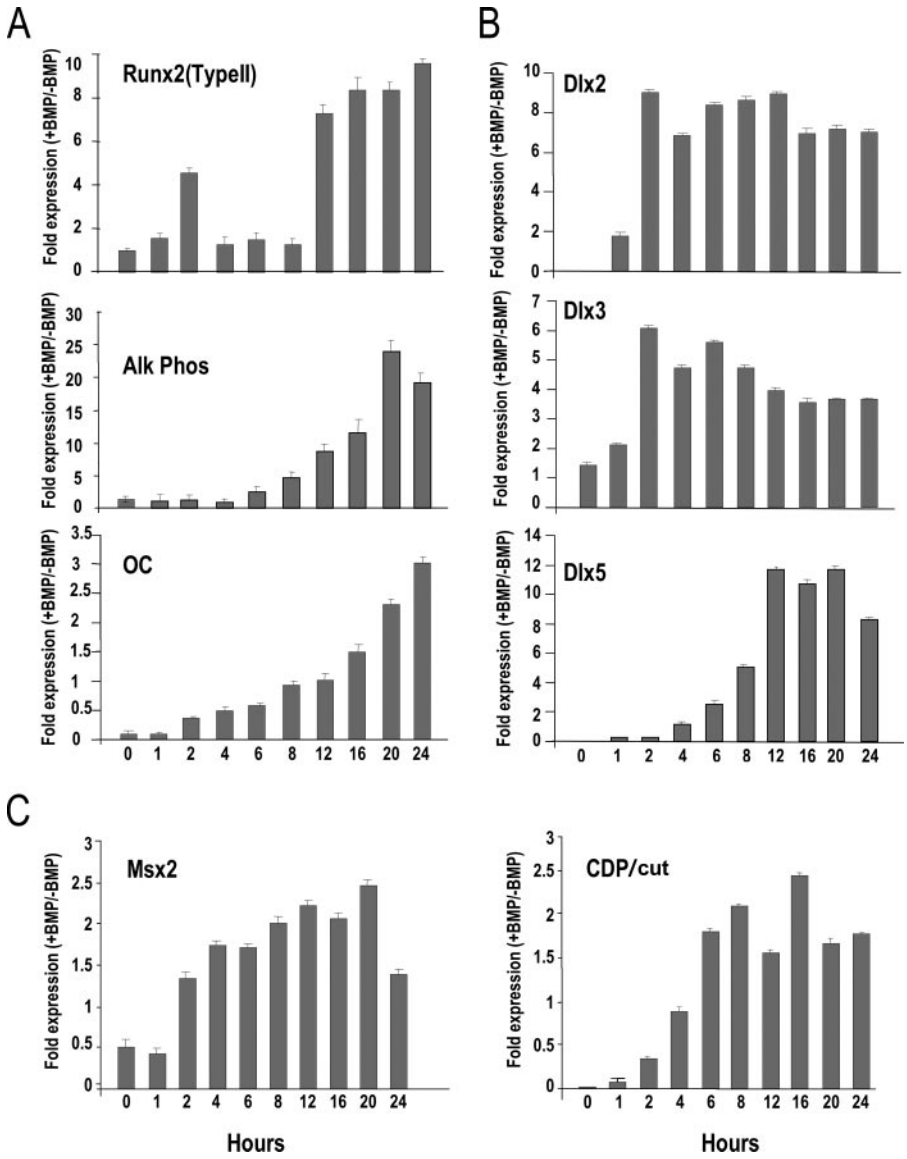

FIGURE 1. **Expression profile of osteogenic markers and homeodomain proteins during the initial 24 h of BMP2-treated C2C12 cells.** Premyogenic C2C12 cells were treated with 100 ng/ml BMP2 for 24 h. Total RNA was isolated at different time points (0, 1, 2, 4, 6, 8, 12, 16, 20, and 24 h). 5 μg of total DNase I-treated RNA was reverse transcribed with oligo(dT) primer and amplified by gene-specific primers by RT-qPCR to quantitate gene expression. *A*, bone phenotype markers (*Runx2*, alkaline phosphatase (*Alk Phos*), and osteocalcin (*OC*)); *B*, homeodomain proteins (DLX2, DLX3, and DLX5); and *C*, MSX2 and CDP/cut repressor proteins. Relative mRNA expression levels of each gene at each time point were first normalized to glyceraldehyde-3-phosphate dehydrogenase for control (−*BMP2*) and BMP2-treated (+*BMP2*) samples. The mRNA -fold induction levels (+*BMP*/−*BMP2*) of $n = 3$ assays per time point were plotted (mean ± S.D.).

chem/Sigma), and 1× protease inhibitor (Roche Applied Science). To isolate the nuclei, cells were homogenized in a Dounce homogenizer followed by centrifugation at 1,100 rpm at 4 °C. The nuclei pellets were resuspended in 500 μl (500 μl/100-mm plate) of lysis buffer. Samples were sonicated to shear DNA into 0.2–0.6-kb fragments. Cellular debris were removed by centrifugation at 14,000 rpm for 15 min at 4 °C, and resulting chromatin-containing solutions were distributed into multiple 100-μl aliquots (with equal protein concentrations) that were used as the starting material of all subsequent steps. 900 μl of Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) was added to each immunoprecipitate (100 μl) with 1 mM phenylmethylsulfonyl fluoride, 25 μM MG-132, and 1× protease inhibitor.

Chromatin aliquots were precleared with 60 μl of a 25% (v/v) suspension of protein A/G beads. Samples were used directly for the IP reaction with 3 μg of specific antibody of interest (anti-RUNX2, anti-MSX2, anti-DLX5, anti-DLX3, anti-POL II), and a negative control with normal rabbit/mouse IgG was used. ChIP reactions were allowed to proceed for 4 h to overnight at 4 °C on a rotating wheel. Immune complexes were mixed with 60 μl of 25% (v/v) precoated protein A/G-agarose suspension (Santa Cruz Biotechnology) followed by incubation for 2 h at 4 °C on a rotating wheel. Beads were collected by brief centrifugation, and the immune complexes were washed with low salt (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1), high salt (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1), LiCl salt (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and Tris-EDTA buffer sequentially at 4 °C. The immune complexes were eluted twice by adding 150 μl of freshly prepared elution buffer (100 mM $NaHCO_3$, 1% SDS). After reversal of cross-links at 68 °C overnight, the eluate was treated with 100 μg/ml proteinase K followed by phenol-chloroform extraction and ethanol precipitation using 5 μg of glycogen as carrier. An aliquot (2–3 μl) of each sample was assayed using quantitative PCR for the presence of specific DNA fragments using primers that span the proximal *Runx2* promoter. The rat *Runx2* promoter primers are: forward 5′-CTC CAG TAA TAG TGC TTG CAA AAA AT-3′ and reverse 5′-GCG AAT GAA GCA TTC ACA CAA-3′. The mouse *Runx2* promoter primers are: forward 5′-TGG TAG GCA GTC CCA CTT TAC-3′ and reverse 5′-GGC TGG TAG TGA CCT GCA GAG-3′. The immunoprecipitate samples were analyzed by two methods: 1) hot PCR in which regular amplification was performed with 2.5 μCi of [α-^{32}P]dCTP label or 2) quantitative real time PCR that was carried out using 2× SYBR Green Master Mix (Eurogentec) and a two-stage cycling protocol (60 °C annealing and exten-

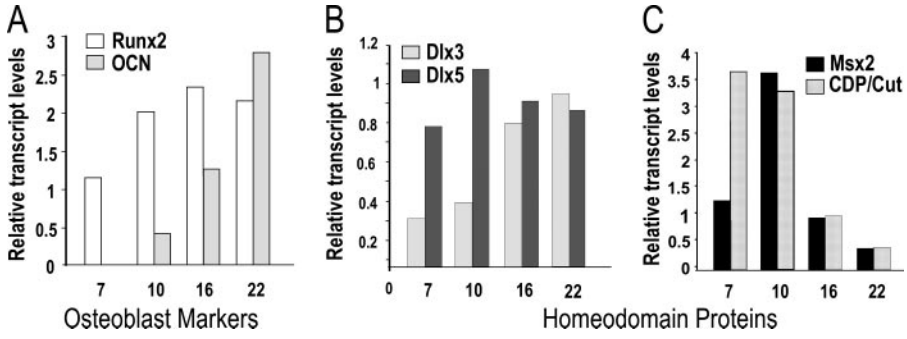

FIGURE 2. **Temporal expression of homeodomain proteins and osteoblast markers during growth and differentiation of preosteoblast MC3T3 cells.** MC3T3 cultures at the indicated days were harvested for the isolation of total cellular RNA and analyzed by RT-qPCR with gene-specific primers (Table 1). The expression values were normalized to glyceraldehyde-3-phosphate dehydrogenase values, and relative expression levels of the specific gene against time were plotted. *A*, osteoblast markers Runx2 and osteocalcin; *B*, the distal-less homeodomain proteins DLX3 and DLX5; *C*, Msh homeodomain protein MSX2 and CDP/cut.

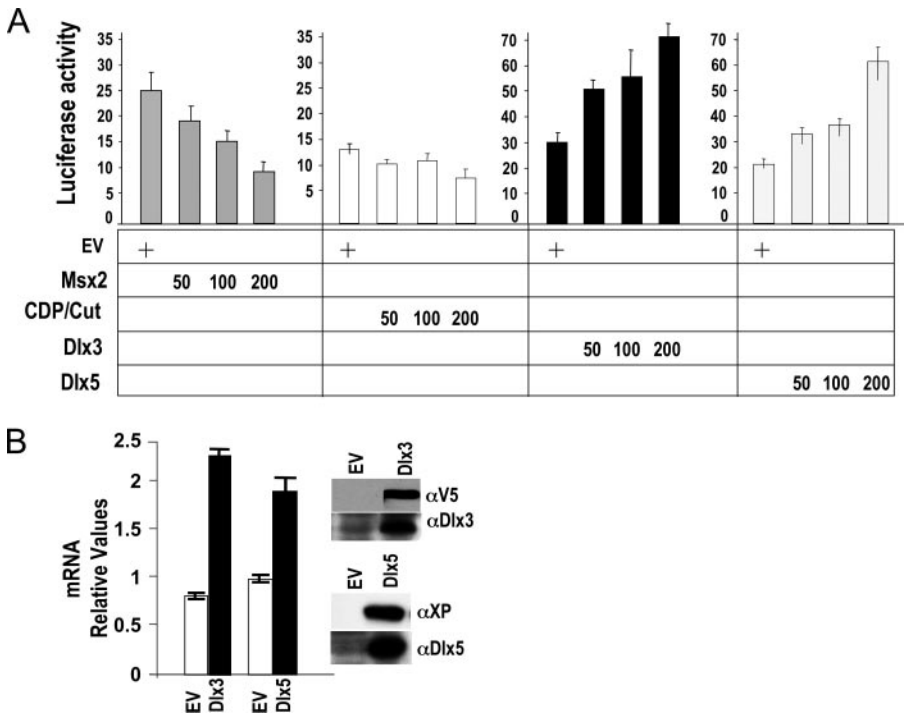

FIGURE 3. **Regulation of endogenous *Runx2* by DLX3 compared with the other HD proteins.** *A*, the 0.6-kb *Runx2* P1 promoter was co-transfected with MSX2, CDP/cut, DLX3, and DLX5 in NIH3T3 cells. The indicated concentration range was evaluated to demonstrate potency of their effects on the *Runx2* promoter. *B*, *left panel*, induction of endogenous *Runx2* mRNA (by RT-qPCR) mediated by *DLX3* and compared with DLX5. Empty vector (*EV*), 400 ng; DLX3/DLX5, 400 ng. The *right panel* shows nearly equivalent expression of DLX3 and DLX5 and the level of endogenous proteins by Western blot analysis.

sion, 94 °C denaturation, 40 cycles). Amplicon specificity was verified by analysis of melting temperature. All data were collected during the linear phase of amplification.

Antibodies used for ChIP assays included the RUNX2 M-70 antibody (Santa Cruz Biotechnology). An affinity-purified polyclonal antibody was raised against a 16-amino acid synthetic peptide (amino acids 242–256) of the murine DLX3 protein as described previously (49). Mouse monoclonal MSX (1 + 2) antibody (4G1) against bacterially expressed *Gallus* Msx2 protein was obtained from Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa. Affinity-purified polyclonal DLX5 antibodies (Y20 and C20) were obtained from Santa Cruz Biotechnology. Both antibodies were raised against a peptide mapping within an internal region (Y20) or the C terminus (C20) of human DLX5. Mouse monoclonal (clone 8WG16) RNA Pol II was purchased from Covance Inc. Because the Msx and Dlx families share many common sequences in addition to their DNA binding domains, the specificities of the different antisera obtained from commercial sources used in these studies were verified with *in vitro* transcribed and translated HD proteins (40).

RNA Interference—The mouse MC3T3-E1 osteoblastic cells at 30–50% confluency were transfected using Oligofectamine (Invitrogen) with small interfering RNA (siRNA) duplexes specific for murine Dlx3 obtained from Qiagen Inc. (Stanford, CA) at different concentrations (50, 100, and 200 nM). The siRNA duplexes were r(CCC UGU GUU GCA AGU CGA A) dTdT and r(UUC GAC UUG CAA CAC AGG G) dAdG. For *Dlx5*, murine *Dlx5*-specific siRNA duplexes (siGENOME SmARTpool reagent from Dharmacon RNA Technologies) were used at the same concentrations as that used for *Dlx3*. To check the transfection efficiency cells were also transfected with siRNA duplexes specific for green fluorescent protein. As a non-specific control non-silencing siRNA duplexes obtained from Qiagen Inc. were used (sense, UUC UCC GAA CGU GUC ACG UdT dT; antisense, ACG UGA CAC GUU CGG AGA AdT dT). Opti-MEM I (a reduced serum medium from Invitrogen) was used to dilute the siRNA duplexes and Oligofectamine and for transfection. After treating the cells with siRNA for 4 h, the cells were supplemented with α-MEM containing 30% fetal bovine serum for a final concentration of 10% in the medium. The siRNA experiment was carried out for 72 h at which time the cells were harvested for total protein and RNA to analyze the knockdown effect of *Dlx3* and *Dlx5* siRNAs on endogenous DLX3 and DLX5 expression on other osteoblast-specific markers by real time qPCR.

RESULTS

Homeodomain Proteins Are Up-regulated at Different Stages of BMP2-induced Osteogenesis in Vitro—The C2C12 mesenchymal cell culture model has been characterized for its respon-

siveness to BMP2, expression of early response genes, and differentiation into osteoblasts by microarray gene profiling (18, 50). Using this system, expression of HD proteins related to the osteogenic process was confirmed by quantitative RT-PCR (Fig. 1). As reported previously (18), we observed a reproducible commitment point to osteogenesis that occurs 8 h after addition of BMP2 and is reflected by appearance of alkaline phosphatase expression, an early marker of bone formation (Fig. 1*A*). *Runx2* is expressed in two waves reaching peak levels after 2 and 8 h. Osteocalcin, an osteoblast-specific marker, is significantly induced by 16 h.

Members of the *distal-less* family of homeodomain transcription factors *Dlx2* and *Dlx3* are induced within 1–2 h of BMP2 treatment and showed sustained expression (Fig. 1*B*). In contrast, *Dlx5* is clearly induced after the 8-h osteogenic commitment point, and its level remains induced from 10- to 14-fold to the 24-h time point. The expression of two classical repressor HD proteins are shown in Fig. 1*C*; *Msx2* is rapidly induced at 1 h (2–3-fold), whereas *CDP/cut* reaches peak expression between 6 and 8 h. Thus homeodomain proteins exhibit selective periods of induction and sustained gene expression in the transition from premyogenic to the osteogenic phenotype.

To explore the biological significance of homeodomain protein expression patterns observed in the C2C12 BMP2-induced osteogenic cell model, we examined BMP2 responsiveness of these HD proteins in two mouse limb bud clonal cell lines that were previously characterized for their osteogenic (clone 14) or chondrogenic (clone 17) response to BMP2 (51, 52) (supplemental Fig. 1). For reference, the osteogenic markers alkaline phosphatase and osteocalcin are clearly up-regulated in response to BMP2 in clone 14 but not clone 17. *Dlx2* was responsive to BMP only in the chondrogenic line, whereas *Msx2*, *Dlx3*, and *Dlx5* were induced in both cell types. However, *CDP/cut* interestingly was slightly down-regulated by BMP2 in the osteogenic clone but up-regulated in the chondrogenic line. These findings support the concept that MSX and DLX class of proteins are involved in BMP-mediated osteogenic or chondrogenic pathways, whereas the CDP/cut protein is down-regulated in osteoblasts, suggesting a function related to cartilage. *Dlx2* regulation by BMP2 in clone 17C is consistent with it role in chondrogenesis (53).

DLX3 Is Continuously Up-regulated during Osteoblast Differentiation—Because the BMP2-induced C2C12 model is limited to commitment to osteogenesis and does not produce a mineralizing matrix, we examined the osteoprogenitor MC3T3 cell line that undergoes further differentiation to a mature osteoblast (Fig. 2). These osteoprogenitors already express *Runx2* in the proliferation stage (Fig. 2*A*, day 7), but *Runx2* levels were increased 40% by day 10 when cells are accumulating an extracellular matrix and have initiated cellular multilayering. By day 10 osteocalcin expression, a marker of mature osteoblasts, can be detected and is continuously up-regulated (Fig. 2*A*). Interestingly *Dlx5*, which was not induced until after the osteogenic commitment point in C2C12 cells, is expressed in the proliferating MC3T3 osteoprogenitor cells and remains at constitutive expression levels during differentiation. *Dlx3*, however, is developmentally up-regulated 2–3-fold higher

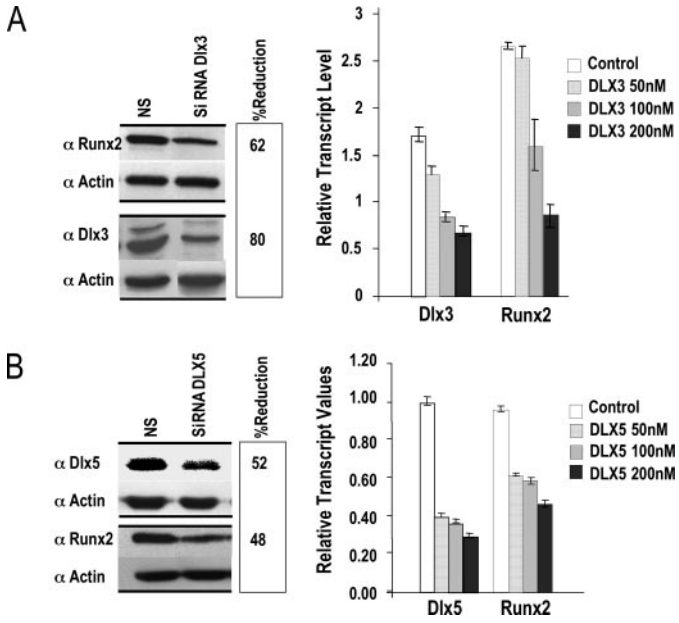

FIGURE 4. **Dlx3 siRNA inhibits *Runx2* gene expression in MC3T3 cells.** *A*, RNA interference effects of *Dlx3* in MC3T3-E1 cells after 72 h of treatment with either nonspecific (*NS*) or specific siRNA duplexes (described under "Materials and Methods") at the indicated doses. *Left panel*, the Western blot of the cell lysate prepared from *Dlx3* siRNA-treated MC3T3 cells probed with anti-DLX3 and -RUNX2 antibodies. The Actin profile is shown for equal loading of protein samples. The percent reduction for DLX3 and RUNX2 proteins upon *Dlx3* siRNA treatment was quantitated by densitometry. The *right panel* shows the expression profiles of *Dlx3* and *Runx2* (mRNA) by RT-qPCR in cells treated with three doses of Dlx3 siRNA. *B*, RNA interference of *Dlx5* in MC3T3-E1 cells for comparison with *Dlx3*. The cells were treated with Dlx5-specific SmARTpool siRNA for 72 h, and total cellular proteins and RNA were isolated and analyzed. *Left panel*, Western blot probed with anti-DLX5, -RUNX2, and -Actin antibodies with densitometric quantitation; *right panel*, expression profiles by RT-qPCR of Dlx5 and Runx2.

during osteoblast differentiation. Both *Msx2* and *CDP/cut*, well characterized repressor transcription factors that suppress bone-specific genes (54, 55), were down-regulated at the stages when mature osteoblast phenotypic markers are expressed.

Molecular mechanisms that activate *Runx2* expression are poorly understood. The continuous up-regulation of *Dlx3* and *Runx2* during MC3T3 osteoblast differentiation (Fig. 2) suggests that *Runx2* and *Dlx3* expression are related. Indeed we observed that the bone-related *Runx2* P1 promoter is potently stimulated by DLX3 (3–3.5-fold) in both NIH3T3 fibroblasts and MC3T3 osteoblasts. Similar activation was observed with promoter segments spanning 3- and 0.6-kb fragments (supplemental Fig. 2A). A Western blot of the transfected cells demonstrates comparable levels of HD protein expression in the two cell types (supplemental Fig. 2B). Also for comparison, the repressor activity of the MSX2 HD protein is shown to be similar for both promoter fragments but less striking in the osteoblastic cells. These data sets demonstrate that DLX3 is a strong activator of *Runx2* gene expression and that the initial 0.6 kb of the P1 promoter mediates the DLX response in both non-osseous and bone cells.

DLX3 Is a Potent Activator of the Runx2 Gene at Multiple HD Regulatory Elements—To further appreciate the relative contribution of DLX3 to regulation of *Runx2* promoter activity in relation to other homeodomain proteins, we examined activity

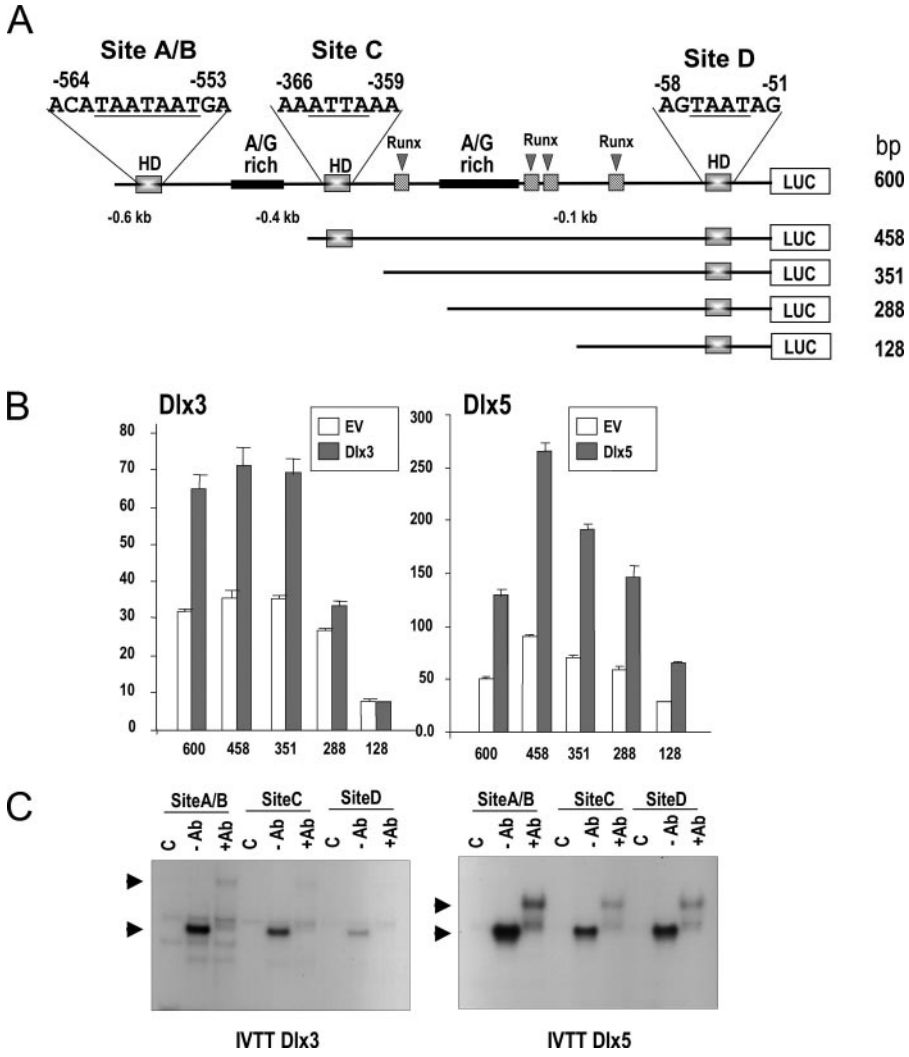

FIGURE 5. **Regulation of the Runx2 promoter activity by multiple HD response elements.** *A*, schematic representation of regulatory elements in the *Runx2* P1 promoter. Four putative homeodomain protein binding sites are indicated: two overlapping HD sites (A/B) positioned from −553 to −564, site C (−361 to −364), and site D (−53 to −56). *Runx2* sites and purine-rich regions are indicated. Deletion mutations of the *Runx2* promoter upstream of the luciferase reporter gene (*LUC*) are indicated. Clone 458 contains two downstream HD binding sites; clones −351 to −128 contain only one HD site. *B*, the deletion mutants of Runx2 promoter-luciferase constructs (400 μg) were co-transfected with 200 ng of either backbone vector (*EV*), *DLX3*, or *DLX5* construct to study the HD responsiveness across the proximal *Runx2* promoter. The promoter activities were expressed in relative luciferase values for MC3T3 cells. All transfections were performed in triplicate. *Error bars* represent $n = 6$ mean ± S.D. *C*, electrophoretic mobility shift assays using ^{32}P-labeled 20-bp probes representing each site with (control (*C*) or with *in vitro* transcribed and translated (*IVTT*) proteins in the absence (−) or presence (+) of the respective antibody (*Ab*), αDLX3 (*left panel*) and αDLX5 (*right panel*).

of the *Runx2* P1 promoter as a function of HD protein concentration comparing MSX2, DLX3, and DLX5 in MC3T3 cells in which these HD proteins are developmentally expressed (Fig. 3). CDP/cut was less effective as a repressor of *Runx2* promoter activity than MSX2, whereas forced expression of DLX3 and DLX5 increased promoter activity to approximately the same extent. The increases in promoter activity by DLX3 and DLX5 are further reflected by endogenous *Runx2* gene expression determined by RT-qPCR (Fig. 3*B*). Twenty-four hours post-transfection, we observed 2.5- and 2-fold increases in *Runx2* by DLX3 and DLX5, respectively, in MC3T3 cells.

To provide additional evidence for DLX3 regulation of *Runx2*, we tested the consequences of *Dlx3* siRNA-mediated depletion in MC3T3 cells (Fig. 4). *Dlx3* mRNA and protein were reduced 50 and 80%, respectively, at a 200 nM dose with two of three Dlx3 siRNAs (Fig. 4*A*, *right panel*, shows the effect of one oligo). Runx2 mRNA at this dose was decreased 70%, and RUNX2 protein was decreased by 62%. We also examined the inhibitory effects of *Dlx5* siRNA duplexes on *Runx2* expression (Fig. 4*B*). The *Dlx5* siRNAs are highly effective in decreasing *Dlx5* mRNA cellular levels (by 75%), but *Runx2* expression was reduced to 48% at the 200 nM dose (Fig. 4*B*). These results indicate that both DLX3 and DLX5 are significant enhancers of endogenous *Runx2* gene expression in the osteoprogenitor cells.

Our studies (Figs. 2 (supplemental) and 3) indicate that HD proteins regulate the *Runx2* gene through the initial 0.6 kb of the P1 promoter. This promoter segment contains four HD consensus sequences (Fig. 5*A*). To address whether specific DLX3- or DLX5-mediated activation occurs at multiple sites equivalently or selectively, a series of promoter deletion constructs were examined (Fig. 5*A*). DLX3 and DLX5 showed a similar stimulation of *Runx2* activity (2-fold) in the −600, −458, and −351 base pair fragments (Fig. 5*B*). However, in contrast to DLX3, DLX5-mediated activation could be readily observed for the −288 and −128 segments of the promoter presumably because of the higher affinity of DLX5 for site D. To further examine this observation of selective functional activities as related to protein-DNA interaction of the homeodomain protein at one of the regulatory sequences, gel shift studies (competition and antibody supershift) were carried out (Fig. 5*C*). We find that recombinant DLX3 and DLX5 bind site A/B (two overlapping sequences) and site C to the same extent, but DLX5 binds more strongly than DLX3 to site D. The combined results of protein-DNA binding activity and deletion mutation studies demonstrate that the *Runx2* HD sites are responsive to DLX3 and DLX5 and that differences in affinities for the *Dlx* consensus motif may favor DLX5 binding to the proximal site D element.

Recruitment of Homeodomain Proteins to the Runx2 Gene at Different Stages of Osteoblastogenesis—The HD proteins exhibited different expression profiles during development of the

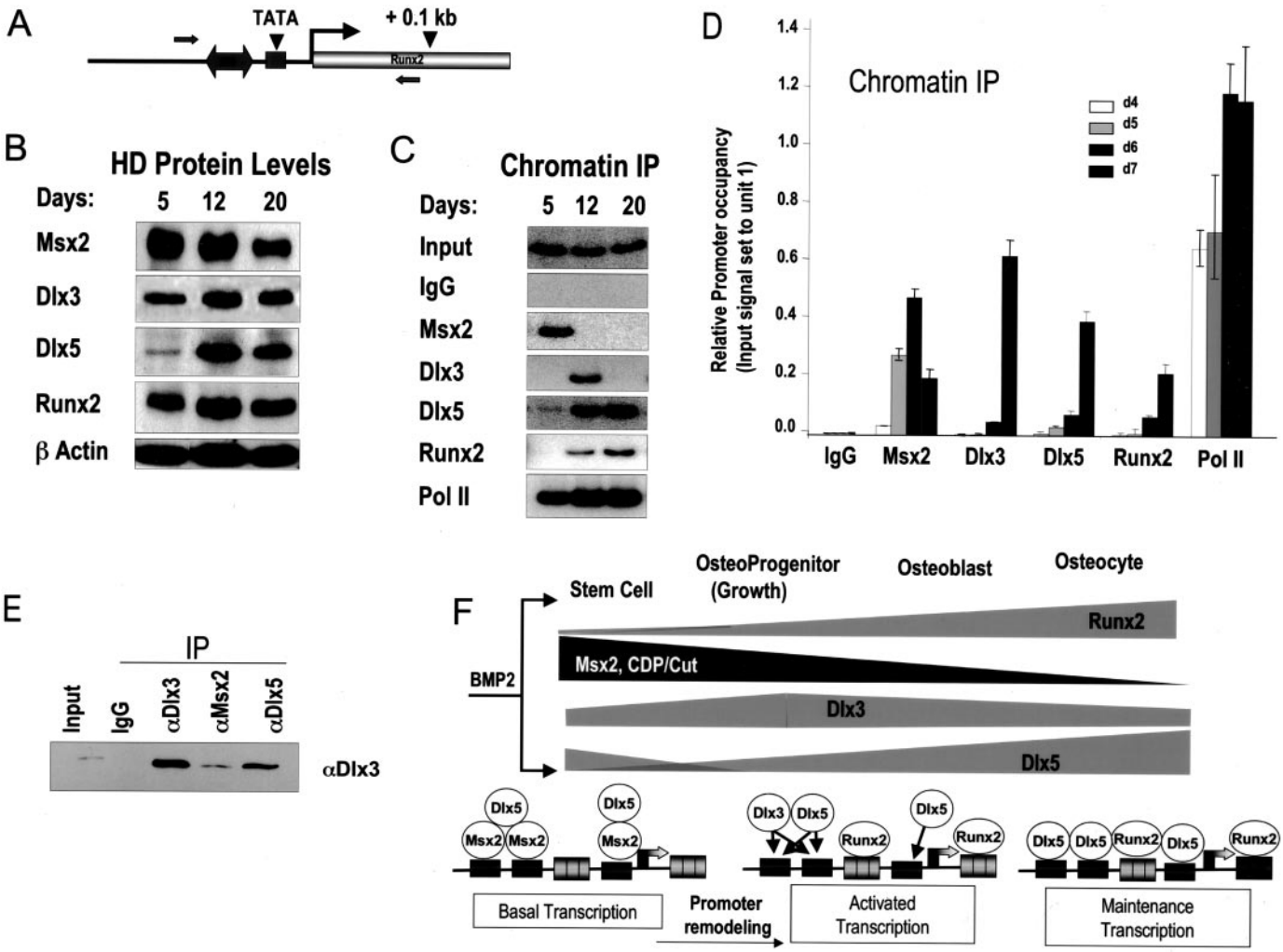

FIGURE 6. ***In vivo* occupancy of HD proteins in the proximal *Runx2* promoter during osteoblast growth and differentiation.** *A*, positions of the primers used to amplify proximal *Runx2* promoter in ChIP assays are indicated by *arrows*. *B*, cell layers from cultures of calvaria-derived primary rat osteoblasts were harvested at the indicated days for homeodomain proteins, and RUNX2 which were analyzed by Western blot analysis. Profiles of β Actin were used as a loading control. *C*, formaldehyde-cross-linked soluble chromatin of primary calvarial osteoblasts harvested at days 5 (proliferation), 12 (matrix maturation), and 20 (mineralization) was immunoprecipitated with 3 μg of the indicated antibodies. The DNA fragments from the immunocomplexes were purified and assayed by PCR using radioactive deoxynucleotides. Normal IgG (2 μg) was used as a control for each time point. Input represents 0.2% of each chromatin fraction used for immunoprecipitation. ChIP data are representative of three experiments in which all three time points were derived from the same osteoblast preparation. *D*, ChIP assays performed on primary calvarial osteoblasts harvested on days 4, 5, 6, and 7. DNA associated with the immunocomplexes of MSX2, DLX3, DLX5, and POL II was purified and assayed as described in *C*. *E*, DLX3 protein-protein interactions with MSX2 and DLX5 were examined in NIH3T3 cells transfected with 5 μg of tagged plasmid (Express-MSX2, Express-DLX5, and V5-DLX3)/100-mm plate. The lysates were immunoprecipitated with MSX2, DLX3, and DLX5 primary antibodies and immunoblotted with antibodies against the V5 tag. *F*, summary of homeodomain protein expression and occupancy of factors on the *Runx2* gene promoter at stages of osteoblast maturation. BMP2 induces a temporal expression of RUNX2 and HD during the differentiation of osteoprogenitors. Illustrated in the *top panels* are HD protein levels in primary osteoblasts. RUNX2 steadily increases during differentiation, whereas MSX2 and CDP/cut decrease, and DLX3 and DLX5 exhibit peak mRNA expression at distinct stages in primary osteoblasts (40). The *lower panel* shows recruitment of RUNX2 and HD proteins to the *Runx2* promoter at stages of osteoblast maturation that contribute to the bone-specific *Runx2* promoter remodeling. Basal transcription is defined as priming the *Runx2* promoter for gene activation, initially regulated by the repressor HD protein MSX2 (day 5) followed by inducer HD proteins, DLX3 transiently (days 5 and 6), and DLX5 for optimal transcription. The model shows DLX5 associated with the proximal site (−56/−53) based on functional activity and DNA binding (Fig. 5, *B* and *C*). RUNX2 provides autoregulation in mature osteoblasts. *IP*, immunoprecipitation; *d*, day.

osteoblast phenotype and like the *Runx2* and osteocalcin genes were temporally regulated during osteoblast differentiation (Fig. 2). Therefore, we further characterized association of the homeodomain proteins in primary calvaria-derived osteoblasts that differentiate similarly to *in vivo* osteoblasts and produce a mineralized bonelike nodule (56) (Fig. 6). HD protein levels were monitored during the differentiation time course (Fig. 6*B*). Although *Msx2* decreased, *Runx2*, *Dlx3*, and *Dlx5* increased to maximal levels with formation of bone nodules (day 12 in primary cells). Chromatin immunoprecipitation studies were performed from the proliferation to differentiation stages, selecting three time points: day 5, the proliferation stage; day 12, the matrix maturation period; and day 20, the mineralization stage. In these primary differentiating cells we find a temporal recruitment of HD proteins in relation to *Runx2* transcription (Fig. 6*C*). We observed that only MSX2 is associated with *Runx2* in the proliferation period (day 5). However, MSX2 is no longer bound to *Runx2* DNA by day 12 despite significant cellular protein levels (Fig. 6*B*). On day 12 both DLX3 and DLX5, as well as RUNX2, are recruited to the *Runx2*

promoter (Fig. 6*C*). The preferential binding of Dlx5 in late stages may be attributable to relatively higher affinity for *Dlx* elements in the *Runx2* promoter. However, we observed that DLX3 association is transient in the immediate postproliferative stage of osteoblast maturation. On day 20, an increased association of RUNX2 to its own promoter was also observed. The increase in RUNX2 protein at this stage is associated with the promoter (Fig. 6*C*), although a slight decrease in cellular protein was observed (Fig. 6*B*). Thus maintenance of RNA POL II binding would be consistent with *RUNX2* autoregulation and sustained transcription by DLX5.

To further define induced *Runx2* transcription (after release from MSX2 repression) by DLX3 and/or DLX5, ChIP studies were performed at earlier time points from day 4 to day 8 (every 24 h). We find low amounts of MSX2 on the promoter on day 4 and no activating transcription factors, but RNA POL II is present (Fig. 6*D*). By day 6 DLX3, DLX5, and RUNX2 are recruited to the *Runx2* gene concomitant with a 2-fold increase in RNA POL II. By day 7 with robust *Runx2* mRNA (data not shown), a 12-fold increase in recruitment of these factors is observed.

MSX2, DLX3, or DLX5 can form co-regulatory complexes with RUNX2 that alter the transcriptional activity of *Runx2* (40, 57, 58). We therefore examined DLX3 interactions with MSX2 and DLX5 by co-immunoprecipitation in NIH3T3 cells transfected with epitope-tagged expression constructs. DLX3 preferentially formed complexes with DLX5 and MSX2 to different degrees (Fig. 6*E*, *lower panel*). Thus recruitment of DLX3 to the *Runx2* promoter may occur as a direct protein-DNA interaction or as a DLX3/DLX5 heterodimer. Taken together, the temporal profiles of HD protein expression in osteogenic cells undergoing differentiation, their association with the *Runx2* promoter, and their ability to form DLX3/DLX5 heterodimers through protein-protein interactions are consistent with a model in which homeodomain protein combinations tightly regulate *Runx2* gene expression to support osteoblast differentiation (summarized in Fig. 6*F*).

DLX3 and DLX5 Promote Osteoblast Differentiation Independently of Runx2 Activation—DLX3 and DLX5 are clearly upstream regulators of *Runx2*, raising the question as to whether their osteogenic effects are solely dependent on RUNX2. To determine the potency of DLX3 and DLX5 in mediating BMP2 bone forming properties in the absence of RUNX2, we examined their ability to activate osteoblast-related genes in a *Runx2* null cell line. We immortalized calvarial cells from embryonic age E17.5 null mice (2, 62) (Fig. 7*A*). As a control for these experiments, the effect of DLX3 and DLX5 on *Runx2* mRNA was quantitated by RT-qPCR. In this study, cells were not treated with BMP2, and thus the induction of gene expression represents the Runx2 mRNA mediated by the HD proteins that were exogenously expressed. *Runx2* mRNA is induced 2-fold in this cell line (Fig. 7*B*), although no functional RUNX2 protein is translated (data not shown). *Runx2* null cells were transfected with either RUNX2, DLX3, or DLX5 and DLX3 or DLX5 in combination with RUNX2 (Fig. 7*C* shows plasmid expression levels). Expression of two osteoblast markers was quantitated in total cellular RNA by RT-qPCR (Fig. 7*C*). Alkaline phosphatase, an earlier marker of osteoblast differentiation, is up-regulated by DLX3 and DLX5 to the same extent as RUNX2. However, in the presence of exogenous RUNX2 and HD protein, a modest suppression of alkaline phosphatase expression mediated by DLX3 and DLX5 is observed. This finding is consistent with the known down-regulation of alkaline phosphatase in more mature osteoblasts. Osteocalcin (*OCN*), a marker of mature osteoblasts, was induced 5-fold by RUNX2, 3.8-fold by DLX3, and 4.5-fold by DLX5. We also observed an induction of collagen type 1α2 (*Col 1α2*) chain but not the *Col 1α1* subunit by DLX3 and DLX5 (supplemental Fig. 3). RUNX2 had no significant effect on expression of either *Col 1α1* or *Col 1α2* genes. The addition of RUNX2 had only a modest effect on the activation mediated by the HD proteins. These studies show that DLX3 and DLX5 can induce expression of osteogenic genes independently of RUNX2 and that both DLX proteins have the same potency as RUNX2. These findings of direct regulation of osteoblast genes provide mechanistic insight for earlier observations that BMP2 induced alkaline phosphatase and osteocalcin in primary calvarial cells from the *Cbfa1* (*Runx2*) null mouse (2). Thus the HD pathways contribute to control of osteoblast genes for bone formation independently of RUNX2 as well as through RUNX2 (Fig. 7*D*).

DISCUSSION

MSX and DLX homeodomain proteins have unique and coordinated roles in patterning and initiating formation of skeletal structures during embryogenesis prior to the onset of bone tissue formation. Here we have identified a molecular mechanism by which homeodomain proteins contribute to osteoblast differentiation and bone formation through regulation of the RUNX2 transcription factor, a requirement for *in vivo* bone formation. Our findings identify DLX3 for the first time as a significant regulator of *Runx2* gene expression at an early stage of osteoblast maturation. We further show that DLX3, as well as DLX5, is competent to promote expression of osteoblast genes in *Runx2* null cells, thus demonstrating that DLX3 and DLX5 can mediate osteoblast differentiation in a RUNX2-independent manner. Our combined results show (i) transcriptional regulation of *Runx2* by DLX3, MSX2, and DLX5 mediated through multiple responsive elements in the bone-specific P1 promoter, (ii) temporal expression patterns of HD proteins in cell models and in primary calvarial cells during differentiation, (iii) functional activities of HD proteins by overexpression and knockdown studies, and (iv) recruitment of HD proteins to the *Runx2* gene during osteoblast differentiation. These results together support our conclusion that DLX3, like DLX5 (37), is a key transactivator of *Runx2* gene expression. DLX3 appears to regulate *Runx2* at early stages of osteoblastogenesis, whereas DLX5 functions later.

We have previously shown that the knockdown of *Dlx3* results in a block of osteoblast maturation of MC3T3-E1 cells by inhibiting expression of HD-regulated phenotypic genes, including collagen type I, osteopontin, bone sialoprotein, and osteocalcin (40). Now that we have demonstrated that DLX3 up-regulates *Runx2* promoter activity in a segment of the promoter that lacks a BMP/SMAD response element, a linear pathway from BMP2 to DLX3 to *Runx2* is defined. Our finding that DLX3, as well as DLX5, can regulate *Runx2* and RUNX2 target

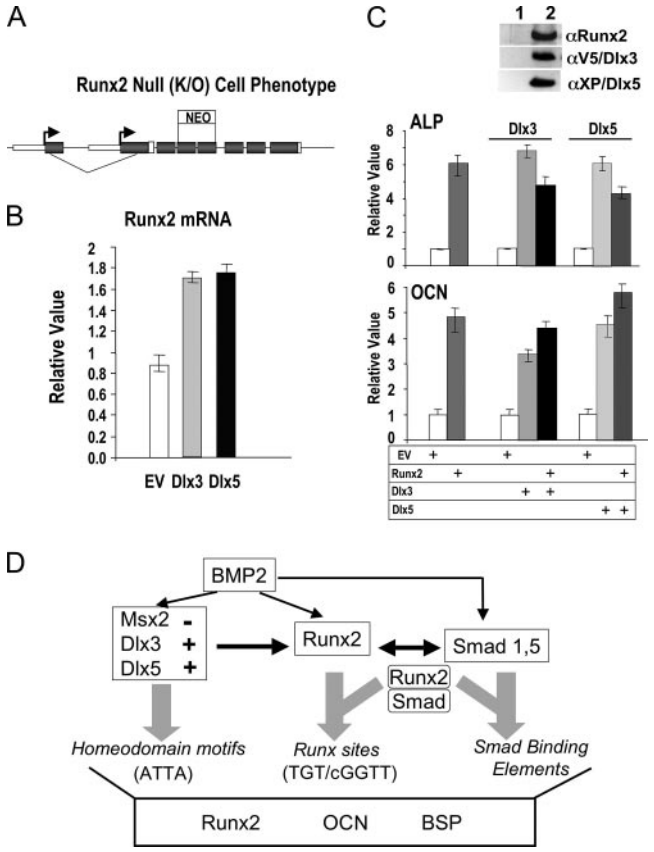

FIGURE 7. **Evidence for DLX3 and DLX5 promoting osteogenic differentiation independent of Runx2.** *Runx2* immortalized null cells were transfected with 200 μg of either RUNX2, DLX3, DLX5, or both RUNX2 and one of the HD expression vectors. Cells were collected after 24 h for total cellular RNA and protein isolation. The expression of osteogenic marker genes was quantitated by qPCR. *A*, schematic of the targeted *Runx2* gene in null cells. *B*, induction of *Runx2* mRNA by DLX3 and DLX5 in the *Runx2* null cells, although no protein is translated by Western blot analyses (data not shown). *C*, *upper panel*, Western analyses of RUNX2 and HD protein expression levels in cell lysates 24 h after transfection. *Lower panel*, activation of alkaline phosphatase (*ALP*) and induction of osteocalcin (*OCN*) by RUNX2, DLX3, and DLX5 by reconstituting the *Runx2* null cells with Runx2. *D*, summary of BMP2 regulatory network for regulation of osteogenesis. As an early event of the BMP2 osteogenic induction pathway, Smad signaling and HD proteins are activated; this will induce *Runx2* expression, and these factors can regulate target genes. There is evidence that formation of RUNX2-SMAD complexes is a critical mediator of osteogenesis. In the absence of RUNX2, DLX3 and DLX5 can contribute to osteogenesis through regulation of osteoblast-related genes (5, 6, 61). *K/O*, knock-out; *EV*, empty vector; *OC*, osteocalcin; *BSP*, bone sialoprotein; *NEO*, neomycin.

genes in the absence of RUNX2 provides additional evidence for a regulatory pathway from BMP2 to commitment to osteogenesis via *Dlx3* gene regulation. The present studies provide evidence that a mechanism contributing to the roles of both DLX3 and DLX5 in bone formation is through activation of *Runx2*. However, DLX3 and DLX5 regulate *Runx2* at different stages of osteogenic differentiation. The contribution of both DLX3 and DLX5 proteins to the regulation of osteoblastogenesis via control of *Runx2* is consistent with *in vivo* studies of *Dlx5* null mice (59) in which normal expression levels of *Runx2* are observed possibly due to compensation by DLX3. *Runx2* and *Dlx3* exhibit similar profiles of expression (both mRNA and protein) during early differentiation of BMP2-treated C2C12 cells (1–6 h), in mouse MC3T3 cells, and in primary rat calvarial osteoblasts. In contrast, *Dlx5* is induced after the osteogenic commitment point (at 8 h) in C2C12 cells. In primary calvarial osteoblasts robust *Dlx3* expression also precedes *Dlx5* during differentiation. Thus, DLX3 appears to have a very specialized transient role in activating osteogenic genes at early stages of differentiation.

The significance of transcriptional regulation of *Runx2* by multiple HD response elements that bind both the repressor and activator HD proteins is underscored by two complementary observations: 1) the temporal expression profiles in response to BMP2 and during osteoblast differentiation and 2) the temporal recruitment and displacement of HD proteins on the *Runx2* promoter related to stages of osteoblast maturation. RUNX2 cellular levels need to be precisely regulated with expression supporting lineage direction in osteoprogenitor cells (60) and up-regulated levels promoting differentiation (12, 15). There appears to be a requirement for multiple HD sequences to regulate both the repressor and enhancer activities of different classes of HD proteins that recognize the core consensus 5′-TAAT motif (this study and Ref. 37). The roles of the repressor MSX2 and enhancer DLX proteins in postnatal bone formation and osteoblast differentiation involve several levels of transcriptional control. Affinities for protein-DNA binding in the context of promoter sequences, cellular levels of the proteins, and formation of HD heterodimeric complexes all contribute to positive and negative regulation of *Runx2* that supports fine tuning of gene expression for physiological control of osteoblast differentiation. We have shown here that DLX3 interacts with DLX5 in addition to the HD protein-RUNX2 interactions reported previously (40, 58, 61). Thus formation of these protein-protein complexes may influence transcription by either preventing DNA binding or modifying activity of a DNA-bound complex.

Our chromatin immunoprecipitation studies reveal a temporal recruitment of MSX2 and the DLX3/DLX5 proteins to the *Runx2* gene during differentiation of primary osteogenic cells. We find exclusive association of MSX2 with the *Runx2* gene in the proliferation period when *Runx2* expression is minimally detected. These results are consistent with strong repression of *Runx2* promoter activity by MSX2 (our results) and with MSX2 tissue distribution (24, 37). MSX2 may function to prime the promoter for transcription by interaction with the phosphorylated POL II-associated transcription factor IIF (TF11F). Towler and co-workers (58) demonstrated that MSX2-mediated repression of the osteocalcin gene promoter can be overcome more than 50% by overexpression of TFIIF in mouse osteoblasts. A molecular switch occurs postproliferatively with loss of MSX2 and appearance of DLX3, DLX5, and *Runx2* at the promoter with increased Pol II. However, DLX3 association with the *Runx2* promoter is transient, whereas DLX5 remains bound to *Runx2* in mature osteoblasts (day 20, mineralized matrix). The increased occupancy of RUNX2 to its own promoter from day 12 to day 20 and the decreased total cellular RUNX2 protein on day 20 are consistent with RUNX2 recruitment to its own promoter for autoregulation (45). Although RUNX2 autoregulates its transcription on day 20, the simultaneous increased association of DLX5 further supports the role of DLX5 as an activator at later stages of differentiation. This finding is consistent with *in vitro* studies (37). Thus, the HD

network along with *Runx2* promoter autoregulation assures optimal physiologic regulation of RUNX2 expression in mature osteoblasts.

In conclusion, our findings suggest a very specific mechanism for regulating *Runx2* transcription by switching from MSX2 occupancy to DLX3 and then DLX5 occupancy of the *Runx2* promoter. We first observed this molecular switch on the osteocalcin gene promoter, which it was mediated through only the HD regulatory element, the tissue-specific osteocalcin box (40). Our findings of the temporal recruitment of the HD proteins to regulate *Runx2* transcription during osteoblast differentiation reveal a regulatory network of HD protein gene regulation operative on the *Runx2* promoter. Equally important, our results demonstrate that both DLX3 and DLX5 contribute to RUNX2-dependent and -independent regulation of osteogenic genes. These findings establish a novel BMP2 response pathway in which homeodomain proteins act both upstream of and parallel to RUNX2 to promote osteogenesis with further convergence of the BMP2 osteogenic signal through RUNX2-SMAD interactions (5, 6) and RUNX2-homeodomain interactions (40, 57, 58, 61).

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