Reciprocal Regulation of Osteocalcin Transcription by the Homeodomain Proteins Msx2 and Dlx5[†]

Elizabeth P. Newberry, Tammy Latifi, and Dwight A. Towler*

Departments of Medicine and Molecular Biology and Pharmacology, Center for Cardiovascular Research, Division of Endocrinology, Diabetes, and Metabolism, Washington University School of Medicine, St. Louis, Missouri 63110

Received August 5, 1998; Revised Manuscript Received September 21, 1998

ABSTRACT: Osteocalcin (OC) is a small calcium binding protein expressed in bones and teeth undergoing mineralization. OC expression in calvarial osteoblasts and odontoblasts is regulated in part via protein−protein interactions between the homeodomain repressor, Msx2, and components of the cell type-specific basal OC promoter. Recent work suggests that homeodomain proteins form heterodimers that confer transcriptional regulation. Since the homeodomain proteins Dlx5 and Msx2 are both expressed by primary rat calvarial osteoblasts, we examined whether Msx2 and Dlx5 functionally interact to regulate the OC promoter. In both primary rat calvarial and MC3T3E1 mouse calvarial osteoblasts, transient expression of Dlx5 only mildly augments basal OC promoter (luciferase reporter) activity, while Msx2 suppresses transcriptional activity by ca. 80%. However, Dlx5 completely reverses Msx2 repression of the OC promoter. Structure−function analyses using far-Western blot and transient cotransfection assays reveal that (i) Msx2 and Dlx5 can form dimers, (ii) Dlx5 residues 127−143 are necessary for dimerization and to reverse Msx2-dependent OC repression, and (iii) intrinsic DNA binding activity of Dlx5 is not required for OC regulation. Msx2 inhibits the DNA binding activity of a third complex, the OC fibroblast growth factor response element binding protein (OCFREB), that supports activity of the basal OC promoter. Dlx5 completely abrogates Msx2 suppression of OCFREB DNA binding activity, and residues required for Dlx5 transcriptional de-repression in vivo are also required for reversing inhibition of OCFREB binding in vitro. Finally, Dlx5 reverses Msx2 inhibition of OC promoter activation by FGF2/forskolin. Thus, Dlx5 regulates the expression of the OC promoter in calvarial osteoblasts in part by de-repression, antagonizing Msx2 repression of transcription factors that support basal OC promoter activity.

Patterning of the mammalian skull is a complex and dynamic process (*1*−*4*). To allow for postnatal growth of the cranium, the calvarial sutures of the skull must remain patent until maturity; therefore, the timing of osteoblast differentiation and mineralization during calvarial morphogenesis is tightly regulated (*1*). Recently, genetic analyses have demonstrated that point mutations in genes encoding Msx2 (*3*) and FGFR-2[1] (*4*, *5*) give rise to craniosynostosis, a developmental disorder characterized by precocious differentiation and mineralization of osteoblasts in the calvarial suture (*1*). In Apert's syndrome, patients possess a mutant FGFR-2 that is constitutively active (*4*, *5*); this results in accelerated calvarial osteoblast maturation, characterized in part by an elevated level of expression of the osteoblast-specific gene, osteocalcin (*5*). The net effects of FGF on osteoblast differentiation are complex and stage-specific; FGF recruits osteoprogenitors from bone marrow stromal cell populations (*6*) but inhibits terminal differentiation of cultured calvarial osteoblasts (*7*, *8*). Moreover, FGF can also directly upregulate osteocalcin (*9*) expression while concomitantly suppressing the expression of other characteristic differentiation markers such as high-level type I collagen (*7*−*9*).

Osteocalcin (OC) is a small, ∼6 kDa γ-carboxylated calcium binding protein that regulates bone formation (*10*) and mineralization (*11*). Cell type-specific expression of this osteoblast matrix protein is regulated at the transcriptional level via protein−DNA and protein−protein interactions assembled in the ∼0.2 kb region immediately upstream of the transcription initiation site (*10*, *12*, *13*). Within this region, two unique protein−DNA interactions have been identified that control basal and FGF-dependent gene expression. Approximately 0.13 kb upstream from the initiation site, the Runt domain transcription factor Osf2/Cbfa1 recognizes and regulates osteoblast-specific gene expression via a CA-rich motif called the NMP2/OSE2 element (*13*, *14*). A second, bipartite element conveys synergistic transcrip-

[†] This work was supported by NIH Grants AR43731 and DK52446 to D.A.T. and the Charles E. Culpeper Foundation. D.A.T. is a Charles E. Culpeper Medical Science Scholar.

* Please address all correspondence to Dwight A. Towler, M.D., Ph.D., Department of Molecular Biology and Pharmacology, Washington University School of Medicine, Box 8103, 660 South Euclid, St. Louis, MO 63110. Phone: (314) 362-9925. Fax: (314) 362-7058. E-mail: dtowler@pharmdec.wustl.edu.

[1] Abbreviations: CMV, cytomegalovirus; FGFR, fibroblast growth factor receptor; FGF2, basic FGF; FSK, forskolin; GST, glutathione *S*-transferase; LUC, luciferase; MEM, modified Eagle's media; OC, osteocalcin; OCFREB, osteocalcin FGF response element binding protein; OCFRE, osteocalcin FGF response element; Osf2/Cbfa1, osteoblast-specific factor 2/core binding factor A1; PCR, polymerase chain reaction; PVDF, poly(vinylidene difluoride); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBP, TATA box binding protein; kb, kilobase(s); TAF, TBP-associated factor; VDRE, vitamin D response element.

10.1021/bi981878u CCC: $15.00
Published on Web 10/29/1998

tional activation in response to FGF2 and forskolin, with regulatory protein−DNA interactions centered at −0.09 and −0.14 kb relative to the start site of transcription (*9*). A FGF2-regulated nucleoprotein complex, called OCFREB, binds to the GCAGTCA motif (OCFRE) at −0.14 kb that mediates FGF2-dependent transcriptional activation (*9*, *15*). The constituents that convey FGF2 activation of the OC promoter have not been cloned; as purified from MG63 osteosarcoma cells, OCFREB is a heterodimeric DNA binding complex of 70 and 80 kDa components (*16*).

A second tier of regulatory protein−protein interactions has now emerged that controls stage-specific OC expression during terminal differentiation of calvarial osteoblasts and odontoblasts. The homeodomain protein Msx2 suppresses the basal OC promoter in part via protein−protein interactions with TFIIF (*17*) and by selectively inhibiting OCFREB DNA binding activity (*16*). Although a HOXBOX homeodomain binding cognate exists within the basal OC promoter (*12*, *18*), intrinsic Msx2 DNA binding activity is not required for suppressor function (*17*). Consistent with its role as a transcription repressor, stable overexpression of exogenous Msx2 in calvarial osteoblasts downregulates endogenous OC expression (*19*), and Msx2 and OC are reciprocally expressed during calvarial osteoblast (*20*) and odontoblast (*19*) differentiation.

Recently, Abate-Shen and co-workers concluded that Msx and Dlx family members can form heterodimeric complexes and that Msx1 can inhibit transcriptional activation by Dlx5 in synthetic promoter contexts (*21*). Most importantly, they concluded that Dlx5 antagonizes suppression of the MyoD enhancer by Msx1, an important regulatory element activated during skeletal muscle differentiation. Concomitantly, Ryoo et al. (*20*) concluded that Dlx5 is expressed in calvarial osteoblasts undergoing terminal differentiation, paralleling the expession of OC. Northern blot analysis of RNA isolated from multiple tissues revealed that Dlx5 expression is readily detected in calvariae and long bone, but not in other tissues, including cardiac and skeletal muscle (*20*). While Dlx5 and Msx2 were shown to repress expression of OC in ROS17/2.8 osteosarcoma cells, functional relationships between Msx2, Dlx5, and transcriptional regulation in the background of calvarial osteoblasts were not described in detail.

We have examined the regulation of the rat OC promoter by Msx2 and Dlx5 in the calvarial osteoblast cell background as a physiologically relevant model for studying Dlx5 function and structure−function relationships. In transiently transfected cultures of primary rat calvarial osteoblasts, Msx2 suppresses OC promoter activity (luciferase reporter); by contrast, Dlx5 completely reverses Msx2-dependent OC promoter repression, and only mildly augments promoter activity in the absence of exogenous Msx2. Virtually identical results are obtained in transient transfection studies carried out with MC3T3E1 murine calvarial osteoblasts, a clonal cell line that closely mimics the phenotypic characteristics of primary rat calvarial osteoblasts in culture (*22*). Structure−function analyses of Dlx5 reveal that the C-terminal domain of the molecule is not required for OC de-repression. However, the Dlx5 homeodomain N-terminal extension is necessary both for dimeric protein−protein interactions with Msx2 and for transcriptional de-repression. Like Msx2 repressor function, Dlx5 activity as a "de-repressor" does not correlate with intrinsic DNA binding activity. Dlx5 reverses Msx2-dependent inhibition of OC-FREB DNA binding, and concomitantly enhances the OC promoter response to FGF2/FSK. In toto, these data demonstrate that OC expression is reciprocally regulated by the homeodomain proteins Msx2 and Dlx5. In calvarial osteoblasts, Dlx5 acts as a transcriptional de-repressor of the OC promoter, antagonizing Msx2-dependent repression of basal promoter activity and inhibition of protein−DNA interactions at the OCFRE.

EXPERIMENTAL PROCEDURES

Reagents. All tissue culture reagents and conditions have been previously described in detail (*9*, *17*). Routine molecular biology reagents were purchased from Promega (Madison, WI) and Fisher Scientific (St. Louis, MO). Advantage Taq polymerase was purchased from Clontech (Palo Alto, CA). Custom synthetic oligodeoxynucleotides were obtained from the Washington University Protein and Nucleic Acid Core Laboratory or from Life Technologies (Gaithersburg, MD). All Western blot reagents and conditions have been previously described in detail (*17*). Anti- FLAG epitope antibody M2 was purchased from VWR Scientific (St. Louis, MO). Anti-T7 epitope antibodies were purchased from Novagen (Madison, WI). Proteins were assessed using the bicinchoninic acid protein assay kit purchased from Pierce (Rockford, IL).

Culture of Primary Rat Calvarial Cells and MC3T3E1 Mouse Calvarial Osteoblasts. All calvarial osteoblast cell cultures were maintained in α-MEM supplemented with 10% fetal calf serum, 2.5 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Fraction 3 primary rat calvarial osteoblastic cells were isolated by timed proteolytic digestion of E20 fetal rat calvaria as previously described in detail (*23*); cells from passage 3 to 7 were used for transfection experiments. MC3T3E1 mouse calvarial cells (*22*, *24*) were maintained as previously described (*9*); cells at passage 10−20 from our frozen stocks were used for transfection experiments.

Dlx5 and Msx2 Eukaryotic Expression Constructs and 0.2 kb OCLUC Reporter Constructs. The pcDNA3-Msx2 expression constructs used in this study have been previously described in detail (*17*). A full-length Dlx5 cDNA clone was obtained by reverse transcription-PCR from mouse E11 whole embryo Quick Clone cDNA (Clontech) using the following amplimers: 5′-GGG GAA TTC ATG ACA GGA GTG TTT GAC AG-3′ and 5′-GGG GAA TTC CTA ATA AAG CGT C-3′. The Dlx5 cDNA was subcloned and sequenced to verify the insert identity. Subsequently, the eight-amino acid FLAG epitope DYKDDDDK was introduced by PCR immediately following the Dlx5 initiator methionine residue to permit monitoring of protein accumulation by Western blot analysis. The epitope-tagged Dlx5 variants were subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) for eukaryotic expression from the CMV promoter and for coupled in vitro transcription and translation from the T7 promoter (*17*). The Dlx5(2−203; Δ127−143) variant lacking residues 127−143 was generated by ligation of PCR products encoding the upstream (Met-FLAG to residue 126) and downstream (residues 144−289) halves of Dlx5, with deleted residues replaced by the Glu-Phe residues

encoded by the *Eco*RI linker restriction site. The pcDNA3-Dlx5(T142A) point mutant was produced by insertion of a cassette spanning residues 129−145 that contains an A to G transition that converts Thr-142 to Ala. A variant encoding the native Dlx5 cassette was generated as the appropriate control for the T142A mutant cassette to verify that the linker-induced alterations (viz., V127E, R128F, and S146E) do not affect Dlx5 function. The 0.2 kb OCLUC construct contains nucleotides −222 to +32 of the rat OC promoter cloned upstream of the luciferase reporter of pGL2 Basic (Promega), and has been previously described (*9*, *12*). The 0.2 kb MUTHOX construct containing the CCAATTAGT to CCAATGGTA mutation in the OC HOXBOX homeodomain binding cognate (*12*) was generated in the context of the −222 to +32 OC promoter fragment by PCR, and subcloned into the promoterless pGL2 Basic luciferase vector (Promega). All plasmids were purified by Qiagen column chromatography (Chatsworth, CA) for transfection experiments. All expression plasmids were sequenced (ABI Prism Dye Terminator Kit, Perkin-Elmer, Foster City, CA) to verify the insert identity. Relevant GenBank accession numbers are U67840 (Dlx5), S60698 (Msx2), and J04500 (OC).

Cellular Transfection, Luciferase Assays, and β-Galactosidase Assays. Calvarial osteoblasts were plated in Costar six-well cluster dishes (35 mm diameter wells, 7×10^5 cells/well). Cells were transiently transfected the following day precisely as previously described (*17*). All transfections incorporate a 0.2 kb OCLUC construct (to monitor OC transcription), a CMV-β-galactosidase construct (to control for transfection efficiency), and varying amounts of pcDNA3-Msx2 and pcDNA3-Dlx5 expression constructs as indicated. The empty pcDNA3 expression plasmid was added to maintain a constant amount of DNA in each precipitation and transfection mixture. Two days following transfection, cultures were re-fed with fresh media containing 10 μM forskolin and analyzed the following day for luciferase and β-galactosidase activities as previously described (*17*). For studies of OC promoter regulation by FGF2, 3 nM FGF2 with 10 μM forskolin was used to stimulate OC promoter activity as previously described (*9*). FLAG-tagged Dlx5 protein was extracted from transfected cell nuclei with high salt concentrations and expression monitored by Western blot analysis as previously detailed (*17*).

Expression of Recombinant Proteins in Bacteria. Recombinant Msx2, Dlx5, and TBP were expressed in bacteria using the pET23a expression vector (Novagen) to introduce a N-terminal T7 epitope tag and a C-terminal hexahistidine tag for monitoring expression and purification, respectively. The pET23a-TBP construct has been previously described (*17*). Dlx5 cDNA was subcloned into the *Bam*HI and *Hin*dIII sites of pET23a using 5′-GAG GGA TCC ACA GGA GTG TTT GAC AG-3′ and 5′-GAG GAA AGC TTA TAA AGC GTC CCG GAG-3′ as PCR amplimers to introduce linkers. Dlx5(2−203; Δ127−143) was made using a similar PCR strategy as described above to introduce the internal deletion. Msx2 cDNA was subcloned into the *Eco*RI and *Hin*dIII sites of pET23a using 5′-AAA GAA TTC GCT TCT CCG ACT AAA GGC GGT GAC-3′ and 5′-GGG GAA GCT TGG ATA GAT GGT AC-3′ as PCR amplimers to introduce linkers. Following verification of the sequence identity, recombinant proteins were expressed and purified using nickel NTA agarose (Qiagen) chromatography as previously described (*17*) and exhaustively dialyzed against gel shift buffer D (*25*) prior to use in gel shift assays. Protein expression was analyzed by Western blot analysis using alkaline phosphatase-conjugated anti-T7 epitope antibody (1:10000 dilution).

Far-Western Interaction Blot Assays. Far-Western blot assays used to assess protein−protein interactions were performed using the vector and methods described by Blanar and Rutter (*26*) as previously described in detail (*17*). The construction of GST−Msx2(55−208) has been previously described (*17*). GST−Dlx5(46−203) was generated by subcloning the core domain of Dlx5 into the *Eco*RI site of pGEX-2TK (*26*) using PCR to introduce in-frame linkers. GST−Dlx5 (46−203) was expressed, purified, and radiolabeled by phosphorylation with protein kinase A and [γ-^{32}P]-ATP as previously described in detail (*17*, *26*) to generate radiolabeled probe. Recombinant proteins used for interaction blot assays were expressed in pET23a and then purified by immunoprecipitation with anti-T7 antibody [0.5 μg of anti-T7 antibody and 25 μL of protein G agarose (Santa Cruz Biotech, Santa Cruz, CA) per 100 μg of bacterial protein extract]. After SDS−PAGE, immunopurified recombinant proteins were electrotransferred to nitrocellulose, renatured from guanidine hydrochloride, and blotted with radiolabeled GST−Msx2(55−208), GST−Dlx5(46−203), or GST precisely as previously described (*17*). Recovery of recombinant protein was verified in separate aliquots by Western blot analysis using alkaline phosphatase-conjugated anti-T7 epitope antibody (vide supra).

Electrophoretic Mobility Gel Shift Assays. Crude nuclear extracts containing OCFREB DNA binding activity were prepared from MC3T3E1 cells treated with 3 nM FGF2 and 10 μM forskolin precisely as previously described (*9*). Msx2-mediated inhibition assays were carried out as previously described in detail (*16*) using 3 μg of crude nuclear extract per binding reaction and the duplex rat OCFRE cognate GGCAGCTGCAGTCACCGGC (OCFRE underlined; *9*, *15*). For OCFREB inhibition and reversal studies, crude nuclear extracts were incubated for 25 min at 20 °C with recombinant Msx2 and Dlx5 as indicated, followed by addition of radiolabeled OCFRE duplex oligo to a standard gel shift binding reaction mixture (*12*). Protein−DNA complexes were identified by native gel electrophoresis and autoradiography precisely as previously described (*9*, *12*, *16*, *17*). The DNA binding activities of Dlx5 and Dlx5(T142A) were assessed in gel shift assays using the high-affinity OCTA26 (*12*, *17*) HOXBOX cogate; Dlx5 proteins for these assays were obtained by coupled in vitro transcription and translation as previously described in detail (*17*).

RESULTS

Dlx5 Antagonizes Msx2 Inhibition of the OC Promoter in Primary Rat and MC3T3E1 Calvarial Osteoblasts. As mentioned above, Msx2, Dlx5, and OC are expressed in distinct temporal patterns in differentiating primary rat calvarial osteoblasts (*20*). To determine functional relationships between Msx2 and Dlx5, we first examined the effect of Dlx5 and Msx2 expression on the activity of the 0.2 kb rat OC promoter in primary rat calvarial osteoblasts. As shown in Figure 1A, expression of mouse Dlx5 has no effect

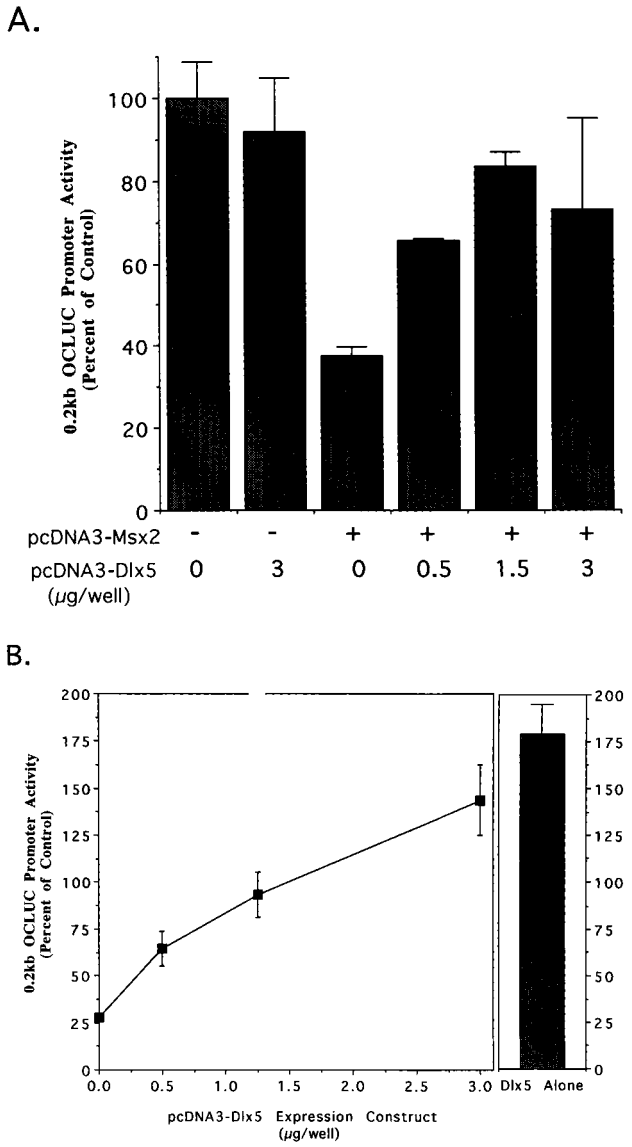

FIGURE 1: Dlx5 reverses Msx2-mediated OC promoter suppression in primary rat calvarial cells and MC3T3E1 calvarial osteoblasts. Calvarial osteoblastic cell cultures were transfected with 0.2 kb OCLUC (1.5 μg/well), pcDNA3-Msx2 (0.3 μg/well), and increasing amounts of pcDNA3-Dlx5 as indicated and detailed in Experimental Procedures. CMV-β-galactosidase was included as an internal control for transfection efficiency. Data are presented as the mean ($\pm$SD) luciferase activity observed in three independent transfections, expressed as a percentage of basal 0.2 kb OCLUC activity in the absence of either Msx2 or Dlx5: (A) results from primary rat calvarial cell cultures and (B) results from MC3T3E1 calvarial osteoblast cultures. Note that while Msx2 suppresses OC promoter activity 70−80%, Dlx5 reverses Msx2-dependent OC promoter suppression in both calvarial cell types. Further note that Dlx5 alone modestly augments basal OC promoter activity in MC3T3E1 cells, and has a minimal effect alone in primary rat calvarial osteoblastic cells. See the text for details.

on basal OC promoter activity, while Msx2 represses basal activity by ca. 70%. However, coexpression of Dlx5 with Msx2 reverses Msx2-induced OC promoter suppression in a dose-dependent fashion. These data suggest that in calvarial osteoblasts, Dlx5 does not function as a repressor as observed in ROS17/2.8 osteosarcoma cells (*20*). To further confirm this notion, we examined the effects of Msx2 and Dlx5 on OC promoter activity in MC3T3E1 cells, a murine calvarial osteoblast cell line that closely mimics the phenotype of primary calvarial osteoblasts in culture. As observed in primary rat calvarial cells, expression of Dlx5 in MC3T3E1 calvarial osteoblasts antagonizes Msx2-induced suppression of the OC promoter in a dose-dependent fashion, while Dlx5 alone mildly augments basal promoter activity (Figure 1B). Thus, in the neural crest-derived calvarial osteoblast, Dlx5 upregulates the OC promoter by acting as a de-repressor, antagonizing Msx2-mediated transcriptional repression.

De-Repression of the OC Promoter by Dlx5 Is Independent of Intrinsic Dlx5 DNA Binding Activity. Our previous analyses of Msx2 function demonstrated that intrinsic DNA binding activity of the homeodomain is not required for Msx2 suppressor function (*17*). Consistent with this, the HOXBOX cognate of the OC promoter is not required for Msx2 suppression; intriguingly, OC promoter de-repression by Dlx5 is also independent of the HOXBOX, suggesting that intrinsic DNA binding activity of Dlx5 is not required for this function (Figure 2A). To test this notion directly, we introduced an Ala for Thr substitution at residue 142 within the Dlx5 homeodomain; as observed in other homeodomain proteins (*17*, *21*), this particular mutation markedly decreases the extent of DNA binding of the Dlx5 homeodomain as assessed by gel shift assay [Figure 2B; compare Dlx5-(T142A) with wild type Dlx5]. However, although the Dlx5-(T142A) mutant exhibits a markedly decreased level of DNA binding (Figure 2B, lanes 6−9), it is as active as wild type Dlx5 in its capacity to reverse Msx2-dependent transcriptional repression (Figure 2C). Thus, the intrinsic DNA binding activity of Dlx5 does not direct de-repression of the OC promoter.

Dlx5 Participates in Protein−Protein Interactions with Msx2 in Vitro, Which Depend upon Dlx5 Residues 127−143 That Encompass the N-Terminal Homeodomain Arm and Extension. Since DNA binding is not required for Dlx5-mediated antagonism of Msx2 repression, we postulated that Dlx5 functions via protein−protein interactions in a manner similar to that of Msx2 (*16*, *17*). We wished to assess whether Dlx5 and Msx2 could interact, as suggested by Abate-Shen (*21*) and our unpublished data demonstrating that Msx2 can form homodimers. Therefore, we examined whether radiolabeled GST−Dlx5 can interact with recombinant purified Msx2 in far-Western interaction blot assays (*17*, *26*). Recombinant purified Dlx5 and TBP were also included as positive and negative controls, respectively. As shown in Figure 3A (lanes 1−3), approximately equivalent amounts of recombinant purified Msx2, Dlx5, and TBP were transferred to nitrocellulose membranes after SDS−PAGE. Radiolabeled GST−Dlx5(46−203) encompassing the Dlx5 core domain binds to both renatured Msx2 and Dlx5 (Figure 3A, lanes 4 and 5) but does not bind TBP (lane 6). Similar results are obtained when full-length GST−Dlx5 is used as a probe (data not shown). By contrast, radiolabeled GST does not bind to any of these three proteins (Figure 3A, lanes 7−9). Thus, Dlx5 can participate in dimeric protein−protein interactions with Msx2 and Dlx5, but not with TBP, in far-Western interaction blot assays.

We previously determined that 17 amino acids encompassing the homeodomain N-terminal arm and extension of Msx2 direct protein−protein interactions (*17*); alignment of the Msx2 and Dlx5 homeodomains reveals significant sequence identity within the N-terminal arm that diverges in the N-terminal extension (*27*). We reasoned that similar

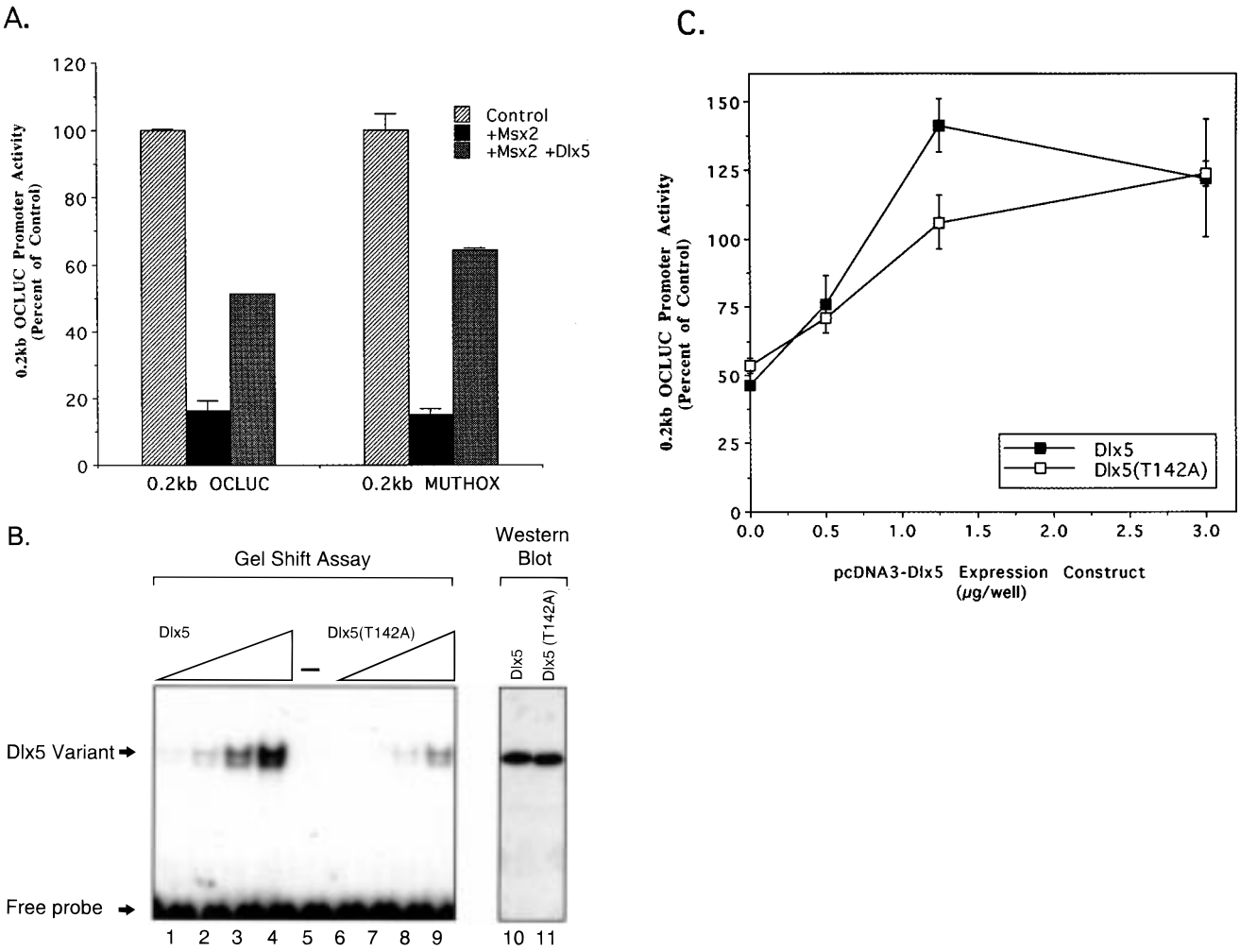

FIGURE 2: Dlx5 does not require the HOXBOX cognate or intrinsic DNA binding activity to antagonize Msx2-mediated OC promoter suppression. (A) MC3T3E1 calvarial osteoblasts were transfected as described in the legend of Figure 1 with pcDNA3-Msx2 (0.5 μg/well), or in combination with pcDNA3-Dlx5 (2 μg/well), using either 0.2 kb OCLUC or 0.2 kb MUTHOX OCLUC (lacking the intact HOXBOX cognate; *12*) as reporters (1.5 μg/well). Data are presented as the mean ($\pm$SD) luciferase activity observed in three independent transfections, normalized to basal activity in the absence of either Msx2 or Dlx5. Basal 0.2 kb MUTHOX activity is ca. 30% of that observed with 0.2 kb OCLUC (*12*). Note that the ability of Dlx5 to reverse Msx2-mediated OC promoter suppression is independent of the presence of the HOXBOX cognate. (B) FLAG-tagged Dlx5 and Dlx5(T142A) proteins were produced by in vitro transcription and translation and binding activities assessed by gel shift assays (lanes 1−9) as outlined in Experimental Procedures. Aliquots were analyzed by Western blot analyses (lanes 10 and 11) with the anti-FLAG epitope M2 antibody to confirm equivalent levels of protein expression. Increasing amounts of Dlx5 (lanes 1−4) and Dlx5(T142A) (lanes 6−9) were assessed for binding activity using the OCTA26 HOXBOX cognate. Note that Dlx5-(T142A) exhibits ~10% of the DNA binding activity of Dlx5, yet equivalent amounts of protein are present in each gel shift assay. (C) The ability of Dlx5(T142A) to reverse OC promoter suppression in MC3T3E1 calvarial osteoblasts was assessed as outlined in the legend of Figure 1, and directly compared with the activity of wild type Dlx5. Data represent the mean $\pm$ range of independent duplicate transfections. Note that although Dlx5(T142A) exhibits markedly decreased DNA binding activity (Figure 2B), it readily reverses Msx2-dependent OC promoter repression (Figure 2C).

regions of Msx2 and Dlx5 homeodomains may be necessary for protein−protein interactions. To test this directly, we compared the interaction of radiolabeled GST−Msx2 with recombinant purified Dlx5(2−203) and the variant Dlx5-(2−203; Δ127−143) lacking the homeodomain N-terminal arm and extension. As shown in Figure 3B (lanes 1 and 2), equivalent amounts of recombinant purified Dlx5(2−203) and Dlx5(2−203; Δ127−143) were transferred to nitrocellulose membranes after SDS−PAGE. Radiolabeled GST−Msx2(55−208), encompassing the Msx2 core suppressor domain (*17*), binds to Dlx5(2−203) (Figure 3B, lane 3) but does not bind to Dlx5(2−203; Δ127−143) (Figure 3B, lane 4). By contrast, radiolabeled GST does not bind to either Dlx5(2−203) or Dlx5(2−203; Δ127−143) (Figure 3B, lanes 5 and 6). Thus, dimeric interactions between Msx2 and Dlx5 require Dlx5 residues 127−143, encompassing the Dlx5 homeodomain N-terminal arm and homeodomain extension.

De-Repression of the OC Promoter by Dlx5 Is Dependent upon Dlx5 Residues 127−143. To assess whether the residues that are required for interaction with Msx2 in vitro are required for Dlx5 function in vivo, Dlx5(2−203) and Dlx5(2−203; Δ127−143) were assayed for their ability to antagonize Msx2-induced suppression of the OC promoter. As shown in Figure 4A, both full-length Dlx5 (residues 2−289) and Dlx5(2−203) reverse Msx2-dependent OC promoter suppression in a dose-dependent fashion. By contrast, Dlx5(2−203; Δ127−143) does not reverse suppression by Msx2. Western blot analysis of these cell extracts demonstrates that the inability of Dlx5(2−203; Δ127−143) to antagonize Msx2-dependent repression is not

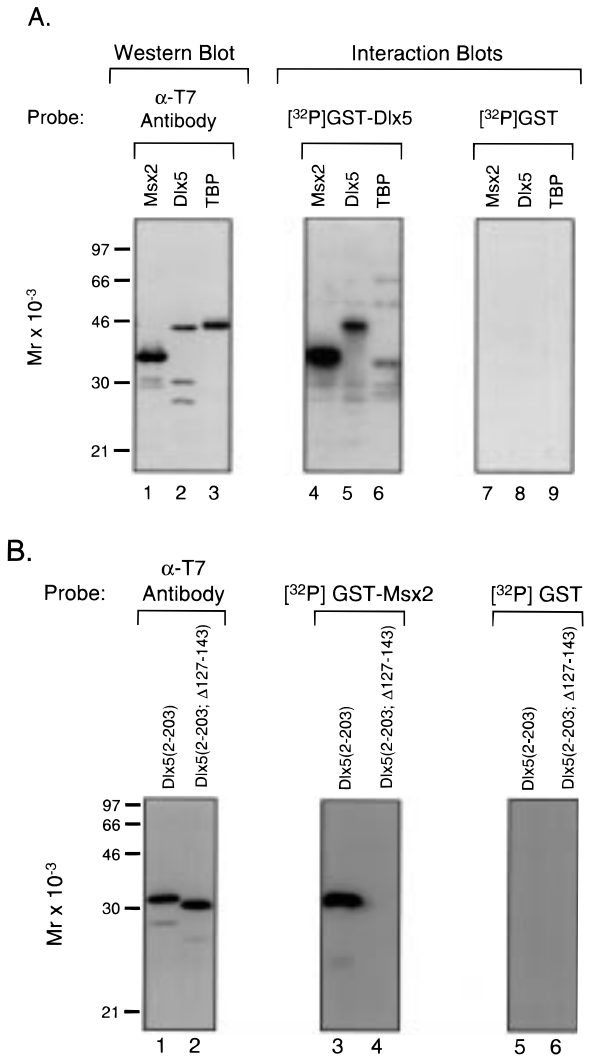

FIGURE 3: Dlx5 binds Msx2 in vitro, dependent upon Dlx5 residues 127−143 that encompass the homeodomain N-terminal arm and extension. T7 epitope-tagged recombinant Msx2, Dlx5, TBP, Dlx5(2−203), and Dlx5(2−203; Δ127−143) were expressed and purified as described in Experimental Procedures. Protein expression was monitored by Western blot for the T7 epitope tag. Protein−protein interactions were assessed by the far-Western interaction blot assay of Blanar and Rutter (*26*) carried out as previously described (*17*). (A) Although equivalent amounts of Msx2, Dlx5, and TBP protein are present (lanes 1−3), radiolabeled GST−Dlx5 binds only to Msx2 and Dlx5 (lanes 4 and 5) but not to TBP (lane 6). By contrast, radiolabeled GST does not bind to any of these proteins (lanes 7−9). (B) Although equivalent amounts of Dlx5(2−203) and Dlx5(2−203; Δ127−143) are present (lanes 1 and 2), radiolabeled GST−Msx2 binds only to Dlx5(2−203) (lane 3) but not to Dlx5(2−203; Δ127−143) (lane 4). Radiolabeled GST does not bind to either of these proteins (lanes 5 and 6).

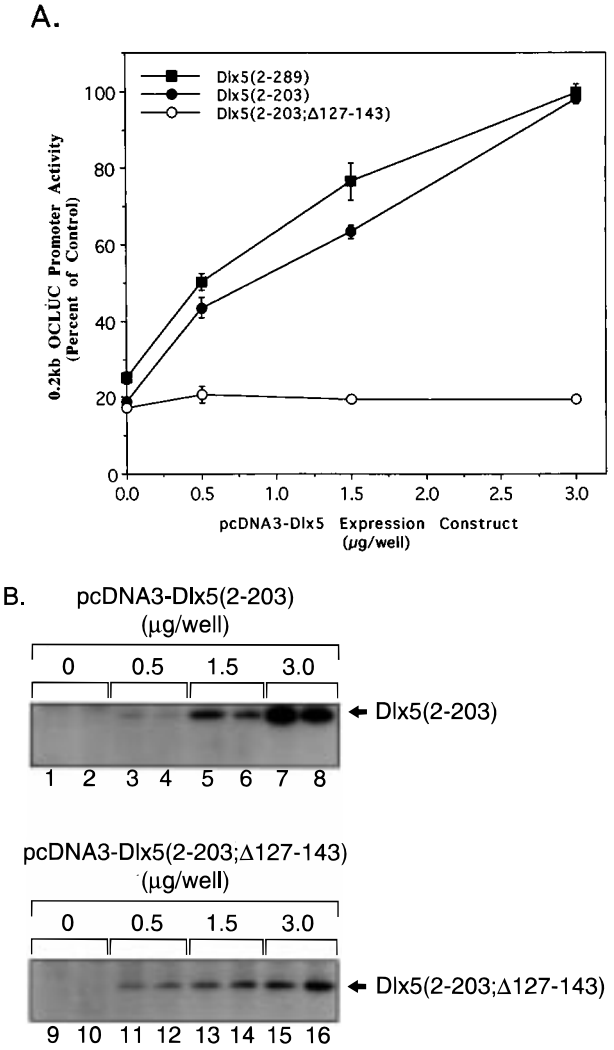

FIGURE 4: Dlx5 residues 127−143 are required to reverse OC promoter suppression by Msx2. (A) The activities of Dlx5, Dlx5-(2−203), and Dlx5(2−203; Δ127−143) in the OC promoter de-repression assay outlined in the legend of Figure 1 were assessed in MC3T3E1 calvarial osteoblasts and directly compared. Data represent the mean (±range) luciferase activity of independent duplicate transfections, expressed as a percentage of the basal OCLUC activity observed in the absence of either Dlx5 or Msx2. Note that while Dlx5(2−203) is as active as full-length Dlx5, Dlx5-(2−203; Δ127−143) cannot reverse Msx2-mediated OC promoter suppression. (B) Western blot analyses of cellular aliquots from these transfected cells indicate that both Dlx5(2−203) and Dlx5-(2−203; Δ127−143) accumulate to significant levels; thus, the inactivity of Dlx5(2−203; Δ127−143) is not due to a lack of Dlx5 variant expression.

due to lack of expression of this variant, since it accumulates to levels roughly equivalent to those of Dlx5(2−203) (Figure 4B). Thus, residues of Dlx5 necessary for interaction with Msx2 in vitro are required for antagonism of Msx2 function in vivo. Moreover, the C-terminal domain of Dlx5 is not required to reverse Msx2-mediated OC promoter suppression.

Dlx5 Antagonizes Msx2-Mediated Inhibition of OCFREB DNA Binding Activity That Is Dependent upon Dlx5 Residues 127−143. Msx2 suppresses the basal OC promoter in part by inhibiting DNA−protein interactions between the OC FGF response element binding protein OCFREB and its cognate, the OCFRE (*9*, *15*). We wished to assess whether Dlx5 antagonizes Msx2 inhibition of OCFREB binding. As shown in Figure 5A, addition of Msx2 to the OCFREB-containing nuclear extract abrogates the interaction of OCFREB with the OCFRE in the gel shift assay. Dlx5 completely reverses Msx2-mediated suppression of OCFREB binding (Figure 5A, lanes 3−5). By contrast, TBP, which does not interact with Msx2 (*17*), has no effect on Msx2-dependent OCFREB inhibition (Figure 5A, lanes 8−10). Dlx5 has no independent effect on OCFREB binding in the absence of Msx2 (Figure 5A, lane 12). Notably, Dlx5 residues 127−143 that are necessary for interaction with Msx2 by far-Western blot assays (Figure 3B) and Dlx5 transcriptional de-repression (Figure 4A) are also required

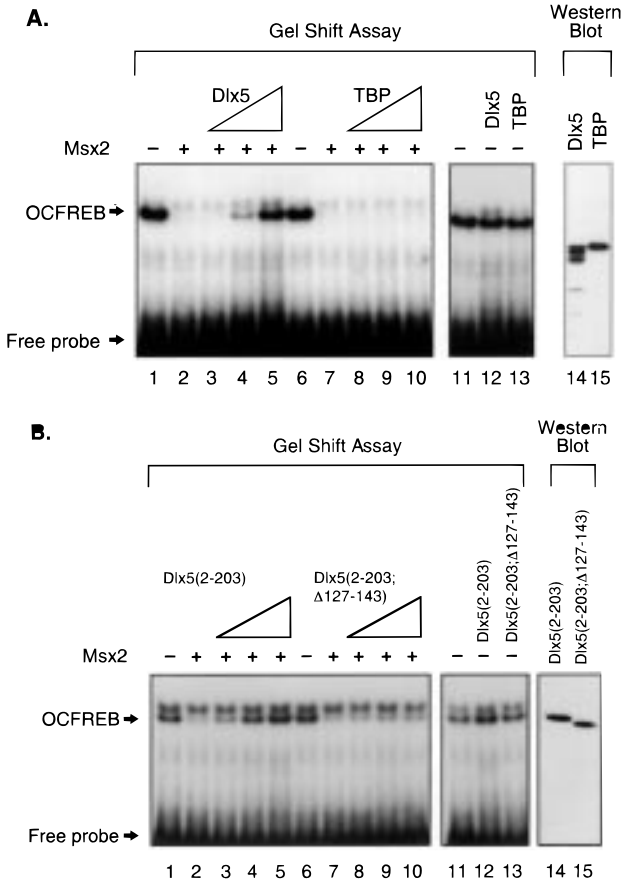

FIGURE 5: Recombinant Dlx5 antagonizes Msx2-mediated inhibition of protein−DNA interactions at the OCFRE. Crude nuclear extracts were prepared from FGF2/FSK-treated MC3T3E1 calvarial osteoblasts as a source of OCFREB activity (*9*). (A) In lanes 1−13, nuclear extracts were incubated either alone (lanes 1, 6, and 11) or with recombinant purified Msx2 (lanes 2−5 and 7−10), Dlx5 (lanes 3−5 and 12), and TBP (lanes 8−10 and 13) proteins as indicated, and then analyzed for OCFREB DNA binding activity by gel shift assay using the OCFRE probe (see Experimental Procedures). Note that as previously described (*16*), Msx2 suppresses OCFREB DNA binding activity (lanes 2 and 7). Further note that Dlx5 completely restores OCFREB binding (lanes 3−5), while TBP does not (lanes 8−10). Dlx5 and TBP have no effect on OCFREB activity in the absence of Msx2 (lanes 12 and 13). Western blot analyses performed as described in the legend of Figure 3 were used to ensure that equivalent amounts of recombinant purified Dlx5 and TBP were added to each admixture (lanes 14 and 15). (B) In lanes 1−13, MC3T3E1 nuclear extracts were incubated either alone (lanes 1, 6, and 11), or with recombinant purified Msx2 (lanes 2−5 and 7−10), Dlx5(2−203) (lanes 3−5), or Dlx5(2−203; Δ127−143) (lanes 8−10). Note that while Dlx5(2−203) can reverse Msx2 inhibition of OCFREB binding (lanes 3−5), Dlx5(2−203; Δ127−143) is inactive (lanes 8−10). Western blot analyses confirm that equivalent amounts of Dlx5 and Dlx5(2−203; Δ127−143) were present in these admixtures (lanes 14 and 15). See the text for details.

to reverse inhibition of OCFREB binding (Figure 5B). Thus, Dlx5 antagonizes Msx2-mediated inhibition of OCFREB DNA binding activity that is dependent upon Dlx5 residues 127−143.

Dlx5 Augments FGF2/FSK Stimulation of the OC Promoter in the Presence of Msx2. We wished to assess whether the effect of Dlx5 on Msx2-inhibited OCFREB binding in vitro corresponds to enhanced regulation of the OC promoter in vivo. Therefore, we examined the effects of Msx2 and Dlx5 expression on OC promoter regulation by FGF2/FSK in MC3T3E1 osteoblasts. As previously demonstrated (*16*),

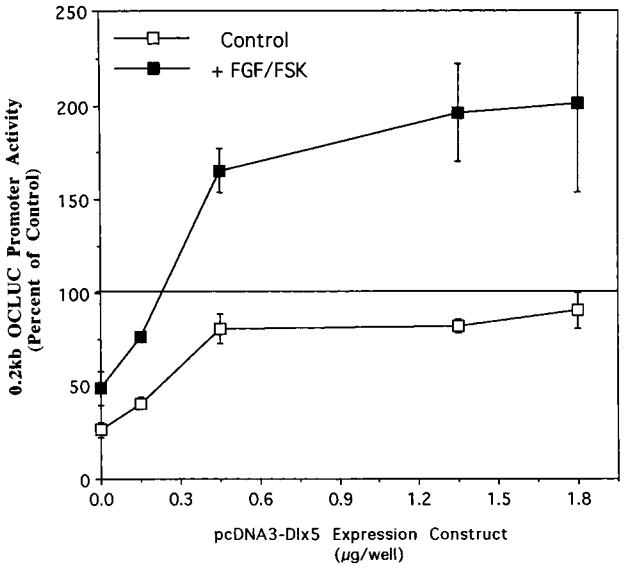

FIGURE 6: Msx2 suppresses and Dlx5 restores FGF2/FSK induction of the OC promoter. Cultures of MC3T3E1 calvarial osteoblasts were transfected with 0.2 kb OCLUC (0.75 μg/well), pcDNA3-Msx2 (0.75 μg/well), and increasing amounts of pcDNA3-Dlx5 (0−1.8 μg/well as indicated) as outlined in the legend of Figure 1. Two days after transfection, cells were refed with media containing either vehicles or 3 nM FGF2/10 μM FSK as previously described (*9*). Cell extracts were assayed 1 day later for luciferase and β-galactosidase activities. Data are presented as the mean (±SD) luciferase activity of three independent transfections, expressed as a percentage of the basal 0.2 kb OCLUC activity (note the line at 100%) in the absence of Msx2, Dlx5, and FGF2/FSK. Note that expression of Dlx5 dose-dependently augments induction of the OC promoter in the presence of Msx2. In the absence of Msx2, FGF2/FSK induces OC promoter activity 3−8-fold (*9*).

expression of Msx2 inhibits both basal and FGF2/FSK-stimulated OC promoter activity (Figure 6). Coexpression of Dlx5 reverses basal OC promoter suppression and augments FGF2/FSK promoter activation in a dose-dependent fashion (Figure 6). Thus, Dlx5 reverses both the Msx2-mediated inhibition of OCFREB binding (Figure 5) and inhibition of FGF2/FSK promoter activation (Figure 6).

DISCUSSION

A transcriptional heirarchy is emerging that controls tissue-specific and stage-specific osteoblast gene expression during skeletal morphogenesis. The Runt domain factor Osf2/Cbfa1 globally regulates skeletal mineralization, conferring transcriptional "competency" that permits developmental expression of osteoblast-specific genes such as osteocalcin, bone sialoprotein, and osteopontin (*13*, *14*, *28*). By contrast, homeodomain proteins such as Msx1, Msx2, Mhox, and Dlx5 control osteoblast gene expression and mineralization in specific craniofacial skeletal structures (*3*, *20*, *29*−*31*). Using a physiologically relevant target of Msx2 action in calvarial osteoblasts, the OC promoter (*12*, *17*, *20*, *32*), we have determined that Dlx5 and Msx2 reciprocally regulate both basal promoter activity and OC induction by FGF2/FSK. Intriguingly, the actions of both homeodomain proteins on OC promoter regulation are independent of their intrinsic DNA binding activities; Msx2 and Dlx5 actions are instead dependent upon protein−protein interactions directed by the homeodomain N-terminal arm and extension. It remains possible that in other promoter contexts, the intrinsic DNA binding activities of Msx2 and Dlx5 may be necessary for

transcriptional regulation. Moreover, our experiments were performed using transient expression assays with promoter−reporter plasmid constructs; reciprocal effects of Msx2 and Dlx5 on endogenous OC gene expression have yet to be tested, and might be dependent upon intrinsic homeodomain DNA binding functions in this chromatin-bound context. However, recent in vivo transgenic analysis of another transcriptional repressor, the glucocorticoid receptor, indicates that suppressors can indeed function independent of DNA binding (*33*). Notably, dimerization of homeodomain proteins via contacts provided by the N-terminal region of the homeodomain is a recurrent theme. The prototypical yeast homeodomain repressor MATα2 interacts with its MADS box corepressor MCM1 via protein−protein contacts conferred in part by the N-terminal extension of MATα2 (*34*). Abate-Shen and co-workers concluded that the N-terminal extension of Msx1 is necessary for interaction with Dlx family members (*20*). Recently, Largman et al. (*35*) demonstrated that the Meis1 N-terminal domain directs selective homeodomain protein−homeodomain protein interactions with paralogs 9−13 of the Hox complex; these dimeric complexes determine DNA binding cognate specificity in binding site selection assays. Thus, this study adds to the recent literature that points to a homeodomain regulatory code directed by combinatorial protein−protein interactions (*36*).

Our biochemical structure−function studies of Msx2 and Dlx5 emphasize a physiologically relevant target, the osteoblast-specific OC promoter (*3*, *5*, *12*, *17*−*19*). From these analyses, we determine that antagonistic interactions between these homeodomain proteins mediate transcriptional regulation in part via a third protein complex, the osteocalcin FGF response element binding protein (OCFREB; *9*, *15*). As purified from MG63 human osteosarcoma cells, OCFREB is a dimer of 70 and 80 kDa constituents (*16*). Intriguingly, the sequences surrounding the OCFRE core form an inverted 8 bp repeat (GGYRRCTGCAGYYRCC; *9*), suggesting recognition by a dimer. Msx2 inhibits the binding of OCFREB to its OCFRE cognate; once bound, OCFREB cannot be displaced (*16*). While Dlx5 has no effect on OCFREB binding alone, it completely abrogates Msx2-mediated inhibition of OCFREB activity. In toto, this indicates that (i) Msx2 acts either to sterically hinder OCFREB−OCFRE interactions or to regulate OCFREB dimerization and (ii) Dlx5 acts to sterically hinder and/or sequester Msx2. It is important to note the similarities between Msx2 and Dlx5 regulation of the OC promoter and Msx1 and Dlx5 regulation of the MyoD enhancer (*21*). Abate-Shen and co-workers noted that Dlx5 can act as a weak transcriptional activator in multimerized, artificial promoter contexts; however, as we observe in the native OC promoter context, Dlx5 functions as a "de-repressor" at the MyoD enhancer (*21*). On the basis of our analyses of OC promoter regulation, it is intriguing to speculate that Msx1 and Dlx5 may regulate the DNA binding activity of a third protein complex that recognizes and regulates the MyoD enhancer.

Ryoo et al. (*20*) determined that Dlx5 and OC are generally coexpressed during rat calvarial osteoblast differentiation in culture, consistent with our results demonstrating that Dlx5 promotes OC promoter activity in two calvarial osteoblast cell culture systems. However, the brief analysis of Dlx5

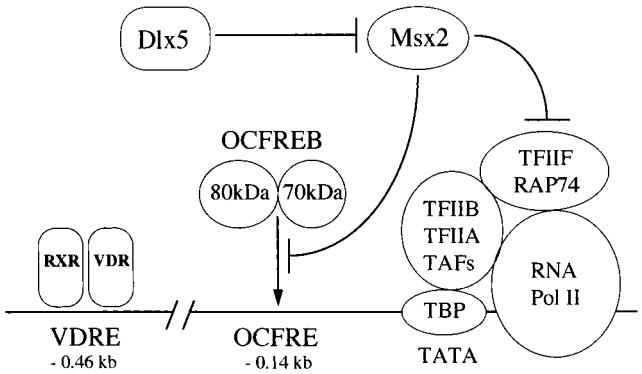

FIGURE 7: Model of rat OC promoter regulation in calvarial osteoblasts by Msx2 and Dlx5. Msx2 suppresses basal OC promoter activity via protein−protein interactions with the TFIIF subunit RAP74 (*17*). Msx2 also suppresses induction of the OC promoter by FGF2/FSK, but not by vitamin D (calcitriol); as outlined in ref *16*, inhibition is achieved in part by selective inhibition of protein−DNA interactions at the OCFRE, but not at the VDRE (vitamin D response element). Dlx5 de-represses the OC promoter in calvarial osteoblasts by functionally antagonizing the inhibition by Msx2 of both basal and FGF2-dependent promoter activity (this study). Whether post-translational modifications and transcriptional co-adaptors regulate calvarial homeodomain protein activity is currently unknown. See the Discussion for details.

function performed in this previous study showed that Dlx5 downregulates OC expression in ROS17/2.8 osteosarcoma cells (*20*). This mineralizing tumor cell line is derived from rat long bone, and exhibits dysregulated, constitutive OC expression (*37*). It may be that oncogenic transformation perturbs Dlx5 function; it is more likely, however, that the neural crest origin of the calvarial osteoblast (*1*, *2*) influences Dlx5-dependent gene regulation. For example, Lichtler and co-workers (*38*) demonstrated that regulation of OC by Msx2 occurs in calvarial osteoblasts, where Msx2 is normally expressed (*3*), but not in osteoblasts derived from long bone. Whether the transformed nature of the ROS17/2.8 osteosarcoma (*37*) or its derivation from long bone accounts for altered Dlx5 action is unknown; however, our studies were carried out in the background of the calvarial osteoblast, a cellular background where Dlx5, Msx2, and OC expression interact during normal development. The temporal coexpression of Dlx5 and OC in cultured calvarial osteoblasts (*20*) and the ability of Dlx5 to promote OC promoter activity in calvarial osteoblasts (this study) suggest that Dlx5 controls the timing of OC gene transcription during terminal differentiation by antagonizing Msx2-dependent repression. Moreover, in preliminary studies, we have determined that a component of Msx2 protein accumulation can be regulated by Dlx5; this proceeds independently of OC promoter repression and/or de-repression and requires the C terminus of Msx2 (unpublished data), a domain dispensable for OC promoter regulation (*17*). It remains to be identified whether this second Dlx5−Msx2 interaction may play a role in the expression of other genes characteristic of the mineralizing calvarial osteoblast, such as high-level expression of the type α1(I) collagen gene (*39*).

Finally, it is interesting to contrast the reciprocal regulation of OC expression by homeodomain proteins (Figure 7) with the recently defined mechanisms utilized by nuclear receptors to inhibit or activate gene expression (*40*). In the absence of ligand, a nuclear receptor acts as a cognate-dependent transcriptional repressor; addition of ligand de-represses

transcription, but also induces transcriptional activation. Ligand binding releases histone deacetylase and recruits histone acetyltransferase complexes that inhibit or promote transcription, respectively, by covalent remodeling of histone protein−DNA complexes (*40*). By contrast, repression of OC expression by Msx2 occurs via inhibitory protein−protein interactions with specific components of the basal OC promoter. Notably, the histone deacetylase inhibitor trichostatin A has no effect on Msx2-dependent promoter repression, but reverses nuclear orphan receptor-dependent promoter repression in osteoblasts (unpublished observations); as predicted from our cumulative data, this indicates that histone acetylation is not a component of Msx2-mediated repression (Figure 7). Dlx5 does not exhibit significant transcriptional activator function, but potently de-represses the basal OC promoter by binding and hindering Msx2. Whether these specific molecular differences reflect a more general mechanistic dichotomy between transcriptional repression by homeodomain proteins and nuclear receptors is as yet unknown. However, glucocorticoid inhibition of Fos/Jun-dependent transcription in vivo is also independent of intrinsic glucocorticoid receptor DNA binding activity (*33*), reminiscent of Msx2 action at the OCFRE, suggesting that mechanistic overlap may occur at mitogen-regulated response elements.

REFERENCES

1. Cohen, M. M., Jr. (1993) *Am. J. Med. Genet. 47*, 581−616.
2. Marks, S. C., Jr., and Hermey, D. C. (1996) in *Principles of Bone Biology* (Bilezikian, J. P., Raisz, L. G., and Rodan, G. A., Eds.) 1st ed., pp 3−14, Academic Press, San Diego, CA.
3. Jabs, E. W., Muller, U., Li, X., Ma, L., Luo, W., Haworth, I. S., Klisak, I., Sparkes, R., Warman, M. L., Mulliken, J. B., Snead, M. L., and Maxson, R. (1993) *Cell 75*, 443−450.
4. Wilkie, A. O. M., Slaney, S. F., Oldridge, M., Poole, M. D., Asworth, G. J., Hockley, A. D., Hayward, R. D., David, D. J., Pulleyn, L. J., Rutland, P., et al. (1995*) Nat. Genet 9*, 165−172.
5. Lomri, A., Lemonnier, J., Hott, M., de Parseval, N., Jaleunie, E., Munnich, A., Renier, D., and Marie, P. J. (1998) *J. Clin. Invest. 101*, 1310−1317.
6. Long, M. W., Robin, J. A., Ashcraft, E. A., and Mann, K. G. (1995) *J. Clin. Invest. 95*, 881−887.
7. Hurley, M. M., Abreu, C., Harrison, J. R., Lichtler, A. C., Raisz, L. G., and Kream, B. E. (1993) *J. Biol. Chem. 268*, 5588−5593.
8. Hurley, M. M., Kessler, M., Gronowicz, G., and Raisz, L. G. (1992) *Endocrinology 130*, 2673−2682.
9. Boudreaux, J. M., and Towler, D. A. (1996) *J. Biol. Chem. 271*, 7508−7515.
10. Ducy, P., and Karsenty, G. (1996) in *Principles of Bone Biology* (Bilezikian, J. P., Raisz, L. G., and Rodan, G. A., Eds.) 1st ed., pp 183−195, Academic Press, San Diego, CA.
11. Romberg, R. W., Werness, P. G., Riggs, B. L., and Mann, M. G. (1986) *Biochemistry 25*, 1176−1180.
12. Towler, D. A., Bennett, C. D., and Rodan, G. A. (1994) *Mol. Endocrinol. 8*, 614−624.
13. Stein, G. S., Lian, J. B., Stein, J. L., van Wijnen, A. J., Frenkel, B., and Montecino, M. (1996) in *Principles of Bone Biology* (Bilezikian, J. P., Raisz, L. G., and Rodan, G. A., Eds.) 1st ed., pp 69−86, Academic Press, San Diego, CA.
14. Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997) *Cell 89*, 747−754.
15. Newberry, E. P., Boudreaux, J. M., and Towler, D. A. (1996) *Mol. Endocrinol. 10*, 1029−1040.
16. Newberry, E. P., Boudreaux, J. M., and Towler, D. A. (1997) *J. Biol. Chem. 272*, 29607−29613.
17. Newberry, E. P., Latifi, T., Battaile, J. T., and Towler, D. A. (1997) *Biochemistry 36*, 10451−10462.
18. Hoffman, H. M., Catron, K. M., van Wijnen, A. J., McCabe, L. R., Lian, J. B., Stein, G. S., and Stein, J. L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12887−12891.
19. Bidder, M., Latifi, T., and Towler, D. A. (1998) *J. Bone Miner. Res. 13*, 609−619.
20. Ryoo, H. M., Hoffmann, H. M., Beumer, T., Frenkel, B., Towler, D. A., Stein, G. S., Stein, J. L., van Wijnen, A. J., and Lian, J. B. (1997) *Mol. Endocrinol. 11*, 1681−1694.
21. Zhang, H., Hu, G., Wang, H., Sciavolino, P., Iler, N., Shen, M. M., and Abate-Shen, C. (1997) *Mol. Cell. Biol. 17*, 2920−2932.
22. Franceschi, R. T., Iyer, B. S., and Cui, Y. (1994) *J. Bone Miner. Res. 9*, 843−854.
23. Rodan, S. B., Wesolowski, G., Thomas, K., and Rodan, G. A. (1987) *Endocrinology 121*, 1917−1923.
24. Sudo, H., Kodama, H.-A., Amagai, Y., Yamamoto, S., and Kasai, S. (1983) *J. Cell. Biol. 96*, 191−198.
25. Dignam, J. D., Lebowitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res. 11*, 1475−1489.
26. Blanar, M. A., and Rutter, W. J. (1992) *Science 256*, 1014−1018.
27. Bell, J. R., Noveen, A., Liu, Y.-H., Ma, L., Dobias, S., Kundu, R., Luo, W., Xia, Y., Lusis, A. J., Snead, M. L., and Maxson, R. (1993) *Genomics 16*, 132−131.
28. Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., Stamp, G. W. H., Beddington, R. S. P., Mundlos, S., Olsen, B. R., Selby, P. B., and Owen, M. J. (1997) *Cell 89*, 765−777.
29. Satokata, I., and Maas, R. (1994) *Nat. Genet. 6*, 348−356.
30. Martom, K. F., Bradley, A., and Olson, E. N. (1995) *Genes Dev. 9*, 1237−1249.
31. Zhao, G. Q., Zhao, S., Zhou, X., Eberspaecher, H., Solursh, M., and de Crombrugghe, B. (1994) *Dev. Biol. 164*, 37−51.
32. Liu, Y. H., Kundu, R., Wu, L., Luo, W., Ignelzi, M. A., Jr., Senad, M. L., and Maxson, R. E., Jr. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 6137−6141.
33. Reichardt, H. M., Kaestner, K. H., Tuckermann, J., Kretz, O., Wessely, O., Bock, R., Gass, P., Schmid, W., Herrlich, P., Angel, P., and Schutz, G. (1998) *Cell 93*, 531−541.
34. Tan, S., and Richmond, T. J. (1998) *Nature 391*, 660−666.
35. Shen, W. F., Montgomery, J. C., Rozenfeld, S., Moskow, J. J., Lawrence, H. J., Buchberg, A. M., and Largman, C. (1997) *Mol. Cell. Biol. 17*, 6448−6458.
36. Simon, K. J., Grueneberg, D. A., and Gilman, M. (1997) *Mol. Cell. Biol. 17*, 6653−6662.
37. Bortell, R., Owen, T. A., Shalhoub, V., Heinrichs, A., Aronow, M. A., Rochette-Egly, C., Lutz, T., Stein, J. L., Lian, J. B., and Stein, G. S. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2300−2304.
38. Dodig, M., Kronenberg, M. S., Sumoy, L., Pan, Z., Upholt, W. B., Gerstenfeld, L. C., and Lichtler, A. C. (1996) *J. Bone Miner. Res. 11* (Suppl. 1), S260 (abstract).
39. Dodig, M., Kronenberg, M. S., Bedalov, A., Kream, B. E., Gronowicz, G., Clark, S. H., Mack, K., Liu, Y. H., Maxson, R., Pan, Z. Z., Upholt, W. B., Rowe, D. W., and Lichtler, A. C. (1996) *J. Biol. Chem. 271*, 16422−16429.
40. Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature 387*, 43−48.

BI981878U