Structure−Function Analysis of Msx2-Mediated Transcriptional Suppression†

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ABSTRACT: Osteocalcin (OC) is a calcium binding protein expressed in mature osteoblasts undergoing mineralization. The OC gene has been identified as a target for transcriptional suppression by Msx2, a homeodomain transcription factor that controls ossification in calvarial bone of the developing skull. We have initiated systematic structure−function analyses of Msx2, using OC promoter suppression (luciferase reporter) in MC3T3-E1 calvarial osteoblasts as an assay. Msx2 variants were epitope ("FLAG")-tagged for monitoring Msx2 protein expression by Western blot analysis. Functional analyses of N- and C-terminally truncated molecules identify Msx2 residues 97−208 as the core suppressor domain. Internal deletion analyses indicate that suppressor function is dependent upon structural features encoded by residues 132−148—upstream of the homeodomain and overlapping the homeodomain N-terminal extension—but not upon residues in the three homeodomain helices. Mutations that enhance DNA binding activity do not proportionally enhance Msx2 suppressor function; moreover, a Msx2 point mutant Msx2(T147A) that completely lacks DNA binding activity is indistinguishable from wild-type Msx2 in its ability to suppress the OC promoter, demonstrating that direct interaction with DNA is not required for Msx2 suppressor function. This suggests that Msx2 suppresses transcription via protein−protein interactions with components of the basal transcriptional machinery, either alone or in concert with co-regulators. Using interaction "Far Western" blotting assays, we systematically tested for protein−protein interactions between Msx2 and components of the basal transcriptional machinery known to mediate transcriptional activation (TBP, TFIIB, and TFIIF). Msx2 binds both components of TFIIF (RAP74, RAP30), but not TFIIB or TBP. Msx2(55−208) encompasses core suppressor domain residues and binds TFIIF; in this context, deletion of the seventeen amino acid residues 132−148 that are required for core suppressor function abrogates interactions with TFIIF components. Co-expression of RAP74 in MC3T3-E1 cells partially reverses (>50%) suppression of OC promoter activity by Msx2, while co-expression of TFIIB or RAP30 has no effect. Thus the core suppressor domain of Msx2 participates in functionally important interactions with RAP74 that regulate OC promoter activity in calvarial osteoblasts.

In the developing skull, calvarial bone formation proceeds via intramembranous ossification, a process whereby osteoblasts form bone without a chondrocyte-elaborated cartilagenous template (*1*). Unmineralized fibrous regions (cranial sutures and fontanelles) between ossification centers contain proliferative osteoprogenitors; these cells subsequently mature to become post-proliferative, mineralizing osteoblasts (*1*, *2*). The transcriptional mechanisms that regulate calvarial osteoblast proliferation, differentiation, and gene expression are only beginning to be understood (*3*). Elegant human and mouse genetic studies have very recently identified two specific homeodomain transcription factors—Msx2 (*4*, *5*) and Mhox (*6*)—as important regulators of intramembranous bone development in the skull. A point mutation in Msx2 has been shown to give rise to Boston-type craniosynostosis syndrome, characterized by precocious differentiation of calvarial osteoprogenitors and accelerated mineralization in the cranial sutures (*4*). Transgenic mice expressing this altered Msx2 protein from the CMV promoter also develop craniosynostosis (*5*), consistent with the dominant inheritance of Boston-type craniosynostosis in humans (*4*). The precise pathophysiologic mechanisms of Msx2 action are currently unknown. Recently, we (*7*) and others (*8*−*10*) identified the bone specific osteocalcin (OC)[1] gene as a target for Msx2 action. Msx2 suppresses the OC promoter in calvarial osteoblasts (*7*−*9*). Moreover, reciprocal patterns of Msx2 and OC expression are observed during differentiation of rat calvarial osteoblasts in culture (*9*), and constitutive over-

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[1] Abbreviations: AP1, activating protein 1; BCA, bicinchoninic acid; CMV, cytomegalovirus; CSPD, disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2′-(5′-chloro)-tricyclo[3.3.1^{3,7}]decan]-4-yl)phenyl phosphate; DTT, dithiothreitol; EDTA, disodium ethylenediaminetetraacetate; FLAG epitope, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; GST, glutathione *S*-transferase; Hepes, 4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl thiogalactoside; LUC, luciferase; MEM, modified Eagle's media; OC, osteocalcin; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; RAP74, RNA polymerase II associated protein TFIIF-74; RAP30, RNA polymerase II associated protein TFIIF-30; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SRF, serum response factor; TAF, TBP associated factor; TBP, TATA box binding protein; Tris, trishydroxymethylaminomethane.

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expression of Msx2 in chick calvarial osteoblasts inhibits OC expression (*10*).

Osteocalcin is a small, ~6 kDa γ-carboxylated calcium binding protein selectively expressed in osteoblasts undergoing terminal differentiation and mineralization (*11*, *12*). In the mouse, an OC gene cluster has been identified; while OG1 and OG2 genes are bone-specific, the OC-related gene ORG is more widely expressed (*11*, *13*). *In vitro* studies indicate that OC binds calcium and inhibits mineral deposition (*14*). Consistent with this result, mice homozygous for targeted disruption of the OC genes exhibit enhanced bone formation and excessive mineralization (*15*). Since osteocalcin regulates bone formation (*15*), mineralization (*14*, *15*), and turnover (*16*), the OC gene represents an important, physiologically relevant target for Msx2 action in differentiating calvarial osteoblasts.

We have initiated systematic structure−function analyses of the Msx2 protein, using OC promoter suppression in MC3T3-E1 mouse calvarial osteoblasts as an assay (*7*). This phenotypically immature calvarial cell line provides a relevant cellular background for these studies since Msx2 affects calvarial osteoblast differentiation and MC3T3-E1 cells recapitulate the differentiation/mineralization program exhibited by primary calvarial osteoblasts (*17*, *18*). Msx2 proteins were epitope-tagged ("FLAG"-tagged; ref 19) to permit uniform monitoring of Msx2 variant protein expression by Western blot analysis. Functional analyses of N- and C-terminally truncated molecules identify residues 97−208 as the major domain contributing to Msx2 suppressor function (see ref 20 for numbering of Msx2 residues). Internal deletion analyses indicate that suppressor function maps to residues 132−148, upstream of the homeodomain and overlapping the N-terminal homeodomain extension; suppressor function is not dependent upon residues in the three homeodomain helices, including DNA recognition helix 3. An Msx2 point mutation (Pro148His) that enhances DNA binding activity does not proportionally enhance Msx2 suppressor function. Furthermore, a different Msx2 point mutant (Thr147Ala) that completely lacks DNA binding activity is indistinguishable from wild-type Msx2 as a transcriptional suppressor of the OC promoter. These data suggest that Msx2 modulates transcription primarily via protein−protein interactions, either in concert with co-regulatory DNA binding proteins or through direct interaction with components of the basal transcriptional machinery. Using the interaction "Far Western" blotting technique (*21*), we systematically tested for protein−protein interactions between Msx2 and components of the basal transcriptional machinery known to mediate transcriptional activation (TBP, TFIIB, and TFIIF; ref 22). Msx2 binds components of TFIIF (RAP74, RAP30), but not TFIIB or TBP (TATA box binding protein). Recombinant RAP74 and RAP30 (*23*) specifically interact with the core suppressor domain of Msx2 in interaction blotting assays. Removal of Msx2 residues 132−148 necessary for core suppressor function abrogates interaction with the two TFIIF components, while removal of residues 119−131 has no effect on either suppressor function or interaction with TFIIF. Finally, transient co-expression of RAP74 in MC3T3-E1 calvarial osteoblasts partially reverses (>50%) suppression of OC promoter activity by Msx2, while co-expression of TFIIB or RAP30 has no effect. To our knowledge, this represents the first example of an interaction between TFIIF and a transcriptional suppressor. Taken together, these data demonstrate that Msx2 suppresses OC transcription independent of homeodomain DNA binding, but via protein−protein interactions. Transcriptional suppression by Msx2 is mediated at least in part via interactions with the TFIIF subunit RAP74, a component of the basal transcriptional machinery previously identified as a target for subsets of transcriptional activators (*24*).

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents. MC3T3-E1 mouse calvarial clonal osteoblastic cells (*17*, *18*) were grown as previously described (*7*, *25*) in 10% fetal calf serum (JRH Biochemicals, Lenexa, KS) and α-MEM (Gibco/BRL Life Sciences, Gaithersburg, MD) supplemented with 2.5 mM glutamine, passaged by subculturing subconfluent stocks 1:5 every three days with 0.25% trypsin/0.25% EDTA. All experiments were carried out using MC3T3-E1 cells between passages 10 to 25 from our frozen stocks. Corning tissue culture plates were obtained from Fisher (St. Louis, MO). Molecular biology reagents were obtained from Promega (Madison, WI), Fisher (St. Louis, MO), and Qiagen (Chatsworth, CA). Protein was determined using the Pierce BCA assay (Rockford, IL) after precipitation and resolubilization of protein as described (*26*).

Oligonucleotide-Directed Mutagenesis of Msx2 and Expression Plasmid Preparation. Synthetic oligodeoxynucleotides were obtained from the Washington University Protein and Nucleic Acid Laboratory or from Gibco BRL Life Technologies (Gaithersburg, MD). The N-terminal initiator Methionine with the Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys FLAG motif (*19*) and homeodomain point mutations were introduced by sequential PCR with a Perkin-Elmer model 9600 thermal cycler (Foster City, CA), using the H8F MSV-mouse Msx2 cDNA expression construct (*27*) as a template, and the high-fidelity Klentaq1 polymerase (ref 28; purchased from Dr. Wayne Barnes,Washington University, St. Louis, MO). See Bell et al. (*20*) and GenBank S60698 for Msx2 residue numbering. Briefly, mutations in the homeodomain were introduced in the first PCR step (15 cycles) using the appropriate mutagenic upstream primer and the "hox 4" (*7*) oligonucleotide primer downstream (introduces *Bam*HI site after stop codon; see below), followed by agarose gel purification of the resulting ~0.4 kb fragment (Mermaid, Bio101, Vista, CA). This duplex fragment was heat denatured, and the antisense strand was utilized as the downstream primer in the second PCR step (15 cycles), using the "MetFLAGMsx2" upstream primer (with *Kpn*I site; introduces the FLAG motif following the initiator methionine residue into the amino terminus of Msx2; *vide infra*). After digestion with *Kpn*I and *Bam*HI and agarose gel purification (Gene Clean II, Bio101, Vista, CA), the resultant 0.8 kb full-length PCR fragments encoding the various Msx2 point mutants were cloned into the *Kpn*I−*Bam*HI site in pcDNA3 (Invitrogen, Carlsbad, CA). N- and C-terminal truncations were performed in a similar fashion by PCR, with all N-terminal amplimers encoding the *Kpn*I site and initiator MetFLAG tag and all C-terminal amplimers encoding a stop codon and *Bam*HI site. Thus, all pcDNA3-Msx2 expression constructs in this study contain the CMV promoter and the bacterial T7 promoter upstream of the initiator methionine in good Kozak consensus (*29*) followed by the FLAG tag and Msx2 coding sequence as outlined in Figure 1. Msx2 internal deletion mutants were generated by ligation of two

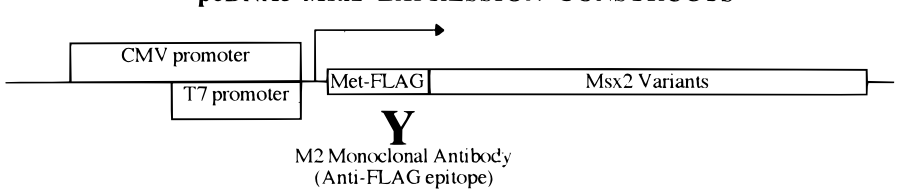


FIGURE 1: Overview of Msx2 expression constructs. Msx2 variants were generated by PCR and cloned into the *Kpn*I−*Bam*HI sites of pcDNA3 (Invitrogen). This vector permits expression of Msx2 protein in eukaryotic cells by transient transfection (driven by the CMV promoter) and *in vitro* by coupled transcription−translation (driven by the T7 promoter). All Msx2 molecules were tagged with the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) immediately following the initiator Met, permitting detection of Msx2 protein by Western blot analysis using the M2 anti-FLAG monoclonal antibody. See text for details.

PCR fragments encoding the 5′-coding region (*Kpn*I−*Xho*I) and 3′-coding region (*Xho*I−*Bam*HI linkers) with stop codon in contiguous open reading frame into the *Kpn*I−*Bam*HI sites of pDNA3 (deleted residues are replaced by two amino acids encoded by the restriction site). All plasmids were purified by Qiagen column chromatography (Qiagen, Chatsworth, CA) and sequenced to verify DNA sequence and the Msx2 protein translated (ABI Prism Dye Terminator Cycle Sequencing Kit; Perkin-Elmer, Foster City, CA). These expression constructs were then used for functional analyses of Msx2 in transient transfections and gel shift assays (after *in vitro* transcription−translation; *vide infra*).

In Vitro Transcription−Translation of Msx2 Variant Proteins, Western Blot Analyses, and Electrophoretic Mobility Gel Shift Assays. *In vitro* transcription−translation was carried out from the T7 promoter with *Xba*I-linearized pcDNA3-Msx2 variant expression plasmids using Promega Reticulocyte TnT system (Madison, WI), precisely following the manufacturer's instructions. For gel shift analyses, samples were then diluted to a total volume of 100 μL with 50 μL of gel shift buffer D (*30*). Oligonucleotide labeling and gel shifts were carried out precisely as detailed (*7*, *31*), using 5 μL of diluted Msx2 transcription−translation reaction and ∼30 000 cpm/0.2 pmol of ^{32}P radiolabeled OCTA 26 duplex oligo (Msx2 DNA binding cognate; ref 7) in a standard 15 μL, 20 min binding assay (*31*). For Western blot analyses, 20 μL aliquots of each transcribed−translated Msx2 protein reaction were treated with 5 μL of 5× Laemmli sample buffer, heated for 3 min at 95 °C, and then subjected to fractionation by denaturing polyacrylamide gel electrophoresis (*21*). After electrotransfer to PVDF membranes (Tropix, Bedford, MA), Western blot analysis was carried out using 1.5 μg/mL M2 anti-FLAG antibody (IBI/Kodak via VWR Scientific, St. Louis, MO; ref 19) to detect the FLAG-tagged Msx2 variants. Immunoreactive proteins were visualized with alkaline phosphatase conjugated anti-mouse IgG secondary antibody (1:5000 dilution) using the chemiluminescent alkaline phosphatase substrate CSPD (WesternLight System; Tropix, Bedford, MA) as per the manufacturer's instructions.

Transfections, Luciferase Assays, and β-Galactosidase Assays. MC3T3-E1 cells were plated into Costar six-well cluster dishes (35 mm diameter wells, 7×10^5 cells/well). Cells were transiently transfected the next day by calcium phosphate precipitation/15% glycerol shock as described (*32*) except that the length of the glycerol shock was shortened to 90 s. Typically, a total of 15 μg of DNA was precipitated in 1.5 mL, and 480 μL of this precipitate was applied per well in 4 mL of growth medium (*7*, *25*). The various pcDNA3-Msx2 expression constructs (up to 9 μg per precipitation) were co-transfected with the osteoblast-specific rat osteocalcin basal promoter fragment−reporter construct 222 OCLUC (−222 to +32; 4.5 μg per precipitation; refs 7 and 31). Empty pcDNA3 expression vector was added as required to maintain a constant amount of total plasmid DNA in each precipitation. CMV−β-galactosidase (0.3 μg per precipitation) was included in every transfection as an internal control for transfection efficiency. Two days following transfection, cells were re-fed with fresh medium containing 10 μM forskolin to stimulate 0.2 kb OC promoter activity, as previously described (*7*). Three days after transfection, cells from each well were rinsed twice with phosphate-buffered saline, harvested by scraping into 300 μL of Promega β-galactosidase enzyme assay lysis buffer, and extracted by vortexing as per the manufacturer's instructions (Promega Technical Bulletin No. 097). After centrifugation to remove insoluble cellular debris, aliquots were analyzed for enzyme activities (*vide infra*) and also for expression of FLAG-tagged Msx2 proteins by Western blot (*vide infra*) after increasing the NaCl concentration of the buffer to 0.45 M to facilitate extraction of nuclear proteins (*30*). Luciferase assays were carried out as previously detailed (*7*, *31*) using a Berthold AutoLumat 953 luminometer (EG & G Instruments, Oak Ridge, TN). Colorimetric β-galactosidase assays were carried out using the Promega β-galactosidase enzyme assay system following the manufacturer's instructions (Promega Technical Bulletin No. 097). In dose−response curves, data are presented as the mean (+/− range) of two independent transfections, in which the independent duplicates did not differ from each other by more than 15% of the mean value. For studies examining the effect of internal deletions on Msx2 function, data are presented as the mean (+/− standard deviation) of three independent transfections. All data sets were repeated in a minimum of two independent experiments to verify results. For studies examining reversal of Msx2 suppression of OC promoter by RAP74 in MC3T3-E1 cells, data are presented as the mean (+/− standard deviation) of six transient transfections. To assess epitope-tagged Msx2 protein expression, aliquots of extracts were fractionated by denaturing polyacrylamide gel electrophoresis (*21*), then electrotransfered per the manufacturer's instructions (Novex blot module; San Diego, CA) to PVDF membrane (Tropix, Bedford, MA) for Western blot analysis using 1.5 μg/mL M2 monoclonal antibody (IBI/Kodak via VWR Scientific, St. Louis, MO) and 1:5000 dilution of alkaline phosphatase-conjugated secondary antibody for chemiluminescent detection with the WesternLight system (Tropix, Bedford, MA) as described above.

RAP74, RAP30, TFIIB, and TBP Protein Expression Constructs. Recombinant human RAP74 and RAP30 (TFIIF components) in pET23d expression plasmids were the kind gift of Dr. Z. Burton (*23*). TFIIB (GenBank M76766; kind gift of P. MacDonald, St. Louis University) and TBP (GenBank M55654; obtained by RT-PCR from MG63 human osteosarcoma cell RNA, ref 33) were subcloned into the prokaryotic expression vector pET23a (Novagen, Madison, WI). The specific amplimer oligonucleotides for obtaining TBP by RT-PCR (*25*) were 5′-GAG GAT CCA TGG ATC AGA AC-3′ and 5′-GAT CGA AGC TTC GTC GTC TTC CTG-3′. Amplimers used to subclone TFIIB into pET23a were 5′-AAG GAT CCA TGG CGT CTA CCA G-3′ and 5′-GAG AAA GCT TTA GCT GTG GTA G-3′. After purification and digestion of amplified fragments with *Bam*HI and *Hin*dIII, these fragments were cloned into the *Bam*HI and *Hin*dIII sites of the pET23a polylinker. This strategy results in proteins in contiguous open reading frame with a carboxy terminal six-residue histidine tag and stop codon supplied by the pET23a vector. Inserts were verified by automated cycle sequencing; inserts corresponded to the sequences reported in GenBank (referenced above). Recombinant proteins were expressed from the T7 promoter of pET23 plasmids in *E. coli* strains JM109(DE3) or BL21-(DE3) as detailed (*23*) and as per Novagen's instructions (pET System Manual TB055, Novagen, Madison, WI). Briefly, pET23/RAP74, pET23/TFIIB, and pET23/TBP plasmids were used to transform BL21(DE3) cells which were grown in M9 minimal media and induced with IPTG. After extraction with 4 M urea in 50 mM sodium phosphate, pH 8.0/300 mM NaCl, recombinant histidine-tagged proteins were purified by nickel chelate chromatography using Ni^{2+}-NTA agarose (Qiagen, Chatsworth, CA) as detailed by the manufacturer as previously described (*23*). Proteins were eluted by pH step gradients between pH 7.0 and 4.0 in the above phosphate-buffered saline solution supplemented with 4 M urea, then analyzed by SDS−PAGE. Recombinant RAP30 was expressed in JM109(DE3) cells as previously described (*23*) but was not purified by nickel chelate chromatography. Recombinant protein expression was verified by Coomassie Blue staining and Western blot analysis of extracts using commercially available polyclonal antibodies to RAP74 (sc-235x; Santa Cruz Biotechnology), RAP30 (sc-236), TBP (sc-225), and TFIIB (sc-204) followed by chemiluminescent detection using the WesternLight system (Tropix, Bedford, MA). The RAP74, RAP30, and TFIIB eukaryotic expression constructs were generated by directional subcloning of cDNA fragments into the pcDNA3 (Invitrogen, Carlsbad, CA) polylinker downstream of the CMV promoter. All plasmids were purified by Qiagen column chromatography for co-transfection studies (Qiagen, Chatsworth, CA). The inserts were verified by DNA sequencing (ABI Prism Dye Terminator Cycle Sequencing Kit; Perkin Elmer, Foster City, CA) and analytic restriction enzyme digestion.

Interaction Blot Assays. "Far Western" analyses of protein−protein interactions between Msx2 and recombinant RAP74, RAP30, TFIIB, and TBP were performed using the interaction blotting technique and vectors of Blanar and Rutter as described in detail (*21*). Briefly, recombinant Msx2 fusion proteins were generated by cloning PCR fragments of Msx2 cDNA coding sequence downstream of the GST−protein kinase A peptide consensus (in continuous open reading frame) at the *Eco*RI site of vector pGEX2T[128/129] (ref 21; kind gift of Dr. M. Blanar, Bristol-Myers-Squibb Pharmaceutical Research Institute, Princeton, NJ). Inserts were sequenced to verify orientation and identity. Recombinant protein was isolated from transformed *Escherichia coli* extracts after IPTG induction by affinity purification over glutathione-agarose and elution with 25 mM glutathione in 0.1 mM DTT/50 mM Tris, pH 8.0. For generating radiolabeled Msx2 proteins, recombinant GST fusion proteins were phosphorylated with bovine heart protein kinase A and γ-[^{32}P]-ATP while bound to the affinity resin, followed by rewashing and subsequent elution in glutathione-Tris buffer with 0.1% SDS. For interaction blots, extracts from control transformed cells or JM109(DE3) *E. coli* expressing recombinant RAP74, RAP30, TFIIB, or TBP were resolved by denaturing polyacrylamide gel electrophoresis (*21*) and transferred to nitrocellulose as described using a Novex blot module (Novex, San Diego, CA). Nitrocellulose-bound proteins were renatured by serial dilution from buffered guanidine hydrochloride (25 mM Hepes, pH 7.9; 25 mM NaCl; 5 mM $MgCl_2$; 6 M guanidine hydrochloride), blocked, and subsequently probed overnight at 4 °C with either radiolabeled recombinant GST, GST-Msx2(55−208), or GST-Msx2(200−267) following the Blanar and Rutter protocol (0.1 μg of labeled protein/mL, 2×10^5 cpm/mL; ref 21). After washing for 1 h at 4 °C, membranes were blotted dry on chromatography paper, covered with polyvinyl chloride wrap, and then exposed to X-ray film overnight at room temperature using an intensifying screen.

RESULTS

Msx2 Residues 97−182 Encompass the Core Transcriptional Suppressor Domain. We previously demonstrated that Msx2 suppresses the rat OC promoter in MC3T3-E1 calvarial osteoblasts (*7*). We wished to identify those structural features of Msx2 that convey transcriptional suppression of OC. Immature MC3T3-E1 calvarial osteoblasts provide a relevant and convenient cellular background for such studies, since Msx2 affects calvarial osteoblast maturation *in vivo* (*4*, *5*) and MC3T3-E1 cells express very little endogenous Msx2 (*7*). Therefore, we analyzed the capacity of systematically truncated Msx2 molecules to suppress the OC promoter (luciferase reporter) in MC3T3-E1 cells. All Msx2 molecules were tagged with the 8-amino acid FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; ref 19) immediately following an initiator Met residue in good Kozak consensus (*29*). This epitope tag permits assessment of Msx2 variant expression and subcellular localization by Western Blot analysis using the commercially available M2 monoclonal antibody (see Experimental Procedures; ref 19). Engineered Msx2 variants were subcloned into pcDNA3 for eukaryotic expression from the CMV promoter (Figure 1), then co-transfected with the osteoblast-specific 0.2 kb rat OC promoter−luciferase reporter construct 222 OCLUC, using CMV−β-galactosidase as an internal control for transfection efficiency. As shown in Figure 2A, the N-terminal domain of Msx2 (residues 2−54; numbered as in ref 20) is not required for OC suppression; the maximal suppression of the OC promoter produced by full-length Msx2, denoted Msx2(2−267), can be achieved by Msx2(55−267). Removal of the 59 C-terminal amino acid residues of Msx2 slightly reduces suppressor activity as revealed by comparison of Msx2 variants Msx2(55−267) and Msx2(55−208) (Figure

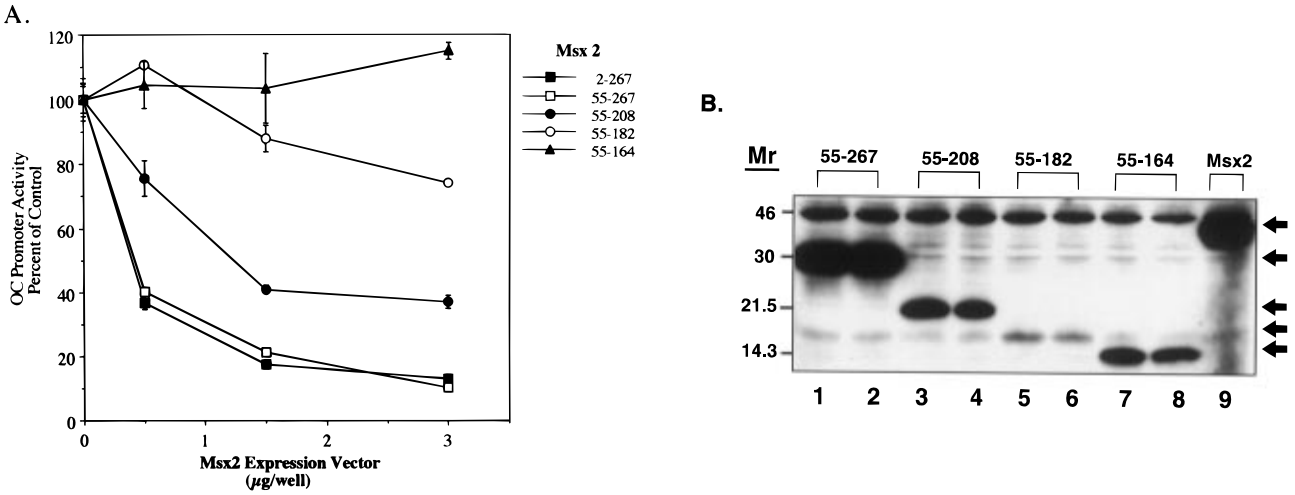


FIGURE 2: Mapping suppressor function in truncated Msx2 variants. Systematically truncated, FLAG-tagged Msx2 variant expression constructs (Figure 1) were co-transfected with the 0.2 kb rat OC promoter−luciferase reporter 222 OCLUC (1.5 μg/well) and CMV−β-galactosidase (0.1 μg/well) in MC3T3-E1 calvarial osteoblasts as described in Experimental Procedures. After 3 days, cells extracts were prepared and aliquots assayed for luciferase (OC promoter activity) and β-galactosidase (transfection efficiency) activities, as well as for Msx2 protein expression by Western blot with M2 anti-FLAG antibody. (A) Activity of Msx2 truncation mutants in OC promoter suppression assay. Results are expressed as percent of OC promoter activity in absence of Msx2 expression. Data points represent the mean (+/− range) of two independent transfections normalized for β-galactosidase activity. Data are presented as a dose−response curve, showing 0.2 kb OC promoter activity as a function of increasing amounts of input Msx2 variant expression construct. (B) Analysis of Msx2 variant expression in transiently transfected MC3T3-E1 cells. Western blots were performed on extracts from cells transfected with the highest level of Msx2 expression plasmid (3 μg/well) using M2 anti-FLAG antibody. The relative mobility of molecular weight standards are shown on the left, and the position of Msx2 truncation variants are indicated by arrows on the right. Subcellular fractionation reveals that Msx2(55−164) does not localize to the nucleus (not shown). See text for details and Bell et al. (ref 20) for murine Msx2 protein sequence.

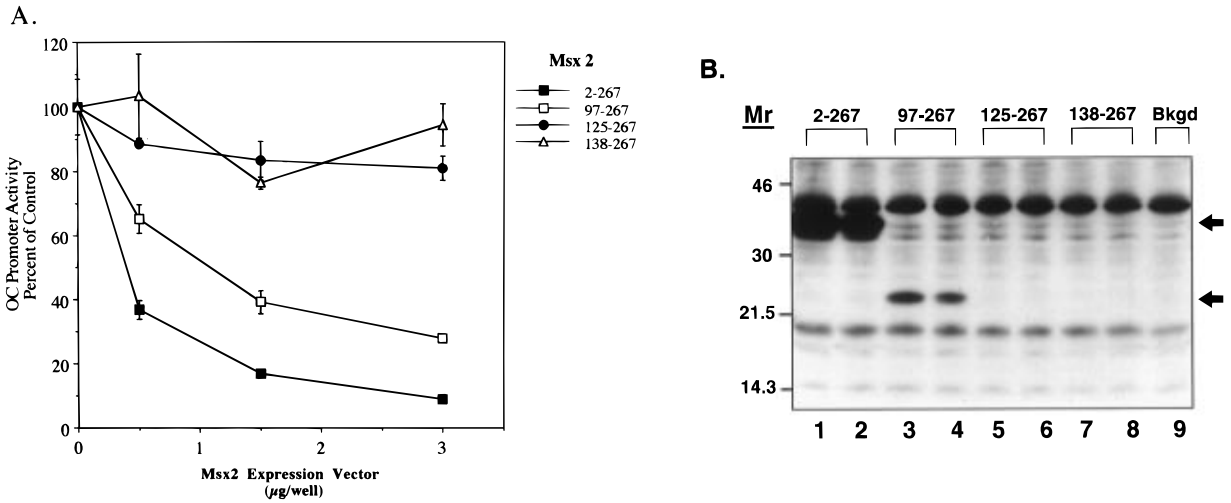
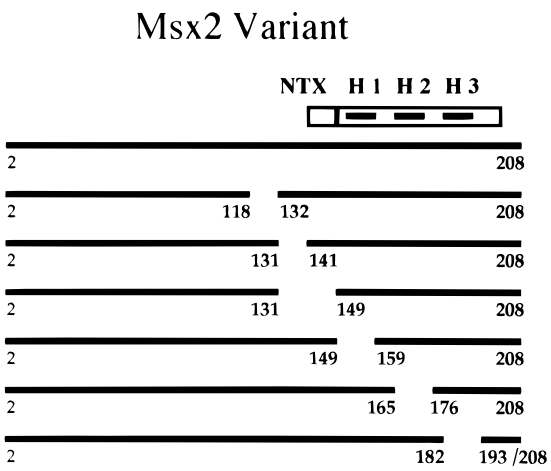


FIGURE 3: N-terminal residues 2−96 of Msx2 are dispensible for suppression of the OC promoter. Full-length and N-truncated, FLAG-tagged Msx2 expression constructs (Figure 1) were analyzed as suppressors of the OC promoter in MC3T3-E1 cells as detailed in the legend to Figure 2 and in the text. (A) Activity of Msx2 mutants in OC promoter suppression assay. Results are expressed as percent of OC promoter activity in the absence of Msx2. Data points represent the mean (+/− range) of two independent transfections normalized for β-galactosidase activity. (B) Accumulation of N-terminally truncated Msx2 variants in MC3T3-E1 cells. Extracts from cells transfected with the highest level of Msx2 expression plasmid (3 μg/well) were subjected to Western blot analysis with M2 anti-FLAG antibody. Arrows mark the position of Msx2(2−267) and Msx2(97−267) variants. Note that Msx2(125−267) and Msx2(138−267) proteins do not accumulate to detectable levels.

2A). However, this reflects slightly lower levels of Msx2-(55−208) protein accumulation, as revealed by M2 anti-FLAG Western blot analyses of extracts from transfected cells (Figure 2B; compare lanes 3 and 4 with lanes 1 and 2). Identical results are obtained when C-terminal domain residues 209−267 are deleted in the context of full-length Msx2 or Msx2(97−267) (data not shown). Msx2(55−182) is much less active, suppressing OC promoter activity at most only 25% (Figure 2A), and accumulates to lower levels in cells (Figure 2B, lanes 5 and 6). Msx2(55−164) completely lacks suppressor function (Figure 2A), even though Msx2-(55−164) protein accumulates in whole cell extracts to levels equivalent to Msx2(55−208) (Figure 2B; compare lanes 7 and 8 vs lanes 3 and 4). Subcellular fractionation and Western blot analyses reveal that, unlike the other Msx2 variants, Msx2(55−164) does not enter the nuclear fraction but accumulates in the cytoplasmic compartment (data not shown; *vide infra*). N-terminal truncation to residue 97 has little effect on suppressor function; Msx2(97−267) is ca. 80% as active as full-length Msx2 (Figure 3A), with reduced suppressor activity corresponding to decreased Msx2(97−267) protein accumulation in cells (Figure 3B). N-terminal truncation beyond amino acid 97, as in Msx2(125−267) and Msx2(138−267), gives rise to Msx2 variant proteins that do



Osteocalcin Promoter Suppressor Activity, %Msx2 [2-208] (+/- s.d.)	Expression
100 (+/- 12)	++
95 (+/- 2)	++
98 (+/- 9)	++
33 (+/- 4)	++
116 (+/- 14)	+
76 (+/- 10)	+
78 (+/- 4)	+

FIGURE 4: Msx2 residues 132−148 encompassing the homeodomain N-terminal extension are required for OC promoter suppressor function; homeodomain helices are dispensible. Expression constructs for internally deleted FLAG-tagged Msx2(2−208) variants (3 μg/well) were co-transfected with the 0.2 kb rat OC promoter−luciferase reporter 222 OCLUC (1.5 μg/well) and CMV−β-galactosidase (0.1 μg/well) in MC3T3-E1 calvarial osteoblasts as described in Experimental Procedures and the legend to Figure 2. After 3 days, cells extracts were prepared and aliquots assayed for luciferase (OC promoter activity) and β-galactosidase (transfection efficiency) activities as well as for Msx2 protein expression by Western blot with M2 anti-FLAG antibody. Results are expressed as the suppressor activity of each internally deleted variant relative to Msx2(2−208). Data represent results obtained (+/− standard deviation) from three independent transfections normalized for β-galactosidase activity. Expression of Msx2 variant proteins was monitored by Western blot analysis as described in Figures 2 and 3. Note that while deletion of residues 119−131 and 132−140 has little effect on suppressor function, deletion of residues 132−148 markedly decreases suppressor function. By contrast, deletion into the three homeodomain helices (150−158; 166−175; 183−192) either has no effect (helix 1) or only slightly hinders (helix 2 or helix 3) Msx2 suppressor function. See text for details and ref 20 for murine Msx2 protein sequence. NTX, homeodomain N-terminal extension; H1, homeodomain helix 1; H2, homeodomain helix 2; H3, homeodomain helix 3, DNA recognition helix.

not accumulate to detectable levels in MC3T3-E1 cells (Figure 3B, lanes 5−8), and thus do not significantly suppress OC promoter activity (Figure 3A). Thus, combined analyses of N- and C-terminal truncation data reveal that residues 2−96 and 209−267 are dispensible for Msx2 suppressor function. Residues 97−208 encode the core suppressor domain of Msx2, overlapping the homeodomain encoded by residues 142−201 (see ref 20).

Msx2 Suppressor Function Is Dependent Upon Residues That Include the Homeodomain N-Terminal Extension and Not Upon Residues in the Three Homeodomain Helices. Western blot analyses indicate that C-terminal truncation beyond residue 208 decreases accumulation of nuclear Msx2 protein in MC3T3-E1 calvarial osteoblasts (*vide supra*). We suspected that the basic amino acid stretch Arg-Arg-Ala-Lys-Ala-Lys-Arg at residues 193−199 downstream of helix 3 within the homeodomain functions as a nuclear localization signal (*34*). Therefore, to dissect structural features of the Msx2 core suppressor domain contributing to suppressor activity, a series of Msx2 internal deletion mutants were constructed that leave these basic residues intact within the context of the larger protein Msx2(2−208). This series of Msx2 internal deletion mutants were then analyzed in the OC promoter suppression assay. The Msx2 expression vectors for these experiments were co-transfected at the 3 μg "dose" that gives maximal suppression (see Figures 2 and 3). As shown in Figure 4, internal deletion of Msx2 residues 119−131 or residues 132−140 has little effect on core suppressor function. However, a deletion that removes Msx2 residues 132−148 markedly decreases Msx2 suppressor function (by 70%), without hindering accumulation of Msx2 protein (Figure 4); this deletion removes the Msx2 homeodomain N-terminal extension ("NTX" in Figure 4; ref 20). By contrast, individual deletion of the three homeodomain helices either has no effect (helix 1) or only slightly hinders (helix 2 or helix 3) Msx2 suppressor function. All internal deletion Msx2 variant proteins accumulated to readily detectable levels in these experiments, as determined by Western blot analyses with anti-FLAG antibody (Figure 4). Thus, residues 132−148 of the Msx2 core suppressor domain play an important role in core suppressor function, residues that include the Msx2 homeodomain N-terminal extension (Figure 4, ref 20).

Suppression of the Rat OC Promoter by Msx2 Does Not Require Msx2 DNA Binding Activity. Residues in both the homeodomain N-terminal extension and helix 3 are known to contribute to DNA binding (for a review see refs 35 and 36; see also Figure 5). The point mutant Msx2(T147A) completely lacks DNA binding activity as assayed by gel shift analysis of *in vitro* transcribed−translated protein (Figure 5A, lane 3). By contrast, Msx2(P148H) has enhanced DNA binding activity relative to wild-type Msx2 (Figure 5A, lane 2 vs lane 1), in good agreement with recent data published by Maxson and co-workers (*36*). C-terminal truncation of Msx2 to residue 208 does not hinder DNA binding (Figure 5A, lane 4), while truncation through helix 3 completely abrogates DNA binding activity (Figure 5A, lane 5). Equivalent amounts of all Msx2 variants are made by *in vitro* transcription−translation, as indicated by Western blot analysis using anti-FLAG M2 antibody (Figure 5B, lanes 1−5). The internal deletion and C-terminal truncation analyses described above suggest that the DNA binding activity of Msx2 is not required for suppressor function, since deletion of the recognition helix 3 (residues 183−192; H3 in Figure 4) does not abrogate suppressor function. To further verify that Msx2 suppression of OC expression is independent of Msx2 DNA binding activity, we carefully compared the OC suppressor activities of Msx2 vs Msx2-(T147A) and Msx2(P148H) point mutants. Although the three Msx2 variants differ dramatically in their DNA binding activities (Figure 5A, lanes 1−3), their dose−response curves as OC suppressors are virtually superimposable (Figure 6);

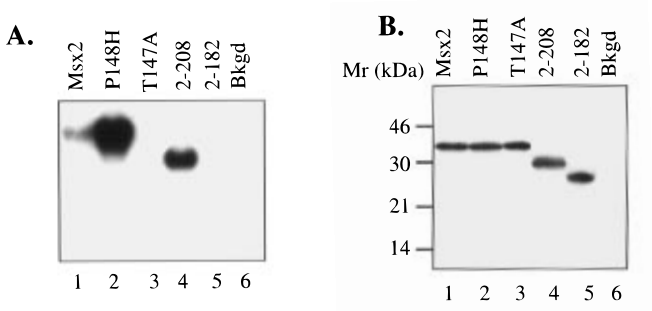


FIGURE 5: DNA binding activity of Msx2 variants produced by *in vitro* transcription/translation. Msx2 variants were expressed *in vitro* from pcDNA3 expression plasmids (Figure 1) by coupled *in vitro* transcription−translation as described in the text, then assessed for Msx2 DNA binding activity by gel shift assay (nondenaturing gel electrophoresis) and Msx2 protein expression by Western blot analysis (denaturing gel electrophoresis). (A) Binding of Msx2 variants to OCTA 26 homeodomain DNA cognate. Aliquots (5 μL) of transcribed−translated Msx2 variants analyzed by gel shift assay using radiolabeled OCTA 26 duplex oligo as described in Experimental Procedures. Lane 6 (Bkgd) contains extract from a transcription−translation reaction performed with empty pcDNA3 vector. Free (unbound) radiolabeled probe has been run off the bottom of the gel, since prolonged electrophoresis is required to emphasize differences in the sizes of DNA binding complexes formed by Msx2 and Msx2(2−208) in native gels. Note that Msx2(P148H) (lane 2) binds OCTA26 to a greater extent than wild-type Msx2 (lane 1). Scatchard analyses indicate that Msx2 binds OCTA26 with an apparent K_d of 12 nM and Msx2(P148H) with an apparent K_d of 4 nM (not shown). Note also that Msx2(T147A) (lane 3) does not bind OCTA26 at all. C-terminal truncation downstream of the homeodomain does not inhibit DNA binding (lane 4), while truncation that removes the DNA recognition helix completely abrogates DNA binding (lane 5). No DNA binding activity is noted in the absence of Msx2 protein expression (lane 6). (B) Relative protein expression of *in vitro* transcribed−translated Msx2 variants. Aliquots of *in vitro* transcribed−translated Msx2 variants (20 μL per lane) were analyzed by Western blot analysis using M2 anti-FLAG antibody. Note that the inability of Msx2(T147A) and Msx2-(2−182) (panel A, lanes 3 and 5) to bind DNA is not due to lack of expression of these variants (panel b, lanes 3 and 5). Note also that no immunoreactive protein is detected in reticulocyte lysate in the absence of *in vitro* transcribed−translated FLAG-tagged Msx2 protein (lane 6). See text and Figure 1 for for details.

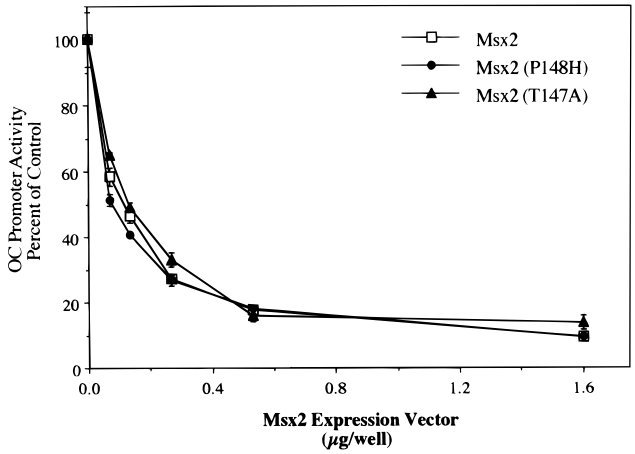


FIGURE 6: Msx2, Msx2(P148H), and Msx2(T147A) suppress the OC promoter equivalently. Full-length, FLAG-tagged Msx2 expression constructs encoding P148H and T147A point mutants were generated as described in Experimental Procedures. Msx2, Msx2-(T147A), and Msx2(P148H) were then assayed for OC promoter suppressor activity as described in the legend to Figure 2. Data points represent the mean (+/− range) of two independent transfections normalized for β-galactosidase activity. Results are presented as a dose−response curve, showing the effect of increasing amounts of Msx2 variant on 0.2 kb OC promoter activity, expressed as a percent of OC promoter activity in the absence of Msx2. Note that while Msx2(P148H) and Msx2(T147A) exhibit increased and decreased (none) intrinsic DNA binding activities, respectively, relative to wild-type Msx2 (Figure 5A), all three variants suppress OC promoter activity equally well. Western blot analyses performed with extracts from transfected cells indicate that these three FLAG-tagged Msx2 variants accumulate to equivalent levels in MC3T3-E1 cells (not shown).

at lower levels of Msx2 protein expression, Msx2(P148H) is only slightly better as a transcriptional suppressor than the nonbinding Msx2(T147A) variant. Maximal OC promoter suppression is not different among the three Msx2 variants. Similar results are obtained in transfected cultures of primary rat calvarial osteoblasts, and Western blot analyses of cell extracts with M2 anti-FLAG antibody show that Msx2, Msx2(T147A), and Msx2(P148H) proteins accumulate to equivalent levels in these titrations (data not shown). Thus these data directly demonstrate that Msx2 suppression of the OC promoter is independent of intrinsic Msx2 DNA binding activity even though one functionally mapped suppressor domain overlaps the homeodomain.

The Core Suppressor Domain of Msx2 Interacts with TFIIF but Not TFIIB or TBP in the Basal Transcriptional Apparatus. The Msx2 point mutant Msx2(T147A) that completely lacks DNA binding activity is indistinguishable from wild-type Msx2 in its ability to suppress the OC promoter (Figure 6), demonstrating that direct interaction with DNA is not required for Msx2 suppressor function. These data suggest that Msx2 modulates transcription primarily via protein−protein interactions, either in concert with co-regulatory DNA binding proteins or through direct interaction with components of the basal transcriptional machinery. We developed the working hypothesis that Msx2 inhibits OC expression in calvarial osteoblasts by antagonizing transcriptional activation of the basal transcriptional machinery (*22*). Using the interaction "Far Western" blotting technique (*21*), we systematically tested for protein−protein interactions between Msx2 and recombinant TBP, TFIIB, and TFIIF, components of the basal transcriptional machinery known to convey transcriptional activation signals (*22*, *24*, *35*−*39*). This interaction blotting technique uses ^{32}P-labeled recombinant protein to probe proteins renatured on nitrocellulose membranes after resolution by denaturing polyacrylamide gel electrophoresis. As shown in Figure 7A, [^{32}P]-Msx2(55−208) binds recombinant RAP74 and RAP30 (lanes 2 and 6; components of TFIIF; refs 22 and 40), but not TFIIB or TBP (Figure 7A, lanes 3 and 4, respectively). By contrast, the C-terminal domain residues 200−267 of Msx2—residues not required for suppressor function (Figure 2)—does not bind TFIIF components (Figure 7A, lanes 8 and 10). Radiolabeled [^{32}P]GST also does not bind any of these recombinant proteins (Figure 7A, lanes 11−16). Recombinant RAP74, RAP30, TFIIB, and TBP protein expression was verified by Western blot analysis (Figure 7B). Recombinant RAP74 is unstable; besides full-length RAP74, a number of immunoreactive RAP74 degradation products are visualized (Figure 7B, lane 1), several of which also bind [^{32}P]Msx2-(55−208) (Figure 7A, lane 2, and data not shown). Thus, Msx2(55−208) encompasses the core suppressor domain and can direct specific protein−protein interactions with RAP74 and RAP30 but not with TFIIB or TBP.

Residues 132−148 of Msx2(55−208) Are Required for Msx2 Interaction with TFIIF Components, while Residues 119−131 Are Not. We wished to test if residues 132−148

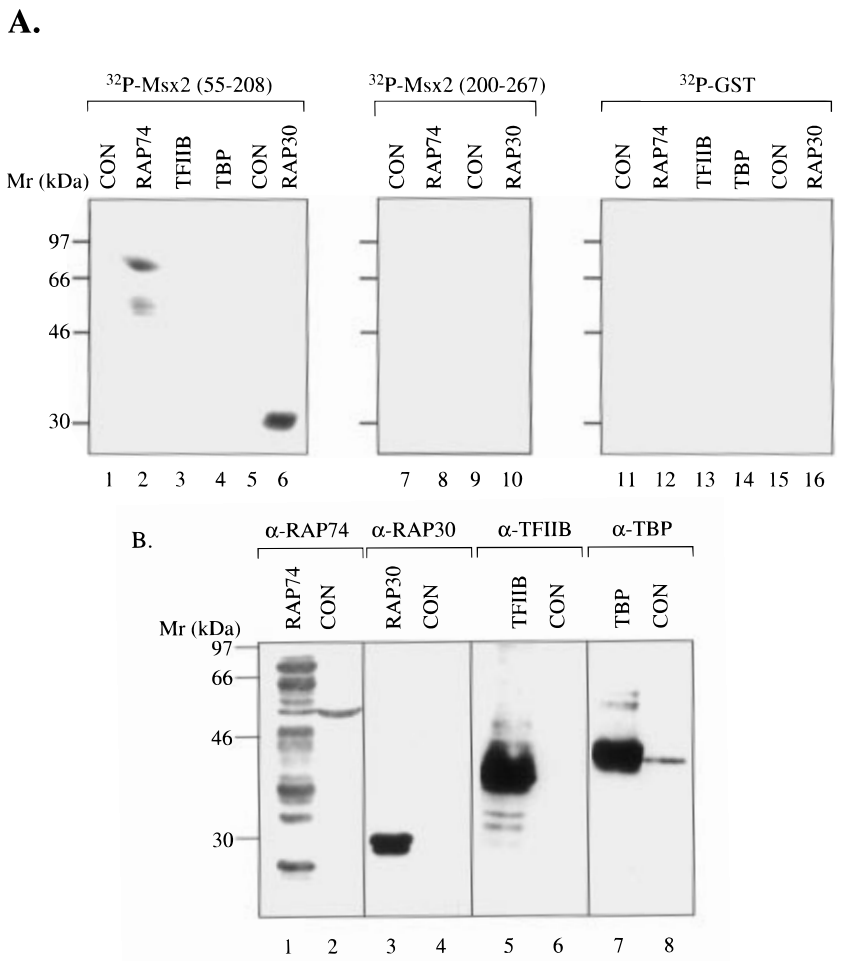


FIGURE 7: Msx2 interacts with RAP74, a component of the basal transcriptional machinery. Recombinant RAP74, RAP30, TFIIB, and TBP were expressed in *E. coli* and partially purified as described in Experimental Procedures. Extracted proteins were resolved by denaturing polyacrylamide gel electrophoresis, then analyzed by interaction "Far Western" blot (with radiolabeled Msx2 proteins; panel A) and by Western blot (with commercially available antibodies; panel B) as described in Experimental Procedures. (A) [^{32}P]Msx2(55−208) interacts with recombinant RAP74 (lane 2) and RAP30 (lane 6), but not with TFIIB(lane 3) or TBP(lane 4). Little endogenous Msx2 binding protein is observed in extracts derived from cells transformed with empty expression vector (lanes 1 and 5). By contrast, [^{32}P]Msx2(200−267) (lanes 7−10) and [^{32}P]GST (lanes 11−16) do not bind these recombinant proteins. (B) Western blot analyses were carried out on aliquots of recombinant protein extacts using commercially available antibodies to RAP74 (lanes 1 and 2), RAP30 (lanes 3 and 4), TFIIB (lanes 5 and 6), and TBP (lanes 7 and 8). Lanes 2, 4, 6, and 8: Western blot data using control *E. coli* protein extract. Lanes 1, 3, 5, and 7: Western blot data using partially purified protein extracted from *E. coli* expressing RAP74, RAP30, TFIIB, and TBP, respectively. Note that all four recombinant proteins were expressed. Further note that recombinant RAP74 is unstable; along with full-length RAP74, a number of RAP74 degradation products were immunovisualized (panel B, lane 1). Some of these breakdown products also bind [^{32}P]Msx2 (55−208) (panel A, lane 2). See text for details.

in the core suppressor domain that are required for full suppressor function (Figure 4) are also required for interactions with TFIIF. Therefore we tested whether deletion of residues 132−148 inhibited Msx2 binding to recombinant RAP30 and RAP74 in the interaction blot assay. A second Msx2 mutant lacking residues 119−131 was included in these analyses as a control; these residues are dispensible for Msx2 suppressor function (Figure 4). As shown in Figure 8, deletion of Msx2 residues 119−131 as in Msx2(55−208; Δ119−131) has no effect on TFIIF subunit binding; this internal deletion variant binds RAP74 and RAP30 as well as Msx2(55−208) (Figure 8, compare lanes 2 and 3 with lanes 7 and 8). By contrast, deletion of Msx2 residues 132−148 as in Msx2(55−208; Δ132−148) completely abrogates binding to RAP74 and RAP30 (Figure 8, lanes 12 and 14). Thus, the Msx2 residues responsible for the majority of core suppressor domain activity (Figure 4) are also required for interaction with TFIIF components (Figure 8).

Co-Expression of RAP74 Partially Reverses Msx2 Suppression of the OC Promoter in MC3T3-E1 Cells. We wished to verify the functional consequences of Msx2-TFIIF interactions in cells. If Msx2 suppression occurs at least in part via protein−protein interactions that sequester TFIIF components, over-expression of functionally important components is predicted to reverse Msx2 suppression of the OC promoter. RAP74, RAP30, and TFIIB (a control) were subcloned into pcDNA3 to permit expression in eukaryotic cells from the CMV promoter, and co-transfected with pcDNA3-Msx2 (suppressor), 222 OCLUC (reporter), and CMV−β-galactosidase (internal control for transfection efficiency) in MC3T3-E1 cells. As shown in Figure 9A, co-expression of RAP74 in MC3T3-E1 osteoblasts partially reverses Msx2 suppression of the OC promoter. By contrast, co-expression of RAP30 or TFIIB pcDNA3 expression constructs has no significant effect on Msx2 suppression of the OC promoter (Figure 9A). Reversal of suppression is dose-dependent with respect to the amount of pcDNA3-RAP74 expression construct co-transfected (Figure 9B). Thus, a significant portion of OC promoter suppression by Msx2 can be reversed by over-expression of the TFIIF

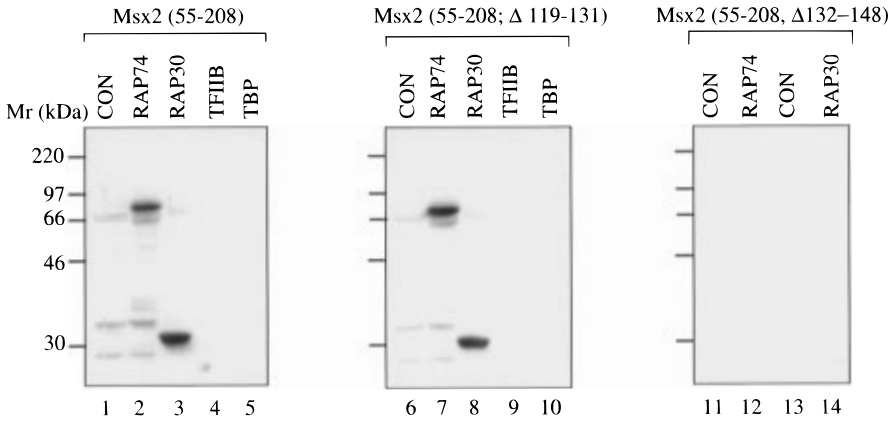


FIGURE 8: Msx2 residues 132−148 are required for interaction with RAP74 and RAP30. Recombinant RAP74, RAP30, TFIIB, and TBP were expressed in *E. coli* and partially purified, resolved by denaturing polyacrylamide gel electrophoresis, and then analyzed by interaction "Far Western" blot as described in the legend to Figure 7 and in Experimental Procedures. [^{32}P]Msx2(55−208) interacts with recombinant RAP74 (lane 2) and RAP30 (lane 3) but not with TFIIB (lane 4) or TBP (lane 5). Deletion of residues 119−131 does not inhibit interaction with either RAP74 or RAP30 (lanes 7 and 8); however, deletion of residues 132−148 abrogates interactions between Msx2 and the two subunits of TFIIF (lanes 12 and 14). Note that this same region is required for Msx2 suppressor function (Figure 4). See text for details.

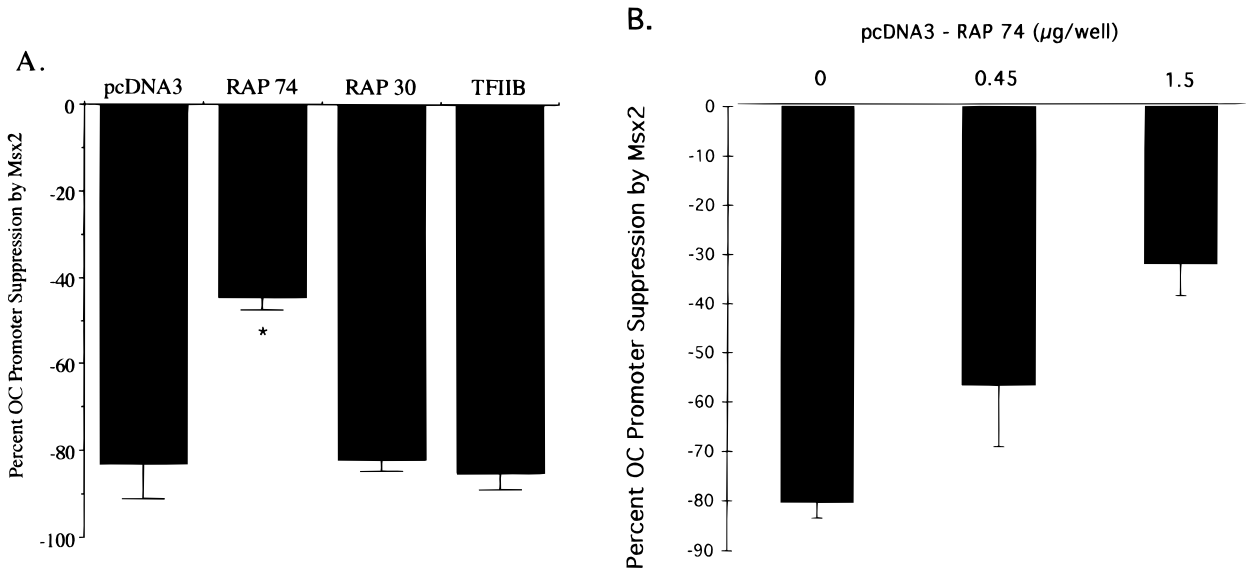


FIGURE 9: Co-expression of RAP74 partially reverses Msx2 suppression of the OC promoter in MC3T3-E1 calvarial osteoblasts. (A) MC3T3-E1 cells were co-transfected with the 0.2 kb rat OC promoter−luciferase reporter 222 OCLUC (1.5 μg/well), CMV−β-galactosidase (0.1 μg/well), pcDNA3-Msx2 (0.5 μg/well), and 1.6 μg/well of either pcDNA3, pcDNA3-RAP74, pcDNA3-RAP30, or pcDNA3-TFIIB as indicated. OC promoter activity was then assessed as described in Experimental Procedures and the legend to Figure 2. Data are presented as percent OC promoter suppression (+/− standard deviation) of three independent transfections. Note that only co-expression of RAP74 (asterisk; $P < 0.01$) significantly reversed OC promoter suppression by Msx2. (B) MC3T3-E1 cells were co-transfected with the 0.2 kb rat OC promoter−luciferase reporter 222 OCLUC (1.4 μg/well), CMV−β-galactosidase (0.1 μg/well), pcDNA3-Msx2 (0.7 μg/well), and increasing amounts of pcDNA3-RAP74 as indicated. Empty pcDNA3 vector was added to keep total DNA constant in all transfections. Data presented are mean values (+/− standard deviation) of six independent transfections and are expressed as percent suppression of control 222 OCLUC activity. Note that co-transfection with CMV-RAP74 partially reverses Msx2 suppression of the OC promoter in a dose-dependent fashion.

component RAP74, indicating that one component of Msx2 suppressor activity in MC3T3-E1 calvarial cells is mediated via a RAP74-dependent process.

DISCUSSION

Very few studies have examined in detail the regulation of physiologically relevant vertebrate gene targets by homeodomain suppressors. Most analyses published to date detail promoter−homeodomain suppressor interactions defined as important by *Drosophila* genetics (*41*−*44*). The bone-specific osteocalcin gene represents an important, physiologically relevant target for Msx2 action in differentiating calvarial osteoblasts (*4*, *5*, *7*−*9*). We have performed systematic structure−function analyses of the Msx2 protein, using OC promoter suppression in MC3T3-E1 calvarial osteoblasts as an assay. We have identified structural features of Msx2 that participate in OC promoter suppression (summarized in Figure 10). The core suppressor domain encoded in Msx2 residues 97−208 overlaps the DNA binding homeodomain (*20*). This core suppressor domain participates in the formation of functionally important interactions with RAP74 that regulate OC expression in osteoblasts, dependent upon residues 132−148 that include the homeodomain N-terminal extension (*20*) but independent of the homeodomain DNA binding activity. To our knowledge, these data provide the first report of a transcriptional suppressor interacting with TFIIF components. Although interactions were detected between Msx2 and both TFIIF

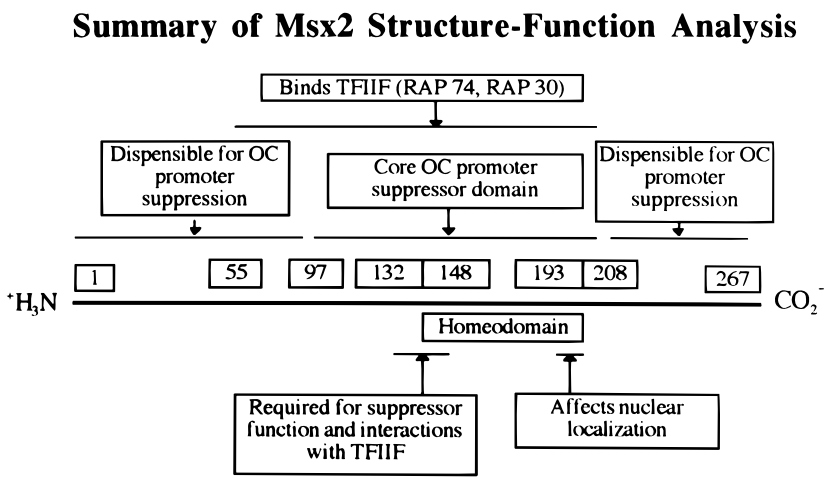


FIGURE 10: Summary of Msx2 structure−function analysis. Numbering of amino acid residues for mouse Msx2 is as outlined by Bell et al. (ref 20). The 60 amino acid homeodomain corresponds to Msx2 residues 142−201. See text for discussion.

subunits, only co-expression of RAP74 partially reversed Msx2 suppression; this may reflect differences in the stoichiometry of endogenous RAP74 and RAP30 expression in MC3T3-E1 osteoblasts or differences in TFIIF subunit function (*24*, *38*, *40*, *45*, *46*) that influence transcriptional responses to Msx2 in cells. The precise amino acid residues participating in protein−protein interactions between Msx2-(55−208) and RAP74 have yet to be defined. Of note, removal of Msx2 residues 132 to 148 markedly decreases but does not completely inactivate core suppressor function; it will be of future interest to identify whether other domains in Msx2 contribute to interactions between Msx2 and the transcriptional machinery assembled by the OC promoter (*2*, *7*, *11*, *25*). The interaction blotting technique can detect and identify domains of interaction between proteins but does not generally permit detailed mapping of multiple contacts occurring at an interaction interface (*21*). However, as done for analysis of Dr1 (*47*, *48*), a two-hybrid system may prove useful in the future to help provide a high-resolution map of the interface between Msx2 and RAP74, and to identify other domains in Msx2 that contribute to or stabilize these interactions.

The basal transcriptional apparatus of TATA box dependent promoters is a large multiprotein complex of 40−50 subunits, organized about the transcriptional initiation site by TATA binding protein (TBP), the core constituent of TFIID (*22*). TBP organizes TFIIA, TFIIB, and TFIIF in steps necessary for subsequent RNA polymerase II recruitment. Transcriptional activators—such as DNA binding transcription factors or co-regulator TAF molecules—upregulate gene expression via protein−protein interactions with the basal machinery (*22*). TFIIB and TBP are well described as targets for transcriptional activation by multiple transcription factors (*22*, *37*). Recently, however, RAP74 has also been identified as a target for transcriptional activation by SRF, TAFII250, and AP1 (*24*, *38*, *49*, *50*). This is intriguing, since RAP74 may not be required for transcription initiation for all promoters (*45*, *46*). Indeed, the minimal transcriptionally competent core complex consists of TBP, TFIIB, RAP30, and RNA polymerase II (*46*). As recently proposed (*38*), these observations suggest that RAP74 may mediate transcriptional activation in response to specific subtypes of transcriptional activators and promoters. Not all promoters are inhibited by Msx2 (e.g., CMV promoter; see also refs 7−10). Therefore, protein−protein interactions between Msx2 and RAP74 may decrease promoter activity supported by activators that target RAP74, but not affect promoter activity supported by activators that target components of the minimal basal promoter (*22*). We propose that transcriptional suppression of the basal OC promoter by Msx2 is mediated at least in part by Msx2 antagonizing interactions between RAP74 and factors that support OC promoter activity in osteoblasts (*3*, *11*, *31*). Preliminary data indicate that Msx2 selectively antagonizes a growth factor-regulated DNA binding activity that recognizes the proximal rat OC promoter; RAP74 stimulates this DNA binding activity (our unpublished observations). Once this OC promoter DNA binding protein complex is purified to homogeneity and its structural features established, it will be possible to biochemically examine its interactions with Msx2 and RAP74. Of note, the suppressor YY1 inhibits vitamin D activation of the OC promoter in part by antagonizing interactions between the vitamin D receptor and TFIIB in the basal transcriptional machinery, and in part by antagonizing vitamin D receptor DNA binding activity (*51*); the model evolving for Msx2-mediated OC promoter suppression is conceptually similar to this model of YY1-dependent regulation.

Features of OC promoter suppression by Msx2 clearly differ from features of *Drosophila* Ultrabithorax (Ubx) promoter suppression by Even-skipped (Eve), one of the best characterized promoter−homeodomain suppressor interactions described to date (*41*−*43*). Suppression is dependent upon Eve binding to the Ubx promoter (*42*, *43*). Eve suppresses gene expression via cooperative binding between upstream high-affinity binding sites and downstream low-affinity binding sites, preventing transcription initiation by inhibiting access of TBP to the TATA box (*42*−*44*). By contrast, binding of the Msx2 homeodomain to a *cis*-"HOXBOX" cognate is not requisite for suppression of the OC promoter; we show that a single homeodomain point mutation in Msx2 which completely abrogates DNA binding activity does not significantly alter Msx2 suppressor function. Mutation of the HOXBOX decreases binding of unidentified positive acting transcription factors that support OC promoter activity in osteoblasts (*9*, *31*); we anticipate that Msx2 will antagonize transcriptional activation by these factors (ref 7; *vide infra*).

Abate-Shen and co-workers (*52*, *53*) recently identified a subdomain of the related homeodomain protein, Msx1, as

necessary for suppression of the SV40 promoter. They demonstrated that protein−protein interactions between Msx1 homeodomain (166−225) and TBP inhibit transcription from the SV40 promoter (*53*), consistent with a report by others describing TBP as a target for suppression of SV40 promoter activity (*47*). Alanine mutations in the N-terminus of the homeodomain that decrease interaction with TBP also decrease Msx1 suppression of the SV40 promoter (*53*), independent of homeodomain DNA binding activity. Our data demonstrate that the core suppressor domain of Msx2 participates in functionally important interactions with RAP74 that contribute to transcriptional regulation of the OC proximal promoter in calvarial osteoblasts; we do not detect interactions with TBP or TFIIB. The specific transcription factors supporting the osteoblast-specific OC promoter in calvarial osteoblasts will differ from those supporting activity of viral SV40 promoter (*7*, *31*) and will likely influence the impact of transcriptional repressors. Moreover, differences between Msx2 and Msx1 proteins in the residues upstream and outside of the homeodomain (*4*, *20*) may influence interactions of these related homeodomain proteins with the basal transcriptional machinery. These notions remain to be tested directly.

Our initial report of Msx2 action in MC3T3-E1 cells described the role of the OC promoter HOXBOX motif in Msx2 action (*7*). However, the HOXBOX mutations we introduced in the OC promoter to abrogate Msx2 binding also inhibit binding of transcriptional activators that support basal OC promoter activity (*8*, *9*, *31*). Our functional analyses of the Msx2 homeodomain itself now directly demonstrate that the DNA binding activity of Msx2 protein is not required for OC promoter suppression, even though core suppressor domain function overlaps the homeodomain. No significant correlation between DNA binding activity and OC promoter suppressor activity is discernible. Even though the Msx2(P148H) homeodomain mutant binds DNA better (Figure 5 above; ref 36) than Msx2 and Msx2(T147A), it is not significantly better as a transcriptional suppressor. Of note, over-expression of either wild-type Msx2 or Msx2-(P148H) from the CMV promoter in transgenic mice gives rise to craniosynostosis (*5*), suggesting that quantitative differences in total Msx2 activity may result in this phenotype and that Msx2 activity must be temporally and spatially regulated for appropriate calvarial bone formation. It remains possible that Msx2 DNA binding activity, augmented by the Pro to His homeodomain mutation, plays an important role *in vivo*, particularly at very low levels of Msx2 protein expression. It remains to be tested whether alteration of Msx2 DNA binding activity influences its ability to control the calvarial osteoblast gene program during murine development or whether the Pro to His homeodomain mutation subtly alters subsets of protein−protein interactions during osteoblast differentiation. Intriguingly, transgenic mice overexpressing the human MSX2 gene under the control of its own MSX2 promoter exhibit *decreased* ossification and aplasia of the interparietal and hyoid bones in the skull (*54*) and no ectopic bone, consistent with the observation that Msx2 suppresses expression of the osteoblast differentiation marker, OC (*7*−*10*). These very recent data (*54*) and the data of Liu et al. (*5*) indicate that the particular calvarial cell background and the stage of osteoblast differentiation influences the biological actions of Msx2 during development. Preliminary data indicate that, besides recognizing TFIIF, Msx2 can form homodimers and bind growth factor-regulated osteoblast nuclear proteins (E. Newberry and D. Towler, unpublished; see also ref 55). Future experiments will examine if these Msx2 binding proteins are transcriptional co-regulators that modulate the activity of Msx2 during calvarial osteoblast differentiation.

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