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Antagonistic Regulation of *Dlx2* Expression by PITX2 and Msx2: Implications for Tooth Development

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The transcriptional mechanisms underlying tooth development are only beginning to be understood. Pitx2, a bicoid-like homeodomain transcription factor, is the first transcriptional marker observed during tooth development. Because *Pitx2, Msx2*, and *Dlx2* are expressed in the dental epithelium, we examined the transcriptional activity of PITX2 in concert with Msx2 and the *Dlx2* promoter. PITX2 activated while Msx2 unexpectedly repressed transcription of a TK-*Bicoid* luciferase reporter in a tooth epithelial cell line (LS-8) and CHO cell line. Surprisingly, Msx2 binds to the *bicoid* element (5′-TAATCC-3′) with a high specificity and competes with PITX2 for binding to this element. PITX2 binds to *bicoid* and *bicoid*-like elements in the *Dlx2* promoter and activates this promoter 45-fold in CHO cells. However, it is only modestly activated in the LS-8 tooth epithelial cell line that endogenously expresses Msx2 and Pitx2. RT-PCR and Western blot assays reveal that two Pitx2 isoforms are expressed in the LS-8 cells. We further demonstrate that PITX2 dimerization can occur through the C-terminus of PITX2. Msx2 represses the *Dlx2* promoter in CHO cells and coexpression of both PITX2 and Msx2 resulted in transcriptional antagonism of the *Dlx2* promoter. Electrophoretic mobility shift assays demonstrate that factors in the LS-8 cell line specifically interact with PITX2. Thus, *Dlx2* gene transcription is regulated by antagonistic effects between PITX2, Msx2, and factors expressed in the tooth epithelia.

PITX2 Dlx2 Transcriptional regulation

THE transcriptional mechanisms of tooth development are beginning to be defined and several key transcription factors have been shown to be involved in tooth morphogenesis (24,33). It is known that homeodomain proteins play a role in tooth development (19–21,24,30,32,36,37). Several of these homeobox genes have been shown to have an overlapping pattern of expression that correlates with tooth development (36). Recent evidence has demonstrated that Pitx2, Msx2, and Dlx2, all homeodomain proteins, are involved in tooth morphogenesis (11,13,19,21, 30,31,36,37).

Pitx2 is expressed very early during tooth development in the tooth bud epithelium (13,21,31). The expression of *Pitx2* is restricted to the dental epithelium and *Pitx2* transcripts can be detected as early as day 8.5 during mouse tooth morphogenesis (21,31). *Pitx2* expression remains specific to the oral epithelium with a progressive restriction to the dental placodes, followed by high-level expression in the dental lam-

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ina and enamel knot in embryonic tooth primordia. Postnatal expression is still detected in relatively undifferentiated epithelial tissue in the tooth germs, in the later developing second and third molar anlage. *Pitx2* transcripts are found in the preameloblasts, although the levels are lower, and it is absent from the fully differentiated ameloblasts (21).

Patients with Rieger syndrome present clinically with missing teeth among other anomalies (30). Rieger syndrome is an autosomal dominant human disorder associated with mutations in *PITX2* (30). The analysis of Rieger syndrome patients provided the first link of PITX2 involvement in tooth development. We have previously shown that some of the naturally occurring *PITX2* mutations associated with Rieger syndrome are defective for either DNA binding or transcriptional activation (4). Thus, the molecular basis of tooth anomalies in Rieger syndrome appear to be the inability of PITX2 to activate genes involved in tooth morphogenesis [for a review see (2)]. Taken together, these data support an early role for PITX2 in tooth morphogenesis.

Dlx2, a member of the distal-less gene family, has been established as a regulator of branchial arch development (26,34). Homozygous mutants of *Dlx2* have abnormal development of forebrain cells and craniofacial abnormalities in developing neural tissue; *Dlx* genes exhibit both sequential and overlapping expression, implying that temporospatial regulation of *Dlx* genes is tightly regulated (17). Within the mandibular and maxillary divisions of the first branchial arch, whose mesenchyme and epithelium eventually form the teeth, *Dlx2* is expressed proximally in the mesenchyme and distally in the epithelium (34). *Dlx* genes are believed to play a role in tooth morphogenesis because homozygous *Dlx1/Dlx2* mutants are missing maxillary molars (37).

A third homeobox protein, Msx2, is also implicated in the development of the teeth and other craniofacial structures (16,19,36). Msx2 is a transcriptional repressor that has been shown to bind to the Msx1 binding motif 5′-TAAT TG-3′ (28). Some evidence indicates that the repressive activity is due to protein–protein interactions rather than direct binding with DNA (22,23,41). Like other homeodomain proteins, *Msx2* expression is both spatially and temporally regulated primarily through interactions between epithelial and mesenchymal tissue (39). In the dental ectoderm, Msx2 expression overlaps with that of *Dlx2* (36).

The signaling factors that regulate *Pitx2* transcription during tooth development have been shown to include BMP4 and FGF8 (16,18,31). However, the target genes of PITX2 in tooth development are not known.

In this study, we demonstrate that the *Dlx2* promoter is a target of PITX2. The *Dlx2* promoter, with 3.8-kb upstream sequence, has been shown to contain the regulatory elements directing expression of *Dlx2* in the epithelium, but not the mesenchyme of the first arch (34). The epithelial specificity of this region is maintained in the late stages of tooth formation (15). PITX2 binds to the *bicoid* element that is present in numerous copies in the *Dlx2* promoter. Interestingly, Msx2, a repressor, also binds to the *bicoid* element and competes with PITX2 for binding. We are using a cell line derived from mouse enamel organ epithelia (LS-8) in an attempt to identify PITX2-interacting proteins and transcription factors involved in tooth morphogenesis (7). We report here that this cell line endogenously expresses Pitx2 and has previously been shown to express Msx2. We have used a PITX2 antibody to demonstrate synthesis of Pitx2 isoforms in LS-8 cells. The transcriptional activity of the *Dlx2* promoter is decreased in the LS-8 cell line transfected with PITX2 compared with CHO cells. Furthermore, Msx2 functionally antagonizes PITX2 activation of the *Dlx2* promoter. We demonstrate the existence of specific PITX2–protein complexes in LS-8 nuclear extract that may attenuate PITX2 activation of the *Dlx2* promoter in the dental epithelium.

MATERIALS AND METHODS

Expression and Purification of GST-PITX2 and GST-Msx2 Fusion Proteins

The human *PITX2* and deletion constructs were PCR amplified from a cDNA clone as described (4). The *PITX2* PCR products were cloned into the pGex6P2 GST vector (Amersham Pharmacia Biotech) as previously described (4,5). The Msx2 construct was PCR amplified from a cDNA clone provided by Dr. YiPing Chen (Department of Cell and Molecular Biology, Tulane University). The 5′ primer contained the initiation codon and a unique *Bam*HI site (5′-GCGGGATCCTACATGGCTTCTC CGACTAAAGGCGGTGAC-3′) and the 3′ antisense primer contained the termination codon and a unique *Eco*RI site (5′-CGGAATTCTTAGGATAGATGG TACATGCCATATCCAAC-3′) to facilitate cloning into the pGex6P2 GST vector. The resulting plasmid pGST-Msx2 was confirmed by DNA sequencing. The plasmids were transformed into BL21 cells. Protein was isolated as described (4). PITX2 and Msx2 proteins were cleaved from the GST moiety using 80 units of PreScission Protease (Pharmacia Biotech) per milliliter of glutathione Sepharose. Purified proteins used in the binding assays have been previously described (5). The cleaved proteins were analyzed on

SDS polyacrylamide gels and quantitated by the Bradford protein assay (BioRad).

Electrophoretic Mobility Shift Assay (EMSA) and Nuclear Extract Preparation

Complementary oligonucleotides containing a Drosophila *bicoid* site (8) and *Dlx2 bicoid* and *bicoid*-like sites with flanking partial *Bam*HI ends were annealed and filled with Klenow polymerase to generate ^{32}P-labeled probes for EMSAs, as described (38). The sequence of the sense oligonucleotide for probe *Dlx* TAATCC was 5′-gatccGCTCATGCCTGTAATCCCAGCACTCAGGg-3′ and antisense 5′-gatccCCTGAGTGCTGGGATTACAGGCATGAGCg-3′, leaving the four-base overhangs (lowercase letters), which were end filled and labeled. The sequence of oligonucleotides for probe *Dlx* TATTCC were sense 5′-gatccACCTGCCTCATTATTCCGCTGTGTGAATg-3′ and antisense 5′-gatccATTCACACAGCGGAATAATGAGGCAGGTg-3′, again leaving a four-base overhang. The Dlx2 *bicoid* and *bicoid*-like sites are underlined. Sequences of the *bicoid* probe and competitor oligonucleotides, all with flanking partial *Bam*HI ends, have been previously described (4).

Standard binding assays were performed as previously described (5). Either 80 or 160 ng of the bacterial expressed and purified PITX2 or Msx2 proteins were used in the assays. PITX2 antibody was incubated with purified protein or nuclear extract for 15 min on ice prior to addition of the probe. Approximately 3 μg of nuclear extract was used in each EMSA. The samples were electrophoresed, visualized, and quantitated as described previously, except quantitation of dried gels was performed on the Molecular Dynamics STORM PhosphoImager (4).

Nuclear extracts (NE) were prepared from LS-8 and HeLa cells using the mini extract protocol as previously described (1,3). Nuclear extracts were dialyzed against 50 mM NaCl with two changes of dialysis buffer.

RT-PCR Assays

LS-8 and CHO cells were harvested by scraping and the polyadenylated mRNA was extracted from LS8 and CHO tissue culture cells using the PolyA-Tract System 1000 (Promega, Madison, WI). RT-PCR was performed using the TAKARA RNA PCR Kit (Panvera/Takara, Madison, WI) utilizing AMV reverse transcriptase and oligo dTs as the primers. Samples of RNA were added to the system per protocol parameters. The RT and PCR cycles were performed on an Eppendorf Mastercycler Gradient Thermocycler.

PCR of the cDNA product was performed using primers specific to *Pitx2* isoforms. The antisense primer used in these studies was (5′-GATTTCTTCGCGTGTGGAC-3′) and was complimentary to the homeodomain of *Pitx2*. The sense primers for *Pitx2A* were: primer#1 (5′-ATGGAGACCAACTGCCGC-3′) and primer#2 (5′-GGAGAGGAGCAGAAAGAAAC-3′) located in the 5′ UTR. The sense primer for Pitx2C was (5′-GACTCCTCCAAACATAGACT-3′). All of these sense primers are specific to the unique N-terminus of each isoform. PCR products were evaluated on a 1% agarose gel in 1× TBE for appropriate size. CHO cells provided a negative control. Sequencing reactions were performed on samples and sequences were analyzed on an Applied Biosystems 373 Sequencer (Perkin Elmer, Foster City, CA). All RT-PCR products were sequenced to confirm their identity.

GST-PITX2 Pull Down and Western Blot Assays

Immobilized GST fusion proteins were prepared as described above and suspended in binding buffer (20 mM HEPES, pH 7.5, 5% glycerol, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% milk, and 400 μg/ml of ethidium bromide). LS-8 nuclear extract or HeLa nuclear extract (~25 μg) was added to 5 μg immobilized GST-fusion proteins or GST in a total volume of 100 μl, and incubated for 30 min at 4°C. The beads were pelleted and washed four times with 200 μl binding buffer. The bound LS-8 nuclear proteins were eluted by boiling in SDS sample buffer and separated on a 12.5% SDS-polyacrylamide gel. Approximately 20 μg of NE and 200 ng of purified PITX2 and 400 ng of Msx2 proteins were analyzed in separate Western blots. Following SDS gel electrophoresis, the proteins were transferred to PVDF filters (Millipore), immunoblotted, and detected using Pitx2 antibody P2R10 (13), Msx2 antibody (Medical and Biological laboratories Co.), and ECL reagents from Amersham.

Expression and Reporter Constructs

Expression plasmids containing the cytomegalovirus (CMV) promoter linked to the PITX2 and PITX2 truncated DNA were constructed in pcDNA 3.1 MycHisC (Invitrogen) (5). The Msx2 expression plasmid was also constructed in pcDNA 3.1 MycHisC. The TK-*bicoid*-luc reporter plasmid has *bicoid* elements (5′-gatccGCACGGCCCATCTAATCCCGTGg-3′ annealed to 5′-gatccCACGGGATTAGATGGGCCGTGCg-3′) ligated into the unique *Bam*HI site upstream of the thymidine kinase (TK) promoter in the TK-luc plasmid (4). TK-*bicoid*-luc contains four inserts: three in the sense and one in

the antisense orientation (++—+). Using the *Dlx2* promoter as a template, primers were designed to create a unique *Bam*HI site at the 5′ end of the construct and a *Hin*DIII site at the 3′ end. The sequence for the sense strand primer to create the full-length promoter construct, Dlx2-3276 Luc, was 5′-CGCGGATCCG CGCCTGGGACCAGCAGCAAG-3′. The sequence of the sense strand primer used to create the minimal promoter, Dlx2-200 Luc, was 5′-CGCGGATCCG CGGCAGTGCTTGTACAC-3′. The sequence of the antisense primer used for both constructs was 5′-TGACTAACTCTAGATAAGCTTGCAAGAACGG TCAGACC-3′. The restriction sites are underlined. The primers were then used to PCR amplify the desired region of the *Dlx2* promoter. The PCR products were then cloned into the TK-luc plasmid in which the TK promoter was deleted and replaced with the *Dlx2* constructs upstream of the luciferase gene. All constructs were confirmed by DNA sequencing. A CMV β-galactosidase reporter plasmid (Clontech) or an SV-40 β-galactosidase reporter plasmid was cotransfected in all experiments as a control for transfection efficiency.

Cell Culture, Transient Transfections, Luciferase and β-Galactosidase Assays

CHO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and penicillin/streptomycin in 60-mm dishes and transfected by electroporation. CHO and LS-8 cells were mixed with 2.5 μg of expression plasmids, 5 μg of reporter plasmid, and 0.5 μg of CMV β-galactosidase plasmid plated in 60-mm culture dishes and fed with 5% FBS and DMEM. Electroporation of CHO cells was at 360 V and 950 microfarads (μF) (Bio-Rad); cells were fed 24 h prior to transfection. LS-8 cells were cultured in DMEM supplemented with 5% FBS and penicillin/streptomycin. LS-8 cells were transfected by electroporation as previously described (7). Transfected cells were incubated for 24 h then lysed and assayed for reporter activities and protein content by Bradford assay (Bio-Rad). Luciferase was measured using reagents from Promega. β-Galactosidase was measured using the Galacto-Light Plus reagents (Tropix Inc.). All luciferase activities were normalized to β-galactosidase activity. Expression of transiently expressed PITX2 proteins has been previously demonstrated (5).

RESULTS

Msx2 Represses Transcription From a TK Promoter Containing Bicoid *Elements*

We have previously shown that PITX2 can transactivate the TK-*bicoid* artificial promoter. This promoter contains four *bicoid* elements (5′-TAATCC-3′) upstream of the TK promoter (4). Because Msx2 is expressed in the dental epithelium along with Pitx2 we asked if Msx2 might interact with PITX2 to regulate transcription from the TK-*bicoid* luciferase reporter plasmid. Surprisingly, we found that Msx2 repressed the TK-*bicoid* promoter twofold when transfected into CHO cells (Fig. 1). PITX2 transactivated this promoter fivefold in transfected CHO, COS-7, and HeLa cells (Fig. 1, and data not shown). However, in CHO cells cotransfection of both PITX2 and Msx2 did not reduce the activity of the TK-*bicoid* promoter compared with PITX2 alone at 4.5-fold (Fig. 1). Both PITX2 and Msx2 had no effect on the TK-luciferase reporter, which does not contain *bicoid* elements. Because transfection of Msx2 in CHO cells revealed a twofold repression in TK-*bicoid* transcription activity, this suggested that Msx2 might be binding to the *bicoid* element to repress transcription from the TK-*bicoid* promoter. Furthermore, Msx2 repression occurs in the absence of PITX2, indicating that these two factors do not physically interact or dimerize for repression to occur.

Msx2 Binds With a High Specificity to the Bicoid *DNA Element*

We next wanted to determine the binding specificity of Msx2 using both its reported DNA binding element (5′-TAATTG-3′) (28) and the *bicoid* probe (5′-TAATCC-3′) in an electrophoretic mobility shift assay (EMSA). Our results demonstrate that bacterial expressed and purified Msx2 binds to both elements with apparently similar activity (Fig. 2A). The purity and quality of our Msx2 protein preparation was confirmed by Western blot. However, using a panel of cold competitor oligonucleotides that correspond to other know factor binding sites, we found that all of these DNA elements can compete for Msx2 binding to the TAATTG probe (Fig. 2A). The competitor oligonucleotides containing the *Msx2* class (5′-TAA TTG-3′), *Bicoid* class (5′-TAATCC-3′), *Ftz* class (5′-TAATGG-3′), *Nkx* class (5′-CAAGTG-3′), a 5′-TAATAT-3′ element, and a 5′-TAATCA-3′ element all were able to bind Msx2 and compete for its binding to the TAATTG probe (Fig. 2A). Furthermore, we demonstrate that only a very small amount of PITX2 binds to the Msx2 probe (Fig. 2A). We have previously shown that these oligonucleotides do not compete for PITX2 binding to the *bicoid* probe (4). However, only *bicoid* and *Nkx* competitor oligonucleotides were able to slightly compete for Msx2 binding to the *bicoid* probe (Fig. 2A). All of the competitor oligonucleotides contain similar flanking sequences. The amount of competition was determined

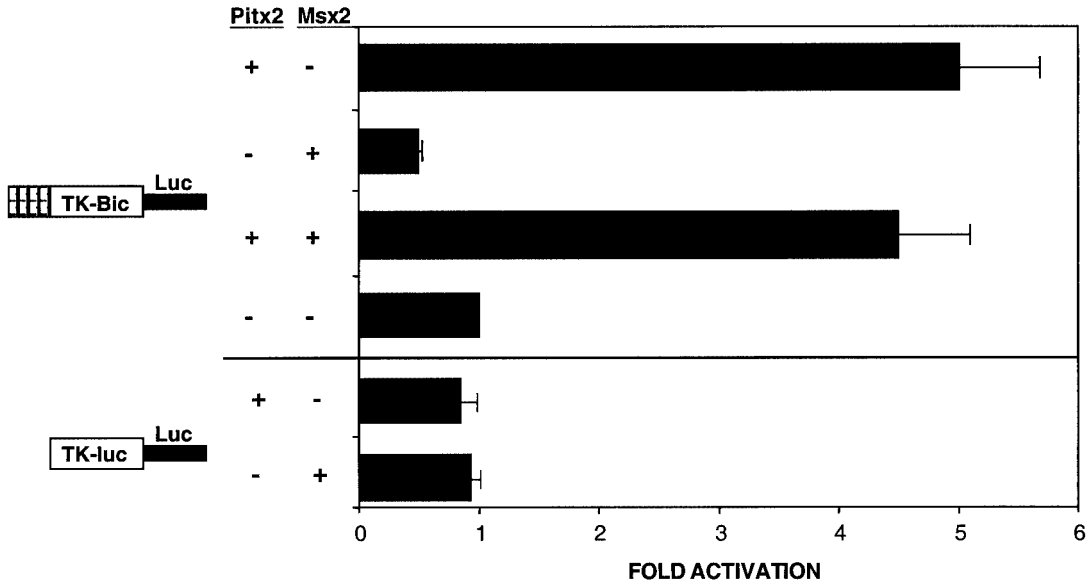

Figure 1. Transcriptional control of the TK-*bicoid* promoter by Msx2 in CHO cells. CHO cells were transfected with either the TK-*bicoid* luciferase reporter gene containing four copies of the Pitx2 binding site (dashed boxes) or the parental TK-luciferase reporter without the *bicoid* sites. The cells were cotransfected with either the CMV-Pitx2 and/or -Msx2 expression plasmids (+) or the CMV plasmid without Pitx2 or Msx2 (–). To control for transfection efficiency, all transfections included the CMV β-galactosidase reporter. Cells were incubated for 24 h, then assayed for luciferase and β-galactosidase activities. The activities are shown as mean fold activation compared with TK-*bicoid* luciferase without Pitx2 expression and normalized to β-galactosidase activity (±SEM from four independent experiments). The mean TK-*bicoid* luciferase activity with Pitx2 expression was about 40,000 light units per 15 μg protein, and the β-galactosidase activity was about 70,000 light units per 15 μg protein.

by quantitation of the bound DNA directly from the polyacrylamide gel. As a control we show that PITX2 binds to the *bicoid* probe and that mixing equal amount of PITX2 and Msx2 proteins demonstrates that both can bind to the *bicoid* probe independent of each other (Fig. 2A). Consistent with the lack of reduction of TK-*bicoid* transcriptional activity seen with the cotransfection of *PITX2* and *Msx2* in Figure 1, we show that PITX2 appears to bind preferentially over Msx2 to the *bicoid* probe (Fig. 2A). Furthermore, our experiments demonstrate that Msx2 binds to the *bicoid* probe with a higher specificity than to its reported DNA element (5′-TAATTG-3′).

To ascertain the binding affinity of Msx2 for the *bicoid* element, an EMSA DNA titration experiment was performed. We analyzed several concentrations of Msx2 protein binding to the *bicoid* sequence and determined an apparent K_D of 65 nM by Scatchard plots (Fig. 2B). These experiments reveal that Msx2 binds the *bicoid* DNA element with a reasonable affinity but somewhat lower than we previously reported for PITX2 binding to the *bicoid* element ($K_D = 50$ nM) (4).

The Dlx2 *Promoter Contains Multiple Elements That Bind PITX2*

The downstream targets of PITX2 in tooth development have not been identified and we were searching for candidate genes that could be regulated by PITX2. The *Dlx2* promoter contains multiple *bicoid* and *bicoid*-like sites as denoted in Figure 3A. The *Dlx2* promoter contains three consensus 5′-TAA TCC-3′ *bicoid* sites and five nonconsensus *bicoid* sites that have one nucleotide substitutions within the DNA element. These *bicoid*-like elements are 5′-TATTCC-3′, 5′-TTATCC-3′, 5′-TAAGCC-3′, and 5′-CAATCC-3′. We made radiolabeled probes using the sequences of the *Dlx2 bicoid* element and the TAT TCC sequence with flanking *Dlx2* sequences. We tested these probes for PITX2 binding at two concentrations of purified PITX2 protein (Fig. 3B). Our experiments reveal that PITX2 binds to the *Dlx* TAATCC probe with a greater activity than the *Dlx* TATTCC probe or our originally reported *bicoid* probe. The only difference between the *Dlx* TAA TCC probe and the *bicoid* probe lies in the sequences flanking the TAATCC sequence. At the higher concentration of PITX2 protein (160 ng), PITX2 binds as a homodimer to the *Dlx* TAATCC probe but does not form dimers on the *bicoid* probe or the *Dlx* TAT TCC probe (Fig. 3B). This represents an approximately twofold increase in binding activity using the *Dlx* TAATCC probe. Overall, PITX2 binds much more efficiently to the *Dlx* probes of either element than to the artificial *bicoid* probe we reported previously (4), although when we use 50-fold molar ex-

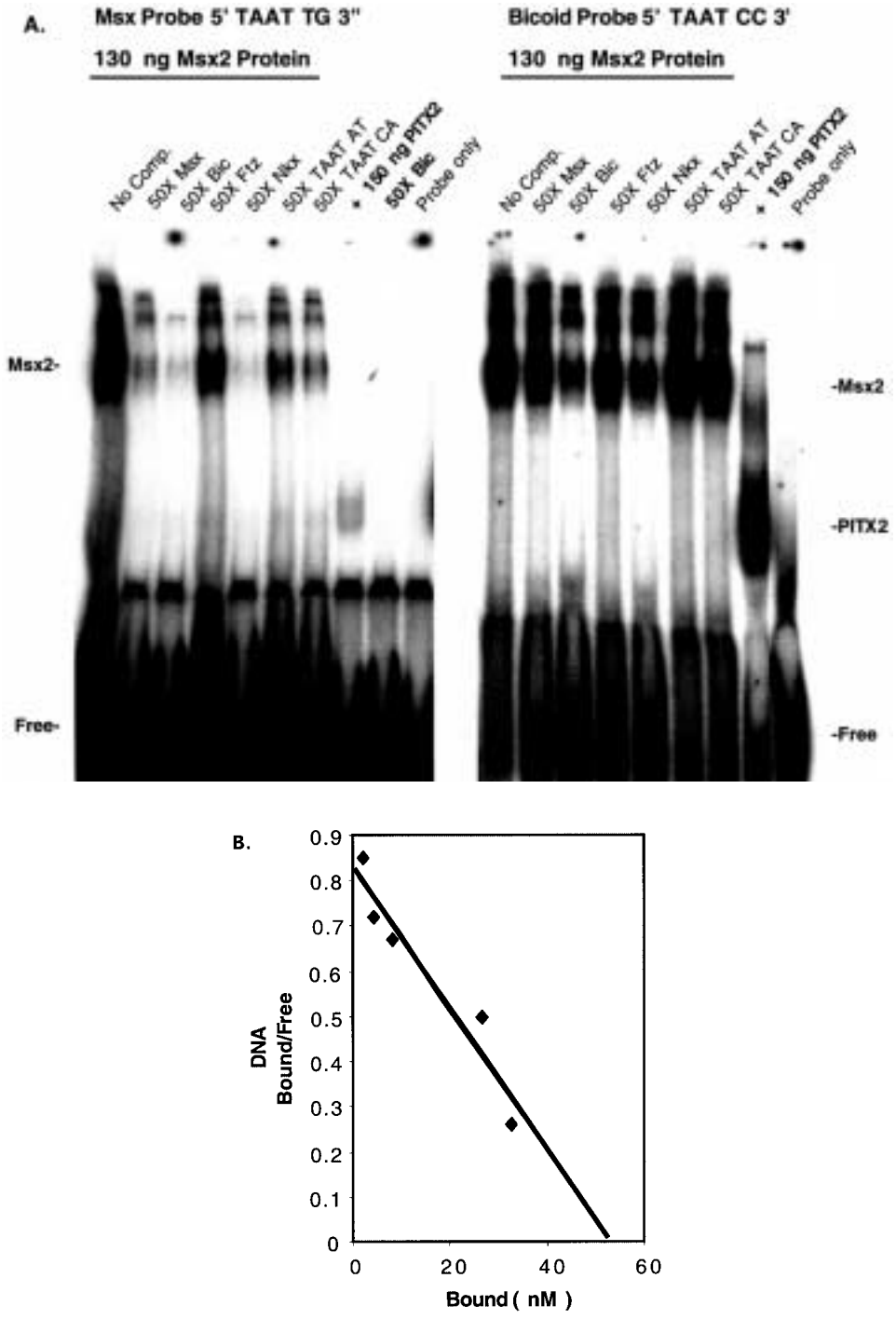

Figure 2. Binding properties of Msx2. (A) Msx2 protein (~130 ng) and PITX2 protein (~150 ng) were incubated with the *Msx* or *bicoid* consensus sequence as the radioactive probe in the absence or presence of 50-fold molar excess unlabeled oligonucleotides as competitor DNAs. The EMSA experiments were analyzed in 8% native polyacrylamide gels. The free probe and bound complexes are indicated. (B) Scatchard plot of Msx2 protein binding to increasing amounts of *bicoid* probe. The free and bound forms of DNA were quantitated using the Molecular Dynamics STORM PhosphoImager.

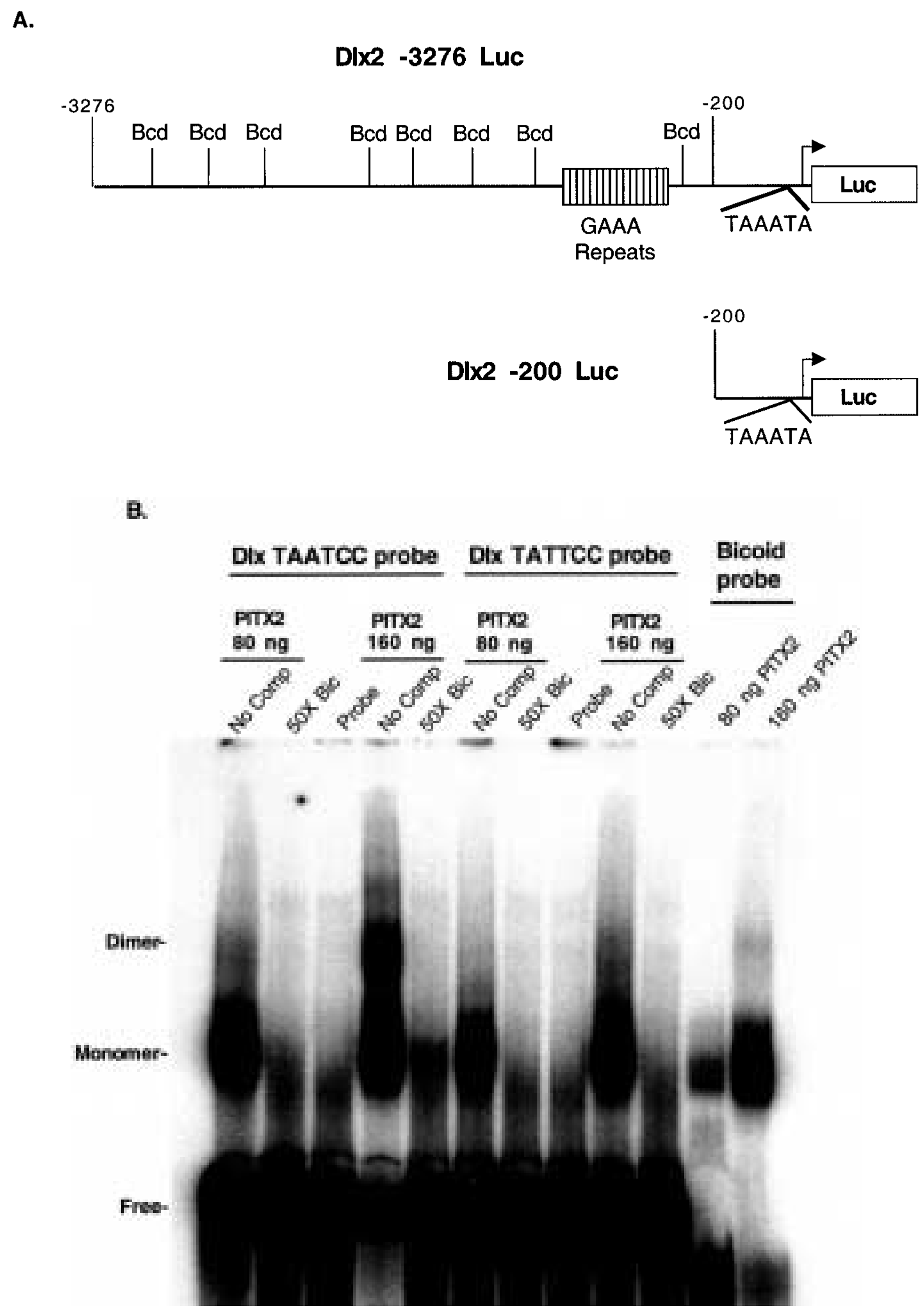

Figure 3. PITX2 binds to DNA elements within the *Dlx2* promoter. (A) Schematic of the *Dlx2* promoter constructs used in transient transfection assays showing the location of *bicoid* and *bicoid*-like DNA elements, Bcd, *bicoid*, and *bicoid*-like sequences. (B) PITX2 protein (80 and 160 ng) was incubated with the *Dlx2 bicoid* consensus sequence (TAATCC), and the *Dlx2 bicoid*-like TATTCC sequence as the radioactive probe or our previously reported *bicoid* probe (4) in the absence or presence of 50-fold molar excess unlabeled *bicoid* element as competitor DNA. The EMSA experiments were analyzed in 8% native polyacrylamide gels. The bound forms of DNA were quantitated as described in Figure 2. The free probe and bound complexes are indicated.

cess of cold competitor *bicoid* oligonucleotide this concentration was able to compete for PITX2 binding to either *Dlx* probe. We conclude from these data that the sequences flanking the *bicoid* site can greatly influence binding of the PITX2 protein. We have also demonstrated that the DNA elements TTATCC, TAAGCC, and CAATCC can compete for PITX2 binding to the *bicoid* element (data not shown).

Msx2 Binds to the Bicoid *and* Bicoid*-Like* Dlx2 *DNA Elements*

Because we have shown that Msx2 binds to the *bicoid* element we next wanted to determine if it also efficiently binds to the *Dlx* probes. Msx2 binds to both *Dlx* DNA probes; however, we observe a two-fold increase in binding to the *Dlx* TATTCC probe

compared with the *Dlx* TAATCC probe (Fig. 4). Msx2 binding to the *Dlx* TAATCC probe was efficiently competed by 50-fold molar excess of the *bicoid*, *Dlx* TAATCC, and *Dlx* TATTCC oligonucleotides (Fig. 4). However, only the self-competitor TATTCC oligonucleotide was able to efficiently compete for Msx2 binding to the *Dlx* TATTCC probe (Fig. 4). The *bicoid* and *Dlx* TAATCC competitor oligonucleotides demonstrated incomplete competition of Msx2 binding to the *Dlx* TATTCC probe (Fig. 4). These data indicate that Msx2 binds more efficiently to the TATTCC element than to the TAATCC DNA sequence.

PITX2 and Msx2 Bind Independently to the Dlx2 Bicoid *DNA Element*

To determine if PITX2 and Msx2 could functionally compete for one DNA binding site, both proteins were mixed together and assayed for binding to the *Dlx* TAATCC probe. We tested two concentrations of PITX2 (40 and 80 ng) with increasing amounts of Msx2 (40, 80, and 160 ng) (Fig. 5). Addition of increasing amounts of Msx2 resulted in an increase in bound Msx2 to the *Dlx bicoid* probe in the presence of PITX2 protein (Fig. 5). However, as the concentration of Msx2 protein increased we did not observe a decrease in PITX2 binding under probe excess. When the reciprocal experiment was performed where Msx2 protein was kept constant with increasing amounts of PITX2, we did not observed a decrease in Msx2 binding (data not shown). Thus, both proteins bind to the *Dlx bicoid* element independent of one another and the levels of binding are based on the relative amount of each protein.

PITX2 Specifically Transactivates the Dlx2 *Promoter*

Having demonstrated that PITX2 binds to elements within the *Dlx2* promoter, we next determined if PITX2 was capable of activating transcription from the *Dlx2* promoter. The *Dlx2* promoter was linked to the luciferase gene (Fig. 3A) and used as the reporter plasmid. We compared the activities of the minimal *Dlx2* promoter (Dlx2-200-luc), which contains only the TAATAA box and 200 bp of 5′ flanking sequences, to the full-length promoter (Dlx2-3276-luc) containing all of the upstream regulatory elements (Fig. 3A). Transfection of CHO cells with Dlx2-

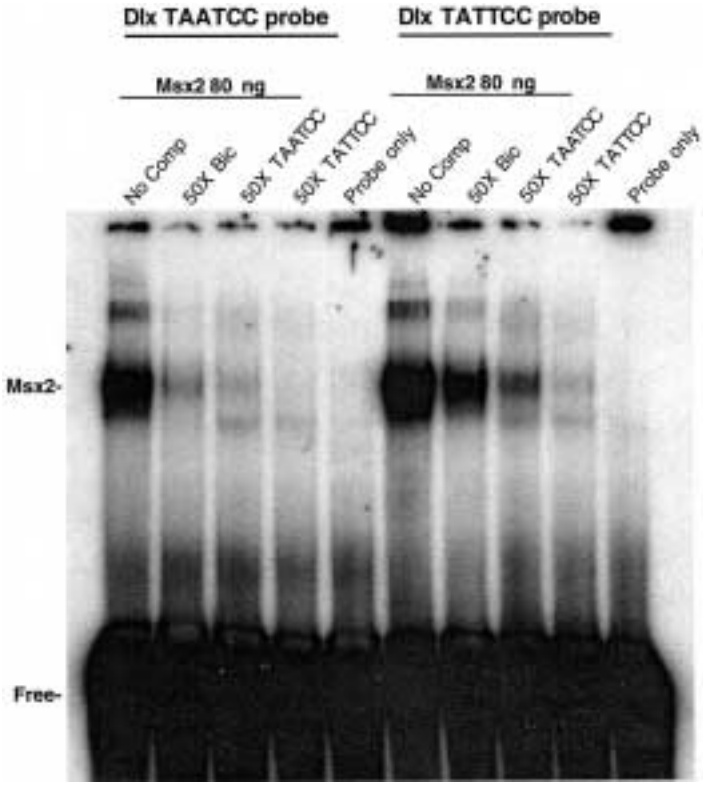

Figure 4. Msx2 binds to DNA elements within the *Dlx2* promoter. Msx2 protein (~80 ng) was incubated with the radioactive *Dlx2* probes described in Figure 3. The competitors used to demonstrate specific binding were our original *bicoid* oligonucleotide (4) and the two *Dlx2 bicoid* and *bicoid*-like oligonucleotides at 50-fold molar excess. The bound forms of DNA were quantitated as described in Figure 2. The free probe and bound complexes are indicated.

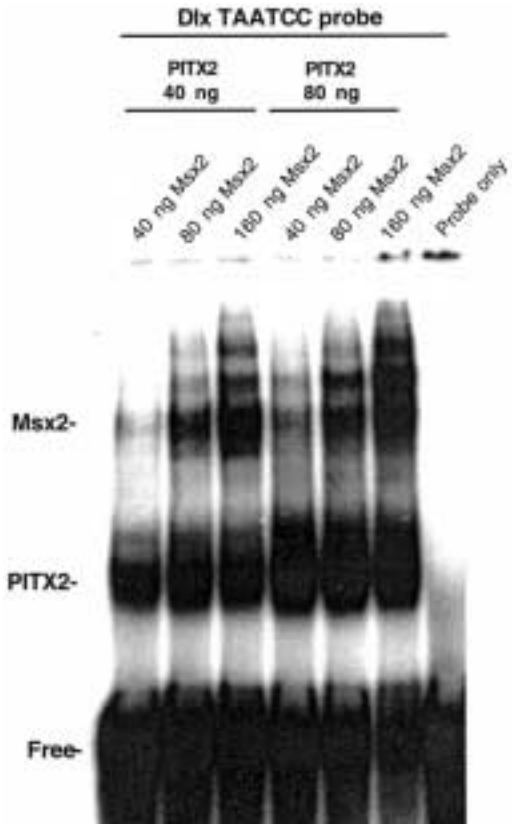

Figure 5. PITX2 and Msx2 proteins bind independently to the *Dlx2 bicoid* DNA element. PITX2 protein (either 40 or 80 ng) was mixed with varying amounts of Msx2 protein and incubated with the radioactive *Dlx2 bicoid* probe described in Figure 3. The bound forms of DNA were quantitated as described in Figure 2. The free probe and bound complexes are indicated.

3276-luc and PITX2 resulted in 45-fold activation of this promoter, which contains multiple *bicoid* and *bicoid*-like elements (Fig. 6A). Although there does not appear to be any *bicoid*-like sites within the minimal promoter of *Dlx2*, we observe an 11-fold activation in the presence of PITX2. Thus, we cannot rule out that PITX2 may be interacting with other transcription factors to activate transcription from this construct or that it is binding to other DNA sites in the minimal promoter. Msx2 minimally repressed transcription of the full-length promoter in CHO cells while about twofold repression was seen with the minimal promoter in the presence of Msx2 (Fig. 6A). Previous results indicate that Msx2 can interact with the basal transcription machinery to affect transcriptional activation (22). In contrast, cotransfections of *PITX2* and *Msx2* with the Dlx2-3276-luc plasmid reduced activation of this promoter from 45-fold with PITX2 alone to 10-fold with both factors present (Fig. 6A). Interestingly, expression of both factors reduced activation of the Dlx2-200-luc plasmid from 11-fold with PITX2 alone to fivefold (Fig. 6A). Thus, we have identified the *Dlx2* promoter as a target for PITX2 and Msx2.

PITX2 and Msx2 Act as Transcriptional Antagonists

Because PITX2 and Msx2 can bind the same DNA element(s) in the *Dlx2* promoter, we then asked if these factors had an antagonistic effect on transcription. The *PITX2* and *Msx2* expression vectors were cotransfected along with the Dlx2-3276 luc reporter plasmid. Cotransfection of equal amounts of each vector resulted in an overall 10-fold activation of transcription in CHO cells (Fig. 6A). This is an intermediate value between the 45-fold activation by PITX2 alone and the approximately twofold repression by Msx2 alone. These results indicate that Msx2 can antagonize PITX2 activation of *Dlx2* and conversely PITX2 can attenuate Msx2 repression. To vary the relative amounts of Msx2 protein compared with PITX2 in the transient transfection assay, we varied the amount of expression vector DNA. Antagonism was increased with higher levels of Msx2 expression vector DNA (Fig. 6B). These results suggest that the relative levels of Msx2 and PITX2 proteins may regulate the activity of *Dlx2* and genes containing the TAATCC DNA element.

PITX2 Protein Isoforms Are Endogenously Expressed in a Novel Tooth Epithelial Cell Line

We used a cell line (LS-8) derived from mouse enamel organ epithelia to study transcription factors and their activities in tooth development. This cell line was prepared from neonatal mouse tooth epithelia, corresponding to later stages in tooth development (7). We prepared a PITX2 antibody, the specificity of which is shown in Figure 7. A Western blot of bacterial expressed PITX2 proteins demonstrates that the Pitx2 antibody P2R10 specifically recognizes the N-terminus of PITX2. Protein PITX2CΔ39 has the C-terminal 39 residues deleted while PITX2NΔ38 has the N-terminus flanking the homeodomain deleted (Fig. 7A). This antibody does not bind to the N-terminal truncated PITX2NΔ38 protein and we have determined that the P2R10 antibody recognizes the peptide sequence DPSKKKR (Fig. 7B) (13). This sequence is present immediately 5′ of the homeodomain and is present in the three major PITX2 isoforms (2,9,29). All of the major PITX2 isoforms have identical homeodomain and C-terminal tails and only differ in the N-terminus. We have identified two major *Pitx2* isoforms in LS-8 NE by RT-PCR (Fig. 7C). We used an antisense primer in the homeodomain of *PITX2* and isoform-specific N-terminal sense primers to detect the *Pitx2* isoform transcripts. The *Pitx2A* and *Pitx2C* isoforms were identified and confirmed

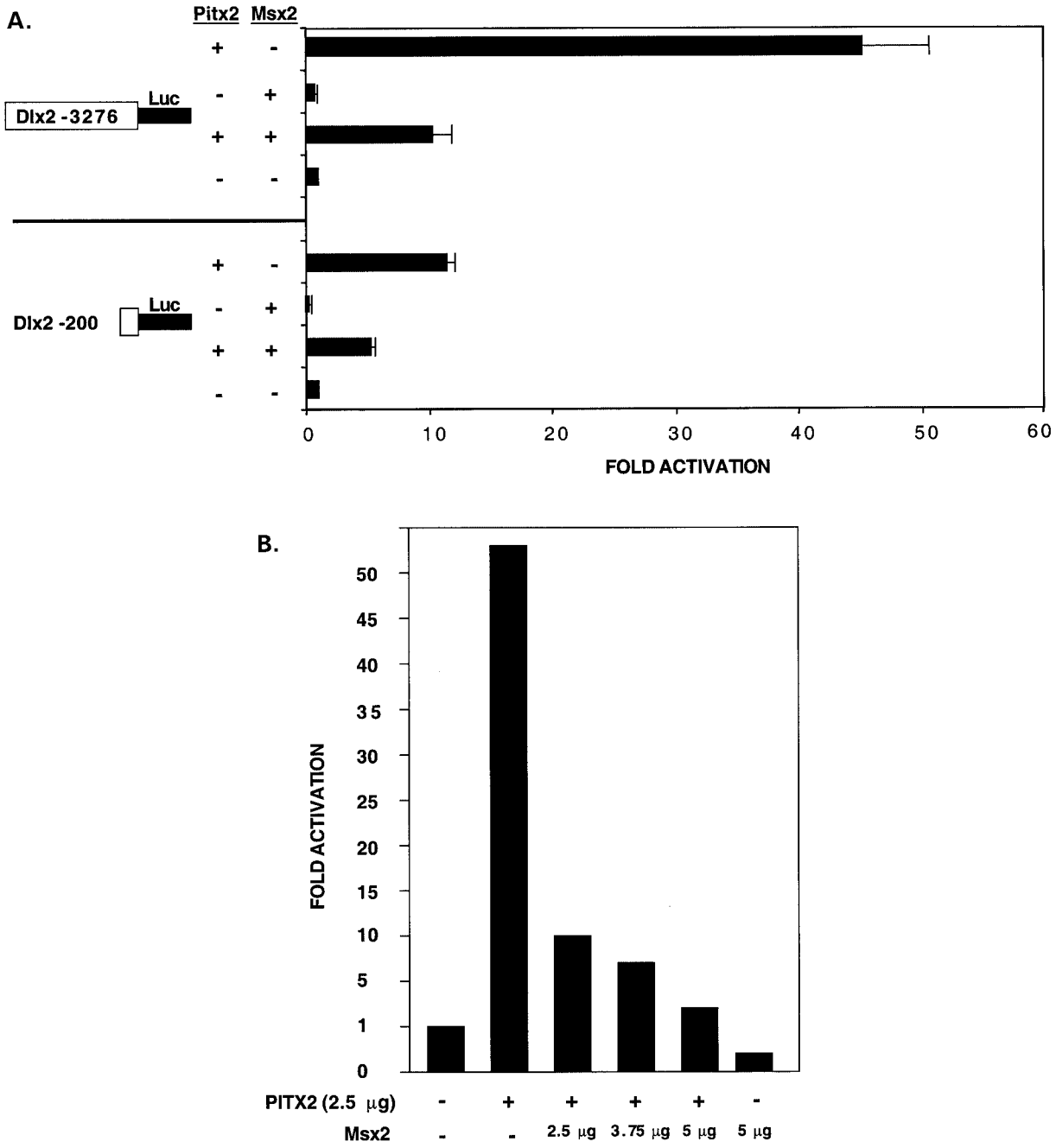

Figure 6. Msx2 antagonizes PITX2 activation of the *Dlx2* promoter. (A) CHO cells were transfected with either the Dlx2-3276 or Dlx2-200 luciferase reporter genes. The cells were cotransfected with either the CMV-PITX2 and/or -Msx2 expression plasmids (+) or the CMV plasmid without Pitx2 or Msx2 (–). (B) CHO cells were transfected with the Dlx2-3276 luciferase reporter and cotransfected with the indicated amounts of CMV-PITX2 and/or -Msx2 expression plasmids (+) or the CMV plasmid without PITX2 or Msx2 (–). To control for transfection efficiency, all transfections included the SV-40 β-galactosidase reporter. Cells were incubated for 24 h, then assayed for luciferase and β-galactosidase activities. The activities are shown as mean fold activation compared with the *Dlx2* promoter plasmids without PITX2 expression and normalized to β-galactosidase activity [±SEM from four independent experiments for (A)]. Repression of the *Dlx2* promoters by Msx2 is shown as being less than the control value set at 1 for fold activation. The mean *Dlx2* promoter luciferase activity with PITX2 expression was about 100,000 light units per 15 μg protein, and the β-galactosidase activity was about 70,000 light units per 15 μg protein.

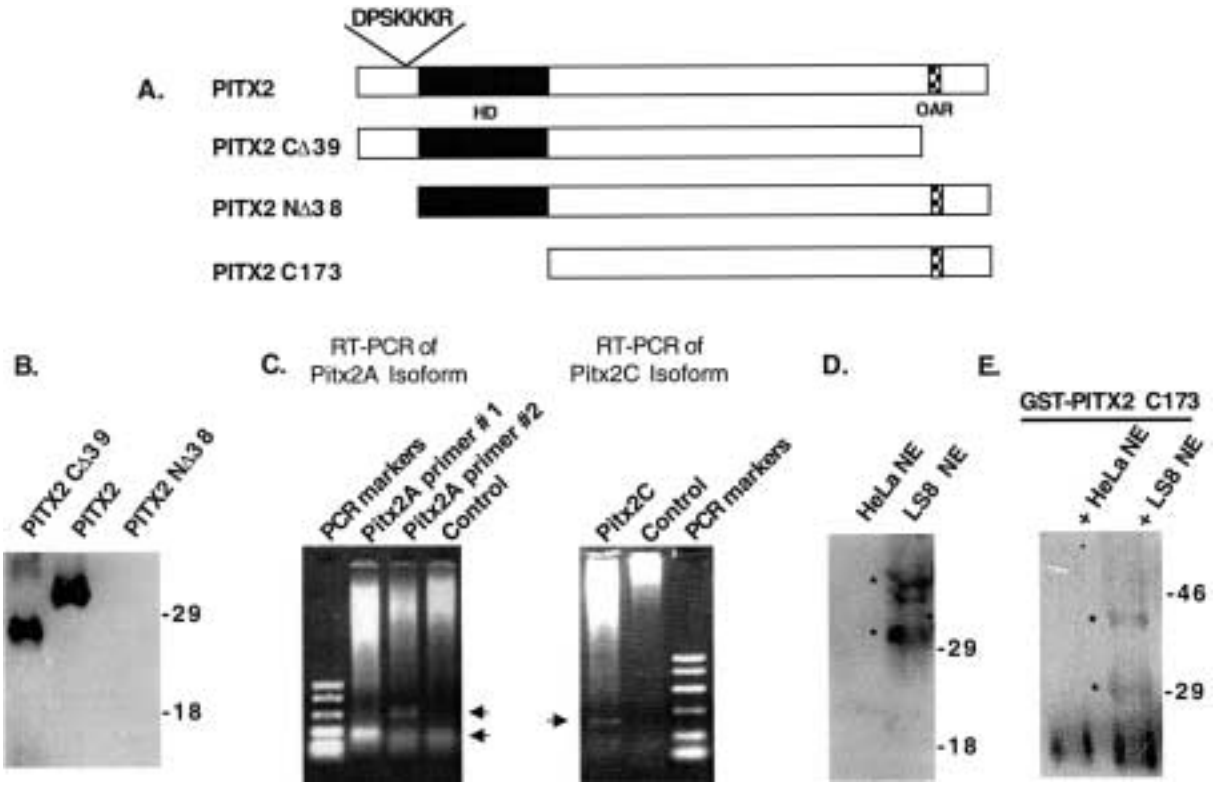

Figure 7. Identification of Pitx2 isoforms in LS-8 tooth epithelial cells. (A) Schematic of bacterial expressed PITX2 protein constructs showing the deleted portions of the proteins used in our assays (DPSKKKR, antibody recognition epitope; HD, homeodomain; OAR, 14 amino acid conserved region). (B) Western blot of purified bacterial proteins demonstrating specificity of the PITX2 P2R10 antibody. (C) RT-PCR of mRNA isolated from LS-8 cells showing the expression of Pitx2A and C isoforms. Primers specific to the N-terminal sequences, which differ in each isoform, were used in combination with an antisense homeodomain primer. Pitx2A and Pitx2C isoforms were detected using specific sense primers indicated by the arrows. All products were confirmed by sequencing the amplified bands. (D) Western blot of HeLa and LS-8 nuclear extracts (approximately 100 μg). No Pitx2 was detected in HeLa nuclear extracts while the two asterisks denote the Pitx2A (~30 kDa) and the Pitx2C (~36 kDa) isoforms in LS-8 nuclear extracts. (E) GST-PITX2 C173 pull-down assay with HeLa and LS-8 nuclear extracts. The endogenous Pitx2A and Pitx2C isoforms bind to the C-terminus of PITX2 and were detected using the PITX2 antibody by Western blot. This demonstrates that PITX2 can form homodimers through interaction within its C-terminal tail.

by sequencing the amplified products. We further characterized the two major Pitx2 isoforms expressed in LS-8 cells by Western blot (Fig. 7D). The two major isoforms detected are noted by the asterisks and represent Pitx2A, which is the lower molecular weigh (MW) species, and Pitx2C as the higher MW protein. Another isoform, presumably isoform Pitx2B (located between Pitx2A and C isoforms, Fig. 7D), may be present; however, it was not detected by RT-PCR. Conversely, this band could correspond to a Pitx2C degraded protein. To further confirm the existence of the protein isoforms, a GST-PITX2 C173 pull-down assay was performed with LS-8 NE. We were able to pull down both Pitx2A and Pitx2C isoforms but did not pull down Pitx2B (Fig. 7E). This assay confirms that the Pitx2A and 2C isoforms are present in the LS-8 NE but also demonstrate for the first time that Pitx2 proteins can interact with each other through their C-terminal tails. We have previously shown that PITX2 can form homodimers by interactions through the homeodomain (5). As a control we show that HeLa NE did not contain endogenous Pitx2. Furthermore, Pitx2 immunostaining of LS-8 cells demonstrates that endogenously expressed Pitx2 is localized to the nucleus (Hjalt, unpublished observation). Pitx2 immunostaining of PITX2 transfected cells also reveals that PITX2 is localized to the nucleus in those cells (Hjalt, unpublished observation).

Msx2-Mediated Repression of the TK-Bicoid *Promoter Is Dominant Over PITX2 Activation in LS-8 Cells*

Because we identified endogenous expression of Pitx2 in the LS-8 cells our next experiment was to determine the transcriptional activity of the TK-*bicoid* promoter in these cells. We show in this report that Msx2 protein is also expressed in LS-8 cells as determined by Western blot analysis (Fig. 8A). Transfection of *PITX2* into the LS-8 cells revealed a

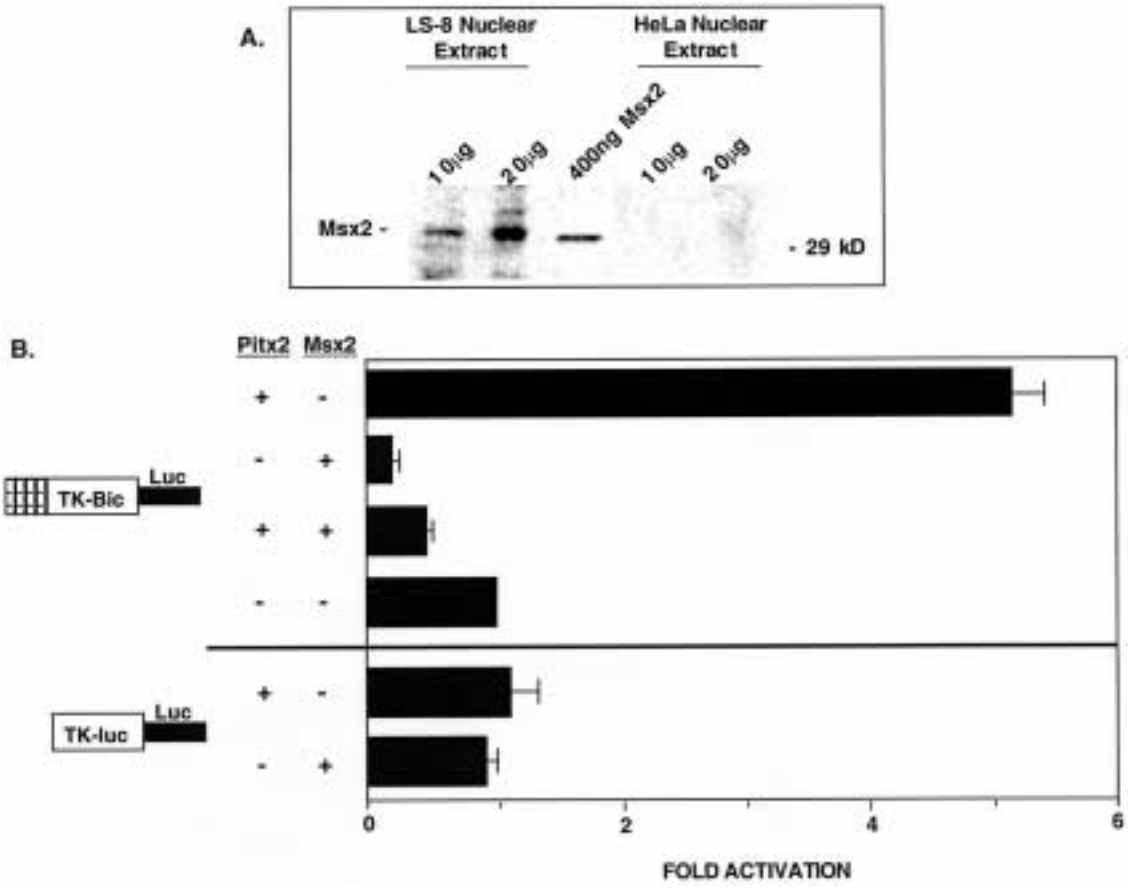

Figure 8. Msx2 attenuates PITX2 activation of the TK-*bicoid* luciferase reporter in LS-8 cells. (A) Western blot of LS-8 nuclear extract using a Msx2 antibody. LS-8 nuclear extract (10 and 20 μg) was tested for Msx2 protein expression. As a control, 400 ng of bacterial expressed Msx2 was used to show the correct migration of endogenous Msx2 and to demonstrate the purity of our Msx2 protein preparation. HeLa nuclear extract was used as a negative control. (B) LS-8 cells were transfected with either the TK-*bicoid* luciferase reporter gene or the parental TK-luciferase reporter without the *bicoid* sites. The cells were cotransfected with either the CMV-Pitx2 and/or -Msx2 expression plasmids (+) or the CMV plasmid without Pitx2 or Msx2 (–). To control for transfection efficiency, all transfections included the CMV β-galactosidase reporter. Cells were incubated for 24 h, then assayed for luciferase and β-galactosidase activities. The activities are shown as mean fold activation compared with TK-*bicoid* luciferase without Pitx2 expression and normalized to β-galactosidase activity (±SEM from five independent experiments). The mean TK-*bicoid* luciferase activity with Pitx2 expression was about 7000 light units per 15 μg protein, and the β-galactosidase activity was about 40,000 light units per 15 μg protein.

fivefold activation of the TK-*bicoid* promoter (Fig. 8B). However, we saw an enhanced Msx2-mediated repression of the TK-*bicoid* promoter in this cell line compared with CHO cells, shown in Figure 1. Transfection of the LS-8 cells with Msx2 and the TK-*bicoid* promoter revealed a fourfold repression (Fig. 8B). More importantly, cotransfection of both PITX2 and Msx2 demonstrated continued repression at twofold of control activity (Fig. 8B). Thus, in the LS-8 cell line PITX2 was unable to overcome the repression of the TK-*bicoid* promoter caused by Msx2.

Activation of the Dlx2 *Promoter by PITX2 Is Attenuated in the Tooth Epithelial Cell Line*

Surprisingly, transfection of *PITX2* and the Dlx2-3276-luc reporter in this cell line revealed only low levels (twofold) of promoter activation by PITX2 (Fig. 9). However, transfection of the *Msx2* expression plasmid and Dlx2-3276-luc reporter caused a fourfold repression of this full-length *Dlx2* promoter (Fig. 9). Cotransfection of both *Msx2* and *PITX2* with the full-length promoter reduced activation to only 1.5-fold (Fig. 9). The minimal *Dlx2* promoter, Dlx2-200-luc, was neither activated nor repressed by PITX2 or Msx2 (Fig. 9). These data suggest that the LS-8 cells contain additional factors that either by themselves or in concert with PITX2 act to attenuate *Dlx2* gene expression. Conversely, this cell line may not contain cofactors required for PITX2 activation. This would seem unlikely because Pitx2 is endogenously expressed in this cell line. We speculate that factors in the LS-8 cells interact with PITX2 to negatively regulate its transcriptional activity.

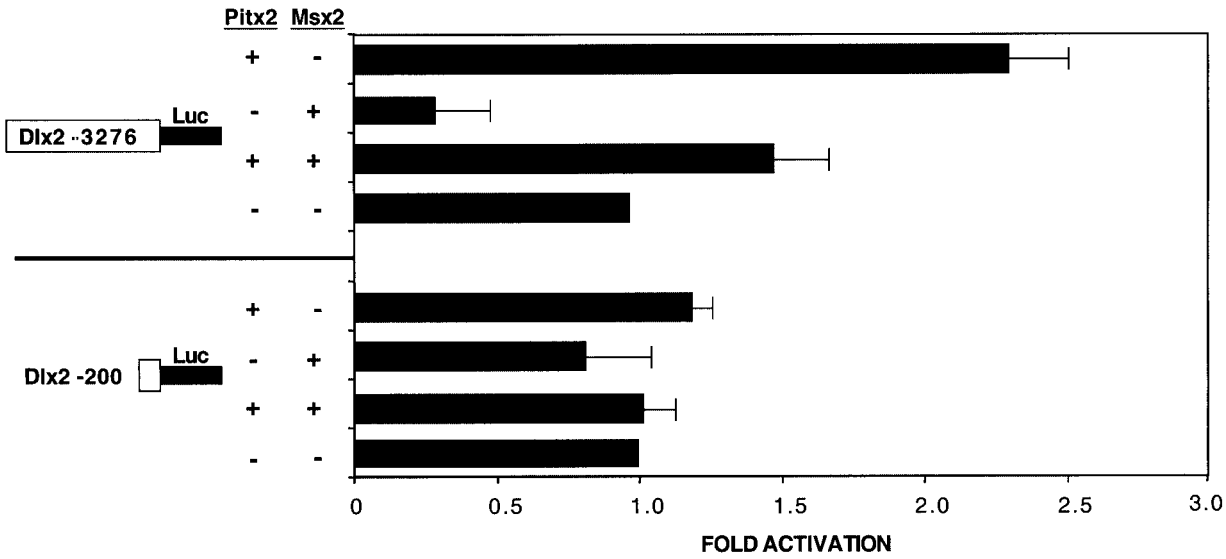

Figure 9. Transcriptional activity of the *Dlx2* promoter is decreased in the LS-8 tooth epithelial cell line. LS-8 cells were transfected with either the Dlx2-3276 or Dlx2-200 luciferase reporter genes. The cells were cotransfected with either the CMV-Pitx2 and/or -Msx2 expression plasmids (+) or the CMV plasmid without Pitx2 (−). To control for transfection efficiency, all transfections included the CMV β-galactosidase reporter. Cells were incubated for 24 h, then assayed for luciferase and β-galactosidase activities. The activities are shown as mean fold activation compared with the *Dlx2* promoters without Pitx2 expression and normalized to β-galactosidase activity (±SEM from three independent experiments). Repression of the *Dlx2* promoters by Msx2 is shown as being less than the control value set at 1. The mean Dlx2-3276 luciferase activity with Pitx2 expression was about 5000 light units per 15 μg protein, and the β-galactosidase activity was about 40,000 light units per 15 μg protein.

Factors in LS-8 Cells Interact With PITX2 and Form Specific Protein–Protein Complexes

We prepared nuclear extracts from LS-8 cells in an attempt to identify PITX2 interacting factors that could explain the attenuation of the *Dlx2* promoter. LS-8 NE was assayed by EMSA using our *bicoid* probe to identify other factors binding to the *bicoid* elements or interacting directly with PITX2. The *bicoid* probe containing the *bicoid* DNA element (5′-TAATCC-3′) was used to demonstrate Pitx2 binding in nuclear extracts. Endogenous Pitx2 occurs as a homodimer in LS-8 cells as seen by EMSA (Fig. 10A). PITX2CΔ39, which we have shown readily forms dimers, was assayed to show the location of the dimer species (Fig. 10A) (5). Two large complexes were formed on the *bicoid* probe using LS-8 NE (Fig. 10A). These complexes were shown to be specific for the *bicoid* probe because 50-fold molar excess of cold competitor *bicoid* DNA was able to compete for these complexes binding to the probe (Fig. 10A). However, two faster migrating bands were not competed by the cold competitor, suggesting that they are nonspecific DNA binding proteins. To further demonstrate that these complexes contained Pitx2 and interacting proteins, we used the Pitx2 antiserum in the EMSA experiments. Addition of the antiserum to purified PITX2 protein after binding to the probe demonstrated a characteristic supershift of the bound PITX2 protein (Fig. 10B). When the antiserum was added to the LS-8 nuclear extract binding reactions this disrupted complex 1 and 2 as well as the Pitx2 homodimer binding to the *bicoid* probe (Fig. 10B). Thus, complex 1 and 2 contain factors expressed in the LS-8 cells that interact with Pitx2. The antiserum binding to Pitx2 apparently inhibits the LS-8 factors from interacting with Pitx2. Preimmune serum had no effect on endogenous Pitx2 binding or Pitx2 complex formation (data not shown). As controls we show that HeLa NE does not contain these complexes and that the PITX2 antiserum does not bind to the probe (Fig. 10B).

DISCUSSION

PITX2 and Msx2 Act to Regulate Transcription of Promoters Containing Bicoid *and* Bicoid*-Like Elements*

The regulation of tooth development by transcription factors is just beginning to be understood. Most homeodomain proteins bind to DNA at a site that contains a TAAT core (12). Homeodomain proteins are known for binding to specific DNA sequences despite the promiscuity of TAAT motifs. The specificity for which protein binds to a promoter is provided by the bases immediately 3′ to the TAAT core (25,40). For example, members of the *Fushi tarazu* class bind to a 5′-TAATGG-3′ motif. *Bicoid*-like pro-

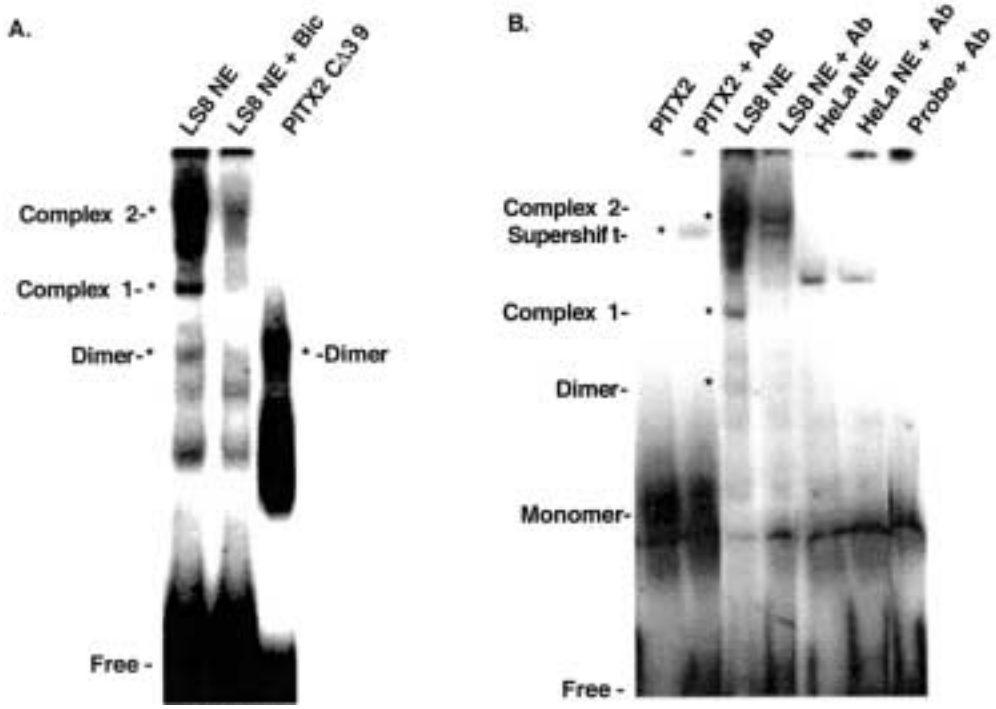

Figure 10. LS-8 nuclear extract forms specific Pitx2–protein complexes. (A) LS-8 nuclear extract (NE) (~3 μg) was incubated with the *bicoid* consensus sequence as the radioactive probe in the absence or presence of 50-fold molar excess unlabeled *bicoid* oligonucleotide (Bic) as competitor DNA. Approximately 80 ng of PITX2 CΔ39 was used in the EMSA to identify where the endogenous LS-8 Pitx2 homodimer migrates. We have previously shown that this protein readily forms homodimers (5). The EMSA experiments were analyzed on native 7% polyacrylamide gels. The free probe, dimer species, and bound complexes are indicated. (B) Purified bacterial expressed PITX2 was incubated with the *bicoid* probe in the absence and presence of PITX2 antibody (first two lanes on the left). The characteristic supershift caused by the PITX2–antibody complex is shown (supershift). LS-8 NE was incubated with the antibody prior to addition of the *bicoid* probe. HeLa NE was used to demonstrate the specificity of the LS-8 complexes. The last lane (far right) demonstrates that the PITX2 antibody does not bind to the probe. Asterisks denote the dimer and specific complexes.

teins bind to a 5′-TAATCC-3′ motif (8,40). Binding of a transcription factor to DNA can result in either activation or repression of the promoter.

This study has focused on the earliest marker of tooth morphogenesis, Pitx2, and the role it plays in the regulation of *Dlx2* using a novel tooth epithelial cell line. To our knowledge, *Dlx2* is the first reported downstream target of Pitx2 identified in tooth morphogenesis. Our previous work has shown that Pitx2 binds to the *bicoid* motif (5′-TAATCC-3′) (4). Here we show that another transcription factor, Msx2, which is a known repressor (28), can also bind to this element. In fact, both Pitx2 and Msx2 protein can bind to *bicoid* elements, competing with each other for binding. This has several implications on the regulation of transcription by Pitx2 in vivo. First, Pitx2 can activate genes that contain *bicoid* elements in their promoters, such as *Dlx2*. Second, competition for these binding sites with a repressor would reduce or disrupt this activation. Msx2 could serve in this capacity in cell lines, such as the LS-8 tooth epithelial cell line we use, in which both Pitx2 and Msx2 are expressed endogenously.

Competition for the *bicoid* site is seen as a reduced activation by PITX2 when Msx2 is cotransfected with both a TK-*bicoid* promoter and the full-length *Dlx2* promoter. As was shown with Msx2 and Dlx5, Msx2 can functionally antagonize Dlx5, a transcriptional activator, through protein–protein interactions, but not through competitive binding to sites within the promoter (23). Other evidence demonstrates that Msx and Dlx proteins functionally antagonize each other, and this antagonism may be the result of the dimerization of the proteins preventing DNA binding of the proteins (41). In this report we demonstrate that Msx2 and Pitx2 have differential binding specificities for consensus and nonconsensus *bicoid* sites, allowing DNA interactions to dictate the transcriptional regulation by these proteins. We were unable to demonstrate heterodimerization between these two proteins. These results are similar to another report describing transcriptional antagonism between an activator and a repressor for a shared DNA binding site (6). Hmx1 was identified as repressing transcription from a promoter containing 5′-CAAGTG-3′ elements while Nkx2.5 activated this promoter. Hmx1 can an-

tagonize Nkx2.5 activation of this promoter and conversely Nkx2.5 can attenuate Hmx1 repression (6). The expression levels of these two gene products determine the level of promoter activity. Therefore, during development our results suggest that the activity of the *Dlx2* promoter would be regulated by the dosage of PITX2 and Msx2 gene products.

Immunoflourescence studies have shown Pitx2 protein expression in the tooth epithelium but not in the mesenchyme at day E13.5 (13). This supports in situ hybridization experiments that indicate, in primordial tooth cells, *Pitx2* is expressed as early as day E8.5, but restricted to the epithelium by day E11.5 (21). Pitx2 may be required for the specification of dental epithelium and in the initiation of tooth formation (21). During tooth morphogenesis, as the level of Msx2 rises, it may act to reduce the activation caused by PITX2, which is expressed earlier. In this case, the response of Pitx2 is changed, not due to heterodimerization with Msx2 but due to the binding of both factors to the promoter. Interestingly, in Rieger syndrome, a haploinsufficiency disorder, the variability in defects associated with mutations in PITX2 has been attributed to PITX2 tissue-specific dosage dependence (10). Because Rieger syndrome patients present with tooth abnormalities, the low level of PITX2 expression would be counteracted by higher levels of Msx2 expression and result in the continued repression of the *Dlx2* promoter.

Several other genes encoding transcription factors important for tooth development are expressed at E10.5, including *Dlx2* (27), *Lef1* (14), and *Msx2* (19) in the dental epithelium. Recently, it has been shown that *Dlx2* is expressed in the epithelium as early as E9.0 (34). The expression of these genes appears to be later than that of Pitx2 in the presumptive dental epithelium. In the later stages of tooth morphogenesis, *Pitx2, Dlx2*, and *Msx2* expression is restricted to the inner enamel epithelium, which forms the ameloblast layer (21,27,35). Because *Pitx2* expression occurs before *Dlx2*, our results are consistent with PITX2 activating *Dlx2* expression.

LS-8 Cells Contain Factors That Regulate PITX2 Transcriptional Activity

We have shown that a cell line derived from mouse enamel organ epithelia endogenously expresses Pitx2 isoforms Pitx2a and Pitx2c. Interestingly, the activity of the full-length *Dlx2* promoter is significantly reduced in LS-8 cells compared with CHO cells transfected with PITX2. We have also shown that LS-8 NE contains factors that interact with PITX2. We speculate that these proteins may act to inhibit PITX2 transcriptional activity. We have previously shown that the C-terminus of PITX2 can interact with the POU homeodomain protein Pit-1 (5). The Pit-1 interaction increases the binding capacity of PITX2 for the *bicoid* element and results in a synergistic transcriptional activation of the prolactin promoter (5). In this report we demonstrate that PITX2 can form homodimers through its C-terminus as well. Interestingly, PITX2 dimerization is enhanced upon binding to the consensus *bicoid* element located in the *Dlx2* promoter. Thus, our data support a model where PITX2 can form dimers with itself and other proteins by interactions through the homeodomain and C-terminus. We speculate that factors in the LS-8 cell line interact with PITX2 to attenuate its activity. However, this cell line may not contain cofactors necessary for PITX2 activation of the *Dlx2* promoter. In either case we can use this cell line to study the regulation of PITX2 activity in tooth development. In CHO cells a simple model of mutual exclusion may exist where either PITX2 or Msx2 bind and activate or repress *Dlx2* expression, respectively. However, in the tooth epithelial cell line PITX2 overexpression has little effect on *Dlx2* promoter activity. In contrast, Msx2 overexpression appears to exert more of a repressive effect on the *Dlx2* promoter. This may be due to Msx2 interacting factors that enhance Msx2 binding activity or simply higher levels of Msx2 protein expression compared with Pitx2 protein in the LS-8 cell line. Western blot analysis has demonstrated an increase in Msx2 protein compared with Pitx2 in this cell line. Alternatively, the tooth epithelial cell line may express factors complexing with PITX2 to inhibit its transcriptional activity. The PITX2–protein complexes appear to enhance PITX2 binding to the *bicoid* probe similar to Pit-1; however, PITX2 transcriptional activity is repressed while Pit-1 causes transcriptional synergism. Because Pitx2 is endogenously expressed in the LS-8 cell line, it is unlikely that these cells do not possess Pitx2 interacting factors whether they are negative or positive acting. Thus, we have identified a cell line that may express factors that attenuate PITX2 transcriptional activity. This is the first report describing factors that interact with PITX2 to repress its activity. We are using a variety of experimental approaches to identify these Pitx2 interacting factors.

The data presented here establish *Dlx2* as a target of PITX2 in tooth morphogenesis. While Msx2 can attenuate *Dlx2* expression, factors in the tooth epithelium at later stages may interact with PITX2 to attenuate its transcriptional activity. Based on the expression patterns of *Pitx2, Msx2*, and *Dlx2*, it would seem possible that Msx2 is regulating *Dlx2* to a reduced level in the distal region of the mandible. However, PITX2 may increase *Dlx2* expression specifically at the sites of tooth development. In Rieger syndrome

patients with tooth abnormalities these defects may be linked to PITX2 mutant proteins that cannot transactivate the *Dlx2* gene.

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