PITX2, β-catenin and LEF-1 interact to synergistically regulate the *LEF-1* promoter

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Summary

PITX2, β-catenin and lymphoid enhancer factor (LEF-1) are required for the inductive formation of several epithelial-derived organs, including teeth. *Lef-1* is expressed in the dental epithelium after *Pitx2*, and both factors have overlapping expression patterns in the tooth bud and cap stages. Our analysis of Pitx2$^{-/-}$ mutant mice showed reduced *Lef-1* expression in facial tissues by RT-PCR and quantitative RT-PCR. Consistent with these results we show that the human 2.5 kb *LEF-1* promoter is activated by PITX2. Furthermore, the *LEF-1* promoter is differentially activated by PITX2 isoforms, which are co-expressed in dental epithelium. The 2.5 kb *LEF-1* promoter contains two regions that act to inhibit its transcription in concert with PITX2. The proximal region contains a Wnt-responsive element (WRE) that attenuates PITX2 activation. LEF-1 cannot autoregulate *LEF-1* expression; however co-transfection of PITX2 and LEF-1 result in a synergistic activation of the 2.5 kb *LEF-1* promoter. LEF-1 specifically interacts with the PITX2 C-terminal tail. Deletion of a distal 800 bp segment of the *LEF-1* promoter resulted in enhanced PITX2 activation, and increased synergistic activation in the presence of LEF-1. Furthermore, β-catenin in combination with PITX2 synergistically activates the *LEF-1* promoter and this activation is independent of the Wnt-responsive element. β-catenin directly interacts with PITX2 to synergistically regulate *LEF-1* expression. We show a new mechanism where *LEF-1* expression is regulated through PITX2, LEF-1 and β-catenin direct physical interactions. LEF-1 and β-catenin interactions with PITX2 provide new mechanisms for the regulation of PITX2 transcriptional activity.

Key words: LEF-1, β-catenin, PITX2, Gene expression, Transcription

Introduction

PITX2 and *LEF-1* are two transcription factors whose expression can be regulated by early signaling events involved in numerous developmental programs. *PITX2* expression appears to be regulated by a wnt/dvl/β-catenin pathway, and *LEF-1* can be activated by BMP and Wnt signaling (Filali et al., 2002; Kioussi et al., 2002; Kratochwil et al., 1996). Although these two factors are differentially expressed in many tissues, they show overlapping expression during tooth development. PITX2 is the earliest transcription marker observed in tooth development and is specifically restricted to the developing dental epithelium (Hjalt et al., 2000; Mucchielli et al., 1997). We have shown that PITX2 mutants associated with patients with Axenfeld-Rieger syndrome (ARS) cause defective transcription. ARS is an autosomal-dominant human disorder characterized by dental hypoplasia, mild craniofacial dysmorphism, ocular anterior chamber anomalies causing glaucoma and umbilical stump abnormalities (Amendt et al., 2000; Semina et al., 1998). The dental hypoplasia is manifested as missing, small and/or malformed teeth (Semina et al., 1996). Teeth anomalies occur as abnormally small teeth (microdontia), giving rise to spaces between teeth, misshapen teeth and missing teeth (hypodontia). The clinical presentations of ARS patients with regard to tooth anomalies are varied and may include all of the aforementioned anomalies or only one. The analysis of ARS patients provided the first link to a role for *PITX2* in tooth development. While the precise role of PITX2 in this process is not yet known, it is expressed in the appropriate tissues and times to be playing an instructive role in tooth morphogenesis, and the dental hypoplasia of ARS patients supports a key role for PITX2.

We are working to establish the regulation of *PITX2* expression and protein interactions that modulate PITX2 function. In tooth formation, as in all organs, developmental programs are usually initiated by more than one gene and cell type, acting in concert to promote cell proliferation, migration and/or differentiation. Tooth development is arrested in *Pitx2*$^{-/-}$ mice (Gage et al., 1999a; Lin et al., 1999; Lu et al., 1999a). *Fgf8* and *Bmp4* expression patterns are disturbed and the enamel knot fails to develop. It was suggested that tooth development proceeds through the initial signaling and determination phases, but that the emergence, migration and expansion of distinct cell types in the developing ectoderm fail to progress past the full bud stage (Lu et al., 1999a).

Lymphoid enhancer-binding factor 1 (LEF-1) is a cell type-specific transcription factor expressed in lymphocytes of the adult mouse and in the neural crest, mesencephalon, tooth germs, whisker follicles and other sites during embryogenesis

(Kratochwil et al., 1996; Oosterwegel et al., 1993; Travis et al., 1991; van Genderen et al., 1994; Waterman et al., 1991; Zhou et al., 1995). LEF-1 is a member of the high mobility group (HMG) family of proteins; it activates transcription only in collaboration with other DNA-binding proteins and may promote the assembly of a higher-order nucleoprotein complex by juxtaposing nonadjacent factor binding sites (Carlsson et al., 1993; Giese and Grosschedl, 1993; Giese et al., 1995).

Recently, transgenic mice expressing *LacZ* under the control of the *LEF-1* promoter showed expression in the dental epithelium at an early stage (E12.5) of incisor development (Liu et al., 2004). The expression of *LEF-1* in this transgenic mouse directly overlaps that of *Pitx2* and occurs approximately 1.5-2 days later than *Pitx2* expression. These data provided the basis for this report on the *LEF-1* promoter and its regulation by PITX2, LEF-1 and β-catenin. *β-catenin* is expressed at the same time as *Lef-1* in the developing tooth bud and, similar to *Pitx2* and *Lef-1*, it is restricted to the epithelial tissues (Fausser et al., 1998). Because β-catenin has been implicated in the regulation of Pitx2 transcriptional regulation we asked if it played a role in regulation of *Lef-1* expression in concert with PITX2. β-catenin has been shown in previous reports to play a role in *Lef-1* expression (Filali et al., 2002).

In this report we show reduced *Lef-1* expression in *Pitx2*$^{-/-}$ mutant mice and activation of the human *LEF-1* promoter by PITX2. The *LEF-1* promoter is differentially regulated by PITX2 isoforms and we have identified two regions of the *LEF-1* promoter that repress its transcriptional activation by PITX2. Both LEF-1 and β-catenin can synergistically activate the *LEF-1* promoter in combination with PITX2. These factors directly interact with PITX2 to regulate its transcriptional activity. Furthermore, the combination of PITX2, LEF-1 and β-catenin can combinatorially regulate the *LEF-1* promoter. Thus, we have identified *LEF-1* as a downstream target of PITX2 and show interactions between PITX2 and LEF-1 and β-catenin. These data reveal new mechanisms for the regulation of PITX2 transcriptional activity during development.

Materials and Methods

Expression and reporter constructs

Expression plasmids containing the cytomegalovirus (CMV) promoter linked to the *PITX2* cDNA were constructed in pcDNA 3.1 MycHisC (Invitrogen) (Amendt et al., 1999; Amendt et al., 1998; Cox et al., 2002). LEF-1 and β-catenin S37A expression plasmids have been previously described (Filali et al., 2002). The human *LEF-1* promoters have been previously described and were PCR amplified and cloned into the luciferase vector as previously described (Amendt et al., 1999; Filali et al., 2002). All constructs were confirmed by DNA sequencing. A SV-40 β-galactosidase reporter plasmid was cotransfected in all experiments as a control for transfection efficiency.

Western blot assays

Expression of transiently expressed PITX2, LEF-1 and β-catenin proteins was shown using the PITX2 P2R10 antibody (Hjalt et al., 2000), or Lef-1 and β-catenin antibodies (Upstate). Approximately 10 μg of transfected cell lysates were analyzed in western blots. Following SDS gel electrophoresis, the proteins were transferred to PVDF filters (Millipore), immunoblotted and detected using specific antibodies and ECL reagents from Amersham Biosciences.

Cell culture, transient transfections, luciferase and β-galactosidase assays

CHO cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 5% fetal bovine serum (FBS) and penicillin/streptomycin in 60 mm dishes and transfected by electroporation. CHO cells were mixed with 2.5 μg of expression plasmids, 5 μg of reporter plasmid and 0.5 μg of SV-40 β-galactosidase plasmid plated in 60 mm culture dishes and fed with 5% FBS and D-MEM. Electroporation of CHO cells was at 360 V and 950 microfarads (μF) (Bio-Rad); cells were fed 24 hours before transfection. Transfected cells were incubated for 24 hours then lysed and assayed for reporter activities and protein content by Bradford assay (BioRad). Luciferase was measured using reagents from Promega. β-galactosidase was measured using the Galacto-Light Plus reagents (Tropix). All luciferase activities were normalized to β-galactosidase activity.

Expression and purification of GST-PITX2A fusion proteins

The human *PITX2A* and *PITX2A* deletion constructs were PCR amplified from cDNA clones as described (Amendt et al., 1999; Amendt et al., 1998). The *PITX2A*, *LEF-1* and *β-catenin* PCR products were cloned into the pGex6P2 GST vector (Amersham Pharmacia Biotech) as previously described (Amendt et al., 1999; Amendt et al., 1998). The plasmids were transformed into BL21 cells. Protein was isolated as described (Amendt et al., 1998). PITX2A proteins were cleaved from the GST moiety using 80 units of PreScission Protease (Pharmacia Biotech) per ml of glutathione Sepharose. Purified proteins used in the pulldown assays have been previously described or reported in this manuscript (Amendt et al., 1999). The cleaved proteins were analyzed on SDS polyacrylamide gels by silver stain or coomassie blue stain and quantitated by the Bradford protein assay (BioRad). All stained gels were directly quantitated using image analysis programs.

Isolation of mouse tissue and RT-PCR assays

Timed pregnancies were established between adult male and female *Pitx2*$^{+/-}$ mice (generation N6-7) and embryos harvested from females, after cervical dislocation, into cold PBS (pH 7.4). The morning of plug identification was designated as embryonic day 0.5. E12.5 embryos were harvested, amniotic sacs collected and DNA isolated and processed for genotyping using previously described PCR primers and conditions (Gage et al., 1999a). Facial tissues were removed by manually separating the nasal, maxillary and mandibular prominences from more caudal and dorsal structures. Fresh tissues were homogenized and processed for RNA using Trizol (Invitrogen). Total RNA was isolated as previously described (Amendt et al., 1994).

Reverse transcription was preformed using 2 μg of total RNA, random primers and AMV RT (Takara Mirus Bio) in a total volume of 20 μl. The reaction was incubated at 42°C for 50 minutes. Products were analyzed on an agarose gel, and bands were isolated and sequenced to confirm their identity.

Real-time PCR was carried out using a Smart Cycler thermal cycler (Cepheid, Sunnyvale, CA). Separate cDNA reactions were used for each RNA preparation analyzed. Each PCR reaction contained the appropriate components, and SYBR Green I (Epicenter Technologies). PCR cycling conditions were 94°C for 1 minute, 60°C for 2 minutes and 72°C for 2 minutes. Optical data was collected during the annealing step. A melting curve was generated at the end of every run to ensure product uniformity. All primers were tested using standard RT-PCR protocols and the products sequenced to ensure and confirm their specificity. The *Lef-1* primers were previously described and standard β-actin primers were used in the PCR reactions (Zhou et al., 1995).

Optical data was exported from the Cepheid Smart Cycler as comma separated values files (*.csv) and imported into MS Excel. A Visual Basic Excel macro was used that facilitates determination and

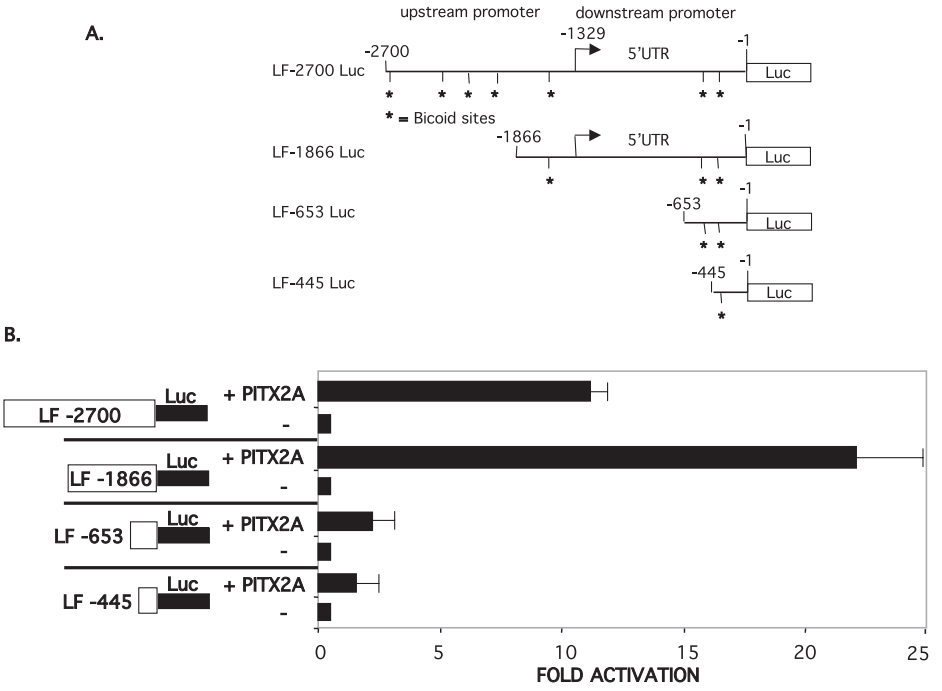


Fig. 1. Activation of the *LEF-1* promoter by PITX2. (A) Human *LEF-1* promoter elements used in the transfection assays. PITX2 binding sites (*Bicoid* sites) are denoted by an asterisk (*). (B) CHO cells were transfected with 5 μg of the appropriate human *LEF-1* luciferase reporter constructs. The cells were co-transfected with 2.5 μg of either the *CMV-PITX2A*, or the CMV plasmid without *PITX2* (vector control). CHO cell lysates transfected with empty vector were used as a control to show lack of endogenous PITX2 protein in CHO cells. To control for transfection efficiency, all transfections included the SV-40 β-galactosidase reporter. Cells were incubated for 24 hours, and then assayed for luciferase and β-galactosidase activities. The activities are shown as mean fold activation compared with the *LEF-1* promoter plasmids without *PITX2* expression and normalized to β-galactosidase activity (± s.e.m. from four independent experiments). PITX2 expression did not change the levels of β-galactosidase activity in the transfected cells.

conversion of the appropriate Smart Cycler optics data to a logarithmic format for subsequent analysis (Marino et al., 2003). Ct values were obtained from three separate experiments and the *Lef-1* expression values were normalized to β-actin values for each preparation. The normalized values from the *Pitx2* homozygous mouse were compared with the wild-type mouse. The differences in Ct values are shown as fold-decrease in transcript levels (Marino et al., 2003).

GST-PITX2 pulldown assays

Immobilized GST-PITX2A fusion protein was 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). Purified bacteria expressed LEF-1 or β-catenin proteins (50-200 ng) were added to 5 μg immobilized GST-PITX2A fusion proteins or GST in a total volume of 100 μl, and incubated for 30 minutes at 4°C. The beads were pelleted and washed four times with 200 μl binding buffer. The bound proteins were eluted by boiling in SDS-sample buffer and separated on a 12.5% SDS-polyacrylamide gel. Approximately 75 ng of purified LEF-1 or β-catenin proteins were analyzed in separate western blots. Following SDS gel electrophoresis, the proteins were transferred to PVDF filters (Millipore), immunoblotted and detected using appropriate antibodies and ECL reagents from Amersham.

Results

PITX2 activation of the LEF-1 promoter and identification of a distal inhibitory region

Quantitative RT-PCR experiments using RNA extracted from facial tissue of E12.5 *Pitx2*$^{-/-}$ mice revelaed a twofold decrease in *Lef-1* transcripts (data not shown). *Lef-1* transcripts in *Pitx2* mutant mice were compared with wild-type tissue and normalized to *β-actin* transcripts. These data suggested that Pitx2 regulated endogenous *Lef-1* expression.

Four *LEF-1* promoter constructs (Filali et al., 2002) were cloned into the luciferase vector and co-transfected with PITX2A into CHO cells. The LF-2700 luc promoter contains 2700 bp of sequence upstream of the translation initiation site and comprises upstream and downstream promoter elements (Fig. 1A). It contains seven *PITX2* binding sites or *bicoid* elements. LF-1866 luc is a 5′ truncated promoter, with 834 bp deleted from the distal region of the promoter compared with LF-2700 luc. This promoter construct removes four *bicoid* sites (Fig. 1A). Two minimal *LEF-1* promoter constructs, LF-653 luc and LF-445 luc, were made as controls for PITX2-activated transcription and contain 2 and 1 *bicoid* sites, respectively (Fig. 1A). PITX2A activated transcription from the LF-2700 promoter construct by approximately 11-fold (Fig. 1B). By contrast, the LF-1866 promoter showed enhanced activation by PITX2 (~22-fold) in comparison to the full-length promoter construct (LF-2700) (Fig. 1B). This increase in PITX2 activation compared with the full-length promoter would suggest the presence of an inhibitory sequence within the distal portion of the promoter. This was an unexpected result because the LF-1866 promoter removed four *bicoid* sites and was expected to decrease PITX2 activation of this promoter construct. Thus, the inhibitory activity of this region appears dominant to the activation by PITX2. The LF-653 promoter showed a significant loss in PITX2 activation at only threefold, and a further decrease in activation was seen with the LF-445 promoter (~twofold) as expected with the loss of the PITX2 binding sites (Fig. 1B). Overall, these data show that PITX2 activates the *LEF-1* promoter.

Electrophoretic mobility shift assays show that PITX2 specifically binds to the *bicoid* and *bicoid*-like sequences (TAATCC, TATTCC, CAATCC, TGATCC, AAATCC) within the *LEF-1* promoter (data not shown).

Differential activation of the LEF-1 promoter by PITX2 isoforms

Because the three major *PITX2* isoforms are co-expressed in

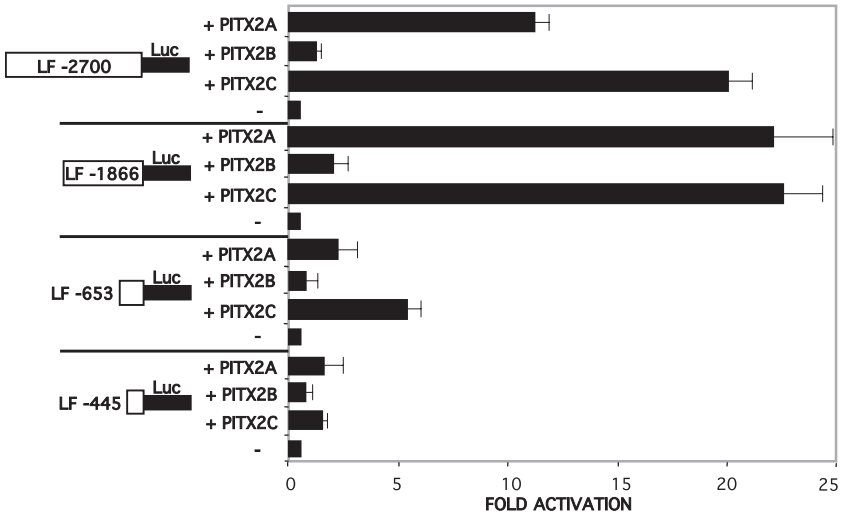


Fig. 2. PITX2 isoforms differentially regulate the *LEF-1* promoter. CHO cells were transfected as described in Fig. 1, using the three major PITX2 isoforms. The activities are shown as mean fold activation compared with the *LEF-1* promoter plasmid without *PITX2* expression and normalized to β-galactosidase activity (± s.e.m. from four independent experiments).

the dental epithelium along with *LEF-1*, we asked if they all activated the *LEF-1* promoter. Transfection of CHO cells with LF-2700 luc and PITX2 isoforms revealed differences in the activation of this promoter. PITX2A as shown in Fig. 1 activated the promoter at 11-fold; however, PITX2B was only minimally active and PITX2C activated the LF-2700 promoter at 20-fold (Fig. 2). Co-transfection of the LF-1866 promoter with the PITX2 isoforms revealed a twofold increase in PITX2A activation; however, only a slight increase was seen for both PITX2B and PITX2C (Fig. 2). The minimal LF-653 and LF-445 promoter showed low activation by the PITX2 isoforms as expected (Fig. 2). We have previously reported the differential activation of other promoters by these PITX2 isoforms (Cox et al., 2002), and we observed similar differences using the *LEF-1* promoter in CHO cells. Thus, the expression levels and activities of these isoforms appear to regulate the expression of PITX2 target genes. PITX2 isoforms are differentially expressed in LS-8 and C3H10T1/2 cell lines, but CHO cells do not express PITX2 (Ganga et al., 2003; Green et al., 2001). A comparison of PITX2 isoform activation of specific promoters reveals differences in their transcriptional activities in these cell lines; however, these differences may correlate with the expression of other factors.

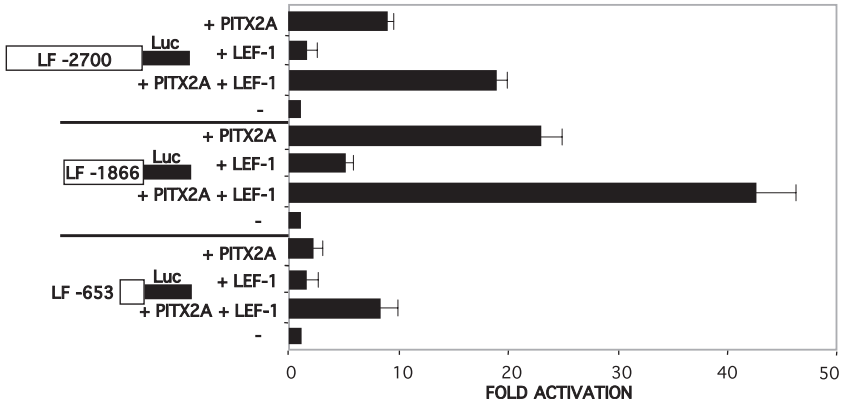


Fig. 3. LEF-1 and PITX2 synergistically activate the *LEF-1* promoter. CHO cells were transfected as in Fig. 1, with CMV-PITX2A or CMV-LEF-1, or both, and the CMV empty expression vector. The activities are shown as mean fold activation compared with the *LEF-1* promoter plasmid without *PITX2* expression and normalized to β-galactosidase activity (± s.e.m. from four independent experiments). LEF-1 expression did not change the levels of β-galactosidase activity in the transfected cells.

LEF-1 and PITX2 synergistically regulate the LEF-1 promoter

We asked if LEF-1 could autoregulate its own promoter in our transfections assays. LEF-1 was unable to activate the LF-2700 promoter, but it did activate the LF-1866 promoter at low levels (~fivefold) in CHO cells (Fig. 3). This activation of the LF-1866 promoter is consistent with the LF-2700 promoter containing a transcriptional inhibitory element that affects both PITX2 and LEF-1 activation. However, co-transfection of PITX2A and LEF-1 synergistically activated the LF-2700 promoter at 20-fold, the LF-1866 promoter at 43-fold and LF-653 at 9-fold (Fig. 3). PITX2A was used initially for these studies because it is the predominately expressed isoform in the LS-8 tooth epithelial cell line (Green et al., 2001). Because LEF-1 alone cannot activate the full-length or minimal promoters these data suggest that LEF-1 and PITX2 physically interact to synergistically regulate the *LEF-1* promoter.

PITX2 and LEF-1 physically interact

To determine if PITX2 and LEF-1 physically interact we performed pull-down assays using bacteria expressed purified proteins. Our initial experiments used immobilized GST-LEF-1 on beads and incubation with 200 ng pure PITX2A. Western blot analysis of the bound protein revealed that PITX2A interacted with LEF-1, but not GST beads alone (Fig. 4A). A faint faster migrating nonspecific band was observed in panel A using GST-LEF-1 without PITX2. This band was not PITX2 or a degradation product and was not observed in other pull-down assays. The reciprocal experiment using immobilized GST-PITX2A and incubation with 50 ng pure LEF-1 protein showed that LEF-1 directly binds to PITX2A (Fig. 4B). As a positive control β-catenin was immobilized on GST beads and incubated with LEF-1 protein. Because LEF-1 and β-catenin are known to interact, we asked if the PITX2A/LEF-1 interaction was similar to the β-catenin/LEF-1 interaction. LEF-1 bound to both proteins at similar levels (Fig. 4B).

LEF-1 binds to the PITX2 C-terminal tail

The PITX2 C-terminal tail has been identified as a region for protein interactions (Amendt et

al., 1999). PITX2 N-terminal and C-terminal deletion proteins and the homeodomain peptide were used to map the LEF-1 interaction to the C-terminal tail (Fig. 5A). LEF-1 bound to the full-length protein and PITX2 C173, which expresses the complete C-terminal tail of PITX2, at similar levels (Fig. 5B). However, LEF-1 did not bind to the PITX2 homeodomain (HD) or to the PITX2A Δ173 protein, which contains the homeodomain and N-terminus (Fig. 5B). These data reveal a direct LEF-1 interaction with the PITX2 C-terminal tail, and because all PITX2 isoforms contain identical C-terminal tails, LEF-1 can presumably interact with all isoforms.

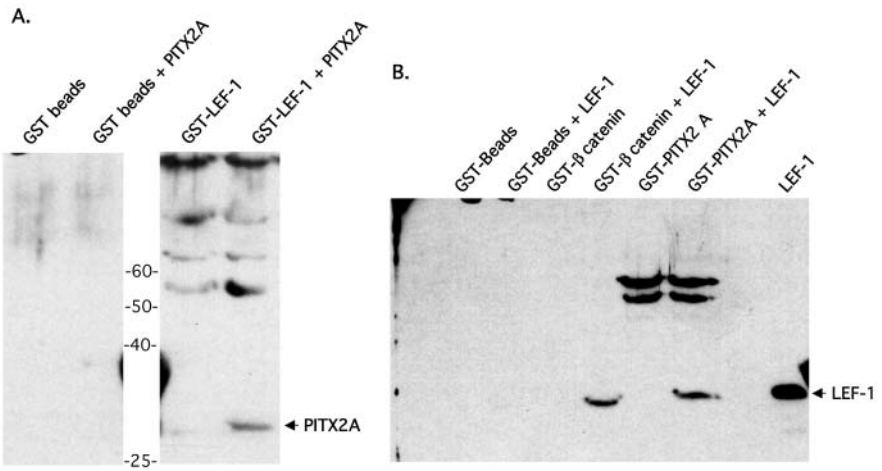


Fig. 4. LEF-1 physically interacts with PITX2. (A) GST-LEF-1 pull-down assay with bacterial expressed and purified PITX2A protein (200 ng). PITX2A binds to immobilized GST-LEF-1, showing that PITX2A can physically interact with LEF-1. The bound protein was detected by western blot using the PITX2 antibody, P2R10. As a control GST-beads were incubated with purified PITX2A to show the specificity of binding to LEF-1. (B) GST-β-catenin and GST-PITX2A pull-down assay with bacterial expressed and purified LEF-1 protein (50 ng). LEF-1 binds to immobilized GST-β-catenin as expected and used as a positive control. LEF-1 binds to GST-PITX2A in a reciprocal experiment shown in A. The bound protein was detected by western blot using a LEF-1 antibody. As a control, GST-beads were incubated with purified LEF-1 to show the specificity of binding to β-catenin and PITX2A.

A LEF-1 Wnt responsive element (WRE) in the proximal promoter inhibits PITX2 activation

A Wnt responsive element (WRE) was previously identified in the LEF-1 proximal promoter that was responsive to Wnt3a activation (Filali et al., 2002). This WRE element is in the downstream promoter of the 5′UTR region (Fig. 6A). When this element was deleted (LF-2700 ΔWRE), PITX2 isoform activation increased approximately twofold in transfected CHO cells (Fig. 6B). PITX2A activated the LF-2700 ΔWRE promoter at 28-fold compared with 12-fold activation of the full-length promoter (Fig. 6B). PITX2B only minimally activated the LF-2700 promoter but demonstrated an eightfold activation of the LF-2700 ΔWRE promoter (Fig. 6B). PITX2C activation was increased from 20-fold to ~38-fold when the WRE element was deleted (Fig. 6B). These data suggest that Wnt signaling is repressing PITX2 activation of the *LEF-1* promoter or factors binding to this site inhibit PITX2 activation.

PITX2 and β-catenin synergistically regulate the LEF-1 promoter

The overlapping expression patterns of *Pitx2*, *β-catenin* and *Lef-1* led us to determine if PITX2 and β-catenin could regulate the *LEF-1* promoter in transfected cells. β-catenin S37A only minimally activated the LF-2700 promoter; however, in combination with PITX2C, the promoter was synergistically activated 40-fold in CHO cells (Fig. 7A). To determine if the WRE element was required for this synergistic activation, the LF-2700 ΔWRE promoter was co-transfected with β-catenin S37A. β-catenin S37A did not activate the LF-2700 ΔWRE promoter independently, but PITX2C activated the LF-2700 ΔWRE promoter at 42-fold (Fig. 7A). However, co-transfection of PITX2C and β-catenin S37A synergistically activated the LF-2700 ΔWRE promoter at 67-fold (Fig. 7A). These data suggest that β-catenin is acting independently of the WRE and directly interacting with PITX2.

Because β-catenin synergistically activated the *LEF-1* promoter in combination with PITX2, we asked if they physically interacted. PITX2A binds to immobilized GST-β-

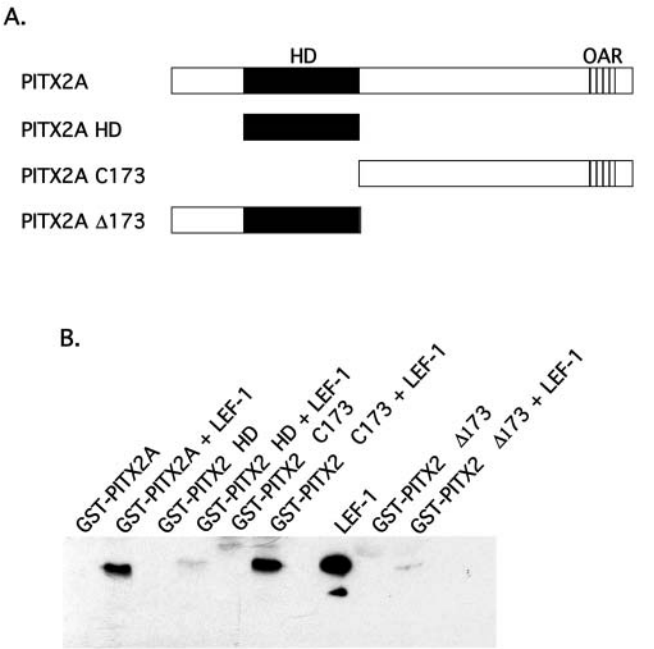


Fig. 5. LEF-1 binds to the C-terminal tail of PITX2. (A) The PITX2 deletion constructs used to map the LEF-1 interaction. (B) GST-PITX2A, GST-PITX2 HD (homeodomain only), GST-PITX2 C173 (C-terminal tail only) and GST-PITX2 Δ173 (deletion of the C-terminal tail) pull-down assay with bacterial expressed and purified LEF-1 protein (50 ng). LEF-1 binds to GST-PITX2A and GST-PITX2 C173 but not to GST-PITX2 HD or GST-PITX2 Δ173. The bound protein was detected by western blot using a LEF-1 antibody. As a control GST-beads were incubated with purified LEF-1 to show the specificity of binding to PITX2A.

catenin in a pull-down assay (Fig. 7B). Thus, these data indicate that PITX2 and β-catenin directly interact to regulate the *LEF-1* promoter.

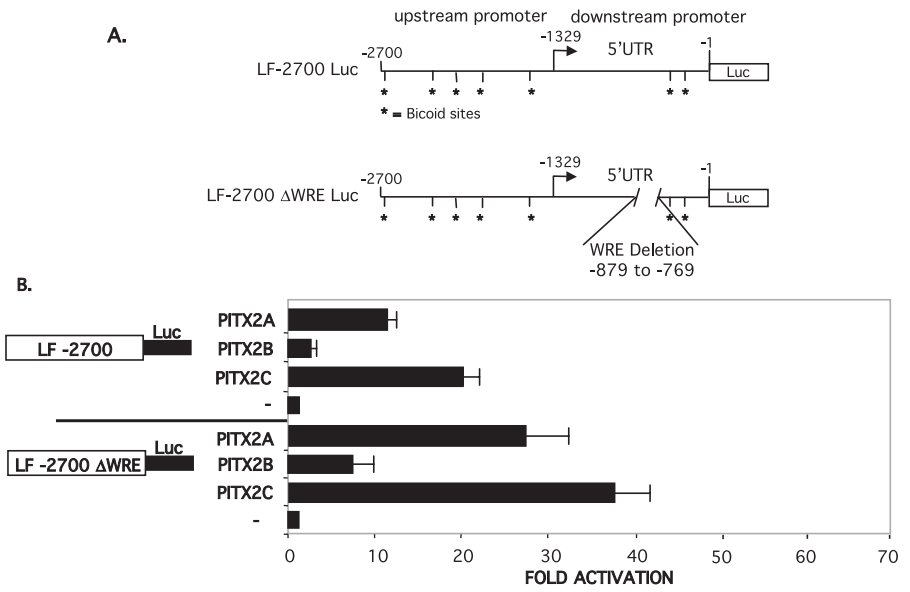


Fig. 6. The *LEF-1 WRE* represses PITX2 activation. (A) The WRE deletion in the *LEF-1* promoter compared with the full-length *LEF-1* promoter. (B) CHO cells were transfected as in Fig. 1, with CMV-PITX2 isoforms and the CMV empty expression vector with the appropriate *LEF-1* luciferase reporter constructs. The activities are shown as mean fold activation compared with the *LEF-1* promoter plasmid without PITX2 expression and normalized to β-galactosidase activity (± s.e.m. from four independent experiments).

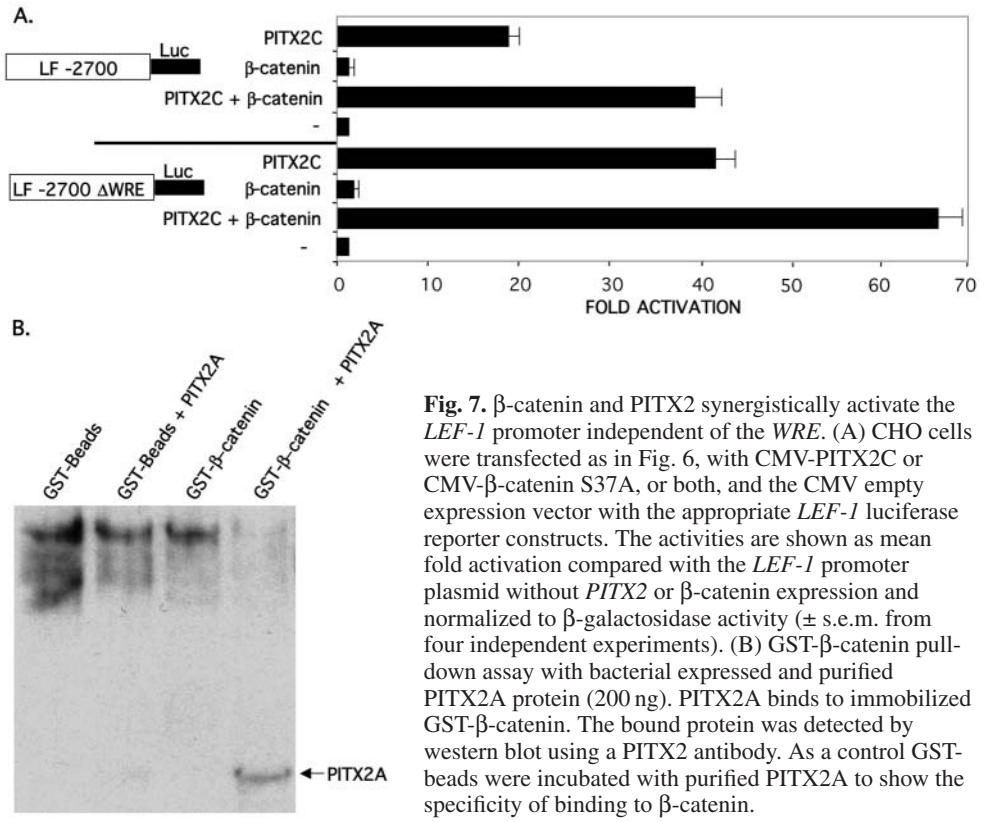


Fig. 7. β-catenin and PITX2 synergistically activate the *LEF-1* promoter independent of the *WRE*. (A) CHO cells were transfected as in Fig. 6, with CMV-PITX2C or CMV-β-catenin S37A, or both, and the CMV empty expression vector with the appropriate *LEF-1* luciferase reporter constructs. The activities are shown as mean fold activation compared with the *LEF-1* promoter plasmid without *PITX2* or β-catenin expression and normalized to β-galactosidase activity (± s.e.m. from four independent experiments). (B) GST-β-catenin pull-down assay with bacterial expressed and purified PITX2A protein (200 ng). PITX2A binds to immobilized GST-β-catenin. The bound protein was detected by western blot using a PITX2 antibody. As a control GST-beads were incubated with purified PITX2A to show the specificity of binding to β-catenin.

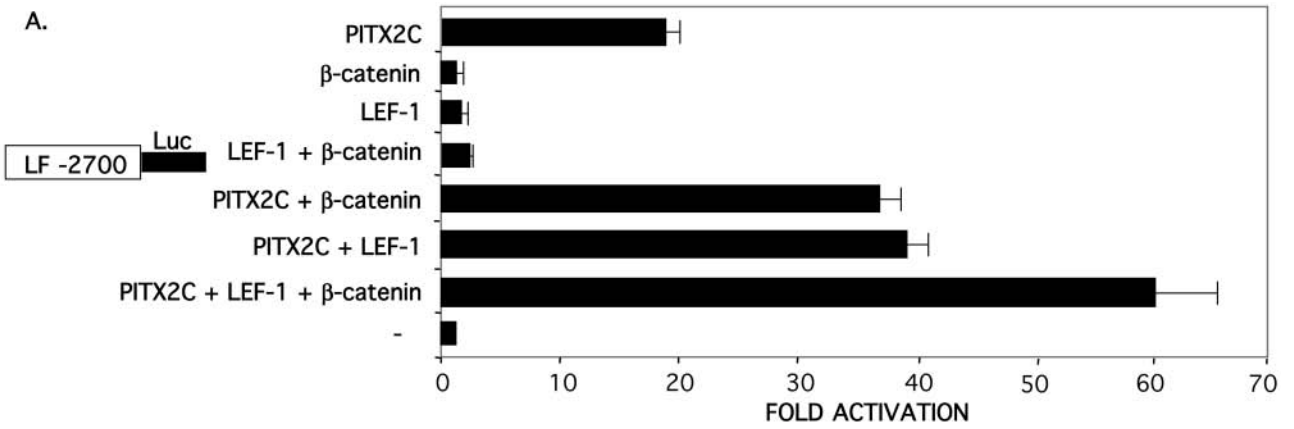
PITX2, LEF-1 and β-catenin dramatically increase LEF-1 expression

We have shown that combinations of PITX2 and LEF-1, and PITX2 and β-catenin S37A synergistically activate the LF-2700 promoter. We next asked if LEF-1 and β-catenin S37A, and PITX2, LEF-1 and β-catenin S37A co-expression would increase *LEF-1* promoter activity. Interestingly, LEF-1 and β-catenin S37A co-expression did not activate the LF-2700 promoter (Fig. 8A). However, PITX2C and β-catenin S37A, and PITX2C and LEF-1 co-expression both synergistically activated the LF-2700 promoter at similar levels (Fig. 8A). More importantly, co-expression of all three factors resulted in a further increase in *LEF-1* promoter activity to 60-fold (Fig. 8A). While either LEF-1 or β-catenin in combination with PITX2 can synergistically activate *LEF-1* expression the combination of all three factors contributes to high levels of *LEF-1* expression.

To determine if β-catenin and/or LEF-1 increased PITX2 expression either from the CHO cell genome or from the transfected PITX2 plasmid, a western blot was preformed using transfected CHO cell lysates. LEF-1 and β-catenin did not activate PITX2 expression (Fig. 8B). Furthermore, transfected PITX2 expression was unaffected by β-catenin and LEF-1 (Fig. 8B). Thus, the increased *LEF-1* promoter activity was not due to increased PITX2 expression by β-catenin and/or LEF-1.

Discussion

Targeted inactivation of the *Lef-1* gene in the mouse resulted in developmentally impaired teeth, whiskers, hair follicles and mammary glands (van Genderen et al., 1994). Tooth development is initiated in *Lef*$^{-/-}$ mouse embryos, but it is arrested before the formation of a mesenchymal dental papilla at E13, after formation of the epithelial tooth bud and mesenchymal condensation but before morphogenesis (van Genderen et al., 1994). From E10 to E12, *Lef1* transcripts are detected initially in the epithelium and subsequently in the mesenchyme, consistent with the change in the developmental



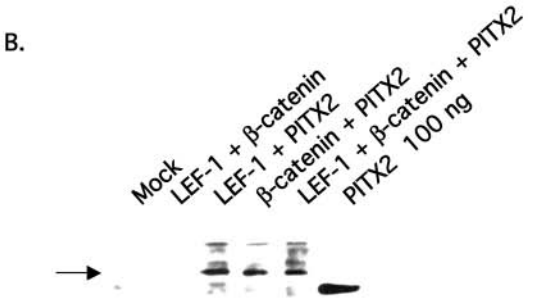


Fig. 8. Combinatorial effect of PITX2, LEF-1 and β-catenin on LEF-1 promoter activity. (A) CHO cells were transfected as in Fig. 1, with CMV-PITX2C, CMV-LEF-1 or CMV-β-catenin S37A, or combinations of each, and the CMV empty expression vector. The activities are shown as mean fold activation compared with the *LEF-1* promoter plasmid without protein expression and normalized to β-galactosidase activity (± s.e.m.from four independent experiments). (B) Expression of PITX2 in transfected CHO cell lysates; approximately 10 μg of lysate was used in the western blot. PITX2 expression was similar in cells transfected with LEF-1 or β-catenin, or both. Bacteria expressed PITX2 protein (100 ng) was used as a control; the protein expressed in transfected cells migrates slower than the bacterially purified protein due to the presence of a Myc/His C-terminal tag.

dominance of these tissues (Kratochwil et al., 1996). The role for *Lef1* in the mesenchyme is unclear since an essential function for *Lef1* expression could be shown only in the dental epithelium between E13 and E14, corresponding to the presence of *Lef1* transcripts in the epithelial tooth bud (Kratochwil et al., 1996).

LEF-1 is a member of the family of high mobility group (HMG) proteins and has been reported to activate transcription only in collaboration with other DNA-binding proteins (Carlsson et al., 1993; Giese and Grosschedl, 1993). The transcriptional regulation of *LEF-1* has been shown to include BMP-4 and wnt/Tcf/β-catenin (Atcha et al., 2003; Filali et al., 2002; Kratochwil et al., 1996). During tooth development BMP-4 activates *Lef-1* expression and tooth development (Kratochwil et al., 1996). However, the regulation of *Lef-1* expression by specific transcription factors has not been determined. In this report we show that PITX2 regulates the *LEF-1* promoter, which provides the first evidence of regulated *LEF-1* expression by a homeodomain transcription factor.

LEF-1 is a downstream target of PITX2

The human *LEF-1* promoter was used to show specific activation by PITX2. A previous report identified the distal region of the *LEF-1* promoter as having a repressive effect on its activation (Filali et al., 2002). The repressive effect of the promoter was observed in transfected HEK 293 cells and with β-catenin expression. Interestingly, the distal region contains four *bicoid* sites, which should bind more molecules of PITX2 and lead to increased activation over a promoter that does not contain these sites. This region inhibits *LEF-1* promoter activity in a variety of cells and transcription factors. We speculate that this region binds a general cellular factor to repress *LEF-1* expression.

PITX2 isoforms differentially regulate the *LEF-1* promoter similar to other PITX2 target genes (Cox et al., 2002). The transcriptional activities of the PITX2 isoforms are both cell/tissue and promoter dependent. Because these isoforms are all expressed in the dental epithelium we have previously shown that they can directly interact with one another to synergistically activate several promoters (Cox et al., 2002; Ganga et al., 2003). The inhibitory region of the *LEF-1* promoter does not repress PITX2C activation, as the levels of *LEF-1* promoter activity are unchanged when this region is deleted. This is a new response to PITX2 isoform regulation that has not been previously reported. It provides the direct method for regulated gene expression by separate PITX2 isoforms, where PITX2C appears to be unaffected by a negative-acting promoter element that inhibits the activation by the other two major PITX2 isoforms. In other tissues, which express only one or two PITX2 isoforms, this could dramatically change *LEF-1* expression during development.

LEF-1 and PITX2 interactions as a mechanism to regulate gene expression

Several groups have shown that LEF-1 activates transcription only in concert with other factors. β-catenin can interact with the TCF/LEF family of transcription factors to change them from repressors to activators (Fisher and Caudy, 1998). Consistent with previous reports we find that LEF-1 cannot activate its own promoter. We show that LEF-1 is a new co-factor in the regulation of PITX2 transcriptional activity. Through our initial mapping of the LEF-1 interaction with PITX2, LEF-1 protein binds to the C-terminal tail of PITX2. The PITX2 C-terminal tail has been shown to interact with other proteins and appears to be a major site of regulation through protein interactions (Amendt et al., 1999; Kioussi et

al., 2002; Tremblay and Drouin, 1999). Our results provide another mechanism for the regulation of LEF-1 and PITX2 transcriptional activities through direct protein interactions.

A new C-terminal PITX2 mutation associated with ARS results in the deletion of a T nucleotide at position 1261 of PITX2A (Brooks et al., 2004). Deletion of this T creates a new reading frame change in the 3′ end of the C-terminal tail. Starting at residue 226 the reading frame is changed and a premature stop codon is created 12 codons downstream. Thus, this mutant protein (PITX2A ΔT1261) is only 237 amino acids compared with 271 for PITX2A, and completely disrupts the PITX2 C-terminal OAR domain. This is the most distal C-terminal mutation reported to date and results in a small deletion of the PITX2 C-terminal tail, including the OAR domain. Recently, we reported the inability of this ARS mutant protein to interact with the POU homeodomain protein, Pit-1 (Espinoza et al., 2004). This mutation corroborates our earlier studies showing a role for this part of the PITX2 C-terminal tail in modulating its activity through a direct interaction with Pit-1 (Amendt et al., 1999). This is the first demonstration of PITX2 protein interactions in regulating normal human development. In this report PITX2 transcriptional regulation is controlled through a similar mechanism involving a LEF-1 interaction with the PITX2 C-terminal tail. These data show the importance of the PITX2 C-terminal tail in regulating gene expression through protein-protein interactions.

A LEF-1 promoter Wnt responsive element (WRE) represses PITX2 activation

A previous report identified a novel element in the *LEF-1* promoter from –884 to –768 bp that might bind repressor proteins that are responsive to Wnt3A (Filali et al., 2002). Our results show that this element strongly represses PITX2 activation of the *LEF-1* promoter. Furthermore, this repressive effect can occur without Wnt signaling in transfected CHO cells; however, Wnt signaling may enhance this repressive response. Wnt induced nuclear extracts from 293 cells show specific protein complexes binding to the WRE (Filali et al., 2002).

Interestingly, expression of constitutively active β-catenin S37A in transfected CHO cells minimally activated the *LEF-1* promoter and deletion of the WRE did not affect β-catenin activation of the *LEF-1* promoter. Thus, our results also indicate that the WRE does not work through a Wnt-induced β-catenin/LEF-1 pathway and deletion of the WRE enhances the PITX2/β-catenin synergistic response. Clearly, factors must be complexing with this element to negatively regulate the *LEF-1* promoter, and these factors appear to be present in both CHO and 293 cells. Experiments are underway to determine the factors regulating this element.

PITX2 and β-catenin directly interact to regulate LEF-1 expression

We have shown that transfection of constitutively active β-catenin does not activate the *LEF-1* promoter; however, in combination with PITX2 it can synergistically activate the *LEF-1* promoter. This synergism results from a direct physical interaction between PITX2 and β-catenin. A previous report identified a PITX2/β-catenin complex by co-immunoprecipitations (Kioussi et al., 2002). Furthermore, β-catenin has been implicated in the regulation of Pitx2 transcriptional activity (Kioussi et al., 2002). These researchers have suggested that a Wnt pathway induces *Pitx2* expression and that β-catenin directly interacts with PITX2 to derepress its transcriptional activity. They propose that LEF-1 activates *Pitx2* expression through Wnt signaling. Interestingly, we were unable to observe increased PITX2 expression by β-catenin and/or LEF-1.

A Gal4/Pitx2 fusion protein showed repressor activity and it was proposed that β-catenin acts in Pitx2 derepression (Kioussi et al., 2002). While we have not observed repression of gene expression using PITX2 and multiple target genes and cell lines, our results are similar in that β-catenin acts to increase PITX2 transcriptional activity. It was proposed that β-catenin interacts with HDAC1 to inhibit its activity and allow for Pitx2 derepression and activation of target gene expression (Kioussi et al., 2002). The data presented in this report further support a role for β-catenin in regulating PITX2 transcriptional activation.

β-catenin can bind to the TCF/LEF family of transcription factors, displacing a corepressor and changing them to activators of transcription (Fisher and Caudy, 1998). Interestingly, our results show that LEF-1 does not activate its own promoter, even though there are multiple LEF-1 binding sites in the *LEF-1* promoter (Filali et al., 2002). In addition, cotransfection of β-catenin and LEF-1 did not activate the *LEF-1* promoter, suggesting that other factors are required for β-catenin/LEF-1 activation of the *LEF-1* promoter. PITX2 appears to be a major factor in the actions of these two factors as shown by their ability to synergistically activate the *LEF-1* promoter in the presence of PITX2. While β-catenin may interact with a corepressor to allow PITX2 to become transcriptionally active, we further show that *LEF-1* expression results in a similar synergistic activation of the *LEF-1* promoter with PITX2. Could LEF-1 also act as a derepressor of PITX2 transcriptional activity? We speculate that β-catenin and LEF-1 may be acting through similar mechanisms to complex with PITX2 to enhance its transcriptional activity. Interestingly, coexpression of PITX2, β-catenin and LEF-1 further activated the *LEF-1* promoter, suggesting a cooperative interaction among all three factors.

In summary, these data reveal that PITX2 activates the *LEF-1* promoter, which would correlate with the temporal and spatial expression patterns of these two factors in the dental epithelium. PITX2 activates and may be required for the sustained expression of *LEF-1*, which is absolutely required for later stages of tooth development. We speculate that PITX2 and LEF-1 may synergistically activate other genes in the tooth developmental pathway. Because *β-catenin* and *Pitx2* are both expressed early during development, they could form a complex capable of regulating other genes similar to LEF-1. *Dlx2* is a PITX2 target gene and is expressed 1.5 days after *Pitx2* in the dental epithelium; it will be of interest to determine if a PITX2/β-catenin/LEF-1 complex can activate its expression similar to LEF-1. We are working to identify other factors in the transcriptional hierarchy and coordinated control of tooth development and determine the mechanisms of these factors in regulating morphogenesis.

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