MOLECULAR AND CELLULAR BIOLOGY, Aug. 2007, p. 5765–5775 Vol. 27, No. 16
0270-7306/07/$08.00+0 doi:10.1128/MCB.01848-06

Nuclear Factor 1 and T-Cell Factor/LEF Recognition Elements Regulate *Pitx2* Transcription in Pituitary Development▽

Di Ai, Jun Wang, Melanie Amen, Mei-Fang Lu, Brad A. Amendt, and James F. Martin*

Institute of Biosciences and Technology, Texas A&M System Health Science Center, 2121 Holcombe Blvd, Houston, Texas 77030

Received 29 September 2006/Returned for modification 17 January 2007/Accepted 21 May 2007

***Pitx2*, a paired-related homeobox gene that is mutated in Rieger syndrome I, is the earliest known marker of oral ectoderm. *Pitx2* was previously shown to be required for tooth, palate, and pituitary development in mice; however, the mechanisms regulating *Pitx2* transcription in the oral ectoderm are poorly understood. Here we used an in vivo transgenic approach to investigate the mechanisms regulating *Pitx2* transcription. We identified a 7-kb fragment that directs LacZ expression in oral ectoderm and in many of its derivatives. Deletion analysis of transgenic embryos reduced this fragment to a 520-bp region that directed LacZ activity to Rathke's pouch. A comparison of the mouse and human sequences revealed a conserved nuclear factor 1 (NF-1) recognition element near a consensus T-cell factor (TCF)/LEF binding site. The mutation of either site individually abolished LacZ activity in transgenic embryos, identifying *Pitx2* as a direct target of Wnt signaling in pituitary development. These findings uncover a requirement for NF-1 and TCF factors in *Pitx2* transcriptional regulation in the pituitary and provide insight into the mechanisms controlling region-specific transcription in the oral ectoderm and its derivatives.**

The primitive oral ectoderm, originally derived from head ectoderm, gives rise to teeth, salivary glands, and the pituitary (9). As development proceeds, signaling interactions between oral ectoderm and the underlying mesoderm or neural ectoderm promote organogenesis. In the mandible, signals from the oral ectoderm are known to instructively pattern the underlying mesenchyme. Regional expression of signaling molecules, such as Bmp4 and Fgf8, imposes pattern information on the mandibular mesenchyme and establishes the initial proximodistal pattern of the mandible (29, 32, 45). While much work has focused on epithelial-mesenchymal signaling, less is known about the molecular mechanisms that define region-specific gene transcription during oral ectoderm diversification.

The *Pitx* (pituitary homeobox) family of homeobox genes contains three genes, *Pitx1*, *Pitx2*, and *Pitx3*, within the larger paired-related superfamily of homeobox genes (17, 37). The *Pitx* group, a remarkably important gene subfamily, has been implicated in human development, disease, and evolution (39–41). For example, *Pitx2* is the gene mutated in Rieger syndrome I that results in ocular, tooth, and body wall defects (40). A regulatory mutation in *Pitx1* has been implicated in the evolution of the pelvis in threespine sticklebacks (41). The last member of the family, *Pitx3*, is the gene mutated in patients with congenital cataracts and ocular anterior segment defects (39).

Previous studies have shown that *Pitx2* is the earliest marker of the oral ectoderm (33); moreover, as the oral ectoderm diversifies into an organ-forming epithelium, such as the dental epithelium or Rathke's pouch (RP), *Pitx2* expression becomes restricted to this maturing epithelium. Consistent with the *Pitx2* expression pattern, loss-of-function experiments in mice uncovered an important function for *Pitx2* in tooth and pituitary development (16, 24, 25, 28, 30); moreover, *Pitx2* was critical for left-right asymmetry, a fundamental component of vertebrate organ morphogenesis, in addition to ocular and abdominal wall morphogenesis (26, 27, 30).

Pitx2 played a critical role in pituitary development through a mechanism that involves both proliferation and cell survival (10, 23). *Pitx2* has been shown to regulate *Pit-1*, *PLOD-1*, and *LEF-1* (1, 20, 46). Other work has also indicated that the *Hesx1*/*Rpx1* homeobox gene is a direct target of *Pitx* genes in the pituitary (11, 16).

Because of its critical role as a late effector of the left-right asymmetry signaling pathways, *Pitx2* transcriptional regulation in the left lateral plate mesoderm has been studied. Transgenic analysis of *Pitx2* gene regulation uncovered separable mechanisms controlling *Pitx2* in the lateral plate and in maturing organs. In the lateral plate, *Pitx2* expression was dependent upon a *Nodal-FoxH1* pathway that functioned through an intergenic enhancer termed the asymmetric element (ASE). In the organ primordia, *Pitx2* maintenance was regulated by an *Nkx* element also contained within the ASE (42, 43). It is unknown whether similar two-step mechanisms apply to *Pitx2* regulation in the oral ectoderm and organ-forming epithelium.

Although *Pitx2* transcriptional regulation in oral ectoderm has not been studied directly in vivo, bead implantation experiments indicated that Fgf8 has a positive influence, while Bmp4 restricts *Pitx2* in the forming of dental epithelium (44). Whether the influence of Fgf8 and Bmp4 on *Pitx2* expression is direct or distant, downstream events are unknown. Other experiments, performed in tissue culture, implicated *Pitx2* as a direct target of Wnt signaling in pituitary and skeletal muscle cells (23). A clear picture of the mechanisms regulating *Pitx2* expression in the oral ectoderm and organ-forming epithelium within the whole embryo is lacking.

* Corresponding author. Mailing address: Institute of Biosciences and Technology, Texas A&M System Health Science Center, 2121 Holcombe Blvd., Houston, TX 77030. Phone: (713) 677-7558. Fax: (713) 677-7512. E-mail: jmartin@ibt.tamhsc.edu.

▽ Published ahead of print on 11 June 2007.

To gain insight into the mechanisms regulating *Pitx2* expression in the oral and dental epithelium, we used a series of transgenic reporter genes to define a *Pitx2* oral ectoderm enhancer element. We uncovered a 7-kb region 3′ of *Pitx2* coding sequences that directed LacZ expression in oral ectoderm.

A deletion analysis of this oral ectoderm element uncovered a subregion that directed LacZ expression in RP, an RP enhancer; moreover, in the RP enhancer we found that nuclear factor 1 (NF-1) and T-cell factor (TCF)/LEF binding sites were required for enhancer activity. These findings reveal that NF-1 and TCF/LEF factors regulate *Pitx2* in RP and implicate *Pitx2* as a direct target of Wnt signaling in early pituitary development.

MATERIALS AND METHODS

Generation of reporter constructs. A *Pitx2* genomic clone was isolated from a mouse 129SV genomic bacterial artificial chromosome library by using the *Pitx2* cDNA as a probe. Construct tg1k is a 16-kb *Pitx2* genomic fragment encompassing the *Pitx2c* promoter (P2) and 4.2 kb downstream of exon 6 (Fig. 1A). Construct tg3k was generated by adding a 7-kb endogenous genomic fragment that was amplified from a bacterial artificial chromosome using a high-fidelity PCR system (Roche) with two oligonucleotides, 5′-GTTTAAACGGCCGGCC GAGACTAATTAGTTGCTCCC and 3′-GTTTAAACTGGCGCGCCCTGAT TGAGTAAAATATCTTTG (two PmeI cutting sites flanking the 7-kb fragment were introduced for subcloning). tg2k was generated by deleting an upstream 2.8-kb fragment of tg3k. Construct tg3k-Del:5821-9918 was made by deleting a 4-kb fragment in the middle of the 7-kb fragment from tg3k by recombineering (see below). A promoterless *LacZ* cassette, which has the *LacZ* coding sequence and poly(A), was inserted into the 5′ untranscribed region of *Pitx2* exon4 (the first exon of *Pitx2c*) in tg1k, tg2k, tg3k, and tg3k-Del:5821-9918 as a reporter. Other constructs were amplified using a high-fidelity PCR system (Roche) and subcloned into the *Hsp68 LacZ* hybrid reporter cassette. All PCR products were sequenced to make sure no mutations were introduced.

Recombineering. Recombineering was used to delete a 4-kb fragment from tg3k to make tg3k-Del:5821-9918. The targeting vector has two 500-bp homologous arms with a floxed chloramphenicol cassette in the middle. Two homologous arms were amplified by PCR, 5′ arm oligonucleotides (CTCGAGCTGTG ACTGCCCACCGATCCC and AAGCTTCGCTGGGATAGCTGCTATGAG) and 3′ arm oligonucleotides (GGATCCGATGCCAGACAAGTGCTAGC and GCGGCCGCCATCTGTAGTGGAATCTGATGCCC). tg3k was first electroporated into the EL350 cell (12). A single colony was inoculated and cultured overnight at 32°C. The next morning, the culture was diluted (1:50) and cultured until the optical density at 600 nm was 0.5. Then the cells were activated at 42°C for exactly 15 min, cooled down, and washed with ice-cold double-distilled water three times. Three hundred nanograms of targeting vector DNA was electroporated into activated cells, and positive clones were selected on plates containing chloramphenicol (25 μg/ml). Targeted clones were screened and induced to express Cre recombinase by a 1-h culture with 0.1% L-arabinose in LB to remove the chloramphenicol cassette.

Generation and analysis of transgenic mice. All transgenic constructs were digested and gel purified using a spin column kit (QIAGEN) and eluted in 1× microinjection buffer (10 mM Tris, 0.1 mM EDTA, pH 7.4). One nanogram per fertilized zygote of purified transgene was injected into the male pronuclei of embryonic day 0.5 fertilized zygotes from FVB mice. Injected zygotes were transferred back to ICR pseudopregnant females, and embryos were harvested at the desired embryonic time points indicated in the text. DNAs of transgenic founders were analyzed by Southern blotting, and DNAs of embryos were analyzed by PCR with either tail snip or yolk sac genomic DNA. *LacZ* genotyping primers were the 5′ primer, 5′-GCA TCG AGC TGG GTA ATA AGG GTT GGC AAT-3′, and the 3′ primer, 5′-GAC ACC AGA CCA ACT GGT AAT GGT AGC GAC-3′, producing a 700-bp *LacZ*-specific product. Embryos from sacrificed mice were dissected. Yolk sacs were removed for *LacZ* PCR genotyping. Embryos were fixed in 2% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline, washed at room temperature, and then stained overnight in staining buffer [1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 5 mM K3Fe(CN)6 and 5 mM K4Fe(CN)6 in phosphate-buffered saline]. For sections, X-gal-stained embryos were dehydrated in a series of 70, 95, and 100% ethanol, embedded in paraffin, sectioned at a thickness of 8 μm, and counterstained with eosin.

Site-directed mutagenesis. Site-directed mutagenesis of the NF-1 and LEF-1 sites was achieved by using the QuikChange II XL site-directed mutagenesis kit (Stratagene). The DNA fragments used for mutagenesis were cloned into the pGEM T-Easy vector (Promega) first, and mutated fragments were then excised and subcloned into the vector with the *Hsp68 LacZ* cassette to make tg5k-NF-1 M and tg5k-LEF-1 M (see Fig. 6). The sense-strand sequences of the oligonucleotides used for mutation were 5′-CCCTTACTGTCTTCTATCGCACATCG ATCGGTTTTACTTTG-3′ for NF-1 and 5′-CCCAGCCAAGGTTTTAGCTA GTTTTCTTTTGCTG-3′ for LEF-1 (underlined letters indicate the mutant NF-1 and LEF-1 sites). All mutations were confirmed by DNA sequencing. The same mutations were introduced into the NF-1 and LEF-1 sites for the electrophoretic mobility shift assay (EMSA).

EMSA. The NucBuster protein extraction kit (Novagen) was used to prepare αT3-1 cell nuclear extracts, and a TNT-coupled reticulocyte lysate system (Promega) was used to synthesize NF-1X protein in vitro. Purified LEF-1 protein was used for the TCF/LEF gel shift. Briefly, double-stranded oligonucleotides were annealed and end labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase (NEB). Labeled probes were purified using a Micro Bio-Spin 6 column (Bio-Rad). Binding reaction mixtures were incubated in ice in 1× binding buffer (100 mM KCl, 20 mM HEPES, 0.5 mM dithiothreitol, 0.2 mM EDTA) containing 10 μg nuclear extract or 2 μg recombinant NF-1X protein or 0.2 μg purified LEF-1 protein, 1 μg of poly(dI-dC) for 5 min and another 5 min after competitor oligonucleotides (100-fold excess) were added. The reaction mixtures were incubated in ice for an additional 15 min after the addition of the probe and another 30 min after the addition of the antibodies. Reaction mixtures were electrophoresed on a 5% nondenaturing polyacrylamide gel. The sense-strand sequences of the oligonucleotides used for EMSAs were 5′-CTTCTTTGTACCCAGCCAAGGTTTTAC-3′ for the wild-type NF-1 site, 5′-CTTCTATCGCACATCGATCGGTTTTAC-3′ for the mutant NF-1 site, 5′-CCCAGCCAAGGTTTTACTTTGTTTTCTTTTGC TG-3′ for the wild-type LEF-1 site, and 5′-CCCAGCCAAGGTTTTAGCTA GTTTTCTTTTGCTG-3′ for the mutant LEF-1 site (underlined letters indicate the wild-type and mutated binding sites).

ChIP analysis. The chromatin immunoprecipitation (ChIP) analysis was performed using a ChIP assay kit (Upstate) as previously described (14). The two primers for amplifying the LEF-1 and NF-1 binding sites in the *Pitx2c* promoter were sense, 5′-GCTCCCCAATCACTGTGTGTACGTGT-3′, and antisense, 5′-TTGGGGGCTGCAACCAGCTGCTGGCTGAAGG-3′. All the PCR products were evaluated for appropriate size (390 bp) on a 2% agarose gel in 1× Tris-borate-EDTA and were confirmed by sequencing. As controls, the *Pitx2c* primers were used without chromatin, normal rabbit immunoglobulin G was used as a replacement for the LEF-1 (Upstate) or NF-1 antibody (Santa Cruz) to reveal nonspecific immunoprecipitation of the chromatin, and primers to an unrelated gene were used to demonstrate the specificity of the LEF-1 or NF-1 antibody-immunoprecipitated chromatin.

RESULTS

A *Pitx2c* oral ectoderm enhancer element in the 3′ flanking region. The *Pitx2* gene encodes three isoforms, Pitx2a, Pitx2b, and Pitx2c, which are generated by a combination of alternative splicing and alternative promoter usage and have overlapping expressions in oral ectoderm (24, 38). To identify *cis*-acting elements regulating *Pitx2* in oral ectoderm, we used a plasmid containing 16 kb of *Pitx2* genomic sequences containing *Pitx2* exons 4 through 6. We introduced a LacZ cassette into the *Pitx2c*-specific exon 4 to generate a construct, tg1k, that directed expression in the heart but not the oral ectoderm (Fig. 1A) (42, 43).

To scan sequences downstream of *Pitx2* for elements that direct *Pitx2c* expression in oral ectoderm, we added a 7-kb fragment to the 3′ end of tg1K, to generate a plasmid referred to as tg3k (Fig. 1A). We also tested a construct that contained the 7-kb downstream sequences in the context of a small (2.8 kb) 5′ deletion (referred to as tg2K) (Fig. 1A). Both tg2k and tg3k directed LacZ activity in the oral ectoderm with high efficiency. To better define the oral ectoderm enhancer, we

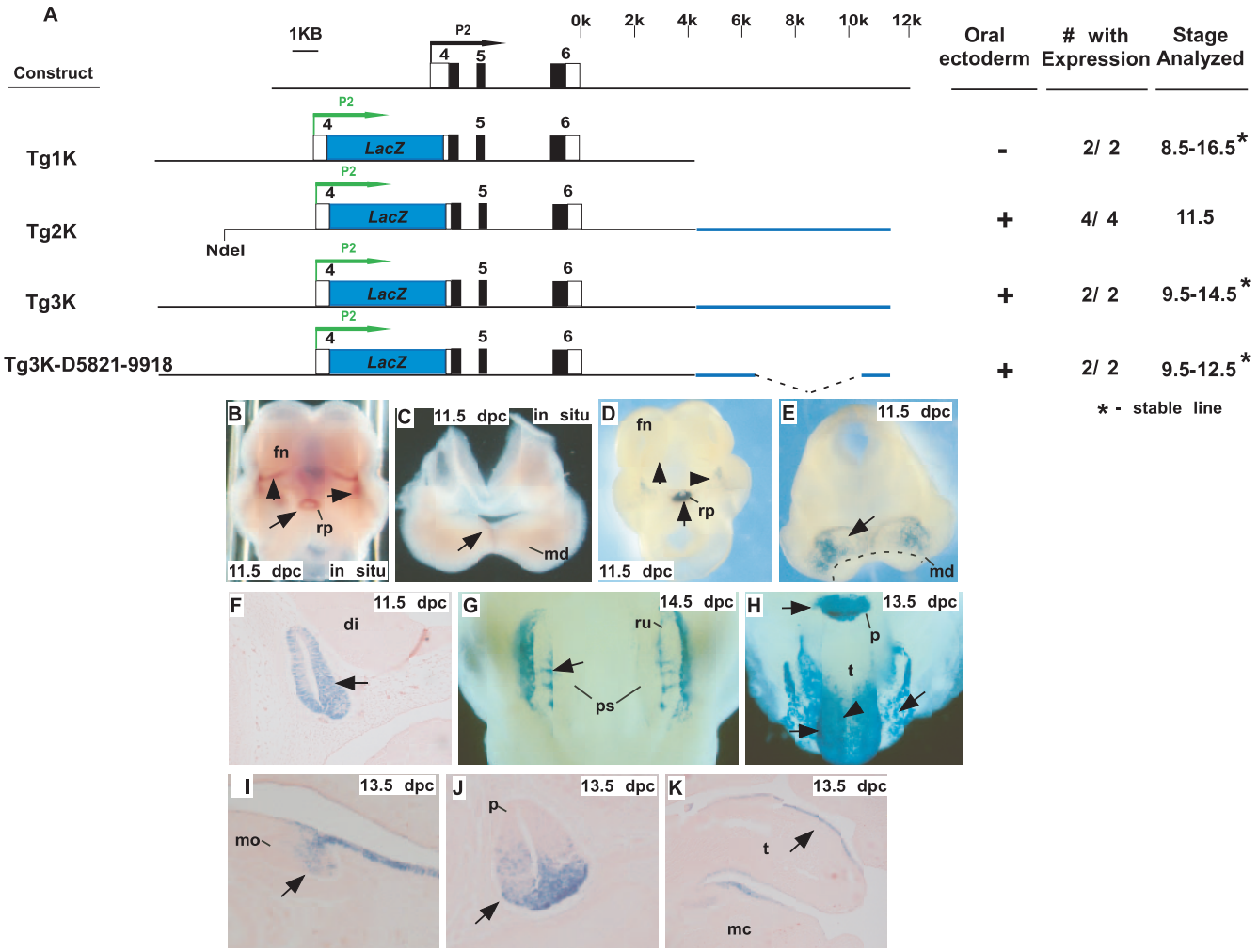

FIG. 1. Identification of a *Pitx2c* oral ectoderm and RP enhancer. Shown at the top of panel A is a representation of the genomic fragment that covers the *Pitx2c* promoter region (P2); exons 4, 5, and 6; and flanking regions. Construct names are indicated on the left, and the corresponding expression patterns are summarized on the right. The "# with expression" column shows the number of transgenic lines with positive RP and oral ectoderm staining per total number of transgenic lines. Asterisks indicate stable lines that were analyzed in multiple stages. Founder embryos were analyzed in other lines. In constructs tg1k to tg3k-Del:5812-9918, a promoterless *lacZ* cassette was inserted into the 5′ untranscribed region of exon4, so that it was controlled by *Pitx2c* regulatory elements. Construct tg1k spans *Pitx2c* with about 6 kb of upstream flanking sequence and 4 kb of downstream flanking sequence. Construct tg3k was generated by adding a 7-kb genomic fragment (blue line) downstream to tg1k. Construct tg2k was generated by deleting a 3-kb fragment upstream of P2 from tg3k. Construct tg3k-Del:5812-9918 was generated by deleting a 4-kb fragment in the middle of a 7-kb fragment in tg3k. Numbers (0k through 12k) show the relative location of the deleted fragment downstream to exon 6. Expression patterns from tg1k, -2k, and -3k indicated that the 7-kb fragment is essential for *Pitx2c* pituitary expression and the pattern from tg3k-Del:5812-9918 indicates that the deleted 4-kb fragment is dispensable for *Pitx2c* pituitary expression. (B) Ventral-caudal view of *Pitx2c* in situ hybridization of the head of a wild-type embryo (11.5 dpc). The mandible was cut off and is shown in panel C. Arrows indicate oral ectoderm and RP hybridization signals. (D to K) Whole-mount X-gal staining and sagittal sections of different stages of tg3k transgenic embryos. (D and E) X-gal staining of tg3k embryos at 11.5 dpc. The mandible was cut off and is shown in panel E. Arrows indicate LacZ staining in RP and oral ectoderm with LacZ absence in the ectoderm of the frontonasal process and most distal mandible (dotted line). (F) Sagittal section of an embryo (11.5 dpc) showing LacZ in RP (arrow). (G to H) Whole-mount X-gal staining of oral cavities of tg3k embryos at 14.5 dpc (G) and 13.5 dpc (H). Sagittal sections showed that LacZ was expressed in molar tooth (I), pituitary (J), and tongue epithelium (K) in 13.5-dpc embryos. Abbreviations: fn, frontal nasal process; md, mandible; di, diencephalon; ru, rugae; ps, palatal shelf; t, tongue; p, pituitary; mo, molor; mc, meckel cartilage. −, absence of oral ectoderm expression; +, presence of oral ectoderm expression.

introduced a 4-kb deletion in the 7-kb fragment in the context of the tg3k construct (tg3k D5821-9918). Embryos with tg3k D5821-9918 still expressed LacZ in the oral ectoderm, indicating that the enhancer element was located at either end of the 7-kb fragment (Fig. 1A).

Expression of the *Pitx2c* oral ectoderm enhancer during development. To determine whether the *Pitx2c* oral ectoderm enhancer completely recapitulated endogenous *Pitx2c* expression, we performed a developmental time course using the stable tg3k and tg3k D5821-9918 lines. Two stable transgenic lines were evaluated for both the tg3k and the tg3k D5821-9918 constructs. All stable lines gave comparable results, although the levels of LacZ activity varied and there was more ectopic LacZ activity in the tg3k D5821-9918 lines, suggesting that the

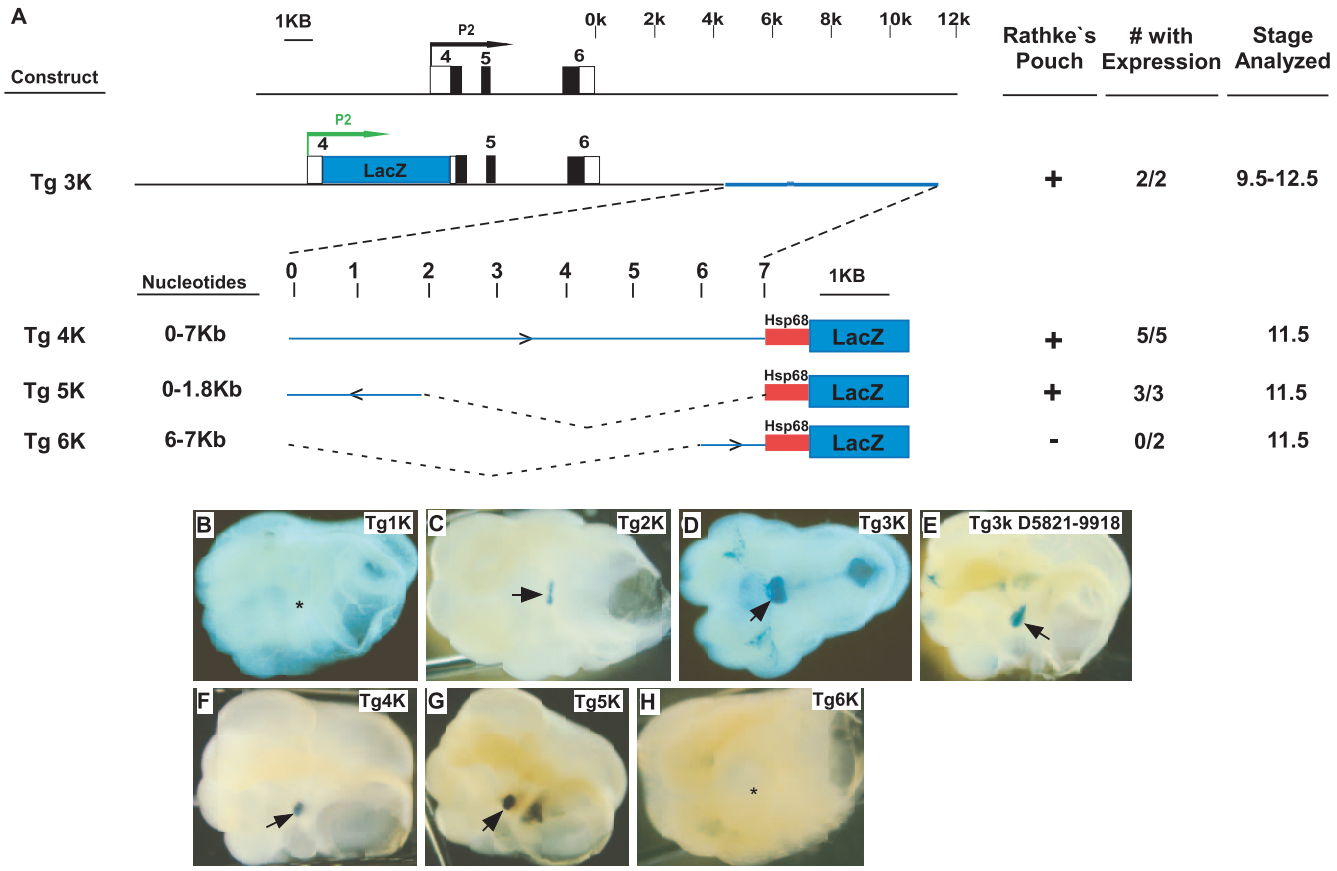

FIG. 2. Localization of the *Pitx2c* RP enhancer. (A) Construct names are indicated on the left, and the corresponding expression patterns are summarized on the right. The "# with expression" column shows the number of transgenic lines with positive RP and oral ectoderm staining per total number of transgenic lines. Constructs tg4k, -5k, and -6k were generated by fusing fragments from the 7-kb fragment to the *Hsp68 LacZ* reporter. X-gal expression of tg4k, -5k, and -6k indicated that the first 1.8-kb fragment of 7 kb was sufficient to initiate *Pitx2c* expression in RP. (B to H) Whole-mount X-gal staining of heads (11.5 dpc) of mice from transgenic lines, with mandibles removed. Constructs are labeled. Arrows denote RP, and asterisks denote no LacZ staining in RP. −, absence of RP expression; +, presence of RP expression.

deleted region may have a negative regulatory role (not shown).

Pitx2 is expressed in the stomatodeal ectoderm that gives rise to RP, beginning at 8.5 days postcoitum (dpc) (33). At 11.5 dpc, *Pitx2c* is expressed in the oral ectoderm of the maxillary and mandibular processes, as well as RP that will give rise to the anterior pituitary (Fig. 1B and C). LacZ staining indicated that tg3k directed LacZ activity in oral ectoderm and RP at 11.5 dpc (Fig. 1D, E, and F). Notably, oral ectoderm expression was biased toward the proximal aspect of the branchial arch (Fig. 1D and E). At 13.5 and 14.5 dpc, LacZ activity was detected in the oral ectoderm, rugae of the secondary palate, dental epithelium, and pituitary (Fig. 1G to K). LacZ activity was also detected in the epithelium of the tongue, as has also been shown by immunohistochemistry (Fig. 1H and K) (21). In dental epithelium and the pituitary, LacZ staining was confined to a subpopulation of cells in each organ, indicating that the tg3k transgene lacked elements that direct endogenous *Pitx2c* expression throughout the dental epithelium and anterior pituitary. Taken together, these data indicate that the tg3k construct contains elements that direct *Pitx2c* expression in oral ectoderm and RP but lack other elements that are necessary for complete *Pitx2c* expression in dental epithelium and anterior pituitary.

Identification of a *Pitx2c* RP enhancer element. We next investigated whether the 7-kb fragment was sufficient to direct LacZ activity in oral ectoderm and RP. The 7-kb fragment was inserted upstream of the *Hsp LacZ* construct that contained the Hsp heterologous promoter element to generate the tg4k construct. An analysis of founder embryos carrying the tg4k construct indicated that the 7-kb region was sufficient to direct LacZ activity in the RP but not in the oral ectoderm (Fig. 2A to F). Next, we wanted to narrow down the critical region for RP expression. Based on data from the tg3k Del 5821-9918 construct (Fig. 1) that indicated that the *Pitx2c* enhancer resided at either the 5′ or 3′ end of the 7-kb fragment, we subcloned the 5′ and the 3′ regions from the 7-kb fragment upstream of *Hsp LacZ* and tested for LacZ activity in founder embryos. This analysis indicated that the RP enhancer element was contained in the 1.8-kb sequence at the 5′ end of the 7-kb fragment (Fig. 2A, G, and H).

Fine mapping the RP enhancer element. To further define the region that directs the expression of LacZ in RP, we performed a deletion analysis. The *Pitx2c* 1.8-kb fragment was

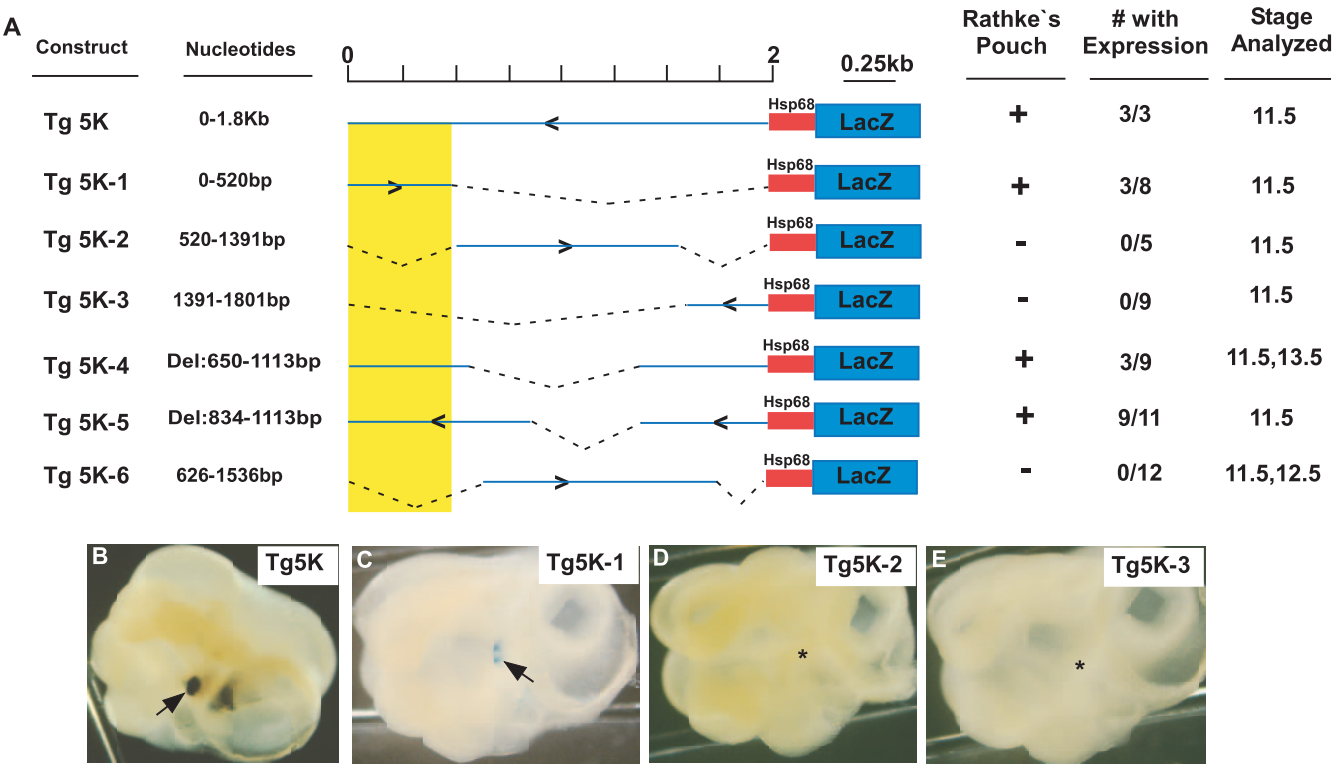

FIG. 3. Fine mapping of *Pitx2c* RP enhancer. (A) A 1.8-kb *Pitx2* downstream fragment was fused to *Hsp68 LacZ* to generate tg5k. Different regions of a 1.8-kb fragment were fused to *Hsp68 LacZ* to generate constructs tg5k-1 through tg5k-6. In the nucleotides column, the relative location in or deletion from tg5k of each construct is shown. (B to E) Whole-mount X-gal staining of heads (11.5 dpc) of mice from transgenic lines, with mandibles removed and constructs labeled. >, sense orientation; <, antisense orientation. Arrows denote RP, and asterisks denote no LacZ staining in RP. −, absence of RP expression; +, presence of RP expression.

subdivided into three fragments and subcloned upstream of *Hsp LacZ* (Fig. 3A). Three out of eight transgenic embryos carrying tg5K-1, which contained 520 bp at the 5′ end of the 1.8-kb fragment, had robust LacZ activity (Fig. 3A, B, and C). In contrast, embryos carrying tg5K-2 and tg5K-3 failed to show LacZ activity in RP; moreover, we tested three other constructs containing deletions within the 1.8-kb fragment (tg5K-4, tg5K-5, and tg5K-6) and the analysis of these three lines revealed that the 520 bp at the 5′ end of the 1.8-kb fragment was common to all constructs with activity in RP. Taken together, these findings indicate that the *Pitx2c* RP enhancer is contained in the 520-bp fragment in tg5K-1 (Fig. 3A). We noted that construct tg5K-5 directed very efficient LacZ expression, indicating that there may be sequence elements located outside the 520-bp element that contribute to *Pitx2c* pituitary expression. Future experiments will be required to evaluate this possibility in more detail.

LEF-1 and NF-1X bind to the RP enhancer. We used the rVISTA software program to compare human and mouse sequences and identified an NF-1 binding site at positions 175 to 189 of the 520-bp enhancer. We also found a consensus TCF/LEF binding site that was just downstream of the NF-1 site in the mouse sequence but that was weakly conserved in the human sequence (Fig. 4A). Next, we used Clustal X to analyze potential conservation of the NF-1 and TCF/LEF sites among different species (mouse, rat, human, and chimp). Alignments indicated that the NF-1 site was well conserved, while the TCF/LEF site was weakly conserved (Fig. 4B).

To determine whether the NF-1 and TCF/LEF binding sites in the 520-bp enhancer were capable of binding to NF-1 and TCF family members in vitro, we first performed EMSAs. As shown in Fig. 5, in vitro-translated NF-1X was capable of binding to the NF-1 element with high affinity; moreover, the binding of NF-1X to the NF-1 element was efficiently competed by the wild-type NF-1 site but not by the mutated NF-1 site, indicating that binding was specific (Fig. 5A and B). The identity of the NF-1 binding complex was further confirmed by a supershift assay with anti-NF-1 antibody (Fig. 5B). The EMSA also showed that purified LEF-1 protein efficiently bound to the TCF/LEF binding site in the *Pitx2c* RP enhancer and was specifically competed by wild-type competitor but not by a mutant oligonucleotide (Fig. 5C). We noted a second TCF/LEF binding site embedded within the 5′ end of the NF-1 element (Fig. 4B). However, gel shift experiments using this site failed to show specific binding activity (not shown). Therefore, we focused on the more 3′ TCF/LEF element.

To establish that NF-1 and TCF/LEF factors bound the 520-bp enhancer element in the native chromatin environment, we performed ChIP assays in αT3 pituitary cells. We used antibodies specific for NF-1 and LEF-1 in ChIP assays as described previously (14); moreover, αT3 cells endogenously express NF-1 and LEF-1, allowing us to investigate transcrip-

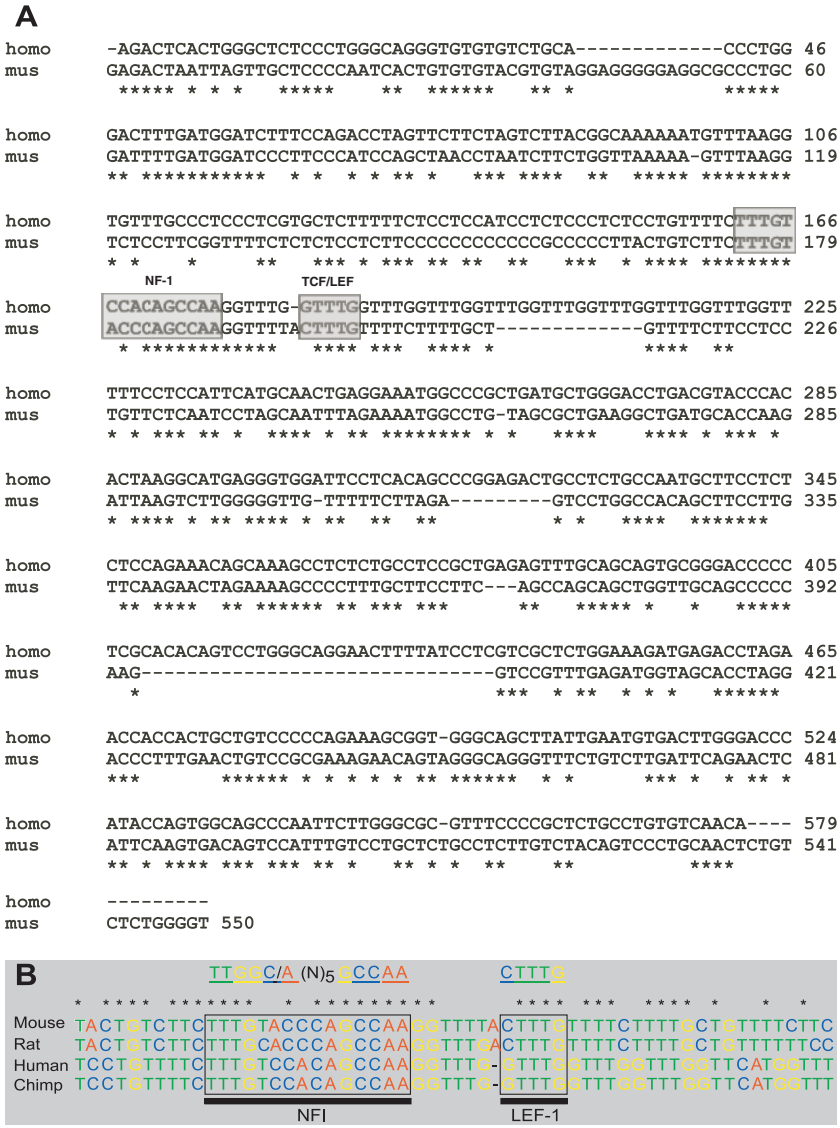

FIG. 4. Multispecies alignment of NF-1 and LEF-1 binding sites in *Pitx2c* pituitary enhancer. (A) One conserved NF-1 and one TCF/LEF binding site were identified in tg5k-1 between mouse (mus) and human (homo). (B) NF-1 and TCF/LEF binding site alignments among more species. Asterisks indicate conserved nucleotides.

tion factor binding to the *Pitx2c* enhancer using endogenous levels of protein. ChIP assays revealed that both NF-1 and LEF-1 can specifically bind to the *Pitx2c* RP enhancer in the native chromatin environment (Fig. 6A to C).

TCF-dependent and NF-1-dependent regulation of *Pitx2c* in RP. To determine whether the NF-1 and TCF/LEF sites were required for transgene expression in vivo, we generated constructs with mutations in the NF-1 (tg5k-NF-1 M) and TCF/LEF (tg5k-TCF/LEFM) sites in the context of the 1.8-kb enhancer element. Of 12 embryos transgenic for the tg5k-NF-1 M construct, 6 failed to show LacZ staining, while another 5 had very weak LacZ activity after 48 h of staining for LacZ (Fig. 7A and B and data not shown). One embryo expressed LacZ in RP at a level similar to that of the wild-type construct (not shown). This embryo also had LacZ expression in other regions of the embryo, such as the limb muscle and eye, suggesting that the integration site was in a region that was permissive for high levels of transcriptional activity. These findings indicate that the NF-1 site is required for optimal transcriptional activity of the *Pitx2c* RP enhancer in most chromatin environments. However, as revealed by the one embryo that expressed LacZ in RP at a level similar to that of the wild type, the NF-1 site is dispensable for LacZ activity in chromatin environments that are permissive for transcriptional activity.

In six embryos transgenic for the tg5k-TCF/LEFM construct, all embryos failed to show LacZ activity even after prolonged incubation in LacZ staining buffer (Fig. 7A and B). This result indicates that the TCF/LEF site is required for transcriptional activity of the *Pitx2c* RP enhancer.

NF-1 has also been implicated in regulating the pituitary-specific POU factor, Pit-1, and the growth hormone gene, and NF-1X was expressed in the mouse gonadotrope cell line

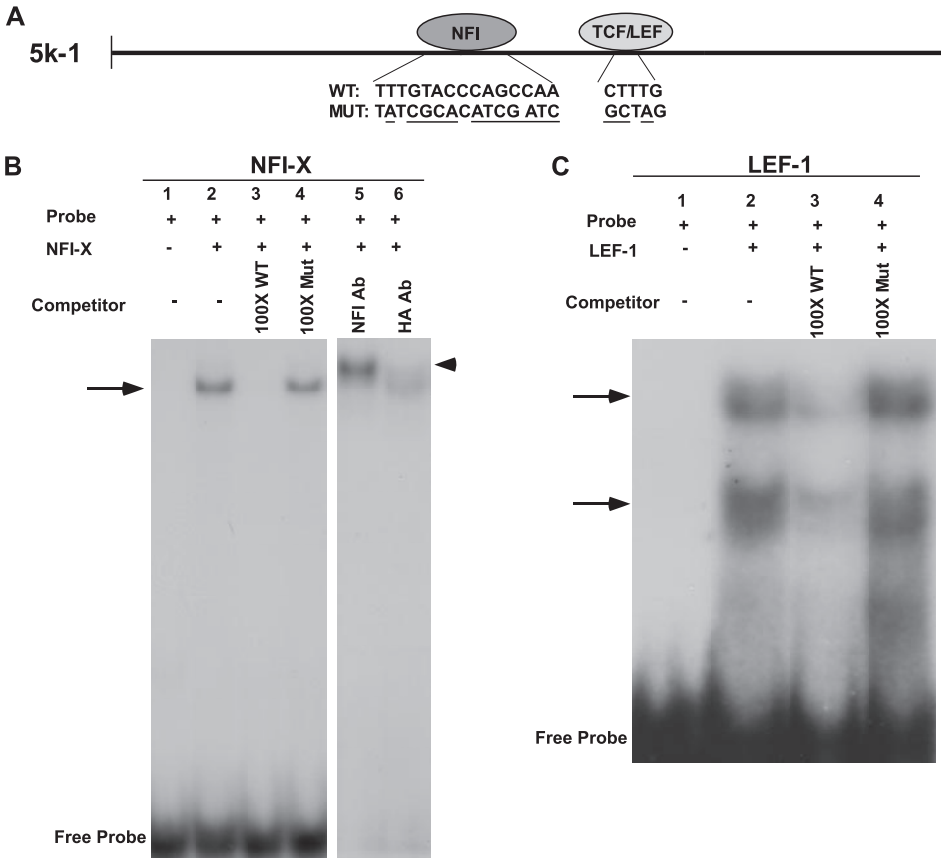

FIG. 5. NF-1X and LEF-1 bind to the *Pitx2c* RP enhancer in vitro. (A) Schematic representation of the 525-bp minimal *Pitx2c* pituitary enhancer region (tg5k-1). γ-^{32}P-labeled oligonucleotides for the NF-1 binding site and LEF-1 binding site of the *Pitx2c* pituitary regulatory region were used as probes in gel mobility shift assays. Underlined nucleotides indicate changes from the consensus binding sequence. (B) In vitro-translated NF-1X protein was used in lanes 2 to 4, and nuclear extracts from αT3-1 cells were used in lanes 5 and 6. (C) Purified LEF-1 protein from cell lysates was used in lanes 2 to 4. Ab, antibody; Mut, mutant; WT, wild type; −, absence of; +, presence of.

αT3-1 (data not shown) (13, 36). However, it is unknown whether NF-1 genes are expressed in RP. To address this issue, we performed in situ analysis with all four *NF-1* genes and found that *NF-1B* was weakly expressed in the oral ectoderm surrounding RP while *NF-1X* was weakly expressed in RP (Fig. 7C). This result suggests that *NF-1X* is the *NF-1* gene that regulates the *Pitx2c* RP enhancer.

DISCUSSION

Pitx2 is an early marker of undifferentiated oral ectoderm, while at later stages, *Pitx2* expression becomes restricted to dental epithelium and RP. The identification of factors acting upstream of *Pitx2* will provide insight into the mechanisms that establish oral ectoderm and define the organ-forming epithelia of teeth and the pituitary. We identified a 520-bp region of the *Pitx2* gene that directs LacZ expression in RP. EMSA, ChIP, and mutational analysis in transgenic embryos indicated that both the NF-1 and TCF/LEF elements were critical for normal levels of *Pitx2c* transcription in RP. Our findings uncover a role for NF-1- and TCF-mediated regulation of *Pitx2c* transcription in the developing pituitary and provide insight into the mechanisms regulating regional gene expression in the oral ectoderm.

Regulation of the *Pitx2c* RP enhancer by NF-1. The NF-1 family contains four homologous members, *NF-1A*, *NF-1B*, *NF-1C*, and *NF-1X*. In vitro experiments have shown that NF-1 factors act both as transcriptional repressors and activators, depending on the context (19). More recent work has shown that NF-1 can influence transcription by remodeling chromatin (15). Our findings indicate that the requirement for NF-1 binding to the *Pitx2c* RP enhancer was necessary for optimal transcriptional activity. Some embryos transgenic for the NF-1 mutant construct showed low transcriptional activity; moreover, the requirement for NF-1 binding could rarely be overcome, for example, when the transgene integrated into a region of high transcriptional activity.

The transcriptional regulatory functions of NF-1 have been studied in the context of the mouse mammary tumor virus promoter. In this system, NF-1 is required for full transcriptional activity by stabilizing the chromatin structure and allowing other factors to bind (15). In *Xenopus* oocytes, NF-1 cooperates with Oct-1 to stabilize an intermediate chromatin structure and likely acts as a platform to recruit other factors (3, 4). Consistent with this notion, NF-1 has been shown to occupy the glial fibrillary acidic protein promoter prior to the differentiation of astrocytes and cooperates with AP1 in astrocytes to regulate gene expression but fails to activate expression

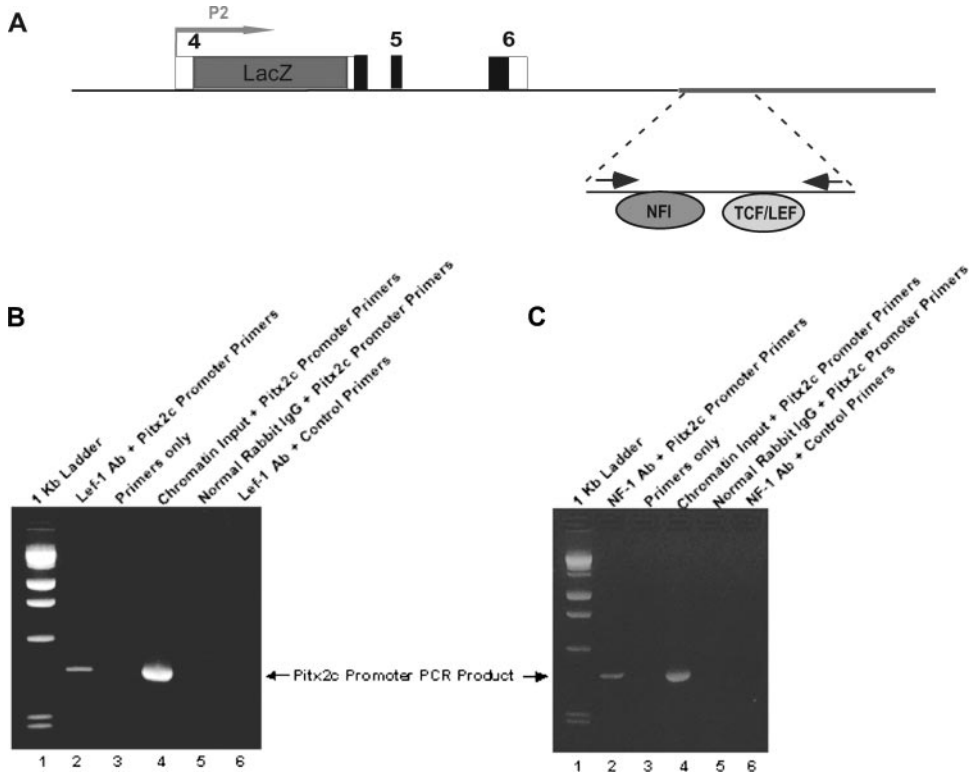

FIG. 6. LEF-1 and NF-1 bind to the *Pitx2c* pituitary enhancer in vivo. (A) Schematic of the Pitx2c promoter and pituitary enhancer with the LEF-1 and NF-1 binding sites noted. The locations of the sense primer and the antisense primer are indicated by arrows. (B) ChIP assays were performed using αT3 cells. Lane 2 shows the LEF-1 immunoprecipitated chromatin amplified using the specific *Pit2c* promoter primers. The product was the correct size (390 bp). Lane 3 shows Pitx2c primers only. Lane 6 shows the LEF-1 immunoprecipitated chromatin amplified with primers to an unrelated gene. (C) ChIP assays were performed as described for panel B, except the NF-1 antibody was used to immunoprecipitate the chromatin.

on its own (7, 18). Our data indicate that NF-1 may have an analogous function as a potentiator of *Pitx2c* transcription in RP.

***Pitx2c*, a target of Wnt signaling in pituitary development.** In the presence of Wnt signaling, β-catenin is stabilized and enters the nucleus, where it interacts with TCF factors, such as LEF-1 or TCF4, to induce downstream gene expression. In the absence of Wnt signaling, β-catenin is targeted for destruction by the *APC*, *Axin*, *Gsk3b* complex that phosphorylates β-catenin and directs it to a destruction pathway (22). Canonical Wnt signaling has been shown to be important during pituitary lineage diversification by promoting *Pit1* and repressing *Hesx1* expression (34). Other experiments indicated that *TCF4* had a role in pituitary expansion by influencing anterior pituitary growth while the noncanonical Wnt, Wnt5a, played a role in regulating cell shape (6, 8).

Canonical Wnt signaling is functional in the pituitary during the time window (11.5 to 14.5 dpc) that we focused on in our study, supporting the idea that *Pitx2c* in RP is regulated by a canonical Wnt signal (34). Previous work showed that *TCF3* and *TCF4* are expressed in RP at the 11.5 and 12.5 dpc stages that we studied in our analysis of the *Pitx2c* RP enhancer, suggesting that they are the TCF factors regulating *Pitx2c* expression. Our data also indicate that LEF-1 binds efficiently to the *Pitx2c* RP enhancer in gel shift and in ChIP assays. In addition, *LEF-1* is expressed transiently in RP, supporting the notion that LEF-1 is involved in the early induction of *Pitx2c* RP expression (34).

The loss of the TCF/LEF recognition element in the *Pitx2c* RP enhancer resulted in a complete loss of transcriptional activity, revealing that this is a functional binding site. In our interspecific sequence alignments, we noted that the TCF/LEF binding site was weakly conserved between mouse and human. Phylogenetic sequence conservation is an extremely powerful tool for determining functional transcription factor binding sites (35). However, there is strong evidence that evolutionary genetic drift can result in point mutations or deletions that disrupt functional transcription factor binding sites and also introduce compensatory mutations that maintain normal gene expression (31). The result of such events would be failure to conserve the location of a functional transcription factor binding site.

Previous studies suggested that *Pitx2a* was regulated in pituitary cells by canonical Wnt signaling (23). Consensus TCF/LEF binding sites identified upstream of *Pitx2a* were shown to respond to Wnt signals and bind LEF-1 in tissue culture cells (23). Although it is unknown whether this element directs *Pitx2a* expression in RP, it will be important in the future to determine whether the different TCF/LEF binding elements interact to direct the expression of the Pitx2a and Pitx2c isoforms in RP. Taken together, our data implicate Wnt signaling

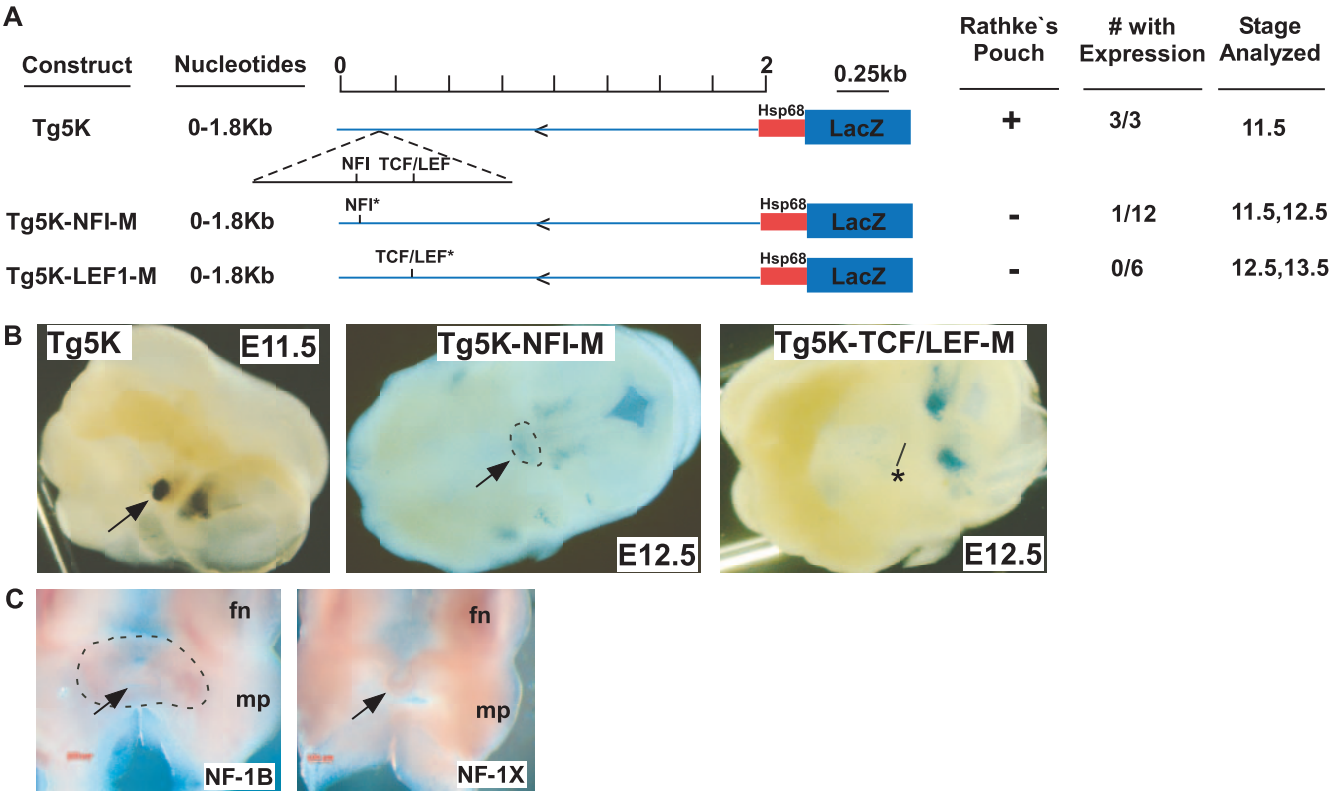

FIG. 7. Mutations within the NFI or TCF/LEF recognition elements in transgenic embryos. (A) NF-1 and LEF-1 individual mutations were made on the background of tg5k (1.8-kb *Pitx2* element). An asterisk indicates a mutant recognition element. −, absence of RP expression; +, presence of RP expression. fn, frontal nasal process; mp, maxillary process. The "# with expression" column shows the number of transgenic lines with positive RP staining per total number of transgenic lines. (B) Whole-mount X-gal staining of heads (11.5 dpc) of mice from transgenic lines, with mandibles removed and constructs labeled. (C) Whole-mount in situ with NF-1B and NF-1X probes at 11.0 dpc. Arrows indicate signal in oral ectoderm (for NF-1B) or RP (for NF-1X). The asterisk indicates lack of expression in Rathke's pouch.

in the diversification of the oral ectoderm into the organ-forming epithelium of RP.

Implications for mechanisms regulating *Pitx2* transcription. Previous studies uncovered a two-step mechanism that functioned through the ASE, regulating *Pitx2c* in the lateral plate mesoderm and individual organ primordia. The induction of *Pitx2c* was dependent upon the Nodal-signaling cascade that works through the FoxH1 effector. The second phase of *Pitx2c* transcription, dependent upon an Nkx binding site in the ASE, was not required for induction but was essential for *Pitx2c* transcriptional maintenance. Therefore, the mechanism involved in *Pitx2c* regulation through the ASE likely reflects sequential binding of transcriptional activators, since transgenes with either element still express at the appropriate developmental stage (42, 43).

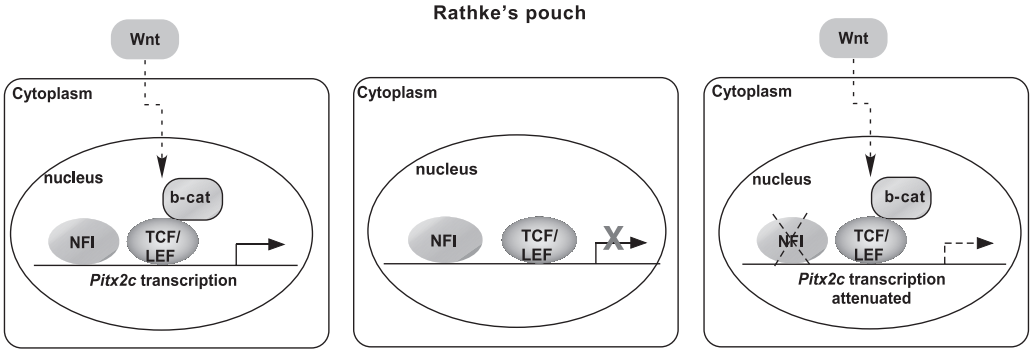

FIG. 8. A model for the regulation of *Pitx2c* transcription in RP. Schematized model showing factors that regulate *Pitx2c* transcription in RP. NF-1 and TCF/LEF-1 binding and canonical Wnt signaling are required for optimal Pitx2c RP enhancer activity (left panel). A function for NF-1 in potentiation of Wnt-induced *Pitx2c* transcriptional activation in RP is proposed. The absence of Wnt signaling or the mutation of the TCF/LEF element results in a loss of Pitx2c RP enhancer activity (middle panel). The loss of NF-1 binding, through mutation of the NF-1 element, results in inefficient Pitx2c RP enhancer activity (right panel).

Although both NF-1 and TCF/LEF factors are expressed in RP, neither NF-1 nor TCF factors are pituitary specific, suggesting the possibility of a cooperative interaction between these two factors in *Pitx2c* RP regulation. We propose a model in which NF-1 binding to the *Pitx2c* RP enhancer induces a potentiation of transcription by NF-1, followed by signal-induced transcriptional activation mediated through the TCF/LEF binding site (Fig. 8). TCF/LEF elements have often been shown to function cooperatively with other elements in developmental systems (2, 5); moreover, in other systems, NF-1 is known to function as a transcriptional potentiator in certain contexts (3). Alternative models are also possible. For example, it is possible that the NF-1 and TCF/LEF elements function independently. In this model, there may be other pituitary-specific factors that function cooperatively with the NF-1 or TCF/LEF elements to direct *Pitx2c* transcription. Further experiments are warranted to investigate this possibility.

ACKNOWLEDGMENTS

We thank R. Behringer for reagents, Pamela Mellon for the mouse gonadotrope cell line αT3-1, Richard Gronostajski for the NF-1X expression plasmid, and E. Olson for *Hsp LacZ*. We thank A. Baldini and W. Klein for comments on the manuscript and discussions. We also thank the TAMHSC-IBT mouse core facility for microinjection.

This work was supported by NIH grants R01 DE16329-01 (J.F.M.) and DE13941 (B.A.A.).

REFERENCES

1. **Amendt, B. A., L. B. Sutherland, E. V. Semina, and A. F. Russo.** 1998. The molecular basis of Rieger syndrome. Analysis of Pitx2 homeodomain protein activities. J. Biol. Chem. **273:**20066–20072.
2. **Barolo, S., and J. W. Posakony.** 2002. Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. Genes Dev. **16:**1167–1181.
3. **Belikov, S., C. Astrand, P. H. Holmqvist, and O. Wrange.** 2004. Chromatin-mediated restriction of nuclear factor 1/CTF binding in a repressed and hormone-activated promoter in vivo. Mol. Cell. Biol. **24:**3036–3047.
4. **Belikov, S., P. H. Holmqvist, C. Astrand, and O. Wrange.** 2004. Nuclear factor 1 and octamer transcription factor 1 binding preset the chromatin structure of the mouse mammary tumor virus promoter for hormone induction. J. Biol. Chem. **279:**49857–49867.
5. **Brannon, M., M. Gomperts, L. Sumoy, R. T. Moon, and D. Kimelman.** 1997. A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. Genes Dev. **11:**2359–2370.
6. **Brinkmeier, M. L., M. A. Potok, K. B. Cha, T. Gridley, S. Stifani, J. Meeldijk, H. Clevers, and S. A. Camper.** 2003. TCF and Groucho-related genes influence pituitary growth and development. Mol. Endocrinol. **17:**2152–2161.
7. **Cebolla, B., and M. Vallejo.** 2006. Nuclear factor-I regulates glial fibrillary acidic protein gene expression in astrocytes differentiated from cortical precursor cells. J. Neurochem. **97:**1057–1070.
8. **Cha, K. B., K. R. Douglas, M. A. Potok, H. Liang, S. N. Jones, and S. A. Camper.** 2004. WNT5A signaling affects pituitary shape. Mech. Dev. **121:**183–194.
9. **Chai, Y., and R. E. Maxson, Jr.** 2006. Recent advances in craniofacial morphogenesis. Dev. Dyn. **235:**2353–2375.
10. **Charles, M. A., H. Suh, T. A. Hjalt, J. Drouin, S. A. Camper, and P. J. Gage.** 2005. PITX genes are required for cell survival and Lhx3 activation. Mol. Endocrinol. **19:**1893–1903.
11. **Chou, S. J., E. Hermesz, T. Hatta, D. Feltner, H. M. El-Hodiri, M. Jamrich, and K. Mahon.** 2006. Conserved regulatory elements establish the dynamic expression of Rpx/HesxI in early vertebrate development. Dev. Biol. **292:**533–545.
12. **Copeland, N. G., N. A. Jenkins, and D. L. Court.** 2001. Recombineering: a powerful new tool for mouse functional genomics. Nat. Rev. Genet. **2:**769–779.
13. **Courtois, S. J., D. A. Lafontaine, F. P. Lemaigre, S. M. Durviaux, and G. G. Rousseau.** 1990. Nuclear factor-I and activator protein-2 bind in a mutually exclusive way to overlapping promoter sequences and trans-activate the human growth hormone gene. Nucleic Acids Res. **18:**57–64.
14. **Diamond, E., M. Amen, Q. Hu, H. M. Espinoza, and B. A. Amendt.** 2006. Functional interactions between Dlx2 and lymphoid enhancer factor regulate Msx2. Nucleic Acids Res. **34:**5951–5965.
15. **Di Croce, L., R. Koop, P. Venditti, H. M. Westphal, K. P. Nightingale, D. F. Corona, P. B. Becker, and M. Beato.** 1999. Two-step synergism between the progesterone receptor and the DNA-binding domain of nuclear factor 1 on MMTV minichromosomes. Mol. Cell **4:**45–54.
16. **Gage, P. J., H. Suh, and S. A. Camper.** 1999. Dosage requirement of Pitx2 for development of multiple organs. Development **126:**4643–4651.
17. **Gage, P. J., H. Suh, and S. A. Camper.** 1999. The bicoid-related Pitx gene family in development. Mamm. Genome **10:**197–200.
18. **Gopalan, S. M., K. M. Wilczynska, B. S. Konik, L. Bryan, and T. Kordula.** 2006. Nuclear factor-1-X regulates astrocyte-specific expression of the alpha1-antichymotrypsin and glial fibrillary acidic protein genes. J. Biol. Chem. **281:**13126–13133.
19. **Gronostajski, R. M.** 2000. Roles of the NFI/CTF gene family in transcription and development. Gene **249:**31–45.
20. **Hjalt, T. A., B. A. Amendt, and J. C. Murray.** 2001. PITX2 regulates procollagen lysyl hydroxylase (PLOD) gene expression: implications for the pathology of Rieger syndrome. J. Cell Biol. **152:**545–552.
21. **Hjalt, T. A., E. V. Semina, B. A. Amendt, and J. C. Murray.** 2000. The Pitx2 protein in mouse development. Dev. Dyn. **218:**195–200.
22. **Huelsken, J., and W. Birchmeier.** 2001. New aspects of Wnt signaling pathways in higher vertebrates. Curr. Opin. Genet. Dev. **11:**547–553.
23. **Kioussi, C., P. Briata, S. H. Baek, D. W. Rose, N. S. Hamblet, T. Herman, K. A. Ohgi, C. Lin, A. Gleiberman, J. Wang, V. Brault, P. Ruiz-Lozano, H. D. Nguyen, R. Kemler, C. K. Glass, A. Wynshaw-Boris, and M. G. Rosenfeld.** 2002. Identification of a Wnt/Dvl/β-catenin→Pitx2 pathway mediating cell-type-specific proliferation during development. Cell **111:**673–685.
24. **Kitamura, K., H. Miura, S. Miyagawa-Tomita, M. Yanazawa, Y. Katoh-Fukui, R. Suzuki, H. Ohuchi, A. Suehiro, Y. Motegi, Y. Nakahara, S. Kondo, and M. Yokoyama.** 1999. Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism. Development **126:**5749–5758.
25. **Lin, C. R., C. Kioussi, S. O'Connell, P. Briata, D. Szeto, F. Liu, J. C. Izpisua-Belmonte, and M. G. Rosenfeld.** 1999. Pitx2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. Nature **401:**279–282.
26. **Liu, C., W. Liu, M. F. Lu, N. A. Brown, and J. F. Martin.** 2001. Regulation of left-right asymmetry by thresholds of Pitx2c activity. Development **128:**2039–2048.
27. **Liu, C., W. Liu, J. Palie, M. F. Lu, N. A. Brown, and J. F. Martin.** 2002. Pitx2c patterns anterior myocardium and aortic arch vessels and is required for local cell movement into atrioventricular cushions. Development **129:**5081–5091.
28. **Liu, W., J. Selever, M. F. Lu, and J. F. Martin.** 2003. Genetic dissection of pitx2 in craniofacial development uncovers new functions in branchial arch morphogenesis, late aspects of tooth morphogenesis, and cell migration. Development **130:**6375–6385.
29. **Liu, W., J. Selever, D. Murali, X. Sun, S. M. Brugger, L. Ma, R. J. Schwartz, R. Maxson, Y. Furuta, and J. F. Martin.** 2005. Threshold-specific requirements for Bmp4 in mandibular development. Dev. Biol. **283:**282–293.
30. **Lu, M. F., C. Pressman, R. Dyer, R. L. Johnson, and J. F. Martin.** 1999. Function of Rieger syndrome gene in left-right asymmetry and craniofacial development. Nature **401:**276–278.
31. **Ludwig, M. Z., C. Bergman, N. H. Patel, and M. Kreitman.** 2000. Evidence for stabilizing selection in a eukaryotic enhancer element. Nature **403:**564–567.
32. **Mina, M., Y. H. Wang, A. M. Ivanisevic, W. B. Upholt, and B. Rodgers.** 2002. Region- and stage-specific effects of FGFs and BMPs in chick mandibular morphogenesis. Dev. Dyn. **223:**333–352.
33. **Mucchielli, M. L., T. A. Mitsiadis, S. Raffo, J. F. Brunet, J. P. Proust, and C. Goridis.** 1997. Mouse Otlx2/RIEG expression in the odontogenic epithelium precedes tooth initiation and requires mesenchyme-derived signals for its maintenance. Dev. Biol. **189:**275–284.
34. **Olson, L. E., J. Tollkuhn, C. Scafoglio, A. Krones, J. Zhang, K. A. Ohgi, W. Wu, M. M. Taketo, R. Kemler, R. Grosschedl, D. Rose, X. Li, and M. G. Rosenfeld.** 2006. Homeodomain-mediated beta-catenin-dependent switching events dictate cell-lineage determination. Cell **125:**593–605.
35. **Pennacchio, L. A., N. Ahituv, A. M. Moses, S. Prabhakar, M. A. Nobrega, M. Shoukry, S. Minovitsky, I. Dubchak, A. Holt, K. D. Lewis, I. Plajzer-Frick, J. Akiyama, S. De Val, V. Afzal, B. L. Black, O. Couronne, M. B. Eisen, A. Visel, and E. M. Rubin.** 2006. In vivo enhancer analysis of human conserved non-coding sequences. Nature **444:**499–502.
36. **Rajas, F., M. Delhase, M. De La Hoya, P. Verdood, J. L. Castrillo, and E. L. Hooghe-Peters.** 1998. Nuclear factor 1 regulates the distal silencer of the human PIT1/GHF1 gene. Biochem. J. **333:**77–84.
37. **Schneitz, K., P. Spielmann, and M. Noll.** 1993. Molecular genetics of aristaless, a prd-type homeo box gene involved in the morphogenesis of proximal and distal pattern elements in a subset of appendages in Drosophila. Genes Dev. **7:**114–129.
38. **Schweickert, A., M. Campione, H. Steinbeisser, and M. Blum.** 2000. Pitx2 isoforms: involvement of Pitx2c but not Pitx2a or Pitx2b in vertebrate left-right asymmetry. Mech. Dev. **90:**41–51.
39. **Semina, E. V., R. E. Ferrell, H. A. Mintz-Hittner, P. Bitoun, W. L. Alward,**

R. S. Reiter, C. Funkhauser, S. Daack-Hirsch, and J. C. Murray. 1998. A novel homeobox gene PITX3 is mutated in families with autosomal-dominant cataracts and ASMD. Nat. Genet. **19:**167–170.

40. **Semina, E. V., R. Reiter, N. J. Leysens, W. L. Alward, K. W. Small, N. A. Datson, J. Siegel-Bartelt, D. Bierke-Nelson, P. Bitoun, B. U. Zabel, J. C. Carey, and J. C. Murray.** 1996. Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. Nat. Genet. **14:**392–399.
41. **Shapiro, M. D., M. E. Marks, C. L. Peichel, B. K. Blackman, K. S. Nereng, B. Jonsson, D. Schluter, and D. M. Kingsley.** 2004. Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature **428:**717–723.
42. **Shiratori, H., R. Sakuma, M. Watanabe, H. Hashiguchi, K. Mochida, Y. Sakai, J. Nishino, Y. Saijoh, M. Whitman, and H. Hamada.** 2001. Two-step regulation of left-right asymmetric expression of Pitx2: initiation by nodal signaling and maintenance by Nkx2. Mol. Cell **7:**137–149.
43. **Shiratori, H., K. Yashiro, M. M. Shen, and H. Hamada.** 2006. Conserved regulation and role of Pitx2 in situs-specific morphogenesis of visceral organs. Development **133:**3015–3025.
44. **St. Amand, T. R., Y. Zhang, E. V. Semina, X. Zhao, Y. Hu, L. Nguyen, J. C. Murray, and Y. Chen.** 2000. Antagonistic signals between BMP4 and FGF8 define the expression of Pitx1 and Pitx2 in mouse tooth-forming anlage. Dev. Biol. **217:**323–332.
45. **Tucker, A., and P. Sharpe.** 2004. The cutting-edge of mammalian development; how the embryo makes teeth. Nat. Rev. Genet. **5:**499–508.
46. **Vadlamudi, U., H. M. Espinoza, M. Ganga, D. M. Martin, X. Liu, J. F. Engelhardt, and B. A. Amendt.** 2005. PITX2, beta-catenin and LEF-1 interact to synergistically regulate the LEF-1 promoter. J. Cell Sci. **118:**1129–1137.