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Chang, WY; KhosrowShahian, Farhad; Chang, R; and Crawford, Michael J., "xPitx1 plays a role in specifying cement gland and head during early Xenopus development" (2001). Genesis, 29, 2, 78-90.
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**xPitx1 Plays a Role in Specifying Cement Gland and Head During Early Xenopus Development**

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Accepted 11 December 2000

**Summary:** *Xenopus Pitx1* is a homeobox gene whose family members are structurally and functionally conserved in organisms as diverse as *Drosophila*, chick, mouse, human, and frog. Present as a maternal transcript, the gene is zygotically expressed during gastrulation in a dorsal streak of cells. This streak restricts to a small circular domain underlying the center of presumptive neural plate. Shortly thereafter, a crescent of expression develops at the border of anterior neural ectoderm, and as the central plate domain diminishes, the crescent coalesces to define the presumptive cement gland. Expression remains high throughout cement gland development, and subsequently expands to include ectodermal cells involved in stomodeal invagination. During early organogenesis, expression ensues in developing eye, posterior lateral mesoderm, and first branchial arch derivatives. Ectopic expression of *xPitx1* causes head deformities including enlarged cement gland, ectopic cement glands, and posterior deformities or, in extreme cases, inhibition of recognizable structures posterior to the cement gland. Expression of markers such as *XCG-1*, *xOtx2*, *xPax6*, *neural β tubulin*, and *xTwist* suggest that increases in cement gland and lower mandibular size are likely at the expense of other head tissues. Paradoxically, overexpression is sufficient to partially rescue embryos that are axially perturbed by ultraviolet irradiation or retinoic acid administration. Ectopic expression of *xPitx1* in ectodermal explants directly promotes cement gland development as there was no evidence that mesodermal or neural tissue was present in explants.

**Key words:** *Xenopus laevis*; placode; paired-like homeobox; ectopic expression; cement gland; induction; head; UV; retinoic acid

**INTRODUCTION**

The paired-like class of homeodomain proteins are a family of transcription factors that play a critical role in the early development of flies and vertebrates. They include factors such as *bicoid*, *goosecoid*, and members of the *Otx*, and the *Pitx* families. Recently, three *Pitx* (pituitary homeobox) family members have been characterized that possess high sequence conservation among chicks, frogs, mice, and humans (Crawford et al., 1997; Gage and Camper, 1997; Lamonerie et al., 1996; Lanctot et al., 1997; Semina et al., 1996; Semina et al., 1997). Moreover, in these disparate species they express in similar structures and at similar times throughout development. A related gene, *Ptx1*, is expressed during *Drosophila* development (Vorbruggen et al., 1997). The genes play a role in body patterning in *Xenopus*; *xPitx2* is involved in regulating left-right asymmetry of internal organs such as heart and gut (Ryan et al., 1998; Campione et al., 1999). *Pitx2* knock-out mice however, reveal normal looping of the heart but possess defective cardiac positioning as well as defects in tooth morphogenesis, lung asymmetry, and mandibular and maxillary facial prominences (Gage et al., 1999; Lin et al., 1999; Lu et al., 1999). Mutations at this locus are responsible for Rieger’s syndrome in human (Semina et al., 1996). Another family member, *Pitx3*, expresses in the lens and in dopaminergic neurons in mouse and is involved in other human developmental mutations (Semina et al., 1997, 1998; Smidt et al., 1997).

Recent work on *Pitx1* has similarly revealed an important role for this gene in body patterning. Murine *Pitx1* is expressed in derivatives of the first branchial arch and also in the posterior lateral mesoderm and hindlimb (Lanctot et al., 1997; Shang et al., 1997). Mice mutated at the *Pitx1* locus develop severe mandibular deformities as well as pituitary developmental arrest (Lanctot et al., 1999; Szeto et al., 1999). Anomalies in limb development suggest that the gene plays a role in differentiating fore- from hind-limb identities. This perspective has been consolidated in ectopic expression studies in chick wing buds where leg-like structures have emerged following localized introduction of *Pitx1* (Szeto et al., 1999). Finally, the human homolog, *PTX1*, has been situated in the chromosomal region associated with Treacher Col-
lins Syndrome, a human developmental mutation which results in craniofacial anomalies and sometimes occlusions foot and thumb abnormalities (Crawford et al., 1997, and references therein).

The early expression patterns of Pitx1 and the mandibular deformities that arise in knock-out mice suggest that the gene specifies the anterior-most “segment” of the developing head. Several other of the bicoid-related genes in Xenopus such as goosecoid and Otx are both required for proper head development. For example, goosecoid is expressed primarily in the organizer region and misexpression leads to the formation of an ectopic body axis sometimes with the duplication of head structures (Cho et al., 1991). The expression pattern of xOtx2 partially overlaps that of goosecoid, and xOtx2 overexpression similarly leads to the development of partial secondary axes, additional cement glands, and bent axes (Blitz and Cho, 1995; Pannese et al., 1995). Moreover, Gammill and Sive (1997) have shown that the cement gland marker genes XCG and XAG are targets of xOtx2.

Here we report the cloning of the Xenopus laevis Pitx1 homolog, which was isolated recently by Hollemann and Pieler (1999). xPitx1 is expressed in a conserved manner consistent with a role for the gene in specifying anterior craniofacial development. xPitx1 is expressed early during gastrulation in presumptive head regions and becomes localized to the prospective cement gland anlage. It is then transiently expressed within the optic cup and olfactory epithelium. Throughout development, xPitx1 remains strongly expressed in the cement gland. In ectodermal cap explant experiments, we show that xPitx1 plays a role in cement gland induction. Finally, because recent work on the ectopic expression of xPitx1 has been limited to chick wing bud, we sought to study the effects of xPitx1 overexpression in specification of anterior structures during early embryogenesis.

RESULTS

Sequence of xPitx1

The sequence of xPitx1 (Genbank accession AF217647) contains an open reading frame that encodes a conceptual protein of 305 amino acids. The homeodomain and other regions share high amino acid identity with Pitx family members and, in particular, Pitx1 homologs from other species (Fig. 1A). Like other Pitx sequences, xPitx1 encodes a carboxy-terminal motif produced by other homeobox genes that are thought to play a role in craniofacial development. This 14-amino acid motif is encoded in genes such as Pitx2, Cart1, Chx10, Prx, Otp, and Drg11 (Semina et al., 1996).

Sequence comparison of the recently cloned xPitx1 by Hollemann and Pieler (1999) revealed only minor differences both at the nucleotide and amino acid level (Fig. 1B). Discrepancies are likely the result of the PCR-based approach used by these investigators to isolate the coding region and to allelic variation.

Spatial and Temporal Expression of xPitx1 in Xenopus

Xenopus Pitx1 expression was examined by RT-PCR and by whole-mount in situ hybridization. RT-PCR reveals that xPitx1 is present as a maternal transcript and that expression increases substantially after gastrulation (Fig. 2). In order to determine the spatial distribution of xPitx1 during early development, whole-mount in situ hybridization was performed using three different digoxigenin-labeled antisense fragments: one possessed 5’ untranslated and coding regions including the homeodomain, one contained 3’ regions excluding the homeodomain, the last encoded the complete coding region. Expression patterns for each of the three riboprobes were identical. xPitx1 is expressed in a rapidly changing and dynamic manner. It is first detected during early gastrulation as a faint dorsal streak (Fig. 3A, B). As gastrulation progresses a band of expression appears in the presumptive anterior region (Fig. 3C). A transient dot in the center of the presumptive anterior neural plate forms where it remains until the onset of neural fold development (Fig. 3D). The dot at the center of the neural plate disappears as neural fold closure begins and stronger expression is observed within the anterior band, which presumably defines the cement gland anlage (Fig. 3E). As development progresses, strong expression continues within the cement gland. In addition, expression is also detected within the invaginating stomodeum, optic eminence, and lens placode (Fig. 3F-H).

Expression of xPitx1 in Embryos Subjected to Anteriorizing and Posteriorizing Agents

Treatment of embryos with either ultraviolet light (UV) or retinoic acid (RA) inhibits development of anterior structures such as the cement gland, eye, and other head structures and leads to the differentiation of posteriorized or truncated embryos. The reverse is commonly induced by treatment with lithium chloride: severely affected embryos may lack posterior ventral structures altogether and form radially symmetrical cement gland and eye bands. We investigated the expression of xPitx1 following treatment with these agents. Lithium chloride administration resulted in a broadening of xPitx1 expression to the extent that it would sometimes circumscribe the gastrulating and neurulating embryo (Fig. 4A and A’), respectively. Whole-mount in situ hybridization of UV-treated embryos did not reveal any expression of xPitx1 at late gastrula (stages 12–12.5; Fig. 4B) or during neuralization (stage 19; Fig. 4B’) when it is normally otherwise observed in the cement gland anlage as a crescent anterior to the neural plate. Later stages of UV-treated embryos similarly failed to reveal xPitx1 expression (data not shown). Consistent with the effect of UV-treated embryos, specimens that were anteriorly inhibited by treatment with 1 μM RA (from the two-cell stage until blastula) failed to express detectable xPitx1 during gastrulation or neuralization (Fig. 4C and C’, respectively).

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Microinjection of xPitx1 mRNA

To determine the effects of xPitx1 overexpression, synthetic capped xPitx1 RNA was injected into one-cell embryos at different concentrations near the animal pole. The amount of injected RNA ranged from 60 pg to 1.2 ng. At higher doses (300–600 pg mRNA), injected xPitx1 resulted in several classes of cement gland phenotypes, sometimes yielding some form of head and axis deformity. At low mRNA concentrations, 60 and 120 pg, defects within the head region yield abnormal head structures and cycloptic features. These features always attend a cement gland/lower mandibular region that protrudes prominently. In addition, posterior defects are observed including bent axes, trunk, and tail deformities (Table 1). The phenotypes observed appear to be dose related. Concentrations of 60 and 120 pg do not cause the formation of supernumerary cement glands—these are only obtained with injections of 300 pg or higher. At low concentrations, enlarged cement glands do not extend across the body flank, however, supernumerary cement glands do, and they usually display as pigmented but sometimes dispersed patches (Fig. 5A and B display ectopic flank cement glands, and 5B also retains an enlarged anterior cement gland). Some early-stage head malformations are hard to interpret and could only be
FIG. 3. Expression of *xPitx1* detected by whole-mount in situ hybridization. All gastrula to early neurula stage embryos are positioned with the yolk plug to the rear with the presumptive anterior regions facing up (with the exception of B, anterior indicated and dorsal up). (A, B) At gastrulation, transcript is detectable above the dorsal lip (dark arrows). (C) Expression is detected in the presumptive anterior ectoderm as a band. An earlier forming dot of expression sits in the central region of the presumptive head plate out of view on the top of this stage-12 specimen. (D) Late gastrula/early 12.5 neurula expression of *xPitx1* is detected as a dot in the center of the neural plate as the anterior band forms a crescent shape. (E) High expression of *xPitx1* occurs in the cement gland anlage at stage 16. (F, G) Expression of *xPitx1* continues within the cement gland (cg) and stomodeum (st) as transient expression commences in the optic cup (e, arrow in E) and olfactory epithelia during organogenesis through to tailbud stages.

FIG. 4. Changed *xPitx1* expression in axially manipulated embryos. Expression of *xPitx1* was detected by riboprobe whole mount in situ hybridization in stages 12–12.5 (dorso-anterior aspect) and 19 (dorsal view, anterior oriented to bottom, except A’ which exogastrulated) embryos (A, B, C, and A’, B’, C’, respectively). Lithium chloride (A and A’) treated embryos revealed a band of expression circumscribing the embryo (arrows). Embryos that were vegetally irradiated with ultraviolet light (UV) (B and B’) or treated with retinoic acid (RA) (C and C’) failed to express *xPitx1*. As no superficial staining was detected in the UV- and RA-treated embryos, they were cleared prior to photographing. Slight bluish tinges are an artifact of light diffraction and disappear when the embryos are rotated.
resolved when tadpoles were analyzed at much later stages. For example, when xPitx1-injected embryos with subtly diminished optic protrusions are reared into swimming tadpoles, a small percentage of them develop severe eye deformities (data not shown). Furthermore, a low number of dwarfed tadpoles develop densely pigmented skin.

Two phenotypes that particularly stand out are enlarged cement glands and supernumerary cement glands on the body flank (Fig. 5A, B; see outline in Table 1). Although the primary phenotypes observed appear to be cement gland related, other phenotypes include prominent cement gland/lower face, reduced trunk and tail structures (Fig. 5C-i), bent tail (Fig. 5C-ii), and severe axes, trunk, and tail deformities (Fig. 5C-iii). At a much lower frequency, a grossly enlarged cement gland forms that is attendant with severe anterior and posterior defects (Fig. 5C-iv). Very rarely (only twice during the course of our experiments) embryos lacking identifiable body parts save a giant cement gland were observed (not shown). Furthermore, a low number of dwarfed tadpoles develop densely pigmented skin.

Overexpression of xPitx1 in UV- and RA-Treated Embryos

The effect of xPitx1 in promoting cement gland development and, in some cases, development of enlarged or supernumerary eye structures led us to determine whether xPitx1 played a specific role in specifying anterior structures. Both ultraviolet light and retinoic acid treatment perturb the proper development of anterior structures, though by different means. Early irradiation of the vegetal pole at the one-cell stage inhibits anterior development via inhibition of cortical rotation. The result of UV irradiation at its most severe results in a completely vegetalized embryo, with a DAI (dorso-anterior index) of 0 (Kao and Elinson, 1988). By injecting synthetic xPitx1 mRNA into UV-treated embryos we hoped to ascertain its ability to promote dorso-anterior structures. Exposure to UV light for 7 min results in 73% of embryos displaying a DAI of 0 and only 9% of the embryos exhibiting development of eyes (Fig. 6A-iv; Table 2). xPitx1 injection (300–1,200 pg) into UV-treated embryos did not appear to fully rescue ventralized phenotypes; however, the number of DAI-0 embryos declines as much as three-quarters with xPitx1 injection. More infrequently, xPitx1 could induce cement glands and eyes in the irradiated embryos (Fig. 6A-i to iv). The morphologies in these rare examples are difficult to interpret and might devolve from one of two scenarios. Either ectopic xPitx1 induces eyes and cement gland in embryos that would otherwise be classified as DAI 0–2, or ectopic transcript is posteriorly truncating DAI 3–4.
Embryos injected with 600 pg of specific marker, XCG-1, induced cement glands on the flank, expressing the gland-body (Fig. 5). Ectopic cement glands frequently develop along the side of the glands often fuse the mandibular region to the body flank (Fig. 5A). Largened or ectopic cement glandlike structures form many deformed phenotypes. Stage-33 embryos reveal elongated cement gland structures occurs exclusive of axial defects: presumptive anterior neural and ectodermal blastomeres injected with xPitx1 form an extended cement gland at the expense of eye on the affected side (dark pigmentation seen under the clear arrow is in the contralateral eye and is seen through relatively transparent head tissues). Neither other parts of the head nor the trunk exhibit signs of axial deformity, indicating that head inhibitory activity is not an artifact of gastrula-mentation; CG: anterior cement gland.

**FIG. 5.** Gain-of-function experiments using xPitx1 results in severely anteriorized embryos. Embryos injected with 600 pg of xPitx1 form many deformed phenotypes. Stage-33 embryos reveal enlarged or ectopic cement glandlike structures (A). Elongated cement glands often fuse the mandibular region to the body flank (B). Ectopic cement glands frequently develop along the side of the body (C). Other phenotypes observed exhibited axial, trunk, and tail deformities. Cement glands induced on the flank express the gland-specific marker, XCG-1 (D, and in section E). Inhibition of head structures occurs exclusive of axial defects: presumptive anterior neural and ectodermal blastomeres injected with xPitx1 form an extended cement gland at the expense of eye on the affected side (dark pigmentation seen under the clear arrow is in the contralateral eye and is seen through relatively transparent head tissues). Neither other parts of the head nor the trunk exhibit signs of axial deformity, indicating that head inhibitory activity is not an artifact of gastrula-mentation defects (F, G, H). GFP transcript was coinjected as a lineage tracer. As the embryos develop, one side (left or right) serves as a control for the other. Embryos displaying a high degree of fluorescence within the anterior region of the embryos (on one-side) were isolated and probed with several anterior markers xOtx2 (Blitz and Cho, 1995), xPax6 (Hirsch and Harris, 1997), neural β-tubulin (Richter et al., 1988), and xTwist (Hopwood et al., 1989). Ectopic expression of xPitx1 diminished expres-
sion of several markers of brain and face at both early and late stages of development (early: Fig. 7A–C; late: Fig. 7D–G and D’–G’). Specifically, xOTX2 is diminished in presumptive head (Fig. 7A, A’) and in the optic cup and brain, whereas the uninjected side displayed the normal highly specific expression of xOTX2 within these regions (Fig. 7D, D’). xPax6 expression is also perturbed in xPitx1 injected embryos. xPax6 expression appears to be reduced on the injected side of embryos (the expression of Pax6 on the uninjected and injected sides of Fig. 7E and E’, respectively). Expression of the neural crest marker xTwist is also diminished on the side of Pitx1 injection (uninjected Fig. 7F with injected 7F’). Neural development as revealed by staining with a probe for neural β-tubulin demonstrated a similar dispersion and diminution of activity on the xPitx1-injected side (Fig. 7G and G’). All embryos exhibiting a concentration of anterior GFP fluorescence (and presumably also of coinjected ectopic xPitx1) in the head showed perturbations of these anterior markers.

In contrast to the effects of ectopic xPitx1 in anterior head, effects differed in other regions. For example, under certain circumstances, ectopic xPitx1-induced posterior defects can be associated with increases in xOTX2 and neural β-tubulin expression. For example, induced supernumerary flank cement gland always expresses xOTX2 both within and around the gland (Fig. 8A). Like cement gland in the head, no neural β-tubulin expresses in the ectopic organ itself (Fig. 8B); however, in some cases an additional body axis forms and a neural tract appears to develop that expresses this marker (Fig. 8C, D).

xPITX1 and Cement Gland Formation in Ectoderm Cap Explants

To further investigate the role of xPitx1 in specifying cement gland formation, embryos were injected with xPitx1 or control mRNA near the animal pole of the one-cell embryo. At blastula, the animal caps were removed and cultured for 2 days. Uninjected caps (Fig. 9A), or animal caps injected with β-galactosidase mRNA fail to form cement gland as assessed either by anatomical features or by hybridization with the cement gland-specific riboprobe for XCG-1. When cement gland-specific riboprobe is used as a marker for the cement gland, we found that cement gland is expressed in the ectoderm cap explant (Fig. 9B). Against the eventuality that xPitx1 induction of cement gland was indirect, we assessed injected caps for the presence of differentiated neural tissue and mesoderm using probes for neural β-tubulin and the pan-mesodermal marker Xbra (Fig. 9D, E, respectively). Clearly no mesodermal or neural tissue was present.

DISCUSSION

We have cloned the Xenopus homolog of Pitx1. The cDNA sequence encodes a conceptual protein of 305 amino acids that contains a homeodomain with high homology to previously described Pitx sequences (Camponge et al., 1999; Lamonerie et al., 1996; Semina et al., 1996, 1998). Like other homeoproteins of the Pitx group, xPitx1 possesses a conserved 14 amino acid C-terminal motif that is found in several other homeodomain proteins that are expressed during craniofacial development. Presumably, this domain plays a role in interactions with other head-specific gene products of developmental significance (Semina et al., 1996). A Pitx1 fragment containing the conceptual open reading frame was recently published that is possessed of some sequence differences, particularly at the 3’ and 5’ ends, and which encodes a slightly different amino acid sequence at the N-terminus (Hollemann and Pieler, 1999). While some of the differences might be attributable to allelic variation, we assume that the differences at the 5’ and 3’ ends of the open reading frame reflect the PCR strategy employed by these authors to amplify xPitx1, and that the sequence extracted from our cDNA library is a closer reflection of the actual transcript.

Expression of the gene is similar to patterns described for other vertebrates: the cement gland is the anterior-most structure to develop in frogs and occupies a topographical location similar to the primordia from which the Pitx1-expressing first branchial arch derives in chick and in mouse (Lancot et al., 1997). In all three organisms, this anterior expression domain is displaced ventrally as the cranial vault expands, so that by the time facial structures have developed, the gland and the mouth no longer have the appearance of being the most extreme anterior “segment” of the head. Expression of xPitx1 in the cement gland supports the hypothesis that...
FIGS. 7–9
the amphibian cement gland has a developmental homolog in the buccopharyngeal region of amniotes: these regions are innervated by the trigeminal nerve, and are sites of Otx2 expression (Sive and Bradley, 1996). Just as the cement gland primordia is the first head organ to display Pitx1 expression in frog, the ectoderm of the buccal face of the first branchial arch is the first to express transcript in mice (Lancot et al., 1997). In frog, Otx5 and xOtx2 are related head-specific paired-like homeobox genes that appear to play a role during cement gland formation. However, unlike xPitx1, their expression is first detected within the organizer region during the onset of gastrulation (Blitz and Cho, 1995; Pannese et al., 1995; Kuroda et al., 1999). The expression of xPitx1 in the dorsal axis during gastrulation is quite faint and whether this suggests a role in patterning the dorsal axis and an interaction with specific organizer genes such xOtx, goosecoid, noggin, or chordin remains to be seen. With regard to this early and faint period of expression, and also with respect to the transient expression of xPitx1 observed in the center of the early neural plate, our findings augment those of Hollemann and Pieler (1999). In all other respects, the expression patterns appear identical.

xPitx1 expression within the most anterior region of the developing embryo led us to investigate whether it could be inhibited by posteriorizing manipulations such as treatment with ultraviolet light and retinoic acid. UV and RA both abolish expression of xPitx1 during gastrulation and neurulation. Conversely, anteriorizing treatment using lithium chloride enhances expression of xPitx1. Not only do the expression patterns of xPitx1 suggest a role for the gene in the regulation of head development, but mutated Pitx gene family members have been associated with anomalous craniofacial development in humans (Semia et al., 1996, 1998; Crawford et al., 1997). We sought to test the effects of enhanced and inhibited activity of this gene during craniofacial development in frogs. Paradoxically, ectopic expression appears at first glance to have contradictory effects: injected embryos often suffer axial truncations, particularly in the face and head, whereas xPitx1 injection of RA and UV treated embryos appears to achieve quite the opposite. In this latter case axial deficits in the head are partially rescued. Ectopic expression in presumptive anterior blastomeres indicates that the perturbations of head development occur exclusive of axial anomalies.

**Overexpression of xPitx1**

Overexpression of xPitx1 induces the formation of enlarged or ectopic cement glands and often results in embryos with posterior defects in which trunk and tail structures could be severely reduced (Fig. 5A–E). The most severe phenotypes display the appearance of an embryo transformed into a cement gland-like structure with both severe head and tail deformities (Fig. 5C–iv). Occasionally during gastrulation in ectopically expressing embryos, there is anomalous blastopore closure reminiscent of defects seen in experiments where xOtx2 has been overexpressed (Blitz and Cho, 1995; Pannese et al., 1995). The mildest phenotypes were seen following injection with low quantities of xPitx1 mRNA (60–120 pg). While enlarged or ectopic cement glands head phenotypes appear subtle, some deformities arose where the mandibular region appeared enlarged relative to forehead structures. The variability of effect seen when ectopic xPitx1 perturbs marker gene expression likely reflects variability in synthetic mRNA dispersion and local concentration. Indeed, when we monitored injection dispersion with co-injected FITC-dextran or in vitro transcribed mRNA for GFP, distribution of fluorescent material always correlated well with the xPitx1-induced phenotype.

When in vitro transcribed capped xPitx1 mRNA was injected into either the left or right side of a two-cell embryo, we were able to monitor the effects that ectopic expression had on endogenous gene products such as the transcripts from xOtx2, xPax6, xTwist, XCG-1, and neural β-tubulin. When xPitx1 is introduced, cement gland marker gene expression increases, while markers for other head regions decline. For example, xOtx2, a gene associated with cement gland development increased expression when that organ was forming ectopically, while xOtx2 expression in eye and brain, and Pax6, xTwist, and neural β-tubulin expression domains were dissipated or abrogated. Changes in the early stage expression of xPax6 are consistent with this interpretation as the presumptive midbrain and facial zones appear compressed. Possibly, ectopic xPitx1 recruits cells from the presumptive ectoderm/neurectodermal margin to form cement gland, and the axial and craniofacial defects that arise are caused by an insufficient supply of competent precursor cells. A common phenotype consistent with this interpretation occurred when embryos reflexed inward toward the injection site as if less tissue was being distributed axially on that side, and more was being co-opted to the formation of cement gland(s). xPitx1 appears to induce cement gland directly: neural markers such as neural β-tubulin were not found in association with either enlarged or ectopic cement glands. However, in cases where xPitx1 induced ectopic cement glands, ectopic expression of xOtx2 was induced. This is consistent with a role for xPitx1 acting upstream of xOtx2, assuming that one or the other is not phenocopying the cement gland induction pathway under the artificial conditions of ectopic expression assays. With regard to this last caveat, in our hands xPitx1 is a more potent inducer of cement gland than either xOtx2 or xOtx5.

Alternatively, ectopic xPitx1 homeoprotein might bind factors or sites normally bound by other bicoid-related transcription factors such as goosecoid or members of the xOtx family. Because the Pitx proteins interact directly with certain basic helix-loop-helix factors to activate gene transcription in a manner distinct from the Otx and goosecoid proteins (Poulin et al., 2000), ectopic xPitx protein could be binding cognate sequence without appropriate heterodimeric partners, thereby acting
as a dominant negative during head development. As a consequence, *Pax, twist, Otx*, and *neural β-tubulin* expression domains might be perturbed. Posterior defects are relatively common, and often entail a truncation of tail structures; however, supernumerary axes can also form. These axes stain positively with probe for *neural β-tubulin*, and arise posterior to an ectopic cement gland on the body flank. We cannot determine on the basis of these experiments if the presence of a *xPitx1*-induced ectopic cement gland is causative or whether a supernumerary axis is generated as a secondary consequence of the aforementioned gastrulation anomalies. Possibly this consequence of misexpression indicates a prominent role for *xPitx* during gastrulation.

**Overexpression in Axially Impaired Embryos Preserves Some Dorso-Anterior Attributes**

Because *xPitx1* apparently has the capacity to direct development of the cement gland and mandible, and because the endogenous gene appears to be down-regulated in dorso-antioriely impaired embryos (UV irradiated or RA treated), we decided to test the efficacy of *xPitx1* to alter ventralized phenotypes. Rescue experiments with ectopic *xPitx1* in UV-treated embryos did not result in the complete rescue of dorsal axis; however, they did prohibit the formation of severely vegetalized DAI-0 embryos. This corroborates similar findings seen in UV-treated embryos administered *xOtx2* transcript (Pannese et al., 1995).

Retinoic acid inhibits the development of structures anterior to the hindbrain (Durston et al., 1989), and rescue experiments with overexpressed *xPitx1* in RA-treated embryos also failed to fully rescue head structures. Remarkably, although head structures could not be fully rescued, *xPitx1* overexpression nevertheless induced the formation of cement glands and even eyes and other recognizable anterior head elements in 75% of embryos where none might otherwise have been expected. At the very least, data observed with UV and RA rescue experiments implicate a direct role for *xPitx1* in initiating the pathway toward cement gland formation and possibly in patterning of the head.

Cement gland normally develops in a mesoderm-free zone (Hauser and Riebesell, 1991). *xPitx1* might normally express in the presumptive cement gland ectoderm subsequent to induction by direct contact with underlying endoderm. Consistent with this interpretation is the finding that the dorso-antioriely endoderm possesses a potent cement gland-inducing activity at midgastrulation (Bradley et al., 1996). Possibly ectopic *xPitx1* mimics a late step in this induction pathway with the result that cement gland forms where it should not, and often at the expense of structures that require greater participation from the diverted ectoderm. This explanation also satisfies the paradox embodied in the head-preserving function of *xPitx1* in axially truncated embryos. Early-stage UV irradiation or retinoic acid treatment will have repressed the steps necessary to normal anterior dorsal development, whereas ectopic *xPitx1*, a later participant in the induction pathway, could overcome this patterning deficit and elicit a partial recovery by acting on still-competent anterior ectoderm. It is worth noting that both heart and craniofacial development likely require the participation of pharyngeal endoderm, and both regions are adversely affected by retinoic acid treatment. Furthermore, the endoderm of the second, third, and fourth pharyngeal arches requires a careful modulation of retinoid signal pathways in order for neural crest and mesoderm to pattern normally in mice (Wendling et al., 2000). Alternatively, one might speculate that intercalary regulation would ensue where an *xPitx1*-induced cement gland forms in close juxtaposition to an abnormally posterior (i.e., hindbrain-level RA-truncated) tissue. If the response were intercalary, moreover, the ectopic axis that occasionally arises behind flank-situated cement glands in *xPitx1*-injected embryos should manifest signs of other head structures. This may occur in some instances (Fig. 7C and D).

**Overexpression in Animal Cap Explants Supports a Direct Role for *xPitx1* in Cement Gland Differentiation**

To study possible direct effects of *xPitx1* in specifying cement gland, animal caps of injected embryos were removed at stage 9 and cultured. Ectodermal caps which overexpress *xPitx1* develop a pigmented sticky patch of cells that resembles a cement gland. Hybridization of animal caps with the cement gland-specific marker *XCG-1* confirmed that it was indeed a cement gland and that *xPitx1* along with *xOtx2* and *xOtx5* may be upstream regulators of *XCG-1*. The relationship between *xPitx1* and the *xOtx* genes is unknown; however, both factors induce ectopic cement glands. As in *xPitx1*-injected embryos, injected animal cap explants differentiates cement gland in the absence of differentiated mesodermal and neural tissue. This confirms the hypothesis that *xPitx* acts on ectoderm to specify and differentiate cement gland in a direct manner. Although *xPitx1* induces ectopic cement glands, it is clearly not sufficient to induce formation of a complete head. The extent to which the gene is required for other aspects of anterior development remains to be defined. However, it seems clear that the context necessary to support cement gland formation also actively precludes specification to other anterior phenotypes in the head of otherwise unperturbed embryos.

**MATERIALS AND METHODS**

**Library Screening, Cloning, and Mutation**

A λZAP II cDNA head library prepared from stage 28–30 *Xenopus* tadpole tailpods with a titer of 2.5 × 10<sup>10</sup> pfu (Hemmati-Brivanlou et al., 1991) was screened at moderate stringency with murine *Pitx1* probe. Positive plaques were isolated and the cDNA subcloned into BlueScript® II SK using ExAssist™ Interference-Resistant Helper Phage (Stratagene). Sequence analysis was per-
formed by dideoxy chain termination (Sanger et al., 1977) using an ABI 377 robotic sequencing apparatus. Both DNA strands were analyzed using primers designed to provide nested and overlapping data sets. Several independent clones were isolated, two of which overlapped by 371 nucleotides. These latter two were spliced together at a Pael site to render a contiguous sequence comprising the open reading frame and portions of both the 5' and 3' untranslated regions.

Expression constructs were derived using Vent polymerase (New England Biolabs) and primers that bracketed the open reading frame and possessed restriction sites for Eco RI and Xba I at the 5' and 3' ends, respectively, and which facilitated insertion into pCS2- Constructs were verified by nucleotide sequence analysis.

Embryos

Embryos were fertilized, dejellied in 2% cysteine, and cultured as previously described (Drysdale and Elinson, 1991). Developmental staging was according to Nieuwkoop and Faber (1967). Axial perturbations using lithium, ultraviolet irradiation (UV), and retinoic acid (RA) were performed using previously described protocols (Drysdale and Elinson, 1991; Kao and Elinson, 1988; Kao et al., 1986).

Whole-Mount in situ Hybridization and Histology

Whole-mount in situ hybridization was performed according to Harland (1991). RNA probes were made from linearized cDNA of the 5', 3', and coding regions, respectively, of xPitx1. Probes were labeled with Digoxigenin (Roche) and revealed using antibodies conjugated to alkaline phosphatase. Additional riborobes for XCG-1, xOtx2, xPax6, neuralβ tubulin, and xTwist were synthesized in similar fashion. Histological sections were prepared according to Fagotto and Gumbiner (1994). Embryological sections of 30 μm were prepared with a cryostat.

Microinjection

The coding region of xPitx1 was inserted into the Xba I and Eco RI cloning sites of the pCS2- vector. Synthetic capped mRNA of xPitx1 was made from linearized template using mMessage Machine (Ambion) driven by a SP6 promoter. Capped mRNA was resuspended in water and injected into embryos with a Drummond nanojector. Injections were made into the animal pole of embryos at either the one- or two-cell stages. Concentrations of the capped mRNA injected ranged from 60 pg to 1.2 ng. Injection volumes never exceeded 9.2 nl. Injected embryos were cultured in 0.1X MMR containing 50 μg/ml gentamicin, and 2% Ficoll-400 (Sigma) at 13°C for at least 1 h to allow healing before being removed and allowed to develop at room temperature. At stage 10–10.5, the solution was changed to 0.1X MMR containing only 50 μg/ml gentamicin.

RT-PCR

Embryos were stored in batches of 10 in RNA later (Ambion) until all desired stages had been collected. Purifications of RNA from each of the stages were done in parallel using oligo dt-polystyrene beads (Sigma DMN-10). From each of the sampled stages, mRNA equivalent to one embryo was withdrawn and cDNA synthesized in the presence on RNasin (Promega) using reverse transcriptase according to the manufacturer's instructions (Omniscript, Qiagen). One-fifth volume of this reaction was employed as template for amplification. PCR conditions were determined empirically to establish the linear range of amplification for xPitx1. Reactions were accomplished using a thermo-stable polymerase in 10 mM Tris (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 3 mM MgCl2, 0.2 mM dNTPs, 0.1mM dCTP32P, and 1 μM of each primer (xPitx1—GCC AGT GAG TCT TCT GAT A and EF1α—CAG ATT GGT GCT GTA TTG ATG ACT CCT A). Initial denaturation was for 3 min at 94°C, and cycling parameters were repeated 29 times at 94°C for 45 s, 57°C for 1 min, and 74°C for 45 s. One-tenth of each reaction was run out on 4% polyacrylamide in 0.5× TBE, and then monitored by autoradiography.

Ectodermal Cap Culture

Stage 8-9 embryos were removed to 1X MMR containing 50 μg/ml gentamicin and 2% ficoll. Ectodermal explants were removed and cultured overnight at room temperature in Petri dishes, the bottom of which had been coated with a thin layer of 1% agarose in 1× MMR. Explants were then removed to 0.1× MMR and cultivated until they reached the stage at which sibling intact embryos had developed cement glands (stage 25 or later). Explants were then fixed and processed for in situ hybridization as described.

ACKNOWLEDGMENTS

Thanks to Drs. H. Sive, R. Elinson, M. Zuber, I. Blitz, K. Cho, and T. Drysdale for probes, Dr. R. Harland for a head cDNA library, and to Dr. T. Drysdale for additional reagents and helpful discussion. This work was supported by grant 203549 from the Natural Sciences and Engineering Research Council of Canada to M.J.C., an Ontario Graduate Scholarship in Science and Technology award to F.K., and an Natural Sciences and Engineering Research Council Summer Studentship to R.C.

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