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ARTICLE

xPitx1 Plays a Role in Specifying Cement Gland and Head During Early *Xenopus* Development

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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. genesis 29:78–90, 2001.

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-

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lins Syndrome, a human developmental mutation which results in craniofacial anomalies and sometimes occasions 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 *xPitx-1* 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 neurulation (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 neurulation (Fig. 4C and C′, respectively).

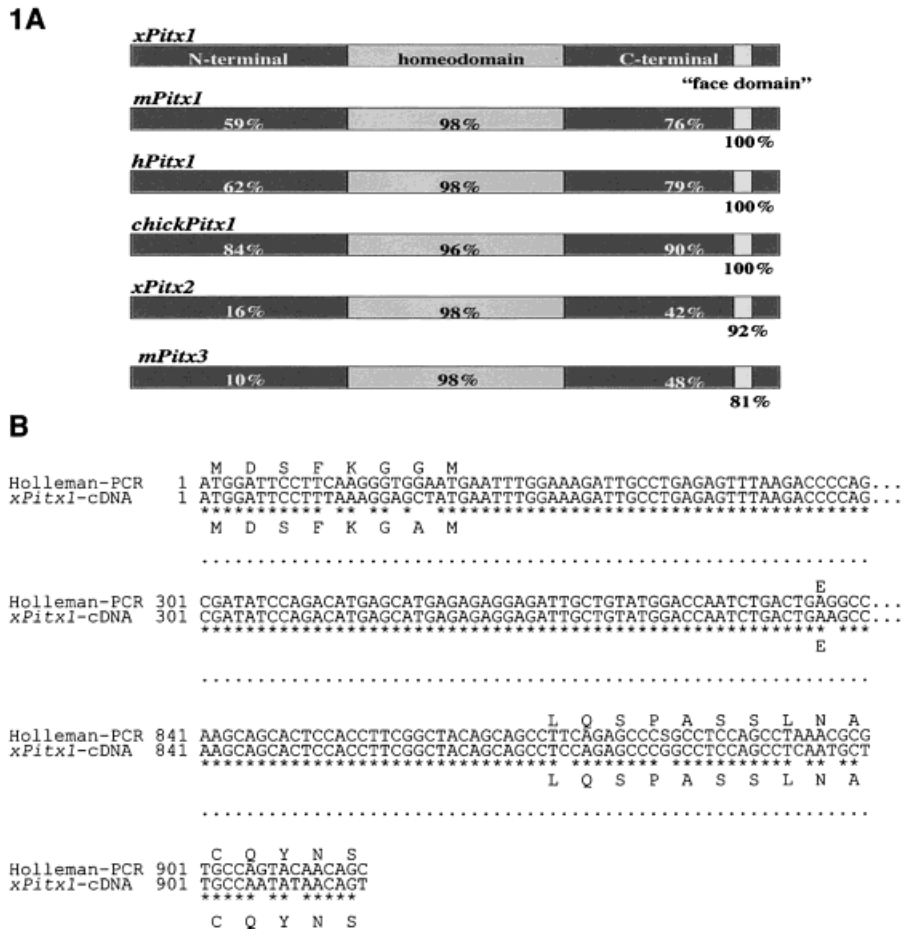


FIG. 1. (**A**) Amino acid comparison of *Xenopus Pitx1* with other *Pitx* genes in mouse, chick, and human. *xPitx1* shares greatest sequence similarity with chick *Pitx1*. However, all *Pitx1* members encode a highly conserved homeodomain (light grey) and 100% identity within the C-terminal "face domain" motif (white). (**B**) Comparison of *xPitx1* cDNA sequence with *xPitx1* fragment previously published (Holleman and Pieler, 1999). For convenience, only portions of the open reading frame are shown: the complete open reading frame, 3′, and 5′ untranslated sequences are available under accession number AF217647. An asterix indicates nucleotide identity. Where nucleotide sequences diverge, the conceptual amino acid sequence is given.

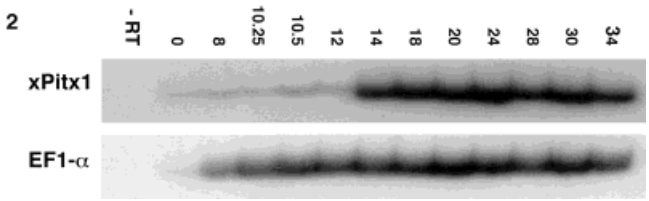
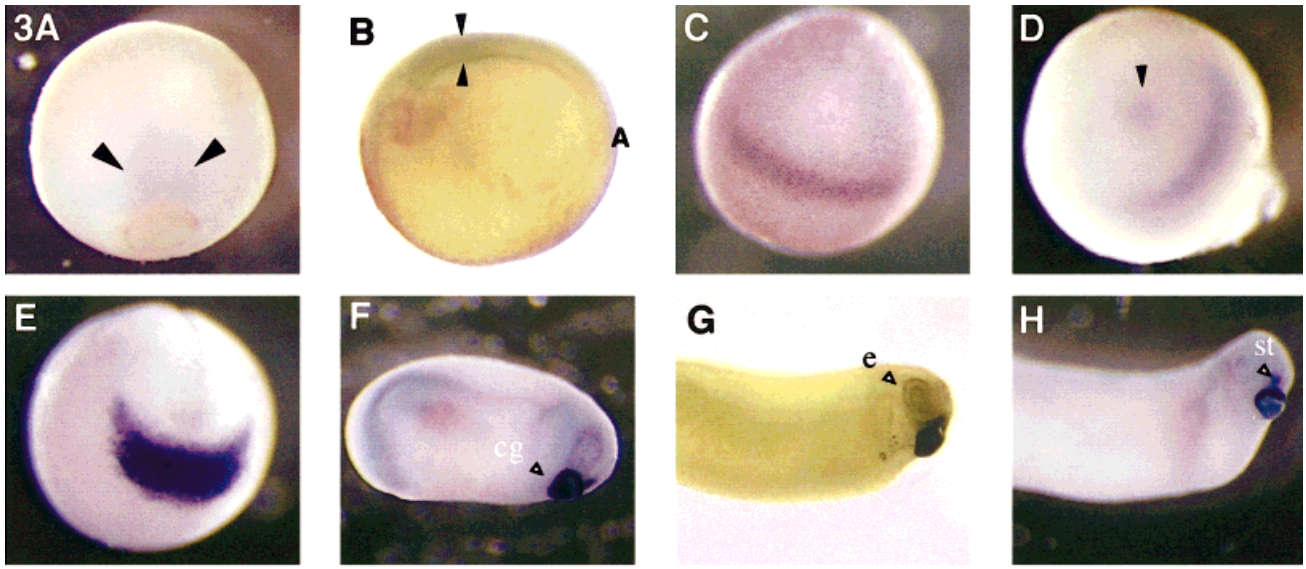


FIG. 2. RT-PCR analysis of *xPitx1* expression during development. Transcript is detectable in unfertilized oocytes and early blastulae. A major increase in expression occurs shortly after completion of gastrulation.

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



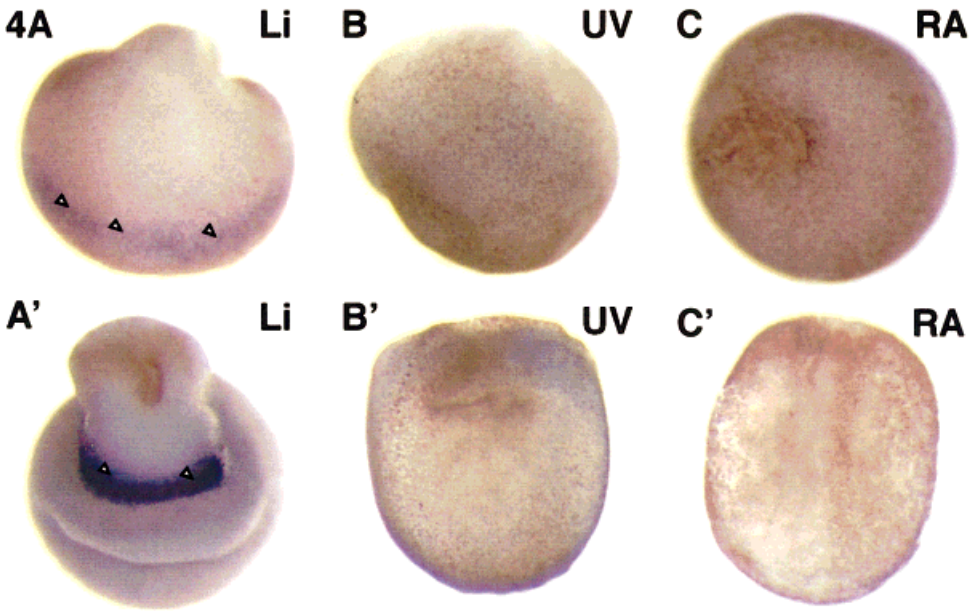


FIG. 3. Expression of *xPitx1* detected by whole-mount in situ hybridization. All gastrula to early neurula stage embryos are positioned with the yolk plug to the rear with the presumptive anterior regions facing up (with the exception of B, anterior indicated and dorsal up). (**A, B**) At gastrulation, 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.

Table 1
Percentage of Phenotypes Observed in Embryos Injected With Wild-Type xPitx1

RNA injected (pg)	*N*	Site	Normal	Extra cement glands (%)	Enlarged cement gland (%)	Other deformities[a] (%)
1,200	54	Animal	15	24	35	5.5 (cement gland only) 5.5 (reduced trunk and tail) 15 (bent axis, trunk, and tail) 42 (blastopore closure)
600	66	Animal	21	14	44	1 (cement gland only) 6 (reduced trunk and tail) 14 (bent axis, trunk, and tail) 32 (blastopore closure)
300	56	Animal	14	16	41	4 (cement gland only) 18 (reduced trunk and tail) 7 (bent axis, trunk, and tail) 20 (blastopore closure)
120	73	Animal	44	4	4	42 (bent axis, trunk, and tail) 5 (extension of mandible) 27 (blastopore closure)
60	69	Animal	43	0	3	49 (bent axis, trunk, and tail) 1 (reduced trunk and tail) 3 (extension of mandible) 33 (blastopore closure)
Uninjected	164	N/A	100	0	0	0
600 pg beta-Gal	52	Animal	100	0	0	0

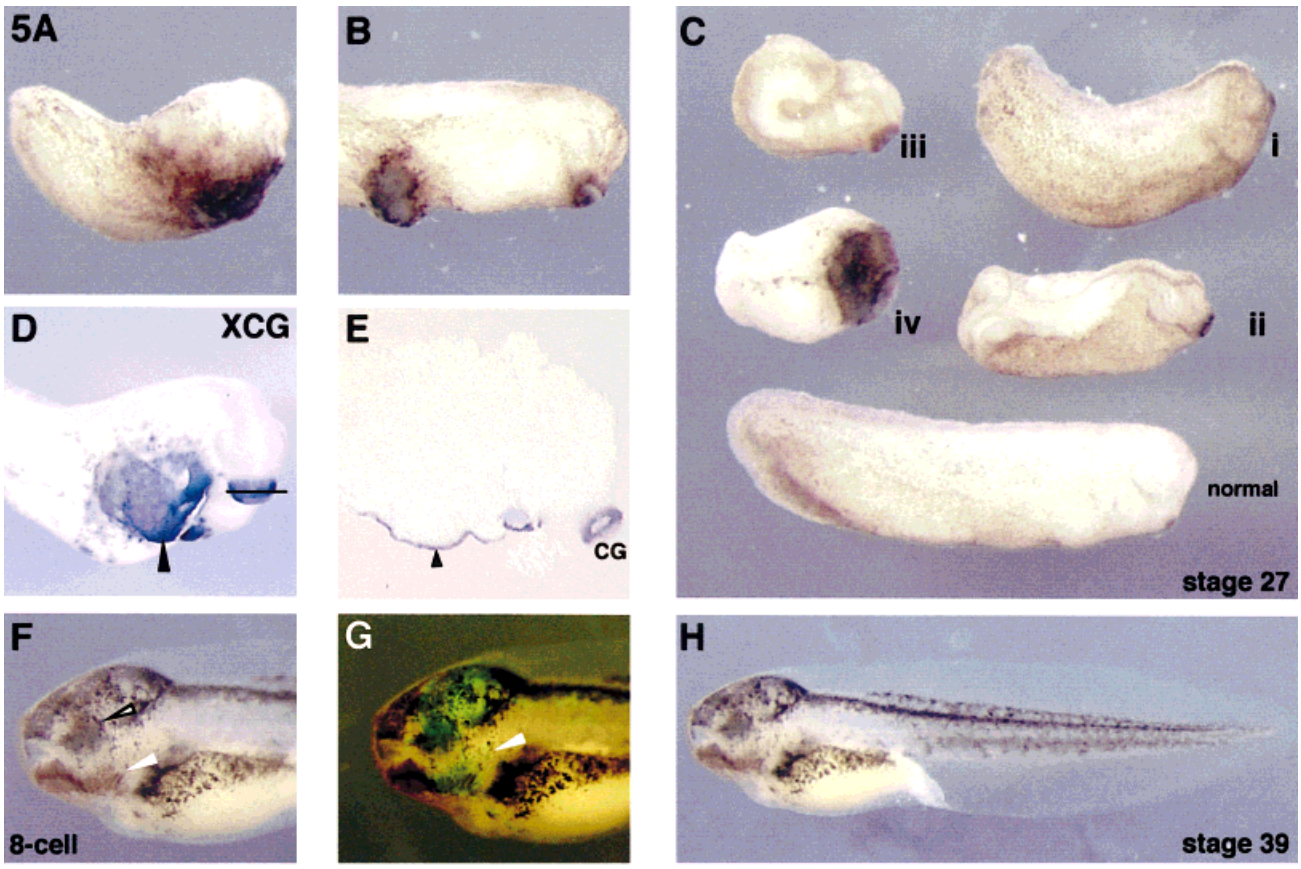
[a]Phenotypes outlined as Other Deformities are shown in Figure 4: cement gland only (Fig. 4C-iv), reduced trunk and tail (Fig. 4C-i), bent axis, trunk and tail deformities (Fig. 4C-ii to iii). As deformities were sometimes compound, row percentages will not sum to 100.

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). The morphological and functional status of ectopic patches of cement gland tissue was confirmed using the cement gland-specific marker XCG-1 (Sive *et al.*, 1989) (Fig. 5D). In section, *XCG-1* is seen confined to the cement gland ectoderm (Fig. 5E). A more common early effect of overexpressing *xPitx1* is anomalous blastopore closure (identical to *xOtx2* phenotype seen in Blitz and Cho, 1995). In addition to the phenotypes described above, rare phenotypes arise such as axes duplications, enlarged and supernumerary eyes. When blastomeres were injected at the 8–32-cell stage, supernumerary or enlarged cement glands formed in the absence of gross axis perturbations. In the head region, enlarged cement glands were often associated with local suppression of head development (Fig. 5F, G, H).

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


5A
B
C
iii
i
iv
ii
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D
XCG
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8-cell
G
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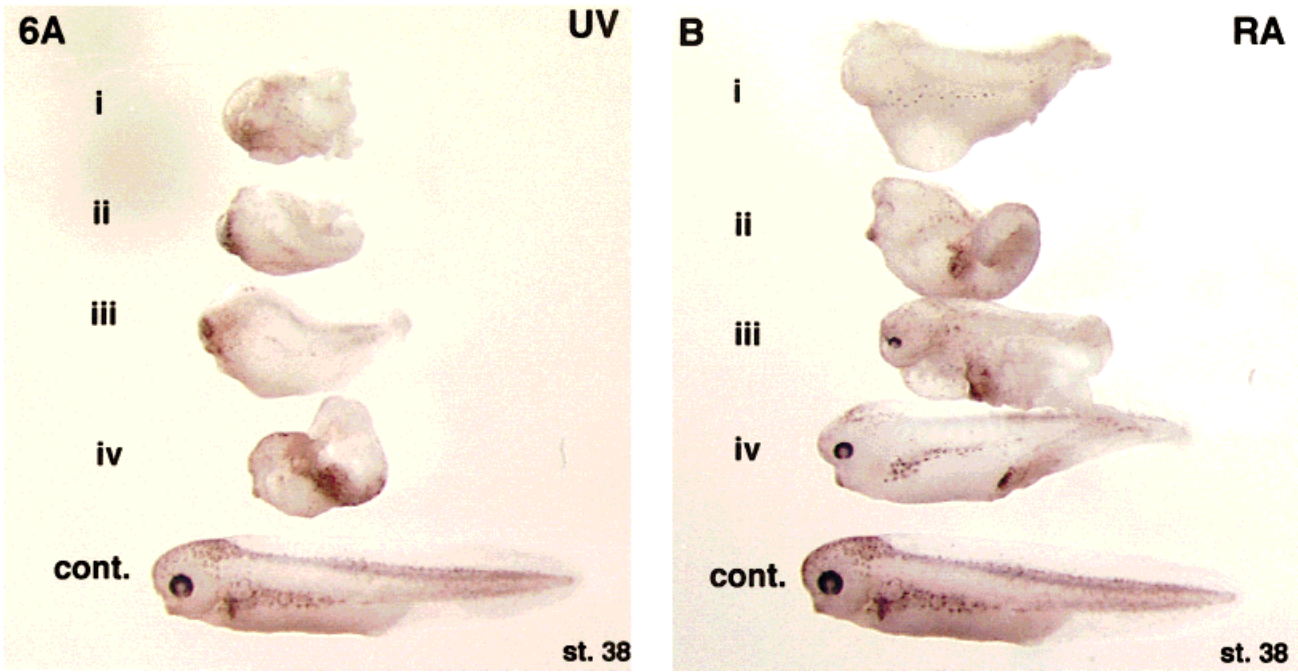
stage 39


6A
UV
i
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FIGS. 5 & 6

Table 2
Effect of xPitx1 Overexpression in Retinoic Acid and Ultraviolet Light-Treated Embryos

RNA injected (pg)	*N*	Site	Normal	Posteriorized (%)	Cement gland and eye (%)	Ectopic cement gland (%)	Other phenotypes[a] (%)
RA treatment alone	49	N/A	0	100	0	0	0
300 + RA treated	105	Animal	0	1	17	61	21 (posterior defects)
600 + RA treated	110	Animal	1	1	23 1% (eyes only)	50	25 (posterior defects)
1,200 + RA treated	63	Animal	0	0	10	79	11 (posterior defects)
			DAI 3–5	DAI 0–2 (no cement gland)	DAI 0–2 (with cement gland and eye)		
UV treatment alone	114	N/A	5 4% (DAI 3–4)	18 (DAI 1–2) 73 (DAI 0)	0	0	0
300 + UV treated	78	Animal	1	5 (DAI 1) 13 (DAI 0)	3	1 (DAI 1) 12 (DAI 0) 33 (posterior defects)	32 (posterior defects)
600 + UV treated	54	Animal	0	4 (DAI 1) 17 (DAI 0)	6	13 (DAI 1) 11 (DAI 0) 33 (posterior defects)	16 (posterior defects)
1,200 + UV treated	66	Animal	0	3 (DAI 1) 23 (DAI 0)	3	1 (DAI 1) 8 (DAI 0) 47 (posterior defects)	15 (posterior defects)
No treatment	74	N/A	100	0	0	0	0

[a]Posterior defects include a variation of axial deformities including bent axes, trunk, and tail, which, though present, are abnormal. Tail defects often resemble those seen in an x0tx2 study (Pannese et al., 1995).

embryos to make them resemble DAI 0–2 specimens at the gross morphological level. The numbers of DAI 0–2 embryos with eyes and cement glands seems to resemble the number of DAI 3–4 embryos in the uninjected but UV-irradiated cohort. Therefore, the second alternative appears the most likely.

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 gastrulation defects (**F**, **G**, **H**). *GFP* transcript was coinjected as a lineage marker for the progeny of the *xPitx1*-injected blastomere (G). Solid arrows: ectopic cement gland; open arrow: contralateral eye pigmentation; CG: anterior cement gland.

FIG. 6. Ectopic *xPitx1* partially preserves dorso-anterior structures in axially perturbed embryos. The ability of *xPitx1* to rescue embryos (**A**) vegetally irradiated with ultraviolet light or (**B**) treated with retinoic acid. Controls are presented on the bottom for purposes of comparison. In uninjected UV treated embryos, 73% of embryos exhibited a DAI of zero (Ai) compared to 13–23% in injected cases. In uninjected retinoic acid-treated embryos, 100% were anteriorly truncated (Bi).

The teratogenic effects of RA include severe truncation of anterior structures from the midbrain forward. Embryos were injected with *xPitx1* mRNA (300–1,200pg) at the one-cell stage and treated with 1μM RA at the two-cell stage through to blastula. Injection into RA-treated embryos failed to rescue anterior structures completely; however, ectopic cement glands (Fig. 6B-ii and iii) and development of anterior structures such as the eye were observed (Fig. 6B-ii to iv; Table 2). Embryos treated with 1μM RA resulted in 100% of the embryos with head truncations, whereas 75% of embryos injected with *xPitx1* and then RA treated developed ectopic cement glands, eye structures, or both (Table 2). The range of phenotypes exhibiting head structures such as cement gland and eyes are shown in Figure 6B. Ectopic cement gland identity was confirmed by hybridization with *XCG-1*.

xPitx1 Induces Misexpression of Anterior Marker Genes

To ascertain the effects of *xPitx1* misexpression on other genes, one blastomere of the two-cell staged embryos was injected with *xPitx1* and coinjected with capped green fluorescent protein (GFP) mRNA as a 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* (Hirsh 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 *xPitx* 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 animal caps are derived from embryos first injected with *xPitx1* mRNA, cement glands develop—sticky, thickened epithelial patches form that hybridize *XCG-1* (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

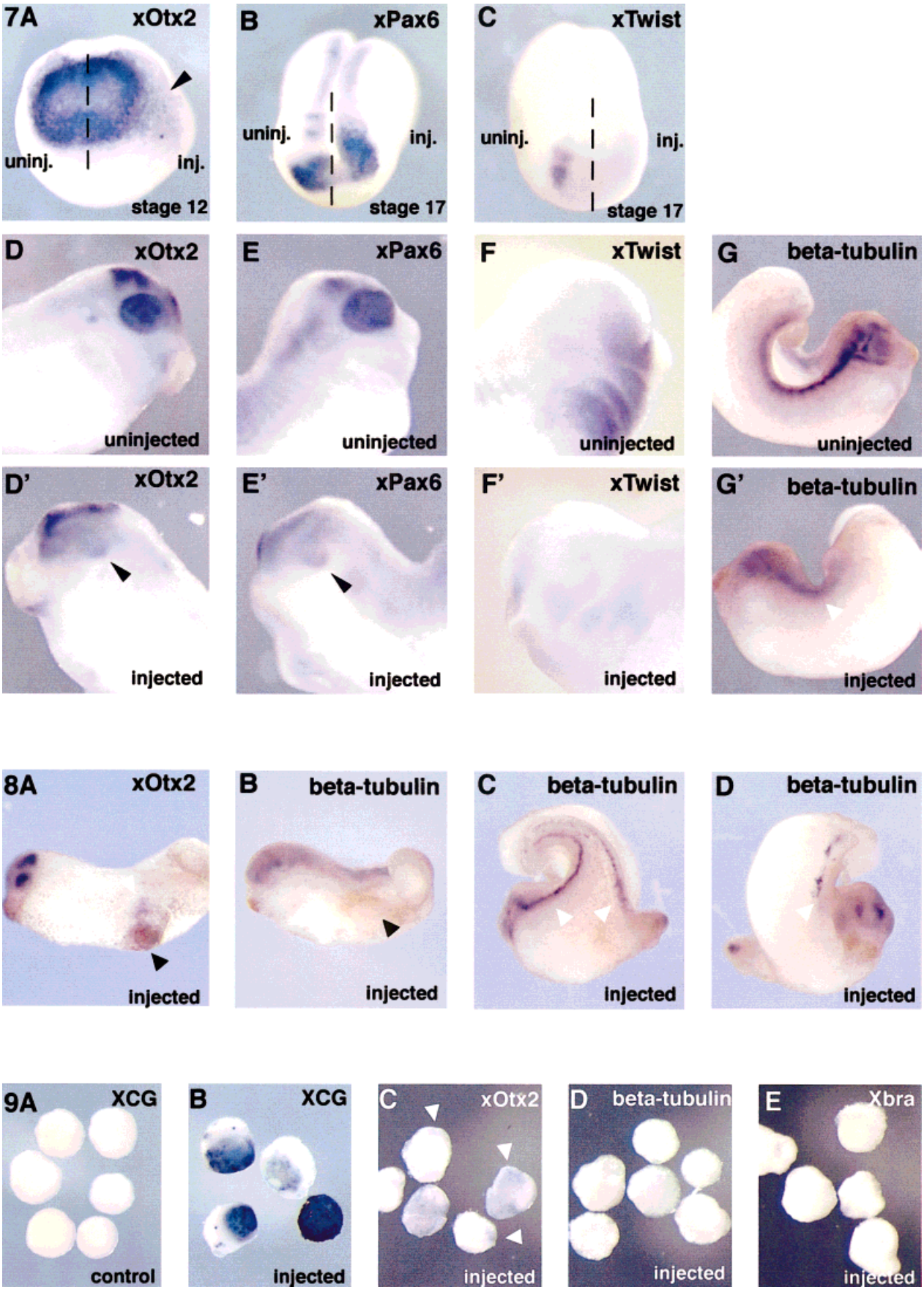
We have cloned the *Xenopus* homolog of *Pitx1*. The cDNA sequence encodes a conceptual protein of 305 amino acids that contains a homeodomain with high homology to previously described *Pitx* sequences (Campione *et al.*, 1999; Lamonerie *et al.*, 1996; Semina *et al.*, 1996, 1998). Like other homeoproteins 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 anteriormost 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 (Lanctot *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

FIG. 7. Ectopic *xPitx1* diminishes expression of several anterior head markers. The effects of *xPitx1* mis-expression on anterior and neural crest markers were assayed by injection of 600 pg of *xPitx1* into a single blastomere at the two-cell stage followed by whole-mount in situ hybridization with *xOtx2* (**A** and **D**-control side; D′–*xPitx1* injected), *xPax6* (**B**, **E**, and E′), *xTwist* (**C**, **F**, and F′), and *neural β-tubulin* (**G**, G′).black arrows: diminished marker gene expression in eye; white arrow: diminished expression of *neural β-tubulin* in dorsal neural structures.

FIG. 8. Ectopic *xPitx1*-induced cement glands reveal *xOtx2* expression, and posterior axis duplications demonstrate the presence of neural tissue. *xPitx1*-induced cement gland displays induction of xOtx2 (**A**) expression but not of *neural β-tubulin* (**B**) Supernumerary body axes sometimes form which demonstrate differentiated neural tissue as indicated by the expression of *neural β-tubulin* in two parallel axes dorsally (**C**), and one not quite coalesced more ventrally in the same specimen (**D**). Dark arrows: ectopic cement gland; white arrows: expression of marker gene.

FIG. 9. Animal cap explants can be induced to form cement gland by injection of *xPitx1* transcript. (**A**) Control explants (uninjected) probed with *XCG-1*; (**B**) Animal caps isolated from embryos that have been injected with *xPitx1* develop sticky cement glandlike structures that hybridize with the cement gland-specific marker *XCG-1*; (**C**) *xPitx1* injection induces expression of *Otx2* near cement gland tissues; (**D**) *neural β-tubulin* staining demonstrates the absence of induced neural tissue in the explants even though cement gland is induced following *xPitx1* injection; (**E**) *xPitx1* does not induce mesoderm as the expression of pan-mesodermal marker *Xbra* is never elicited.


7A
xOtx2
uninj.
inj.
stage 12
B
xPax6
uninj.
inj.
stage 17
C
xTwist
uninj.
inj.
stage 17
D
xOtx2
uninjected
E
xPax6
uninjected
F
xTwist
uninjected
G
beta-tubulin
uninjected
D'
xOtx2
injected
E'
xPax6
injected
F'
xTwist
injected
G'
beta-tubulin
injected
8A
xOtx2
injected
B
beta-tubulin
injected
C
beta-tubulin
injected
D
beta-tubulin
injected
9A
XCG
control
B
XCG
injected
C
xOtx2
injected
D
beta-tubulin
injected
E
Xbra
injected

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 (Lanctot *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 (Semina *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 dispersal with coinjected 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-anteriorly 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 implicates 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 (Hausen 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-anterior 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 *xPitx*-injected embryos, injected animal cap explants differentiate 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* tailbud tadpoles with a titer of 2.5×10^{10} 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 PaeI 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 nanoinjector. 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, 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 synthesised 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 $MgCl_2$, 0.2 mM dNTPs, 0.1mM dCTP-^{32}P, and 1 μM of each primer (xPitx1—GCC AGT GAG TCT TCT GAT A and CAT GCT CAT GCA AGA GAT; EF1-α—CAG ATT GGT GCT GGA TAT G and ACT GCC 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 control embryos had developed cement glands (stage 25 or later). Explants were then fixed and processed for in situ hybridization as described.

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LITERATURE CITED

Blitz IL, Cho KW. 1995. Anterior neurectoderm is progressively induced during gastrulation: the role of the *Xenopus* homeobox gene *orthodenticle*. Development 121:993–1004.

Bradley L, Wainstock D, Sive H. 1996. Positive and negative signals modulate formation of the Xenopus cement gland. Development 122:2739–2750.

Campione M, Steinbeisser H, Schweickert A, Deissler K, van Bebber F, Lowe LA, Nowotschin S, Viebahn C, Haffter P, Kuehn MR, Blum M. 1999. The homeobox gene *Pitx2*: mediator of asymmetric

left-right signaling in vertebrate heart and gut looping. Development 126:1225–1234.

Cho KWY, Morita EA, Wright CVE, De Robertis EM. 1991. Overexpression of a homeodomain protein confers axis-forming activity to uncommitted *Xenopus* embryonic cells. Cell 65:55–64.

Crawford MJ, Lanctot C, Tremblay JJ, Jenkins N, Gilbert D, Copeland N, Beatty B, Drouin J. 1997. Human and murine *PTX1/Ptx1* gene maps to the region for Treacher Collins Syndrome. Mamm Genome 8:841–845.

Durston AJ, Timmermans JP, Hage WJ, Hendriks HF, de Vries NJ, Heideveld M, Nieuwkoop PD. 1989. Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature 340:140–144.

Drysdale TA, Elinson RP. 1991. Development of the *Xenopus laevis* hatching gland and its relationship to surface ectoderm patterning. Development 111:469–478.

Fagotto F. Gumbiner BM. 1994. Beta-catenin localization during *Xenopus* embryogenesis: accumulation at tissue and somite boundaries. Development 120:3667–3679.

Gage PJ, Camper SA. 1997. *Pituitary homeobox 2*, a novel member of the *bicoid*-related family of homeobox genes, is a potential regulator of anterior structure formation. Hum Mol Genet 6:457–464.

Gage PJ, Suh H, Camper SA. 1999. Dosage requirement of *Pitx2* for development of multiple organs. Development 126:4643–4651.

Gammill LS, Sive H. 1997. Identification of *otx2* target genes and restrictions in ectodermal competence during *Xenopus* cement gland formation. Development 124:471–481.

Harland RM. 1991. In situ hybridization: an improved whole-mount method for *Xenopus* embryos. Methods Cell Biol 36:685–695.

Hausen P, Riebesell M. 1991. The early development of *Xenopus laevis*. An atlas of the histology. Tubigen: Verlag der Zeitschrift fur Naturforschung.

Hemmati-Brivanlou A, de la Torre JR, Holt C, Harland RM. 1991. Cephalic expression and molecular characterization of *Xenopus En-2*. Development 111:715–724.

Hirsch N, Harris WA. 1997. Xenopus *Pax-6* and retinal development. J Neurobiol 32:45–61.

Hollemann T, Pieler T. 1999. *xPitx-1*: a homeobox gene expressed during pituitary and cement gland formation of *Xenopus* embryos. Mech Dev 88:249–252.

Hopwood ND, Pluck A, Gurdon JB. 1989. A *Xenopus* mRNA related to *Drosophila twist* is expressed in response to induction in the mesoderm and the neural crest. Cell 59:893–903.

Kao K, Elinson RP. 1988. The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. Dev Biol 127:64–77.

Kao K, Masui Y, Elinson RP. 1986. Lithium-induced respecification of pattern in *Xenopus laevis* embryos. Nature 322:371–373.

Kuroda H, Hayata T, Eisaki A, Asashima M. 2000. Cloning a novel developmental regulating gene, *Xotx5*: Its potential role in anterior formation in *Xenopus laevis* . Develop Growth Differ 42:87–93.

Lamonerie T, Trembley J, Lanctôt C, Therrien M, Gautier Y, Drouin J. 1996. *Ptx1*, a bicoid-related homeo box transcription factor involved in the transcription of the pro-opiomelanocortin gene. Genes Dev 10:1284–1295.

Lanctot C, Lamolet B, Drouin J. 1997. The *bicoid*-related homeoprotein *Ptx1* defines the most anterior domain of the embryo and differentiates posterior from anterior lateral mesoderm. Development 124:2807–2817.

Lanctot C, Moreau A, Chamberland M, Tremblay ML, Drouin J. 1999. Hindlimb patterning and mandible development require the *Ptx1* gene. Development 126:1805–1810.

Lin CR, Kioussi C, O'Connell S, Briata P, Szeto D, Liu F, Izpisua-Belmonte JC, Rosenfeld MG. 1999. *Pitx2* regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. Nature 401:279–282.

Lu MF, Pressman C, Dyer R, Johnson RL, Martin J F. 1999. Function of Rieger syndrome gene in left-right asymmetry and craniofacial development . Nature 401:276–278.

Nieuwkoop PD, Faber J. 1967. Normal of *Xenopus laevis* (Daudin). 2nd ed. Amsterdam: North Holland Press.

Pannese M, Polo C, Andreazzoli M, Vignali R, Kablar B, Barsacchi G, Boncinelli E. 1995. The *Xenopus* homologue of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions. Development 121:707–720.

Poulin G, Lebel M, Chamberland M, Paradis FW, Drouin J. 2000. Specific protein-protein interaction between basic helix-loop-helix transcription factors and homeoproteins of the Pitx family. Mol Cell Biol 20:4826–4837.

Richter K, Grunz H, Dawid IB. 1988 Gene expression in the embryonic nervous system of *Xenopus laevis*. Proc Natl Acad Sci U S A 85:8086–8090.

Ryan AK, Blumberg B, Rodriguez-Esteban C, Yonei-Tamura S, Tamura K, Tsukui T, de la Pena J, Sabbagh W, Greenwald J, Choe S, Norris DP, Robertson EJ, Evans RM, Rosenfeld MG, Izpisua Belmonte JC. 1998. Pitx2 determines left-right asymmetry of internal organs in vertebrates. Nature 394:545–551.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 74:5463–5467.

Semina EV, Ferrell RE, Mintz-Hittner HA. Bitoun P, Alward WL, Reiter RS, Funkhauser C, Daack-Hirsch S, Murray JC. 1998. A novel homeobox gene *PITX3* is mutated in families with autosomal-dominant cataracts and ASMD. Nat Genet 19:167–170.

Semina EV, Reiter R, Leysens NJ, Alward WL, Small KW, Datson NA, Siegel-Bartelt J, Bierke-Nelson D, Bitoun, P, Zabel BU, Carey JC, Murray JC. 1996. Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, *RIEG*, involved in Rieger syndrome. Nat Genet 14:392–399.

Semina EV, Reiter RS, Murray JC. 1997. Isolation of a new homeobox gene belonging to the *Pitx/Rieg* family: expression during lens development and mapping to the aphakia region on mouse chromosome 19. Hum Mol Genet 6:2109–2116.

Shang J, Luo Y, Clayton DA. 1997. *Backfoot* is a novel homeobox gene expressed in the mesenchyme of developing hind limb. Dev Dyn 209:242–253.

Sive H, Bradley L. 1996. A sticky problem: the *Xenopus* cement gland as a paradigm for anteroposterior patterning. Dev Dyn 205:265–280.

Sive H, Hattori K, Weintraub H. 1989. Progressive determination during formation of the antero-posterior axis in *Xenopus laevis*. Cell 58:171–180.

Smid, MP, van Schaick HS, Lanctot C, Tremblay JJ, Cox JJ, van der Kleij AA, Wolterink G, Drouin J, Burbach JP. 1997. A homeodomain gene *Ptx3* has highly restricted brain expression in mesencephalic dopaminergic neurons. Proc Natl Acad Sci U S A 94:13305–13310.

Szeto DP, Rodriguez-Esteban C, Ryan AK, O'Connell SM, Liu F, Kioussi C, Gleiberman AS, Izpisua-Belmonte JC, Rosenfeld MG. 1999. Role of the *bicoid*-related homeodomain factor *Pitx1* in specifying hindlimb morphogenesis and pituitary development. Genes Dev 13:484–494.

Vorbruggen G, Constien R, Zilian O, Wimmer EA, Dowe G, Taubert H, Noll M, Jackle H. 1997. Embryonic expression and characterization of a *Ptx1* homolog in *Drosophila*. Mech Dev 68:139–147.

Wendling O, Dennefeld C, Chambon P, Mark M. 2000. Retinoid signaling is essential for patterning the endoderm of the third and fourth pharyngeal arches. Development 127:1553–1562.