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Anterior neurectoderm is progressively induced during gastrulation: the role of the *Xenopus* homeobox gene *orthodenticle*

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SUMMARY

In order to study the regional specification of neural tissue we isolated *Xotx2*, a *Xenopus* homolog of the *Drosophila orthodenticle* gene. *Xotx2* is initially expressed in Spemann's organizer and its expression is absent in the ectoderm of early gastrulae. As gastrulation proceeds, *Xotx2* expression is induced in the overlying ectoderm and this domain of expression moves anteriorly in register with underlying anterior mesoderm throughout the remainder of gastrulation. The expression pattern of *Xotx2* suggests that a wave of *Xotx2* expression (marking anterior neurectoderm) travels through the ectoderm of the gastrula with the movement of underlying anterior (prechordal plate) mesoderm. This expression of *Xotx2* is reminiscent of the Eyal-Giladi model for neural induction. According to this model, anterior neural-inducing signals emanating from underlying anterior mesoderm transiently induce anterior neural tissues after vertical contact with the overlying ectoderm. Further patterning is achieved when the ectoderm receives caudalizing signals as it comes in contact with more posterior mesoderm during subsequent gastrulation movements. Functional characterization of the *Xotx2* protein has revealed its involvement in differentiation of the anterior-most tissue, the cement gland. Ectopic expression of *Xotx2* in embryos induces extra cement glands in the skin as well as inducing a cement gland marker (*XAG1*) in isolated animal cap ectoderm. Microinjection of RNA encoding the organizer-specific homeodomain protein *goosecoid* into the ventral marginal zone results in induction of the *Xotx2* gene. This result, taken in combination with the indistinguishable expression patterns of *Xotx2* and *goosecoid* in the anterior mesoderm suggests that *Xotx2* is a target of *goosecoid* regulation.

Key words: Spemann's organizer, anterior specification, neural induction, vertical signalling, planar signalling, cement gland, *noggin*, *goosecoid*

INTRODUCTION

How is the anteroposterior axis of an embryo specified? This question has been an area of intense interest since the early days of experimental embryology. In 1924, Spemann and Mangold demonstrated that a region of tissue adjacent to the dorsal lip of the blastopore of an amphibian gastrula, when transplanted into the ventral side of a host embryo, induced a secondary embryonic axis (Spemann and Mangold, 1924). The donor tissue, since referred to as Spemann's organizer, contributed mostly to the notochord with host tissues constituting the bulk of somitic mesoderm and neural tissue. These results and subsequent experiments demonstrated that the mesoderm provides signals directing neural induction and anterior-posterior positional specification during gastrulation (Hamburger, 1988).

Classic embryological studies also provided a framework for understanding the mechanisms governing anterior-posterior specification of the neural plate. Early models suggested that at least two signals were required for patterning of the anterior-posterior (A-P) axis; one signal instructed the development of anterior neural structures and the other instructed posterior neural structures (Waddington, 1940; Yamada, 1950; Nieuwkoop et al., 1952; Toivonen and Saxen, 1955). These two signals were hypothesized to exist as opposing gradients with maximum concentrations at the future anterior and posterior ends of the embryo (Toivonen and Sáxen, 1955). Subsequently, Nieuwkoop and coworkers provided experimental evidence supporting a two-step activation-transformation model (Nieuwkoop et al., 1952). According to this model, naive ectoderm initially receives an activating signal from the anterior mesoderm that instructs it to adopt an anterior neural fate (Sala, 1955). This process is then followed by a transformation step that posteriorizes the ectoderm providing A-P positional values (Nieuwkoop et al., 1952; Toivonen and Saxen, 1955).

The earlier work of Mangold suggested A-P positional information is transmitted by vertical induction from underlying mesoderm (Mangold, 1933). Later, Eyal-Giladi further suggested that the anterior neural-inducing signal emanates from the anterior mesoderm when this mesoderm first makes vertical contact with the overlying neurectoderm. This ectoderm would then subsequently receive caudalizing signals as it comes into contact with more posterior mesoderm passing

beneath it during the subsequent movements of gastrulation (Fig. 1, Eyal-Giladi, 1954). Recently, however, the vertical signalling aspect of these models has been challenged by the discovery of planar signalling (Kintner and Melton; 1987, Ruiz i Altaba, 1992; Doniach et al., 1992; Keller et al., 1992). At the beginning of gastrulation, prior to involution, the organizer mesoderm and prospective neurectoderm exist as a continuous sheet of tissue in the embryo (see illustration in Fig. 8A). When these regions are excised from the embryo and allowed to develop in culture such that no vertical contact between mesoderm and ectoderm is permitted, convergence-extension and A-P specification of the ectoderm is still observed (Keller et al., 1992; Doniach et al., 1992). Since the mesodermal component of the explants is required for the processes of convergence-extension and A-P specification of the ectoderm, it has been suggested that diffusible signals can pass in a planar fashion from the mesoderm to the ectoderm in vivo.

Currently the molecules involved in planar and/or vertical signalling are unknown. However, two candidates have been identified which have neural-inducing activity in vitro and are expressed in prechordal and notochordal mesoderm in vivo during gastrulation. One of these, follistatin, is an inhibitor of activin signalling and is thought to block activin-mediated repression of neural induction (Hemmati-Brivanlou et al., 1994). This allows for the specification of neural ectoderm instead of epidermis, the ground state of the ectoderm. The second molecule, *noggin*, induces general neural markers as well as an anterior neural marker, *otxA* (which is likely to be an alternative genomic copy of *Xotx2*, described in this report), suggesting that *noggin* plays a role in patterning anterior neurectoderm (Lamb et al., 1993).

In addition to the involvement of extracellular factors in neural patterning, homeodomain proteins are important components in the specification of A-P information during the development of the metazoan body plan. Homeodomain proteins, such as *bicoid* in *Drosophila* and *goosecoid* in vertebrates, are involved in axial specification (Nusslein-Volhard, 1991; Cho et al., 1991b). The *Drosophila* homeodomain protein *orthodenticle* (*otd*), and other members of a class of homeodomain proteins, which include *bicoid* and *goosecoid*, is distinguished from other classes of homeodomain proteins by its unique DNA-binding specificity (Hanes and Brent, 1989; Treisman, 1989; Blumberg et al., 1991; Simeone et al., 1993). *Drosophila otd* is expressed as an anterior band at the cellular blastoderm stage (Finkelstein et al., 1990; Finkelstein and Perrimon, 1990). Mutation of *otd* results in a loss of antennal segment-derived sensory organs (Wieschaus et al., 1984). Two mouse homologs of *otd* (*otx1* and *otx2*) have been identified that are expressed in nested fashion in the early mouse brain (Simeone et al., 1992). We sought to identify *Xenopus* homologs of mouse *orthodenticle* to serve as anterior neural markers in our studies of the role of prechordal mesoderm in the specification of anterior neurectoderm. While our work was being conducted, the mouse *otx2* gene was shown to be expressed throughout the embryonic egg cylinder before gastrulation and localized to the anterior primitive streak during gastrulation (Simeone et al., 1993).

In this study, we describe the isolation and expression of the *Xenopus Xotx2* gene, a homolog of mouse *otx2*. *Xotx2* is initially expressed in Spemann's organizer and is subsequently induced in the overlying presumptive posterior neurectoderm. As gastrulation proceeds, *Xotx2* expression is induced in progressively more anterior ectoderm in register with underlying *Xotx2*-expressing mesoderm, in a pattern reminiscent of the Eyal-Giladi model for neural positional specification (Eyal-Giladi, 1954). Later, *Xotx2* is expressed in the midbrain, forebrain, and prospective cement gland anlage from mid-gastrula to early tailbud stages and expression persists in the brain until later stages of embryogenesis. The cement gland resides anterior to the forebrain and is generally regarded as the most anterior ectodermal structure specified by neural-inducing signals (Sive et al., 1989; Drysdale and Elinson, 1993). We demonstrate that ectopic expression of *Xotx2* in the skin induces the differentiation of ectopic cement glands as well as inducing a cement gland marker (*XAG1*) in isolated animal cap ectoderm.

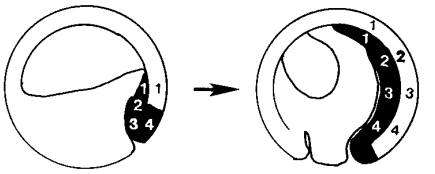

Fig. 1. Schematic representation of Eyal-Giladi's model of neural induction, depicting sagittal sections through early (left) and late (right) gastrula embryos. The numbers (1-4) in involuting dorsal mesoderm (dark area) and neurectoderm (white) indicate relative axial values along the anteroposterior axis, 1 being the most anterior. Involuting dorsal mesoderm first induces anterior neural tissue in the presumptive posterior neurectoderm (left). As gastrulation proceeds, ectoderm further anterior is induced to make anterior neural tissue, and the posterior ectoderm is respecified to make more posterior neural tissue (right). (modified after Doniach, 1993).

MATERIALS AND METHODS

Molecular cloning and characterization of the *Xotx2* cDNA

A mixed gastrulae cDNA library (Cho et al., 1991b) was screened at low stringency with a random-primed probe derived from a 340-bp fragment of the *Drosophila orthodenticle* cDNA containing the homeodomain (Finkelstein et al., 1990). The conditions of hybridization were 42% formamide, 6× SSC, 50 mM sodium phosphate, pH 6.7, 5× Denhardt's, 0.2% SDS, 0.1 mM EDTA, 5% dextran sulfate, and 100 μg torula RNA/ml at 37°C. Filters were washed in 0.5× SSC at 37°C and exposed to Kodak X-ray film. The cDNA inserts from candidate clones were in vivo excised according to the manufacturer's instructions (Stratagene). Nine positive cDNA clones were isolated from a total of 500,000 plaques.

Preparation of RNA and northern blot hybridizations

Total embryonic RNA was isolated from staged *Xenopus* embryos according to Sargent et al. (1986). 20 μg of total RNA was loaded into each lane of a formaldehyde gel and ethidium bromide fluorescence confirmed that all lanes were loaded approximately equally (data not shown). RNA was transferred to nitrocellulose filters according to standard procedures (Sambrook et al., 1989). Filters were hybridized at 65°C in 0.5 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA, and 5% dextran sulfate with a random-primed 1.5-kb *Xho*I fragment of *Xotx2* that lacks the homeodomain.

Whole-mount in situ hybridization

The method of Harland (1991) was used. The *Xotx2* probe consisted of digoxigenin-labelled antisense transcripts derived from the 1.5-kbp *XhoI* fragment of the *Xotx2* cDNA which had been subcloned into pBluescript-KS. The *Krox20* probe was generated by digestion with *Eco*RI and transcription with T7 RNA polymerase. Embryos were sectioned according to the procedure of Norenburg and Barrett (1987).

Embryo manipulations

Preparation of mesoderm-ectoderm conjugates

Embryos were fertilized and microinjected with synthetic mRNAs as described previously (Cho et al., 1991b). Mesoderm-ectoderm conjugates were prepared from a pair of early gastrula stage albino embryos, one member of which was injected at the 2-4 cell stage with lysinated-rhodamine dextran (relative molecular mass of 10×10^3. Molecular Probes Inc., Oregon). Involuted organizer mesoderm from an injected embryo was removed at stage 10.25 and conjugated with animal cap ectoderm derived from an uninjected stage 10.25-10.5 embryo. Conjugates were allowed to develop in 0.3× Barth until sibling embryos reached stages 14-16 (Nieuwkoop and Faber, 1967), and were then fixed for whole-mount in situ hybridization.

Preparation of Keller sandwich explants

Keller sandwiches were prepared essentially as described by Keller and Danilchik (1988), Keller (1991) and Doniach et al. (1992). Early gastrula embryos were lightly stained with Nile Blue and manually dechorionated prior to staging according to Nieuwkoop and Faber (1967), and Keller (1991, and personal communication) as follows. Stage 10–, bottle cell constriction just barely becomes visible on the dorsal side; stage 10.0, bottle cells appear as a straight line and constriction has advanced as is apparent from the intensity of concentration of Nile Blue and the width of the constriction, however the lip has not yet begun to pucker inward significantly; stage 10+, the bottle cells occupy greater width, begin to curve ventrally, and the lip puckers inward substantially; stage 10.25, the bottle cells have progressed laterally to occupy an approximate 180° arc and the dorsal lip occupies an approximate 75° arc; stage 10.5, the bottle cells have progressed to the ventral midline and the dorsal lip occupies an approximate 180° arc. To construct Keller sandwiches, two explants are dissected from two identically staged embryos and sandwiched together with their inner surfaces apposed after removing highly migratory head mesoderm from the inner surface of the explants. Surgeries were performed using eye brow hair knives and hair loops and sandwich explants were allowed to develop in Sater's modified Danilchik's solution (Keller et al., 1992) until sibling embryos reached stage 17-19 (Nieuwkoop and Faber, 1967). Sandwiches were then fixed for whole-mount in situ hybridization.

RT-PCR

Total cellular RNA isolated from animal cap ectoderm was reverse transcribed at 37°C for 60 minutes using random hexamers in a 50 μl reaction volume as described by Makino et al. (1990). cDNA was then PCR amplified in a 10 μl reaction volume containing 300 ng of primer. The conditions for the thermal profile were as follows: 94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute, for 24-30 cycles. A final extension step of 72°C for 10 minutes was included. Amplification of all fragments was within the linear range of the PCR reaction after 30 cycles except histone H4 which was saturated after 24 cycles of PCR. All PCR products were analyzed on a 5% urea-polyacrylamide gel. The oligos used for PCR amplification were as follows: **N-CAM** F:5′-AGATGCAGTCATTATTTGTGATGTC-3′, R:5′-CTGGATGTCC-TTATAGTTGATCTC-3′ (Collett and Steele, 1993); ***Xotx2*** F:5′-GG-AGGCCAAAACAAAGTG-3′, :5′-TCATGGGGTAGGTCCTCT-3′; ***XAG1*** F:5′-GAGTTGCTTCTCTGGCA-3′, R:5′-CTGACTGTC-CGATCAGAC-3′ (H.Sive, personal communication); **histone H4** F:5′-CGGGATAACATTCAGGGTATCACT-3′, R:5′-ATCCATG-GCGGTAACTGTCTTCCT-3′ (kindly provided by B. Blumberg).

RESULTS

Isolation and characterization of the *Xotx2* cDNA

A cDNA library constructed from mixed gastrula stages of *Xenopus laevis* was screened at low stringency with a probe containing the homeodomain region of the *Drosophila orthodenticle* (*otd*) gene (Finkelstein et al., 1990). The longest cDNA isolated was approximately 2.1 kb and analysis of the DNA sequence (GenBank accession numbers U19813 and U19814) revealed an 870-bp open reading frame (ORF) flanked by 207 bp of 5′ and approximately 1 kb of 3′ untranslated sequence, respectively. The homeodomains of all the *orthodenticle* homologs are highly conserved (Simeone et al., 1993), with the *Xenopus* homolog differing at only 3 of 60 amino acid residues from the homeodomain sequence of the *Drosophila orthodenticle* protein. Furthermore, the homeodomain of the *Xenopus* protein differs by a single amino acid substitution from the mouse and human *otx2* proteins and at two amino acid residues from the mouse and human *otx1* proteins. The *orthodenticle* homeodomains, including this *Xenopus* homolog, contain a lysine at position 9 of the third helix, putting them in the same homeodomain class as *bicoid* and *goosecoid.* This lysine residue has been demonstrated to affect DNA-binding specificity of homeodomain proteins (Hanes and Brent, 1989; Treisman et al., 1989). Mouse *otx2* and *goosecoid* have both been demonstrated to bind bicoid-like DNA binding sites (Blumberg et al., 1991; Simeone et al., 1993).

Throughout the remainder of the protein, the conservation in amino acid sequence and the position of corresponding gaps (data not shown, see Simeone et al., 1993) strongly suggest that this *Xenopus* gene is a homolog of the mouse, human and zebrafish *otx2* genes (the *Xenopus* protein is 92% identical to the *otx2* proteins) and not that of *otx1* (at shared residues the *Xenopus* protein is approximately 70% identical to the *otx1* proteins). Comparison of the *Xotx2* amino acid sequence with the available sequence from *Xenopus otxA* (Lamb et al., 1993) demonstrates that these two genes encode related but not identical molecules. These differences occur in the sequence encoding the 5′ untranslated regions of these mRNA's as well

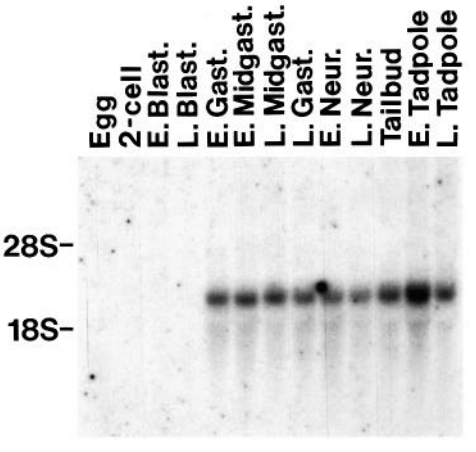

Fig. 2. Northern blot analysis of *Xotx2* transcripts during *Xenopus* embryogenesis. RNA was isolated from embryos of the indicated stages and subjected to northern blot analysis using a 1.5 kb probe lacking the homeobox region. The stages of embryos assayed are: unfertilized egg; 2-cell (stage 2); early blastula (stage 8); late blastula (stage 9); early gastrula (stage 10-10.5); early midgastrula (st.11); mid gastrula (stage 12); late gastrula (stage 12.5); early neurula (stage 13-14); late neurula (stages 18-19); tailbud (stages 24-28); early tadpole (stage 32-34); and late tadpole (stages 43-46) according to Nieuwkoop and Faber (1967).

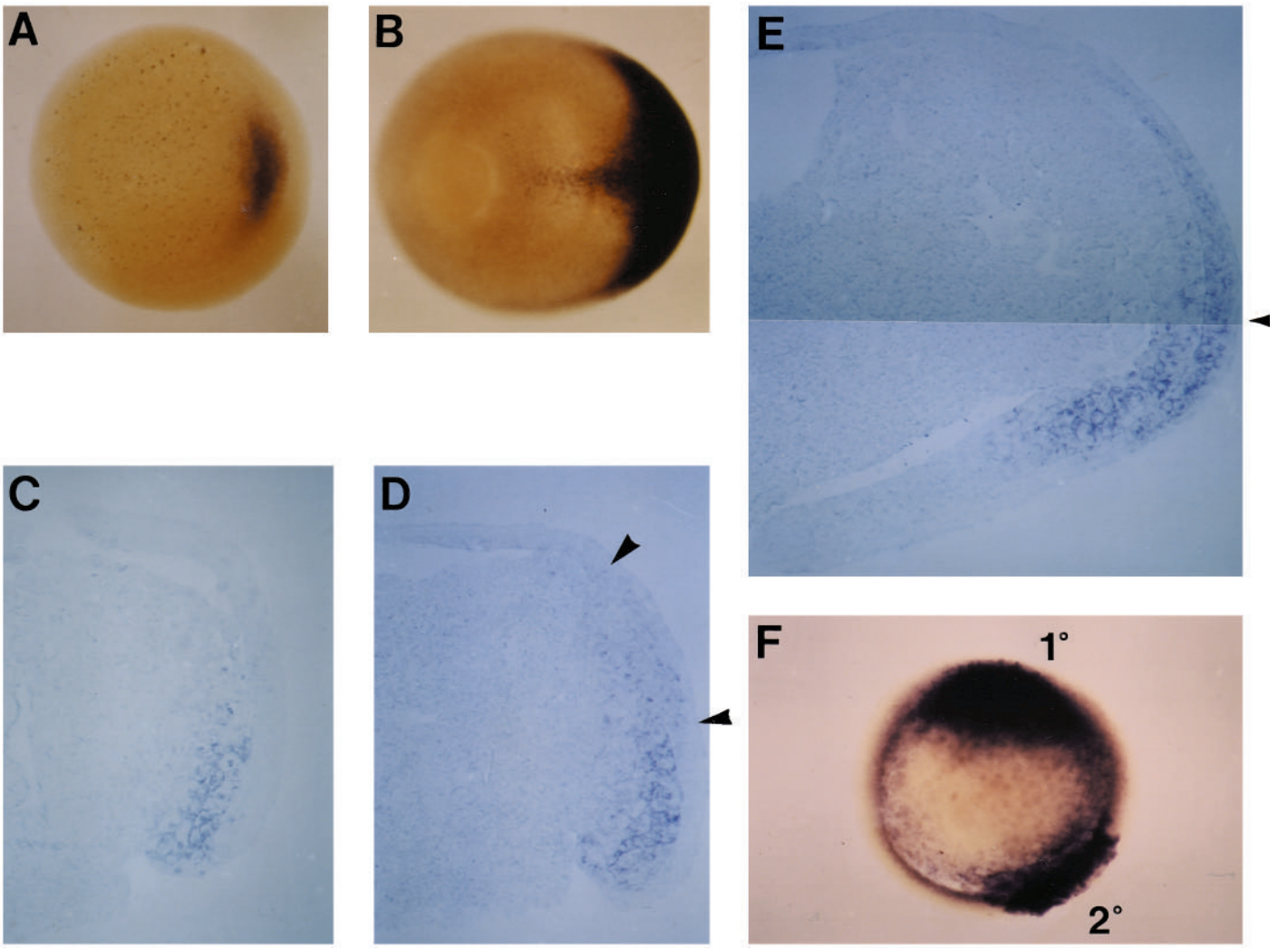

Fig. 3. Whole-mount in situ hybridization of *Xotx2* during *Xenopus* gastrulation. (A) Vegetal view of a stage 10 embryo. (B) Dorsovegetal view of a stage 12 embryo. (C-E) Dorsal portion of midsagittal sections of whole-mount in situ hybridized stage 10+, 10.25, and 12 embryos, respectively. Anterior is to the right in all panels except F. *Xotx2* staining in C is confined to deep mesoderm, while in D and E the expression in ectoderm is apparent (arrowheads in D indicate a region of nuclear ectodermal *Xotx2* staining). In E *Xotx2* expression in both ectoderm and mesoderm are approximately in register. Note the ectodermal thickening marking the presumptive anterior neurectoderm (arrow). The anterior border of ectodermal staining extends further anteriorly into presumptive cement gland (see also Jamrich and Sato, 1989). (F) *Xotx2* is induced in ventrolateral mesoderm expressing *goosecoid*. Single C-tier blastomeres were coinjected with *goosecoid* RNA and the lineage tracer LRD at the 32-cell stage. At the early gastrula stage, embryos containing LRD at a position greater than 120° away from the dorsal midline were subjected to whole-mount in situ hybridization for *Xotx2*. A vegetal pole view is shown and the primary and secondary sites of *Xotx2* expression are indicated.

as in the amino acid sequence. An amino acid sequence that would unambiguously identify *otxA* as an *otx2* homolog is presently not available, but it seems likely that *Xotx2* and *otxA* represent divergent genomic copies of different *otx2* genes present in the pseudotetraploid genome of *Xenopus laevis* (Graf and Kobel, 1991). Since the sequence of the gene described in this study is virtually identical to the *otx2* genes of mouse, human and zebrafish, we refer to our *Xenopus* gene as *Xotx2*.

Spatiotemporal expression of *Xotx2* RNA during early *Xenopus* embryogenesis

We examined the temporal expression of *Xotx2* RNA by northern analysis using a 1.5-kb probe consisting of sequences downstream of the homeodomain and the entirety of the 3′ untranslated region (Fig. 2). A single band of approximately 2.1 kb was detected corresponding to the size of the *Xotx2* cDNA, suggesting that this cDNA is approximately full-length. *Xotx2* RNA was not detected at early stages of development up to and including blastula stages, but is easily detected during gastrula stages and persists at high levels at least until swimming tadpole stages.

The distribution of *Xotx2* RNA in the developing *Xenopus* embryo was examined using whole-mount in situ hybridization (Harland, 1991). *Xotx2* transcripts are first detected in the early gastrula embryo. These results differ slightly from the reported expression pattern of *otxA* in that *otxA* is expressed in the late blastula throughout the marginal zone and quickly becomes

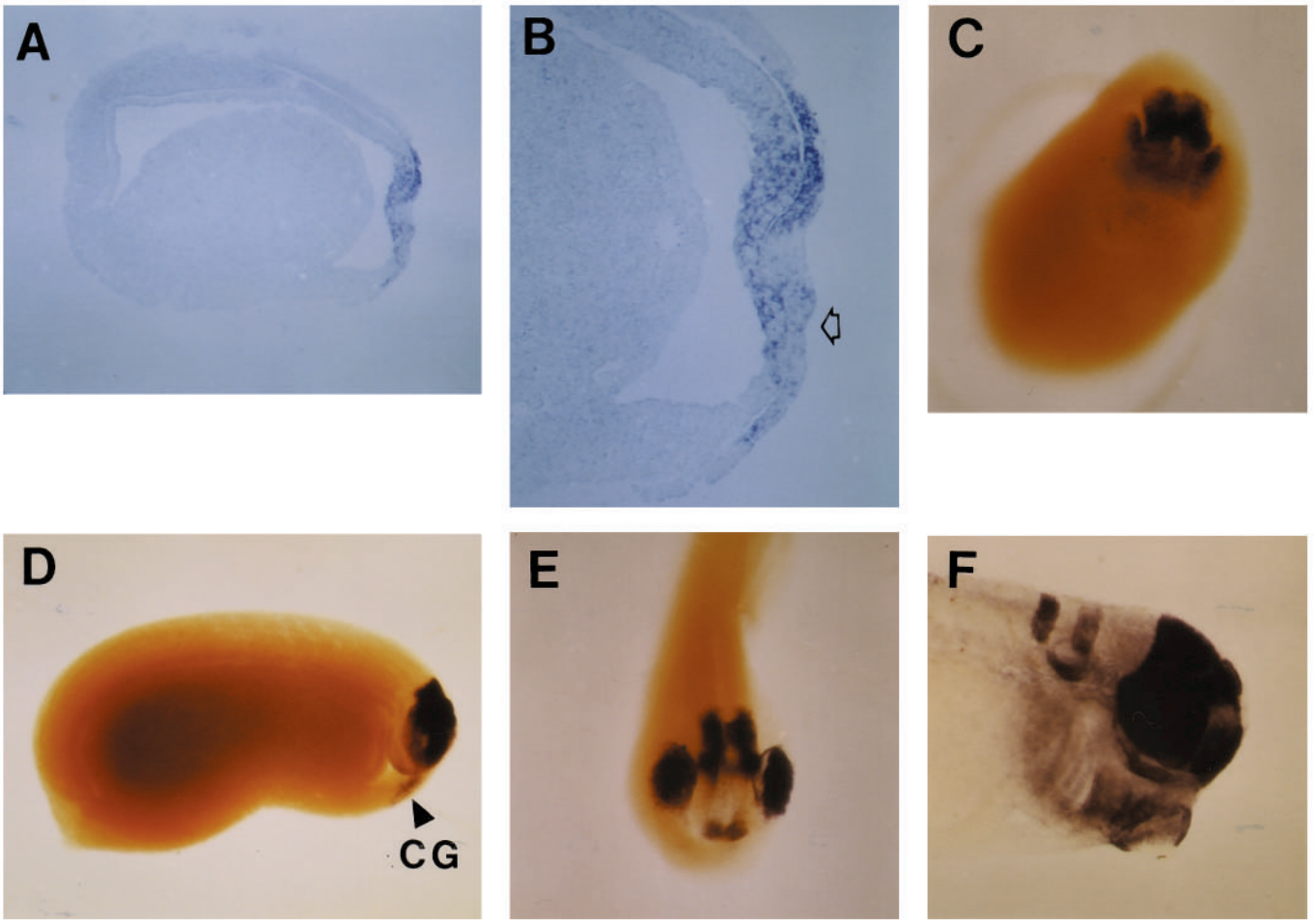

Fig. 4. Spatial expression of *Xotx2* during neurula, tailbud, and swimming tadpole stages. (A,B) Midsagittal sections through a whole-mount in situ hybridized stage 15 neurula. Expression of *Xotx2* in mesoderm and ectoderm is in register. (B) Higher magnification view of A. Open arrow denotes the position of the border separating of the anterior neural plate from the cement gland anlage. (C-E) Staining of stage 19 (neurula), 23 (tailbud) and 32 (tadpole) embryos. Note staining in the early eye anlage in C. (F) Stage 32 embryo double stained with *Xotx2* and *Krox20*. CG, cement gland.

restricted to the superficial layer on the dorsal side (Lamb et al., 1993). We cannot exclude the possibility that *Xotx2* also behaves in this fashion immediately prior to onset of bottle cell constriction. *Xotx2* hybridization in the gastrula is localized to cells of Spemann's organizer in a region consisting of an approximately 45° arc from the mid-sagittal line (Fig. 3A), in a pattern indistinguishable from that of *Xenopus goosecoid* (Cho et al., 1991b; Inoue et al., unpublished data). As gastrulation proceeds further, the abundance of *Xotx2* RNA in the ectoderm increases, moves anteriorly, and by stage 11 expands mediolaterally (data not shown). At later stages of gastrulation, as mesoderm and ectoderm undergo continued gastrulation movements, the *Xotx2* hybridization signal moves both further anteriorly and concomitantly fans out to form a large cap over the anterior end (Fig. 3B).

In order to determine the identity of cells expressing *Xotx2* during gastrulation, whole-mount in situ hybridized embryos were sectioned and further analyzed. Near the beginning of gastrulation, *Xotx2*-expressing cells of the organizer are found in the deep cell layers (Fig. 3C) comprising the presumptive head mesoderm (Keller, 1975, 1976). During gastrulation these cells are the first to invaginate and constitute the leading edge of migrating mesoderm, which travels along the inner surface of the blastocoel roof. At a slightly later stage (approximately stage 10.25) *Xotx2* expression is detectable, albeit weakly but reproducibly, in ectoderm overlying the anterior-most portion of the mesodermal signal (Fig. 3D). In addition, mesodermal *Xotx2* RNA can be detected more posteriorly in cells which constitute the presumptive anterior notochord (Figs 3B and 8).

This pattern of expression is maintained at the anterior end of the embryo throughout the remainder of gastrulation (Fig. 3B,E). In the late gastrula embryo (stage 12) the domain of *Xotx2* expression overlaps a region of columnar cells (Fig. 3E, see arrow) which constitute the future brain (Nieuwkoop and Faber, 1967; Keller, 1980). *Xotx2* expression is also found in a region anterior to the columnar cells (see arrow in Fig. 4B) prior to morphological delineation of the anterior neural plate boundary, which subsequently bisects the *Xotx2* expression domain into anterior neural plate and the prospective cement gland (Figs 3E, 4A,B; for comparison see also Jamrich and Sato, 1989).

In open neural plate stages, the expression of *Xotx2* is found

in ectoderm and mesodermal cells of the prechordal plate (Fig. 4A and B). As in the gastrula, the ectodermal expression continues along the anterior-posterior axis approximately in register with the mesodermal domain of expression. Prechordal mesodermal *Xotx2* expression occupies a narrow strip in the dorsal midline approximately 150 μm in width (data not shown). Ectodermal *Xotx2* expression overlaps the anterior border of the neural plate and persists weakly in the cement gland anlage until early tailbud stages (Fig. 4A-D).

During subsequent development of the neural tube, *Xotx2* is detected in the anterior brain and developing eye anlage (Fig. 4C). This *Xotx2* domain eventually develops into the forebrain and midbrain (Fig. 4D,E), with the posterior border of *Xotx2* expression demarcating the midbrain-hindbrain junction as revealed by staining with *Krox20* (Fig. 4F) and *engrailed-2* (data not shown) probes. *Krox20* is a marker for hindbrain rhombomeres 3 and 5 whereas *engrailed-2* overlaps the midbrain-hindbrain junction (Papalopulu et al., 1991; Hemmati-Brivanlou et al., 1991). Later, expression of *Xotx2* decreases in the presumptive diencephalon prior to tailbud stage 28 and *Xotx2* marks the presumptive telencephalon and mesencephalon (Fig. 4E,F).

Ectopic expression of *Xotx2* induces development of cement glands

A role for ectodermal *Xotx2* expression was examined by microinjection of *Xotx2* RNA into the animal pole region. These experiments demonstrated that up to 76% (Table 1, Exp.

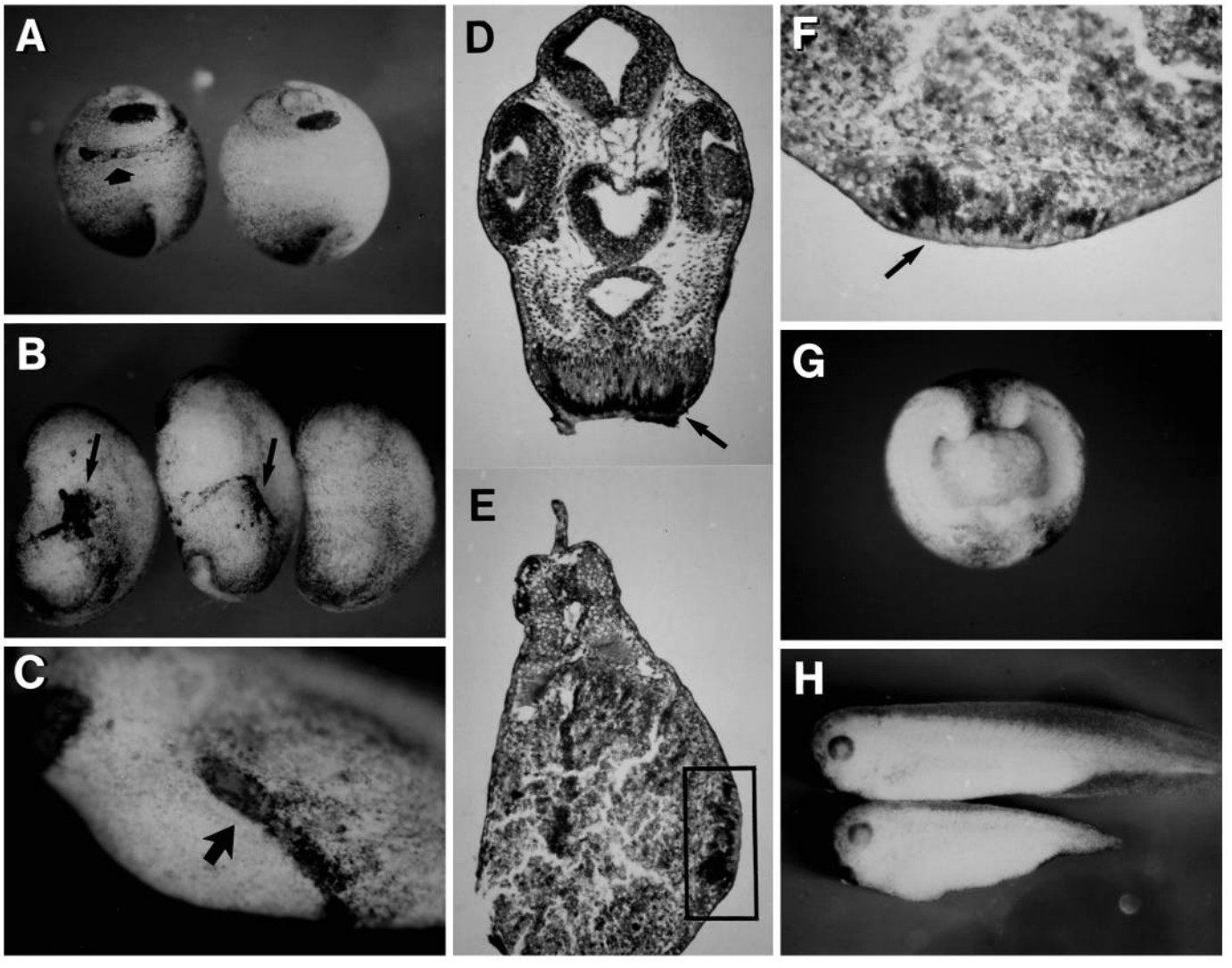

Fig. 5. Ectopic expression of *Xotx2* induces ectopic cement glands in the skin of developing embryos. (A) *Xotx2* injected (left) and uninjected (right) embryos (stage 20). Note the induction of ectopic cement gland (arrow). (B) *Xotx2*-injected embryos (stage 22) are left and middle, and an uninjected embryo is right. Arrows indicate ectopic cement glands. (C) *Xotx2*-injected embryo grown until early tadpole stage. An ectopic cement gland is present in the ventral/lateral ectoderm. (D) Transverse section through head of normal tailbud stage embryo. Note that the normal cement gland shows characteristic columnar cells with dark pigmentation (arrow). (E) Transverse section through the trunk of an *Xotx2*-injected embryo. Box indicates the area of an ectopic cement gland. (F) High magnification view of the boxed area in E. Note the presence of characteristic columnar cells associated with pigment granules (arrow). (G) Incomplete blastopore closure resulting from injection of *Xotx2* into a dorsal blastomere. Such embryos will later develop spina bifida. (H) Uninjected control embryo (top) and short-tail embryo (bottom) resulting from incomplete blastopore closure.

1) of injected embryos developed ectopic cement glands in the skin (Fig. 5A-C). Cement glands were first visible at neurula stages due to concentration of pigment granules during cement gland differentiation. Larger ectopic cement glands secreted mucus (data not shown) and sectioning revealed the presence of columnarized cells containing pigment granules (compare Fig. 5, panels D,E and F) demonstrating bona fide cement gland induction. No other obvious ectopic structures could be identified in the vicinity of ectopically induced cement glands. In contrast, microinjection of transcripts encoding other homeodomain proteins such as *goosecoid* (data not shown, and Cho et al., 1991b), *XlHbox1* (Wright et al., 1989), *XlHbox6* (Cho et al., 1991), and non-homeodomain transcripts such as β-globin (our unpublished observation) and *lac*Z (Inoue et al., unpublished data) into animal pole blastomeres did not induce ectopic cement glands.

To assess the ability of *Xotx2* to induce cement glands in ectoderm in the absence of inductive contributions from other non-ectodermal structures in the embryo, we injected *Xotx2* RNA into the animal hemisphere of 4-cell embryos and dissected animal caps at the blastula stage. These ectodermal explants were cultured until sibling embryos had reached the late neurula stage. Total RNA was isolated and subjected to RT-PCR analysis for relative levels of RNA encoding the cement gland marker *XAG1*. *XAG1* RNA is expressed in vivo at low levels during gastrulation and is dramatically up-regulated in the ectoderm upon cement gland induction (Sive et al., 1989). In uninjected control animal caps *XAG1* was detected at low levels (Fig. 6A, lane 2). However, in animal caps expressing *Xotx2* we found a large increase in *XAG1* RNA levels (Fig. 6A, lane 3), demonstrating that *Xotx2* can induce a marker of cement gland differentiation in isolated animal cap ectoderm.

Table 1. *Xotx2* mRNA injection induces ectopic cement glands and spina bifida

Exp no. and stages	Site injected	Amount injected per blastomere (pg)	Number of embryos		Total (*n*)
			Spina bifida	Cement gland	
Exp 1					
2-cell	ectoderm	800	ND	32 (76%)	42
		400	ND	28 (50%)	56
		200	ND	12 (28%)	44
8-cell	dorsal ectoderm	800	32 (78%)	18 (44%)	41
		400	27 (63%)	12 (28%)	43
		200	10 (36%)	5 (18%)	28
Exp 2					
4-cell	ventral vegetal	800	1 (3%)	0	35
		400	0	0	61
		200	0	0	42
Exp 3					
8-cell	ventral marginal	800	2 (7%)	0	28
		400	2 (7%)	0	27
		200	0	0	22
Exp 4					
32-cell	C4 blastomere (ventral marginal)	400	0	0	105
		200	0	0	65

Xenopus embryos were injected at the 2-, 4-, 8- or 32-cell stage, with the indicated amount of mRNA. Cement gland was identified by morphology and appeared as darkly pigmented group of cells, and secreted mucus. Some were histologically examined (see Fig. 5). Lower doses of RNA had no obvious phenotypic consequences.

Does *Xotx2* play a role in neural induction?

The cement gland is generally considered to be the most anterior structure patterned in response to neural inductive signals (Sive et al., 1989; Drysdale and Elinson, 1993). Since *Xotx2* induces ectopic cement glands in vivo as well as a cement gland marker gene in isolated animal cap ectoderm, and is expressed in neural tissue throughout early embryogenesis, we examined the possibility that *Xotx2* may also play a role in the induction of neural tissue. Induction of *N-CAM* was detected in *Xotx2*-injected animal caps (Fig. 6B, lane 4) although the level of induction is lower than that of *noggin*-injected animal caps (Fig. 6B, lane 3). From these experiments, we conclude that ectopic expression of *Xotx2* appears to induce *N-CAM*, albeit less efficiently than the induction of *N-CAM* by *noggin*. Although *Xotx2* may play a role in neural induction, it is not apparent whether the induction of *N-CAM* observed has any in vivo relevance. Therefore, a more extensive examination using other pan-neural and position-specific markers will be necessary to provide a thorough understanding of the role of *Xotx2* in this process.

Since *Xotx2* is expressed in the forebrain and midbrain, and appeared to induce *N-CAM* in isolated animal caps, we attempted to obtain non-cement gland neural phenotypes by microinjection of *Xotx2* into blastomeres at a variety of developmental stages. Injection into blastomeres contributing to prospective neurectoderm at various stages typically resulted

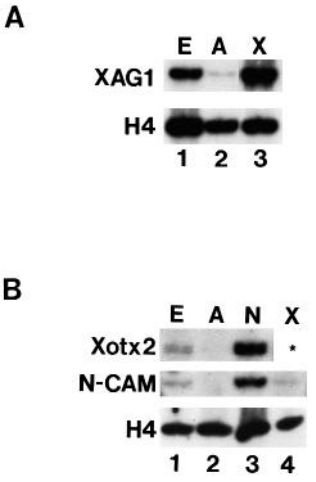

Fig. 6. *Xotx2* induces cement gland marker expression in isolated animal caps. (A) RT-PCR analysis of RNA derived from stage 21 embryos (lane 1) and animal caps isolated from uninjected (lane 2) and *Xotx2*-injected embryos (lane 3) at stage 8 blastula, and developed until sibling embryos reached stage 21. RNA was subjected to RT-PCR analysis using oligonucleotides specific for the cement gland marker *XAG1* and histone H4. (B) RT-PCR analysis of RNA derived from stage 21 embryos (lane 1) and animal caps isolated from uninjected (lane 2), *noggin*-injected (lane 3) and *Xotx2*-injected embryos (lane 4) at stage 8 blastula, and developed until sibling embryos reached stage 21. RNA was subjected to RT-PCR analysis using oligonucleotides specific for *Xotx2*, N-CAM, and histone H4. Lanes marked E, A, N, and X contain PCR products from normal embryos, animal (uninjected control) caps, *noggin*-injected caps, and *Xotx2*-injected caps, respectively.

in a high frequency of embryos exhibiting spina bifida (Table 1, and data not shown), as well as embryos with truncated (or short) tail structures (Fig. 5H) which were often associated with incompletely closed blastopores (Fig. 5G and data not shown). The short-tail phenotype does not appear to reflect a posterior-to-anterior transformation since diminution of the tail is not accompanied by enlargement of the head or the remainder of the trunk. Therefore, we conclude that these phenotypic analyses have failed to demonstrate an in vivo function for *Xotx2* in neural specification.

Ectodermal expression of *Xotx2* can be induced by both vertical and planar signalling routes

Since ectodermal expression of *Xotx2* followed the migration of the underlying anterior mesoderm during gastrulation, we tested the hypothesis that vertical contact by anterior mesoderm was required for the ectodermal expression pattern observed. The lineage tracer lysinated rhodamine-dextran (LRD) was injected into the marginal zone of all blastomeres of two- and four-cell stage embryos (Fig. 7A). At early gastrula stage (stage 10.25), involuting anterior mesoderm was dissected from these embryos and wrapped in unlabelled animal cap ectoderm isolated from sibling embryos at early gastrula stages 10.25-10.5. When these mesoderm-ectoderm conjugates reached early neurula stages (stage 14-16), they were fixed and subjected to whole-mount in situ hybridization using an *Xotx2* probe. No hybridization signal was detected in animal cap ectoderm controls that lacked mesoderm (Fig. 7C), demonstrating that *Xotx2* expression in the ectoderm is not cell autonomous. Conjugates strongly expressed *Xotx2* and most of this signal was present in the unlabelled ectoderm (Fig. 7B,C). In addition, cement glands were also induced by underlying mesoderm (data not shown). We conclude that ectodermal *Xotx2* expression and cement gland differentiation can be induced by underlying anterior mesoderm via vertical signalling.

Since vertical signalling appeared to play an important role in establishing the in vivo pattern of neurectodermal *Xotx2* expression, we sought to examine *Xotx2* expression in the absence of vertical interactions. This is also important as it remained possible that ectodermal *Xotx2* expression may also be induced by signals travelling through the plane of tissue shared by the mesoderm and

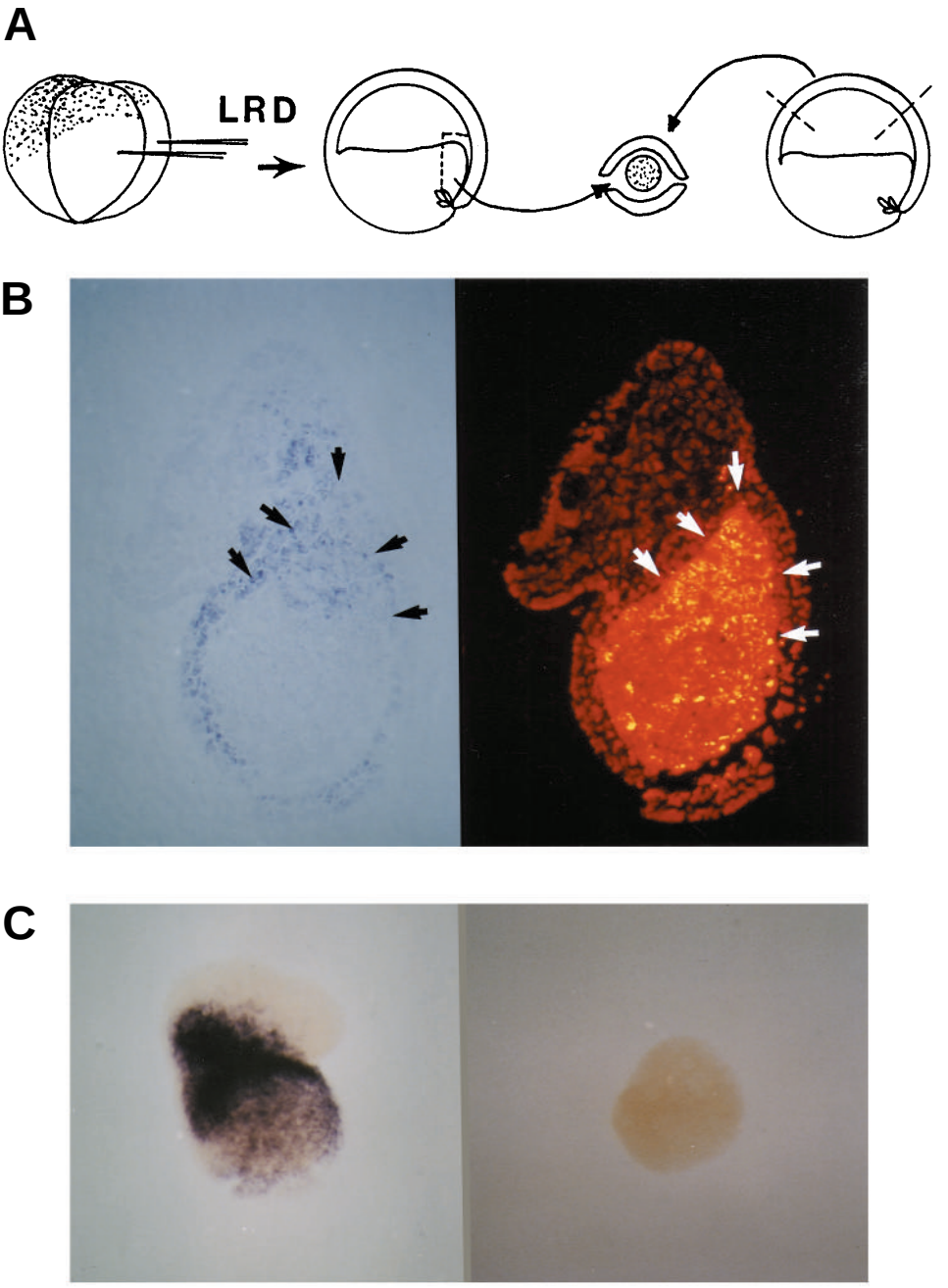

Fig. 7. Ectodermal expression of *Xotx2* can be induced by vertical signals. (A) Schematic representation of construction of mesoderm-ectoderm conjugates. Albino embryos were injected with lysinated-rhodamine dextran (LRD) into the marginal zone of all blastomeres of 2-4 cell stage embryos. At early gastrula stage (10.25), involuted mesoderm was dissected out and wrapped in stage 10.25 gastrula ectoderm from uninjected albino embryos. Conjugates were developed until siblings reached stage 15, fixed, and subjected to whole-mount in situ hybridization using *Xotx2* probe. (B) Light (left) and fluorescence (right) micrographs of sections through the whole-mount in situ hybridized stage 15 equivalent conjugate shown in C (left). Arrows mark the boundary between the labelled mesoderm and unlabelled animal caps. Note the ectodermal expression of *Xotx2* surrounding the mesoderm. Incubation of ectoderm cap alone did not induce ectopic *Xotx2* expression (C, right).

ectoderm at the beginning of gastrulation (Keller and Danilchik, 1988; Doniach et al., 1992). We examined these issues using Keller sandwich explants. Since it has recently been demonstrated that anterior mesoderm can provide vertical signals to overlying ectoderm in the stage 10+ gastrula (A. Posnansky and R. Keller, personal communication), we constructed a series of Keller sandwiches from embryos isolated at stages 10–, 10.0, 10+, 10.25, 10.5 (Nieuwkoop and Faber, 1967; Keller, 1991; and Keller, personal communication) as described in the Materials and Methods. In the stage 10– embryo (Fig. 8A, left), the head and notochordal mesoderm reside as a continuous strip of cells (Keller, personal communication) and have not begun involution. In the stage 10.0 embryo, head mesoderm has begun involution but has not yet contacted ectoderm fate mapping to the posterior neural plate (Keller et al., 1992; Keller, personal communication). During the subsequent stages of gastrulation, head mesoderm and notochordal mesoderm migrate further anteriorly and by approximately stage 11-11.5 the head mesoderm comes to underlie ectoderm of the prospective forebrain (Keller et al., 1992). Sandwich explants were assessed for their pattern of *Xotx2* expression by whole-mount in situ hybridization at equivalent late neurula stages (stages 17-19).

The expression of *Xotx2* could be detected in the mesoderm and the ectoderm at the anterior extremes of the explants (100%, *n*=26, and Fig. 8). The mesodermal staining corresponds to the anterior mesoderm comprising the presumptive prechordal mesoderm and anterior notochord, while the ectodermal stain corresponds to the anterior ectodermal domain of *Xotx2* expression (Fig. 8). Interestingly, Keller sandwiches constructed at different stages of development all had similar patterns of ectodermal *Xotx2* expression (Fig. 8A and B, and data not shown) at late neurula stage, despite the differences in the strength of induction of the hindbrain-specific marker *Krox20* (compare Fig. 8A and B), a marker sensitive to vertical inductive influences (A. Posnansky and R. Keller, personal communication). Therefore, we concluded that both vertical and planar signals may contribute to the ectodermal expression of *Xotx2* in the developing *Xenopus* embryo.

Mesodermal expression of *Xotx2* may be regulated by *goosecoid*

The spatial and temporal expression of *Xotx2* in the anterior mesoderm appears virtually identical to the expression of the homeobox gene *goosecoid* (Figure 3A, Cho et al., 1991b; Inoue et al., unpublished data). In addition, all treatments that affect *goosecoid* gene expression (LiCl, UV, retinoic acid, activin, and *XWnt8*) were shown to affect *Xotx2* expression in a similar manner (data not shown, Cho et al., 1991b; Inoue et al., unpublished data; Christian and Moon, 1993). Since *bicoid* regulates the expression of *orthodenticle* in the cellular blastoderm stage *Drosophila* embryo, it is tempting to speculate that this model for *orthodenticle* gene regulation might be extended to vertebrates. Therefore, synthetic *goosecoid* mRNA was coinjected with a lineage tracer (LRD) into a single C-tier blastomere at the 32-cell stage and then embryos were examined for *Xotx2* expression by whole-mount in situ hybridization at the early gastrula stage. *Xotx2* RNA colocalized with the lineage tracer in all embryos examined (6 of 6 ventrally injected embryos, Figure 3F). We conclude that *Xotx2* may be a target gene regulated by *goosecoid*.

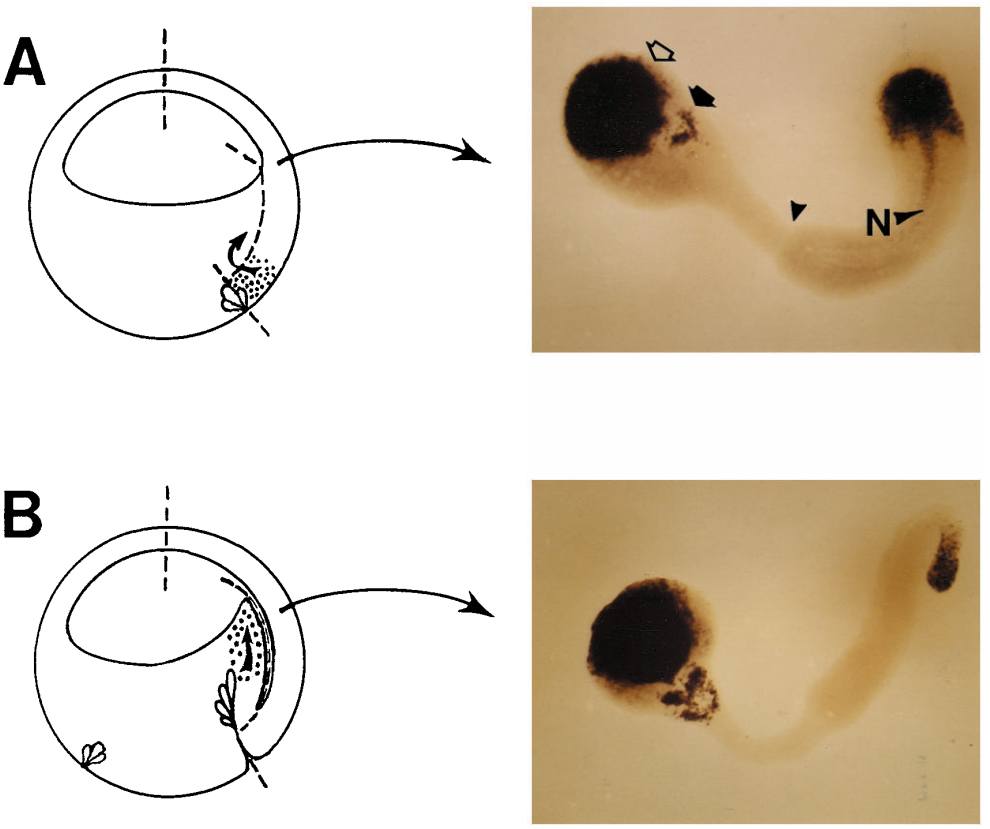

Fig. 8. Planar signals are sufficient to induce *Xotx2* expression in ectoderm. Keller sandwiches were prepared from stage 10–, 10.0, 10+, 10.25 and 10.5 embryos. Sandwich explants were developed to stages 17-19, fixed, and subjected to whole-mount in situ hybridization using *Xotx2* and *Krox20* probes. (A) Schematic diagram illustrating the arrangement of the anterior mesoderm (stippled) of a stage 10- embryo before involution and a representative Keller sandwich derived from stage 10- embryos. (B) Schematic diagram illustrating the arrangement of the anterior mesoderm (stippled) of a stage 10.5 gastrula and a representative Keller sandwich derived from a stage 10.5 embryo. The dotted lines indicate the positions of incisions made to prepare the Keller explants. In both right panels, ectoderm is progressively more anterior toward the left and the mesoderm is progressively more anterior toward the right. Notochord is designated N. The boundary of mesoderm and ectoderm is indicated by the arrowhead. *Xotx2* staining (open arrow) of the ectoderm is anterior to the characteristic striped pattern of *Krox20* (filled arrow). In the stage 10– sandwich (A), there is clear *Xotx2* staining in the anterior notochord.

DISCUSSION

Domains of *Xotx2* expression in the mesoderm and neuroectoderm are in register during gastrulation

Analysis of the spatial expression of *Xotx2* during gastrulation has demonstrated that *Xotx2* expression is initially absent in the ectoderm. But, at a slightly later stage, *Xotx2* is expressed weakly in ectoderm overlying the head mesoderm. This ectodermal signal increases in strength thereafter. Since isolated animal cap ectoderm does not express *Xotx2* in the absence of inducing signals emanating from the mesoderm, the underlying mesoderm appears to be important for the proper induction of expression of *Xotx2* in the ectoderm. Neurectodermal *Xotx2* expression is in approximate register with the underlying *Xotx2*-expressing anterior mesoderm throughout the remainder of gastrulation and neurulation. Although in register expression between the mesoderm and ectoderm has previously been described for other homeodomain genes such as *XlHbox1* (*Xenopus HoxC6*) and *HoxB1* (in mouse), these genes are expressed later in development, after the morphogenetic movements of gastrulation have been completed (De Robertis et al., 1989; Frohman et al., 1990). *Xotx2* is the earliest expressed gene indicating that position-specific determination occurs across germ layers during gastrulation.

Anterior movement of neurectodermal *Xotx2* expression supports the activation-transformation model of neural induction

Morphological features defining position in the ectoderm are absent during the majority of gastrulation and no other molecular markers exist to define the position of prospective forebrain, midbrain, hindbrain, and spinal cord during these stages of development. Therefore a precise analysis relating the position of *Xotx2* expression and the boundaries of these prospective structures in sections of whole-mount in situ preparations is difficult. However, recent fate-mapping experiments by Keller and coworkers have demonstrated that anterior mesoderm initially contacts posterior neurectoderm of the prospective spinal cord during late stage 10+ (A. Poznansky and R. Keller, personal communication) and does not progress anteriorly to underlie and contact the prospective forebrain ectoderm until stages 11-11.5 (Keller et al., 1992). Since *Xotx2* is already expressed in the ectoderm at stage 10.25, well before anterior mesoderm underlies and contacts prospective anterior neurectoderm, the *Xotx2*-expressing ectodermal cells must represent prospective posterior neurectoderm. The observations that *Xotx2* is induced in posterior ectoderm shortly after invagination of anterior mesoderm, remains in register with underlying anterior mesoderm during gastrulation, and is ultimately expressed in the anterior neural plate at the end of gastrulation suggest that a ‘wave’ of transient gene expression moves through the sheet of ectoderm from posterior to anterior.

Sive and coworkers have demonstrated that a wave of inductive activity moves through the ectoderm during gastrulation resulting in the later development of the cement gland (Sive et al., 1989). Our results suggesting a role for *Xotx2* in cement gland induction are consistent with this view and further suggest that the pattern of ectodermal *Xotx2* expression is an early manifestation of the inductive wave described by Sive and coworkers. Expression of the cement gland markers (Sive et al., 1989) and *Xotx2* RNA during gastrulation (our present finding) correlates well with the model for neural induction proposed by Eyal-Giladi (1954; see Fig. 1). This model, a modification of the activation-transformation model of Nieuwkoop et al. (1952), proposes that ectoderm is first activated to an anterior neural state by underlying mesoderm early in gastrulation. Following activation of the overlying ectoderm, the ectoderm undergoes a transformation in response to signals produced by subsequent contact with more posterior mesoderm slightly later in gastrulation. The observed pattern of expression of the anterior brain marker *Xotx2* during gastrulation (and the inductive wave described by Sive and coworkers) may reflect a response to the early activation event proposed by Nieuwkoop (1952) and Eyal-Giladi (1954).

Ectodermal *Xotx2* induction is mediated by both vertical and planar signals

Domains of ectodermal and mesodermal expression of *Xotx2* are in register throughout gastrulation, suggesting that the underlying mesoderm mediates activation of *Xotx2* expression in the ectoderm through vertical signalling as suggested by the model of Eyal-Giladi (1954). Vertical induction of *Xotx2* in the ectoderm was examined by constructing mesoderm-ectoderm conjugates in which lineage-labelled anterior mesoderm from an early gastrula was wrapped in unlabelled naive animal cap ectoderm, also from an early gastrula. *Xotx2* expression was strongly induced in the ectoderm of these conjugates, demonstrating that vertical signalling can induce ectodermally-expressed *Xotx2* during gastrulation.

Induction of numerous neural-specific markers has been demonstrated in the absence of underlying mesoderm in Keller sandwich explants (Doniach et al., 1992; Papalopulu and Kintner, 1993). Under these conditions the mesoderm and ectoderm are in planar contact as attempts by the mesoderm to underlie the ectoderm are mechanically prohibited. One of these markers, a *Xenopus distal-less* homolog expressed in the forebrain, is expressed in Keller sandwiches, indicating that planar signals are capable of patterning expression of anterior markers (Papalopulu and Kintner, 1993). We addressed whether *Xotx2* may also be induced by planar signalling for three reasons. Firstly, it is not yet known whether planar signals alone can induce all anterior marker genes. Secondly, the in-register expression pattern of *Xotx2* during gastrulation suggested that *Xotx2* induction may be mediated by vertical signals in vivo. Thirdly, it has recently been demonstrated that vertical signalling occurs as early as stage 10+ (A. Posnansky and R. Keller, personal communication), suggesting that vertical signalling may have influenced the results of previous studies that concluded a role for planar induction in the A-P patterning of neurectoderm in Keller sandwiches. Therefore, we constructed Keller sandwiches from gastrulae in which mesoderm had not yet involuted as well as later stage gastrulae with varying extents of mesodermal involution. However, all of the Keller sandwiches expressed *Xotx2* strongly in the ectoderm even in the absence of any involuting mesoderm, demonstrating that planar signals may also contribute to the expression of *Xotx2* in the anterior ectoderm.

Although molecules responsible for planar signalling are largely unknown, *noggin* has been suggested to play a role in patterning anterior neurectoderm (Lamb et al., 1993). The fact that *Xotx2* is induced by *noggin* in isolated animal cap

ectoderm, and that *Xotx2* is expressed in the ectoderm of Keller sandwiches, suggest that *noggin* may act as a planar signal to induce *Xotx2*. *noggin* is capable of inducing various anterior neural marker genes as well as cement glands, the most anterior ectodermal structures specified by neural inducing signals (Lamb et al., 1993). Since the cement gland anlage lies at the anterior boundary between the skin and the neural plate and the domain of *Xotx2* expression overlaps this boundary, it is noteworthy that ectopic expression of *Xotx2* in ectoderm induces cement gland differentiation in the skin and induces the cement gland marker *XAG1* in isolated animal cap ectoderm. These results suggest that differentiation of the cement gland at the anterior neural plate border may require overlapping fields of information (Drysdale and Elinson, 1993) specifying both skin and expression of *Xotx2*.

Conservation between mouse and *Xenopus orthodenticle 2* genes

Development of the head, and the rules governing its formation appears to be quite distinct from those of trunk. While the formation of trunk structures is dependent on a combination of HOX gene expression (HOX codes; Hunt and Krumlauf, 1992), head patterning appears to be regulated by other classes of non-clustered homeobox genes such as *orthodenticle* (Finkelstein and Perrimon, 1990; Cohen and Jurgens, 1991). Conservation of *orthodenticle* homeobox sequences among disparate species such as *Drosophila*, *Xenopus*, and mouse and their common localized expression patterns in anterior head regions suggest that the underlying molecular mechanisms of head specification may also have been conserved throughout evolution.

The expression patterns of the *Xenopus Xotx2* and mouse *otx2* genes are quite similar. Both genes are expressed in anterior neurectoderm by the end of gastrulation and later expressed in presumptive fore- and mid-brain. Despite these similarities between *Xenopus* and mouse *otx2* expression patterns, some differences do exist. For example, in mouse, *otx2* transcripts are expressed abundantly in the epiblast of prestreak embryos and *otx2* expression persists in the entire embryonic ectoderm after the onset of gastrulation. However in *Xenopus*, *Xotx2* transcripts in ectoderm, prior to gastrulation, have not been detected by whole-mount in situ hybridization. A difference is also found in their mesodermal expression patterns. Although the expression of *Xotx2* is strongly detected in the organizer mesoderm of gastrulating *Xenopus* embryos, the expression of mouse *otx2* in anterior primitive streak (node), the organizer equivalent in the mouse, was not reported (Simeone et al., 1993). Whether the apparent lack of mouse *otx2* expression in mesoderm is due to insufficiently sensitive methods to detect the transcripts or represents a situation unique to the mouse system has yet to be determined. Despite some dissimilarities in the expression of *Xenopus* and mouse *otx2*, we conclude that *Xotx2* is most closely related to mouse *otx2* due to similarities in their amino acid sequences and overall patterns of expression. Since *Xotx2* is the earliest anterior neural gene currently available in *Xenopus* and this gene appears to be well conserved among vertebrates, further characterization of the function and regulation of *Xotx2* is likely to provide insights into the mechanisms of vertebrate neural induction.

We are grateful to Dr David Finkelstein for providing the cDNA encoding *Drosophila orthodenticle.* We thank Drs N. Papalopulu, and R. Harland for providing us with the *Xenopus Krox20* and *engrailed-2* probes and *noggin* cDNA, respectively. We thank Dr H. Sive for advice on PCR primers for *XAG1* and Dr B. Blumberg for primers to histone H4. We thank Drs C. Hashimoto, K. Inoue, A. Poznansky, D. Gardner and T. Doniach for helpful discussions on whole-mount in situ hybridization. We thank Drs C. Kintner, T. Doniach, A. Posnansky, R. Keller for technical advice on preparation of Keller sandwiches and for sharing data prior to publication and Richard Harland for sharing his *otxA* results prior to publication. We gratefully acknowledge the assistance of Sam Kim with sequencing portions of the *Xotx2* cDNA. We also acknowledge M. Artinger, D. Bittner and M. Selleck for critical readings of the manuscript. This work was supported by NIH grant HD29507-02, Basil O'Connor Starter Research Award No.5-FY93-0795, and grant JFRA-431 from the American Cancer Society to K. W. Y. C. I. L. B. was supported by a NIH training grant.

Note added in proof

While this manuscript was in review, further analyses of the mouse and zebrafish *otx2* expression patterns were published (Ang, S.-L., Conlon, R. A., Jin, O. and Rossant, J. (1994). *Development* **120**, 2979-2989; Li, Y., Allende, M. L., Finkelstein, R. and Weinberg, E. S. (1994). *Mech. Dev.* **48**, 229-244.) In agreement with our results in the frog.

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