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Cloning a novel developmental regulating gene, *Xotx5*: Its potential role in anterior formation in *Xenopus laevis*

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The vertebrate *Otx* gene family is related to *otd*, a gene contributing to head development in *Drosophila*. In *Xenopus*, *Xotx1, Xotx2*, and *Xotx4* have already been isolated and analyzed. Here the cloning, developmental expression and functions of the additional *Otx Xenopus* gene, *Xotx5* are reported. This latter gene shows a greater degree of homology to *Xotx2* than *Xotx1* and *Xotx4*. *Xotx5* was initially expressed in Spemann's organizer and later in the anterior region. Ectopic expression of *Xotx5* had similar effects to other *Xotx* genes in impairing trunk and tail development, and especially similar effects to *Xotx2* in causing secondary cement glands. Taken together, these findings suggest that *Xotx5* stimulates the formation of the anterior regions and represses the formation of posterior structures similar to *Xotx2*.

Key words: head inducer, homeobox, *orthodenticle*, *Xenopus laevis*, *Xotx2*.

Introduction

Two major and distinct classes of homeobox-containing genes have been shown to exert key developmental functions throughout the Metazoa: the HOX genes, which are related to the *Drosophila antennapedia* gene, and those related to the *Drosophila* gene *paired*. A new family of *paired*-class homeobox genes related to the *Drosophila orthodenticle* gene has been described (reviewed in Finkelstein & Boncinelli 1994; Boncinelli & Mallamaci 1995). As for the *Drosophila* gene, the expression pattern of this gene family is strictly related to the development of anterior head structures.

Among the family members, the most conserved genes in vertebrates are *Otx1* and *Otx2* (Simeone *et al.* 1993; Bally-Cuif & Wassef. 1994; Li *et al.* 1994; Pannese *et al.* 1995; Kablar *et al.* 1996). Mouse knock-out experiments have recently confirmed the hypothesis that these genes are possibly involved in anterior brain patterning as *Otx2*$^{-/-}$ mice lack forebrain and midbrain regions (Acampora *et al.* 1995; Matsuo *et al.* 1995; Ang *et al.* 1996), while *Otx1*$^{-/-}$ mice show several brain abnormalities (Acampora *et al.* 1996; Suda *et al.* 1996). After two distinct genes (*Otx1* and *Otx2*) were first characterized in the mouse, a few homologs have been described in zebrafish (*Zotx1*, *Zotx2*, and *Zotx3*; Li *et al.* 1994; Mori *et al.* 1994), in chicken (*Cotx2*; Bally-Cuif *et al.* 1995) and in *Xenopus* (*Xotx1*, *Xotx2*, and *Xotx4*; Pannese *et al.* 1995; Kablar *et al.* 1996). These findings suggest that the occurrence *Otx* genes may not have been exhausted yet. Both *Xenopus* and zebrafish have had three types of *orthodenticle*-like genes isolated. Sequencing analyses have shown that *Xotx1* is similar to *Xotx4*, *Zotx1* and *Zotx3*, while *Xotx2* is similar to *Zotx2*. Here the cloning of the *Xenopus* fourth *orthodenticle*-like gene is reported, and named *Xotx5*.

Activin A has very potent mesoderm-inducing activity on *Xenopus* presumptive ectoderm called animal cap and induces various mesodermal tissues in a dose-dependent manner (Asashima *et al.* 1990; Ariizumi *et al.* 1991a,b). In addition, cells dissociated from animal caps have several prospective cell fates dependent on distinct activin A concentration thresholds (Green *et al.* 1994); for example, a concentration of 1 ng/mL activin A induced only notochord development (Kuroda *et al.* 1999). In the present study, *Xotx5* was cloned using reaggregates made from 1 ng/mL treated animal cap dissociated cells and the degenerated oligonucleotide reverse transcription–polymerase chain reaction (RT-PCR) method.

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Materials and Methods

Embryos and activin A

Xenopus laevis eggs were obtained from females by injecting them with 400 units of human chorionic gonadotropin (Gestron; Denka Seiyaku Co., Kawasaki, Japan), fertilized *in vitro* with minced testis, and cultured in 1× Steinberg's solution (58 mM sodium chloride, 0.67 mM potassium chloride, 0.34 mM calcium nitrate, 0.83 mM magnesium sulfate, 100 mg/L kanamycin sulfate and 4.6 mM timza base; pH 7.4). Staging was according to Nieuwkoop and Faber (1967). The jelly coat was removed with 3% cysteine hydrochloride in Steinberg's solution (pH 7.8), and the vitelline membrane was manually removed with fine forceps. All operations were carried out under sterile conditions. Human recombinant activin A was dissolved at concentrations of 0.05–100 ng/mL in 1× Steinberg's solution containing 0.1% bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO, USA).

Cell dissociation and reaggregation

The cell dissociation and reaggregation method was performed as described by Kuroda *et al.* (1999).

Cloning

Total ribonucleic acid (RNA) was isolated from five reaggregated cells treated with 1 ng/mL of activin A. Full-length cDNA was synthesized using a Clontech Smart PCR cDNA synthesis kit (Clontech, CA, USA). Using SMART II oligonucleotide 5′-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3′ and degenerate oligonucleotide primer 5′-CKNCKRTTYTKRAACCANAC-3′ corresponding to a conserved homeodomain region N-VWF(Q/K)NRR-C of homeobox genes, cDNA was PCR amplified. Amplified fragments were subcloned in pGEM-T EASY Vector (Promega, Madison, WI, USA).

Library screening

Using *Xotx5* primers 5′-CGATTAGTTTAGTGGAGTGG-3′ and 5′-GAGGCTGTTTGATGTAGGAC-3′, approximately 2×10^7 plaque-forming units (p.f.u.) of the phargemid library prepared from stage 12 *Xenopus* embryos were screened using PCR.

Whole-mount in situ *hybridization*

Whole-mount *in situ* hybridization was performed on staged alvino embryos as described by Harland (1991). We used *Xotx2* plasmid T7TSMC19/1 (Pannese *et al.* 1995).

RT-PCR

The oligos used for PCR amplification were as follows: *Xotx5* primers 5′-GTGGCTGTGTTCATTCTTTT-3′ and 5′-GCTCCTCCATCTTGTTGTTG-3′; *Xotx2* primers 5′-GGATGGATTTGTTGCACCAGTC-3′ and 5′-CACTCTCCGAGCTCACTTCTC-3′; *XAG* primers 5′-CTGACTGTCCGATCAGAC-3′ and 5′-GAGTTGCTTCTCTGGCAT-3′; *XCG* primers 5′-GGTGATGTTACTTCCCCAGAGCAG-3′ and 5′-GGGAAGTAACATCAAACAAAGCAACCA-3′; *EF1*α primers 5′-GACAAGAGAACCATCGAGAA-3′ and 5′-GTGCCGGTGATCATGTTCTT-3′. The annealing temperatures were 58°C (*Xotx5* and *Xotx2*) and 60°C (*XAG*, *XCG*, and *EF1*α), and 23 cycles (EF1α), 25 cycles (*Xotx5*, *Xotx2*, *XAG*, and *XCG*) were performed. The RT-PCR products were resolved on a 2% SeaKem GTG agarose gel (Takara, Kyoto, Japan).

Histological examination

Reaggregates were fixed with Bouin's fluid for 3 h, dehydrated with ethanol, embedded in paraffin wax, sectioned at 6 μm and stained with hematoxylin and eosin (HE).

Embryo microinjections

Capped synthetic RNA was generated using *in vitro* transcription of full coding sequences of *Xotx5* and *Xotx2*. We used *Xotx2* plasmid T7TSMC19/1 (Pannese *et al.* 1995). All RNA was resuspended in 88 mM NaCl, 5 mM Tris pH 7.5 and injected into a single cell of 1-cell embryos or two blastomeres of 8-cell embryos. Injected amounts ranged between 0.2 and 1.6 ng. This quantity is not toxic (Rebagliati & Melton 1987). Injected embryos were allowed to develop until uninjected control embryos reached stage 9 for cutting animal caps or stages 36–38 for observing mRNA overexpression embryos.

Results

Cloning and sequencing

Using the degenerate PCR method, four novel homeobox genes were amplified (Table 1). One of the four PCR products of the novel homeobox genes was 60% similar to *Xotx2* DNA sequencing and was named *Xotx5*. A cDNA library prepared from stage 12 *X. laevis* embryos was screened using two primers corresponding to *Xotx5*, and the *Xotx5* full-length clone (2.7 kb) was purified. Figure 1(A) shows the coding region and the deduced peptide sequence of *Xotx5*. Overall, homeodomain, 5′-domain, or 3′-domain sequence similarity between *Xotx5* and the other *Xotx*

Table 1. *Xenopus* homeobox gene isolated using degenerate polymerase chain reaction

Genes	Number of isolates
Previously known genes	
Xlim1	8
goosecoid	7
Mix.1	6
Mixer	4
Hbox10	3
milk	3
Xotx2	3
Xhox3	2
Mix.2	2
Xlim3	2
Xnot	2
Xotx1	2
Xnot2	1
Novel genes	
Xotx5	12
Rat *DRG11*-like gene	1
Zebrafish *Arx*-like gene	1
Newt *Hoxc-10*-like gene	1
Total	135

genes was less than 75, 98, 76, or 68% at the peptide level (Fig. 1B). *Xenopus laevis* is a tetraploid animal. Although *Xotx2* was the most similar gene to *Xotx5*, a similarity of 68% in the 3′-domain was too low to regard *Xotx5* as an *Xotx2* duplicated gene.

Xotx5 *expression in normal embryos*

Reverse transcription–polymerase chain reaction analysis (Fig. 2) detected the presence of low abundance transcripts from stage 1 to approximately stage 9, when the abundance of *Xotx5* transcripts increased. Using whole-mount *in situ* hybridization, *Xotx5* transcripts were confined to dorsal internal regions of the marginal zone at stage 11 (Fig. 3B). As part of this region differentiates into notochord in the future, it might have been possible to isolate *Xotx5*. At this stage *Xotx2* and *Xotx5* expression domains were similar but not identical. Although *Xotx2* transcripts were present in the prechordal plate, bottle cells and presumptive rostral brain (Panesse *et al.* 1995; Fig. 3A), *Xotx2* was slightly localized at the lateral side (Fig. 3A), but *Xotx5* was more localized at the center of the *Xotx2* expressed regions (Fig. 3B). *Xotx5* expression was confined to the cement gland-forming region (Fig. 3C,D) at stages 19–25, and the forebrain, optic vesicle and otocyst at stage 28 (Fig. 3E).

Xotx5 *overexpression phenotype*

Synthetic *Xotx5* mRNA was microinjected into the animal hemisphere of fertilized eggs and two

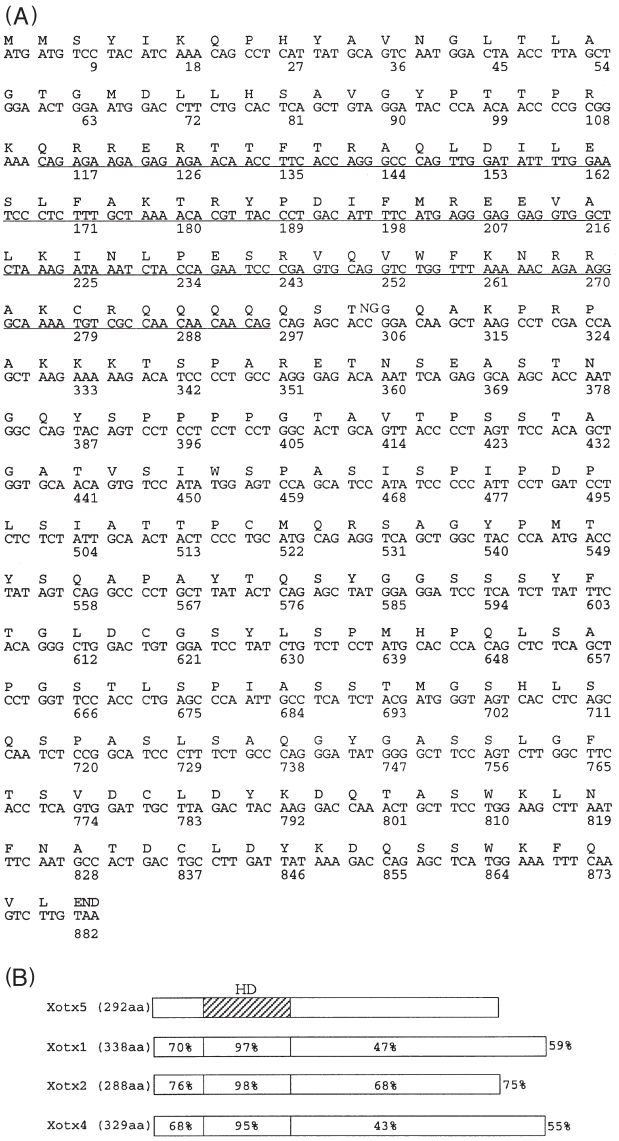

Fig. 1. (A) Sequence of the *Xenopus Xotx5* coding region. The deduced peptide sequence of the *Xotx2* gene product is also shown using the one-letter amino acid code. The homeodomain is underlined. (B) Peptide sequence similarity between *Xotx5* and the other *Xotx*s. HD, homeodomain; aa, amino acids.

Fig. 2. Temporal expression pattern of *Xotx5*. Reverse transcription–polymerase chain reaction (RT-PCR) analysis was performed using early *Xenopus* embryos. The number indicates the developmental stage of the embryos. *Xotx5* expressed maternally, increased at the early gastrula stage (stage 10), and decreased at stage 45. The *EF1* α is the internal positive control and RT− is the negative control.

blastomeres of 8-cell stage embryos to examine the function of *Xotx5*. Embryos injected with *Xotx5* between 400 pg and 1.6 ng into the animal hemisphere of fertilized eggs display posterior deficiencies, shortened tails and additional cement glands (Fig. 4B,D,E). Larger ectopic cement gland sections revealed the presence of columnarized cells containing pigment granules (compare Fig. 4, panels D,E). The frequency of this phenotype increased with increasing amounts of injected *Xotx5* (Table 2). When embryos were injected with 800 pg of *Xotx5* at the two-blastomere stage of 8-cell embryos, only embryos injected at the ventral animal blastomeres displayed additional cement glands (Table 2). When *Xotx5* was injected into the vegetal ventral blastomeres, a different class of phenotype exhibited secondary axes (Fig. 4C; Table 2). No embryo injected at the dorsal blastomeres displayed abnormal phenotypes (Table 2). As these findings resemble the *Xotx2* overexpression phenotype (Table 2), *Xotx5* may be a coworker of *Xotx2*.

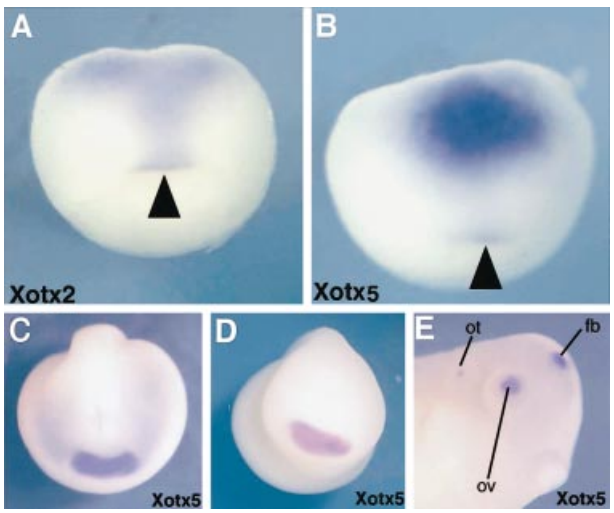

Fig. 3. Localized *Xotx5* expression. (A) Whole-mount *in situ* hybridization, dorsal view at stage 11. An arrowhead indicates blastopore. (B) Dorsal view at stage 11. (C) Anterior view at stage 19. (D) Anterior view at stage 25. (E) Head region view at stage 28. fb, forebrain; ov, optic vesicle; ot, otocyst.

To examine whether *Xotx5* acts as an activator or repressor, the carboxy-terminal domains following *Xotx5* homeodomains were replaced either by the VP16 activator domain (Friedman *et al.* 1988) or the engrailed repressor domain (Han & Manley 1993). Embryos injected with the fusion constructs did not display strange phenotypes (data not shown).

Upstream and downstream of Xotx5

Xotx2 is able to activate the cement gland markers *XCG* and *XAG* (Gammill & Sive 1997). Therefore, examination of whether *Xotx5* also induced cement gland formation in isolated ectoderm was also undertaken.

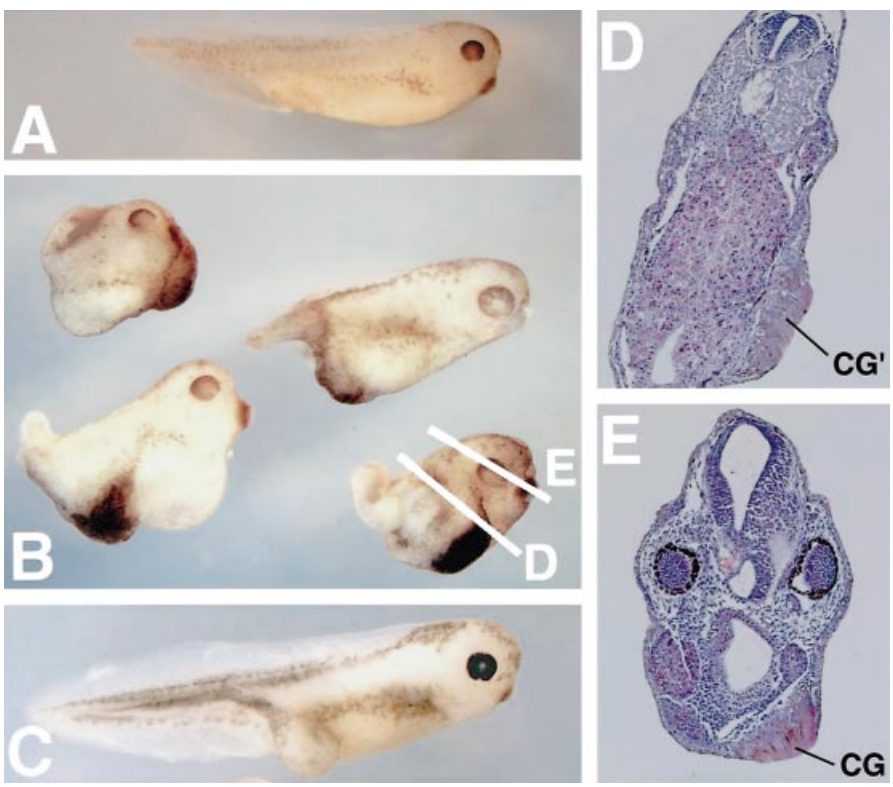

Fig. 4. Phenotypes of embryos injected with *Xotx5*. (A) Normal embryo at stage 36. (B) The embryos shown were injected with 1.6 ng of *Xotx5* at the animal pole of 1-cell embryos. White lines indicate section places. (C) The embryo injected with 0.8 ng of *Xotx5* at two ventral vegetal blastomeres at the 8-cell stage had partial additional axis formation. (D) Transverse section through the trunk of an *Xotx5*-injected embryo. (E) Transverse section through the head of an *Xotx5*-injected embryo. Note that the normal cement gland shows characteristic columnar cells with dark pigmentation. CG, normal cement gland; CG', additional cement gland.

Table 2. External phenotypes of injected embryos

RNA injection (ng)	*n*	Stage	Secondary axis (%)	Secondary cement gland (%)	Posterior defects (%)	Short tail (%)	Minor defects (%)	Normal embryos (%)
Xotx5								
1.6	40	1C ani.	0	17.5	40	32.5	17.5	0
0.8	43	1C ani.	0	48.3	34.9	14	11.6	23.3
0.4	48	1C ani.	0	2.1	18.7	6.3	8.3	66.7
1.6	48	1C veg.	0	0	37.5	6.3	8.3	45.8
0.8	23	4C dor.	0	8.7	13	13	17.4	39.1
0.8	30	4C ven.	0	0	50	26.7	13.3	6.7
0.8	22	8C dor.-ani.	0	0	0	0	0	100
0.8	24	8C dor.-veg.	0	0	0	4.2	0	95.8
0.8	24	8C ven.-ani.	0	66.7	8.3	0	16.7	12.5
0.8	24	8C ven.-veg.	20.8	0	29.2	0	8.3	45.8
Xotx2								
1.6	22	1C ani.	0	63.6	90.1	0	0	4.5
0.8	21	1C ani.	0	42.9	57.1	28.6	4.8	0
0.4	24	1C ani.	0	12.5	29.2	16.7	0	50
0.2	24	1C ani.	0	8.3	4.2	0	0	88
0.8	23	8C ven.-veg.	0	0	26.1	21.7	0	56.4
Water	50	1C ani.	0	0	0	0	2	98

RNA, ribonucleic acid; ani., animal region; veg., vegetal region; dor., dorsal region; ven., ventral region.

Animal caps were removed from *Xotx5*-injected embryos which were incubated until sibling embryos reached at stage 20 or 30. Inductions of *XCG* and *XAG* were detected in *Xotx5*-injected and incubated animal caps until sibling embryos reached at stage 20 (Fig. 5A), and cement gland formation was detected in *Xotx5*-injected and incubated animal caps until sibling embryos reached stage 30 (Fig. 5B). These findings suggested *Xotx5* also induced *XCG* and *XAG* expression and cement gland formation similar to *Xotx2*.

As *Xotx5* was cloned using reaggregates made from activin A-treated animal cap dissociated cells and the degenerated oligonucleotide RT-PCR method, we examined whether activin A induced *Xotx5* expression. Animal caps treated or untreated with activin A were incubated until sibling embryos reached stage 12. Although induction of *Xotx5* was detected in both animal caps, *Xotx5* expression in animal caps treated with 50 or 100 ng/mL of activin A was higher than the others (Fig. 5C). In reaggregates made from animal cap dissociated cells, induction of *Xotx5* was detected in reaggregates treated with 1 ng/mL of activin A. These findings suggested activin A induces *Xotx5* expression.

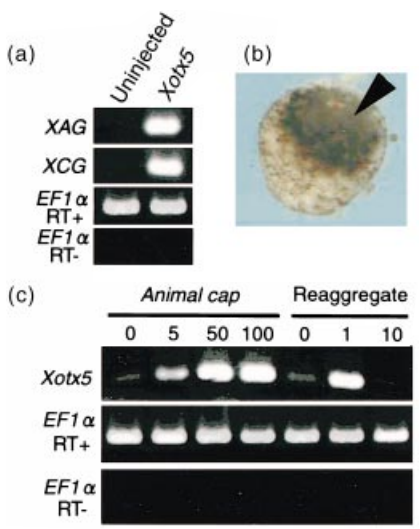

Fig. 5. Reverse transcription–polymerase chain reaction (RT-PCR) analysis of *Xotx5* upstream and downstream. (A) *Xotx5* induced cement gland marker expression, *XAG* and *XCG*, in isolated animal caps. Animal caps isolated from *Xotx5*-injected embryos, and developed until sibling embryos reached stage 20. (B) *Xotx5* induced large cement glands (arrowhead) in animal caps. Animal caps isolated from *Xotx5*-injected embryos developed until sibling embryos reached stage 30. (C) Activin A induced *Xotx5* expression in animal caps and reaggregates. Animal caps treated with 5–100 ng/mL of activin A or untreated developed until sibling embryos reached stage 12.

Discussion

Xotx5, a novel *Xenopus* homeobox gene of the bicoid class homologous to *Otx2* was examined. The *Xotx5* peptide sequence was most similar to *Xotx2*. Its functions resembled *Xotx2*. Its expression patterns at the early embryo stages were a little different from *Xotx2*.

Fourth otx *family gene in* X. laevis

Otx1 and *Otx2* (Simeone *et al.* 1993; Bally-Cuif & Wassef. 1994; Li *et al.* 1994; Pannese *et al.* 1995;

Kablar *et al.* 1996) were first isolated as *orthodenticle* family genes in vertebrates. After two distinct genes (*Otx1* and *Otx2*) were first characterized in the mouse, a few homologs have been described in zebrafish (*Zotx1*, *Zotx2*, and *Zotx3*; Li *et al.* 1994; Mori *et al.* 1994), in chicken (*Cotx2*; Bally-Cuif *et al.* 1995) and in *Xenopus* (*Xotx1*, *Xotx2*, and *Xotx4*; Pannese *et al.* 1995; Kablar *et al.* 1996). Reported here is the cloning of the *Xenopus* fourth *orthodenticle*-like gene, which was named *Xotx5*. In addition, *Otx* family genes have also been described in *Branchiostoma* (*BfOtx*, GenBank 2828715), in *Lampetra* (*LjOTXA,* GenBank 3650205; *LjOtxB*, GenBank 3650207), and in *Petromyzon* (*PmOtx*, GenBank 3941721). Among the several *Otx* family genes that have been reported, the relationship between known *otx* family 3′-domain peptide sequences was clarified (Figs 1,6). The *Otx* family peptide has the following domains: 5′-domain, homeodomain and 3′-domain. As the 3′-domain has much more variety than the 5′-domain and homeodomain, the 3′-domain was regarded as the functional domain. The chordate *Otx* family is divided into two types: 3′-short forms or 3′-long forms (Fig. 6). *Otx2*, *Zotx2*, *Xotx2*, *Xotx5*, Lj*Otx*A, Lj*Otx*B and Pm*Otx* belong to the 3′-short forms, while *Otx1*, *Zotx1*, *Zotx3*, *Xotx1*, *Xotx*4 and *BfOtx* belong to the 3′-long forms. There are two reports about the difference between short and long forms. Mouse knock-out experiments have shown that *Otx2*$^{-/-}$ mice lack forebrain and midbrain regions (Acampora *et al.* 1995; Matsuo *et al.* 1995; Ang *et al.* 1996), while *Otx1*$^{-/-}$ mice show several brain abnormalities (Acampora *et al.* 1996). In *Xenopus*, *Xotx1* overexpression inhibits tail organizer activity, while *Xotx*2 overexpression is able to turn a tail organizer into a head organizer (Andreazzoli *et al.* 1997). In the present study, *Xenopus* showed not only two types of the 3′-long forms isolated from the *otx* family (*Xotx1* and *Xotx4*), but also two types of the 3′-short forms isolated from the *otx* family (*Xotx2* and *Xotx5*). In future studies, if additional *Otx* family genes are isolated, this novel gene should be divided on the point of the 3′-domain sequence of *Xenopus otx* family genes.

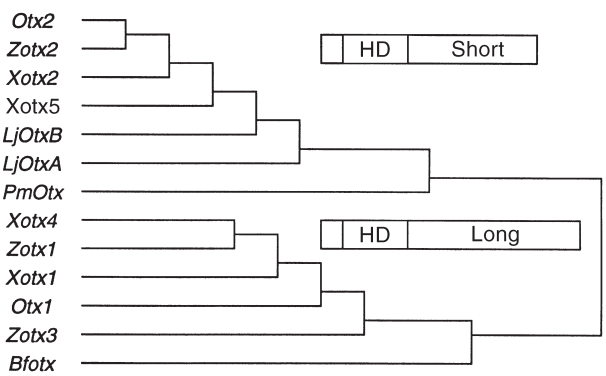

Fig. 6. Relationships between 13 chordate *otx* family genes. The tree is inferred from the 3′-domain peptide sequence. Bf, *Branchiostoma floridae*; Lj, *Lampetra japonica*; Pm, *Petromyzon marinus*.

Potential role of Xotx5 *in anterior formation in* X. laevis

Xotx5 expression was analyzed in normal *Xenopus* embryos. Reverse transcription–polymerase chain reaction analysis (Fig. 2) showed that maternal transcripts of this gene are already detectable in unfertilized eggs. Directly after the midblastula transition, when zygotic transcription starts, *Xotx5* expression increases. Whole-mount *in situ* hybridization experiments revealed early localized expression in the dorsal internal regions of the marginal zone at stage 11 (Fig. 3B). At stages 19 (Fig. 3C) and 25 (Fig. 3D) the major expression site of *Xotx5* was the cement gland. At stage 28 (Fig. 3E) the expression domains were the forebrain, optic vesicle and otocyst. These expression patterns imply that *Xotx5* has a role in anterior formation. Indeed microinjection experiments lend further support to the hypothesis of the role of *Xotx5* in patterning the anterior–posterior axis of the embryo. A major class of shortened embryos with reduced trunk–tail structures and additional cement gland was observed (Fig. 4B). *Xotx2* can activate *XCG* and *XAG* to form the cement gland (Gammill & Sive 1997). In the present study *Xotx5* was also able to activate *XAG* and *XCG* (Fig. 5A). These findings show *Xotx5* could, similar to *Xotx2*, inhibit posterior formation at the early gastrula stage and induce the cement gland at the late gastrula to neurula stage. *Xotx5* was probably also involved in forebain, optic vesicle and otocyst formation at the tail-bud stage.

Moreover, 20.8% of *Xotx5*-injected embryos at the vegetal ventral blastomeres exhibited secondary axes phenotypes (Fig. 4C; Table 2). Although these secondary axes of *Xotx5*-injected embryos were not as complete as *Xwnt8*-injected embryos (Steinbeisser *et al.* 1993), *Xotx5* was able to induce partial secondary axes. *Xotx1* and *Xotx2* were also able to induce partial secondary axes (Table 2; Andreazzoli *et al.* 1997), but the score was lower than *Xotx5*. Thus, *Xotx5*′s ability to induce secondary axes may be more intense than the other *otx* family genes.

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