The Xenopus homologue of Otx2 is a maternal homeobox gene that demarcates and specifies anterior body regions

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INTRODUCTION

We previously reported the cloning and characterization of Otx2 (Simeone et al., 1992, 1993; Boncinelli et al., 1993; Finkelstein and Boncinelli, 1994), a mouse homeobox gene related to orthodenticle (Finkelstein and Perrimon, 1990; Finkelstein et al., 1990), a gene expressed in anterior regions of the developing head of Drosophila. The murine cognate, Otx2, is first expressed in the entire epiblast of prestreak embryos and later in very anterior regions of late-gastrulae, including the neuroectoderm of presumptive fore- and mid-brain. In Xenopus, RNase protection experiments reveal that Xotx2 is expressed at low levels throughout early development from unfertilized egg to late blastula, when its expression level significantly increases. Whole-mount in situ hybridization shows a localized expression in the dorsal region of the marginal zone at stage 9.5. At stage 10.25 Xotx2 is expressed in dorsal bottle cells and in cells of the dorsal deep zone fated to give rise to prechordal mesendoderm, suggesting a role in the specification of very anterior structures. In stage 10.5 gastrulae, Xotx2 transcripts start to be detectable also in presumptive anterior neuroectoderm, where they persist in subsequent stages. Various treatments of early embryos cause a general reorganization of Xotx2 expression. In particular, retinoic acid treatment essentially abolishes Xotx2 expression in neuroectoderm. Microinjection of Xotx2 mRNA in 1-, 2- and 4-cell stage embryos causes the appearance of secondary cement glands and partial secondary axes in embryos with reduced trunk and tail structures. The presence of the Xotx2 homeodomain is required to produce these effects. In particular, this homeodomain contains a specific lysine residue at position 9 of the recognition helix. Microinjected transcripts of Xotx2 constructs containing a homeodomain where this lysine is substituted by a glutamine or a glutamic acid residue fail to cause these effects.

SUMMARY

In this paper we study Xotx2, a Xenopus homeobox gene related to orthodenticle. The murine cognate, Otx2, is first expressed in the entire epiblast of prestreak embryos and later in very anterior regions of late-gastrulae, including the neuroectoderm of presumptive fore- and mid-brain. In Xenopus, RNase protection experiments reveal that Xotx2 is expressed at low levels throughout early development from unfertilized egg to late blastula, when its expression level significantly increases. Whole-mount in situ hybridization shows a localized expression in the dorsal region of the marginal zone at stage 9.5. At stage 10.25 Xotx2 is expressed in dorsal bottle cells and in cells of the dorsal deep zone fated to give rise to prechordal mesendoderm, suggesting a role in the specification of very anterior structures. In stage 10.5 gastrulae, Xotx2 transcripts start to be detectable also in presumptive anterior neuroectoderm, where they persist in subsequent stages. Various treatments of early embryos cause a general reorganization of Xotx2 expression. In particular, retinoic acid treatment essentially abolishes Xotx2 expression in neuroectoderm. Microinjection of Xotx2 mRNA in 1-, 2- and 4-cell stage embryos causes the appearance of secondary cement glands and partial secondary axes in embryos with reduced trunk and tail structures. The presence of the Xotx2 homeodomain is required to produce these effects. In particular, this homeodomain contains a specific lysine residue at position 9 of the recognition helix. Microinjected transcripts of Xotx2 constructs containing a homeodomain where this lysine is substituted by a glutamine or a glutamic acid residue fail to cause these effects.

Key words: Xenopus laevis, homeobox, head, anterior regions, Otx2

MATERIALS AND METHODS

Library screening
Approximately 2×10⁶ pfu of a λgt11 cDNA library prepared from stage 24-25 Xenopus embryos were screened at low stringency by
hybridization with a murine Otx2 probe. Purified phage DNA was subcloned in pGEM3 (Promega Biotech) and sequenced on both strands using a Sequeqene sequencing kit (US Biochemical) according to the supplier’s instructions.

Embryos
Fertilization and embryo culture were performed as described by Newport and Kirschner (1982). Staging was according to Nieuwkoop and Faber (1967).

Whole-mount in situ hybridization, histology and immunostaining
Whole-mount in situ hybridization was performed on staged albino embryos as described by Harland (1991). For histological examination stained embryos were fixed in MEMFA, embedded in Paraplast, cut into 10 μm sections, dried onto slides, dewaxed in Histoclear and mounted in DePex.

Injected embryos, harvested for histological analysis, were fixed, cut and sections were stained with orange G and anilin blue. Some of the controls and Xotx2-injected embryos were fixed in 2% TCA and whole-mount immunostained with a neural specific antibody, 2G9 (Jones and Woodland, 1989) which was visualized with HRP-conjugated secondary antibody according to the procedure of Dent et al. (1989).

RNase protection
The following antisense RNA probes were synthesized using T7 or SP6 polymerase: Xotx2 (220 bp Alll fragment from 3’ untranslated region), rpS8 (90 bp Ddel fragment), Xho3 (210 bp EcoRI-PstI fragment), goosecoid (248 bp PstI fragment), cardiac a1 actin (328 bp Rsal fragment) XHbox-6 (227 bp HindIII-Smal fragment), Xho2.7 (250 bp BglII fragment), N-CAM (700 bp EcoRI-PstI fragment) and Xwnt-8 (260 bp PstI-HindIII fragment). RNase digestion and electrophoresis were carried out as described (Simeone et al., 1993).

Embryo microinjections
Capped synthetic RNAs were generated by in vitro transcription of a full coding sequences of Xotx2 and mouse Otx2, or control constructs. In vitro translation of the synthetic Xotx2 RNA using Promega rabbit reticulocyte lysate kit, yielded a protein of approximately 33×10^3 M_2 in agreement with its predicted size (data not shown). All RNAs were resuspended in 88 mM NaCl, 5 mM Tris pH 7.5 and injected into animal pole region of a single cell of 1-, 2- and 4-cell embryos using a Drummond ‘Nanoject’ apparatus. Injected amounts ranged between 0.3 M LiCl was applied for 10 minutes to the 32-cell embryos. For UV treatment the embryos were supported on a quartz slide and irradiated for 6 minutes with a short wave UV lamp beginning 30 minutes after fertilization. The dorso-anterior index (DAI) ranged between 0 and 4 with an average of 0.5 in UV-treated embryos and between 7 and 10 in LiCl-treated embryos (Kao and Elgin, 1988).

Exogastrulae
Complete exogastrulae were obtained by incubation of embryos upside down, from which the vitelline membrane had been removed, in 1.3x MMR. At stage 24, only complete exogastrulae were used for whole-mount in situ hybridization.

RESULTS
A cDNA library prepared from stage 25 Xenopus embryos was screened at low stringency with a murine Otx2 cDNA and several clones were purified. Fig. 1 shows the coding region and the deduced peptide sequence of Xotx2 together with its comparison with the murine cognate. Overall sequence similarity between the two cognate coding regions is 95% and 82%, at the peptide and nucleotide level, respectively. A similarity of 95% at the peptide level is a very high value, to be compared, for example, to 75% exhibited by the gene products of both goosecoid (Cho et al., 1991) and Brachyury (Smith et al., 1991) in the two species. We have already reported (Simeone et al., 1993) the remarkable conservation of the Otx2 protein, that contains a single conservative (threonine for serine) amino acid substitution between human and mouse.

Xotx2 expression in normal embryos
RNase protection analysis (Fig. 2A) detects the presence of low abundance transcripts from unfertilized egg to approximately stage 10, when the abundance of Xotx2 transcripts increases by one order of magnitude. Isolated animal caps dissected from stage 8 embryos also show low expression levels of Xotx2, which are comparable to those of the entire embryo of this stage (Fig. 2B).

The presence of Xotx2 transcripts in animal caps is confirmed by whole-mount in situ hybridization of stage 8 embryos (not shown). Then, Xotx2 transcripts are progressively confined to dorsal internal regions of marginal zone where they are clearly detectable at stage 9.5 (Fig. 3A,A′,A″). At stage 10.25 (Fig. 3B,B′,B″), the major expression site of Xotx2 is in migratory deep zone cells that are fated to give rise to prechordal mesendoderm (Keller et al., 1992). In addition, it is also expressed in dorsal bottle cells. At stage 10.5, Xotx2 expression persists in these cell types and posteriorly, above the dorsal blastopore lip, this expression clearly respects the boundary between internal deep zone cells and external cell layers, represented by Brachet’s cleft (Keller et al., 1992) (arrowheads in Fig. 3C′). Conversely, in more anterior regions Xotx2 expression extends to cells of presumptive anterior neuroectoderm (Fig. 3C,C′; open arrows in Fig. 3C″), where it
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persists throughout embryogenesis (Figs 3D-F, 4 and data not shown), in keeping with what has been observed for the murine homologue. At stage 14 (Fig. 4), Xotx2 expression is confined to mesendoderm and ectoderm cells of anterior dorsal regions. Xotx2 transcripts are also detectable in a more ventral position, in keeping with the location of the evoking signal between ectoderm and endoderm. The anterior region where Xotx2 is not expressed probably corresponds to presumptive chiasmic regions (Eaglestone and Harris, 1990), in analogy with what we observed in mouse embryos (Simeone et al., 1993).

**Xotx2 expression in embryos subjected to various treatments**

Xotx2 is expressed in restricted regions of late blastula-early gastrula embryos. This expression pattern, similar to that of *goosecoid* (Cho et al., 1991), suggests a role for this gene in mediating events correlated to the Spemann’s organizer activity. To explore this point further, we analysed Xotx2 expression in experimentally treated embryos, by both whole-mount in situ hybridization (Fig. 5A) and RNAase protection (not shown). When *Xenopus* embryos are immersed in a lithium chloride solution during cleavage, a great enhancement of dorso-anterior structures occurs (Kao and Elinson, 1988), whereas irradiation of the vegetal pole of fertilized eggs with ultraviolet (UV) light results in ventralized embryos (Gerhart et al., 1989). This treatment prevents cortical rotation and the correct formation of the Nieuwkoop center (Nieuwkoop, 1973). As a consequence, formation of the organizer is not induced in UV-treated embryos. In whole-mount in situ hybridization experiments, we observed that UV treatment results in an almost total disappearance of Xotx2 expression, whereas lithium chloride treatment appears to redistribute the hybridization signal over all the forming blastopore.

Finally, treatment with 1 µM RA essentially abolished Xotx2 expression in neuroectoderm of gastrulating embryos (Fig. 5A). At stage 10.5, no expression is detectable in neuroectoderm (open arrows in Fig. 5A). Such an expression never reappears at later stages in RA-treated embryos. In fact, Fig. 5C shows that no Xotx2 expression is detectable in neuroectoderm of stage 21 embryos of this type. These embryos lack
most of the rostral brain regions, as already reported (Durston et al., 1989). Notably, we observed the same deleted regions and the corresponding lack of \textit{Xotx2} expression in embryos that had been subjected to a continuous RA treatment starting at different times between the 2-cell stage and stage 9.5. These findings confirmed and extended previous observations (Durston et al., 1989).

RNase protection analysis revealed a comparable overall decrease of \textit{Xotx2} expression in both UV- and RA-treated embryos (not shown). Thus, the main difference between the two treatments is that in RA-treated embryos the expression was still localized in the dorsal mesendoderm, whereas in UV-treated embryos no localization of \textit{Xotx2} transcripts was observed.

\textbf{Xotx2 is not expressed in ectoderm of exogastrulae}

In order to determine whether \textit{Xotx2} is expressed in the neural plate in the absence of the underlying mesoderm, we examined the expression of \textit{Xotx2} in complete exogastrulae by whole-mount in situ hybridization. In exogastrulae, the mesoderm does not involute under the ectoderm but the extension movements of the notochord and posterior neuroectoderm still occur, resulting in the elongation but a lack of apposition of these structures (Ruiz i Altaba, 1992). In complete exogastrulae examined at stage 24, \textit{Xotx2} was present in the region that contains endoderm and mesoderm and its expression was detected in areas corresponding to the prechordal mesoderm but not in the notochord (Fig. 6). In contrast, \textit{Xotx2} RNA was not detected in the ectodermal region of the exogastrulae (Fig. 6). In exogastrulae corresponding to stage 13 \textit{Xotx2} expression is similarly restricted to a mesendodermal region (not shown).

As a control we also studied \textit{Brachyury} expression in exogastrulae of this stage (Smith, et al., 1991). Its transcripts were present in mesodermal regions and absent from the ectoderm of the exogastrulae (not shown). We can conclude from these data that expression of \textit{Xotx2} by cells of the neural plate in normal embryos may therefore depend on inductive signals from the underlying mesoderm, able to establish and/or maintain it.

\textbf{Phenotypes resulting from the microinjection of synthetic Xotx2 RNA}

In order to examine the function of \textit{Xotx2} we injected synthetic \textit{Xotx2} RNA into 1-, 2- and 4-cell embryos.

We first analyzed the translational efficiency and the stability of the injected \textit{Xotx2} RNA. The amount of injected RNA ranged between 1.6 and 6 ng. By a ribonuclease protection assay we observed that after injection of 5 ng of synthetic RNA at the 1-cell stage, the amount of injected RNA remaining at stage 12.5 represented at least a 10-fold excess over the endogenous level of \textit{Xotx2} mRNA.

One of the first effects noticed in most injected embryos is a delay in the gastrulation and a failure in the blastopore to close. We essentially observed four classes of phenotypes after \textit{Xotx2} microinjection, as scored on their external morphology (Fig. 7B-E and Table 1). The best represented class (Fig. 7C), totalling an average of 40%, is comprised of embryos of reduced size showing major posterior defects. In these embryos trunk and tail failed to develop properly. In fact, the size of these structures is considerably reduced and the embryonic axis is bent dorsally. Sometimes a spina bifida is present.

An even more interesting class of phenotypes (Fig. 7D) exhibits, in addition to the posterior defects, an additional cement gland, both in anterior and posterior localizations. In these classes of phenotypes the tail completely fails to develop, being like a protrusion containing a notochord and spinal cord. The trunk is also shorter and is organized in such a way as to accommodate the presence of an additional cement gland. The most effective time of treatment to produce this phenotype was

\begin{figure}
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\caption{Expression of \textit{Xotx2}. (A) RNase protection analysis of \textit{Xotx2} expression during development from unfertilized egg (ue) to stage 25. The \textit{X. laevis} gene for ribosomal protein S8 (rp S8) (Mariottini et al., 1988) is analyzed as an internal standard. P, probes; M, marker. (B) RNase protection analysis of \textit{Xotx2} expression in stage 8 total embryos and in isolated animal caps (AC) of the same stage.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Localized \textit{Xotx2} expression. (A) Stage 9.5. Whole-mount in situ hybridization, animal pole view. (A’) Sagittal section. (A’’) Detailed view of the sagittal section. (B) Stage 10.25. Whole-mount in situ hybridization, vegetal view. (B’) Sagittal section. (B’’) Detailed view of the sagittal section. (C) Stage 10.5. Whole-mount in situ hybridization, vegetal view. (C’) Sagittal section. (C’’) Detailed view of the sagittal section. Hybridization signal is now detectable in presumptive anterior neuroectoderm. Open arrows delimit \textit{Xotx2} expression in neuroectoderm; arrowheads point to Brachet’s cleft. (D-F) Stage 12. Whole-mount in situ hybridization, posterior-dorsal (D), lateral (E) and dorsal (F) view. Embryos in C and F were not cleared. yp, yolk plug; ar, archenteron; bc, bottle cells.}
\end{figure}
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at 1-cell stage as compared to results from microinjections in 2- and 4-cell stage. This treatment also reveals a dose-dependent effect. In fact, the frequency of this phenotype increases by increasing the amount of injected Xotx2 (Table 1).

An average of 4% microinjections resulted in the presence of a partial secondary axis originating from the trunk region and extending anteriorly (Fig. 7E). Finally, embryos showing minor defects (Fig. 7B) such as a little microcephaly or a slightly bent axis were grouped together and amount to 8%. Inspection of results obtained by microinjecting 4-cell embryos (Table 1) reveals that dorsal injection slightly favours axis duplication, while ventral injection privileges formation of a secondary cement gland.

**Xotx2 requires its DNA binding specificity to cause the observed phenotypes**

In order to evaluate the specificity of the observed effects we microinjected a series of control mRNAs (Table 1). Some of these gave comparable negative results. They are: a Xotx2 antisense mRNA, a Xotx2 mRNA encoding a frame-shift mutant that is predicted to generate a mutated protein starting from the last four amino acids of the homeodomain and an in-frame deletion of Xotx2, which basically lacks the third helix of the homeodomain, ΔXotx2. All of them produced minor aspecific defects such as a little microcephaly and bent axis in an average of 25% of the cases, whereas 9% of the embryos showed the posterior defects phenotype described above. In conclusion, Xotx2 requires its DNA-binding domain to produce the observed effects in microinjected embryos.

The homeodomain of Xotx2 contains a specific lysine residue at position 9 of the recognition helix reported to confer the DNA binding specificity. The substitution of this lysine by glutamine in the bicoid homeodomain has been shown to replace its DNA binding specificity with that of the Antennapedia-like homeoproteins (Hanes and Brent, 1989). We produced two Xotx2 mutant constructs (Table 1) carrying an amino acid substitution of this lysine residue.

In the first construct, termed Xotx2 K→Q, the conserved lysine residue is replaced by a glutamine. Its microinjection produced abnormal phenotypes which appear substantially different from those observed microinjecting Xotx2 RNA (Table 1). None of these show an ectopic cement gland. Most of these show various degree of microcephaly and delayed development. Approximately one third of these embryos also show posterior defects.

In the second construct, termed Xotx2 K→E, the conserved lysine residue is replaced by a glutamic acid. This residue is never present at this position in any known homeodomain protein. Microinjection of RNA derived from this construct failed to produce any abnormal phenotype.

These control experiments show that the defects obtained by microinjecting Xotx2 into developing embryos are not the non-specific result of an increased amount of homeobox containing mRNAs. On the contrary, Xotx2 requires its specific DNA-binding properties to produce the observed effects in microinjected embryos.

We further tested the effect of the mouse Otx2 RNA. Its microinjection in 1-cell embryos produced essentially the same results as observed for Xotx2. These findings confirm the extraordinary degree of conservation of Otx2 genes in different species (Table 1).

**Detailed analysis of the phenotypes resulting from the microinjection of synthetic Xotx2 RNA**

Due to the presence in several microinjected embryos of pigmented clusters of cells, in addition to recognizable
Table 1. External phenotypes of injected embryos

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Effect of microinjecting Xotx2 and various alternative constructs in 1-, 2- and 4-cell embryos. Developed embryos were scored for external phenotypes at stage 34 of sibling controls. Next to the name of each construct is shown a schematic representation of the produced protein: the hatched box represents the homeodomain and the capital letter above it, the amino acid in position 9 of the third helix; the stippled box in ΔXotx2 indicates an in frame deletion of the third helix of the homeodomain; the arrow represents antisense RNA; the solid box in Xotx2 Shift indicates the short predicted carboxy terminus downstream from the frameshift mutation; in the Xotx2 K→Q construct the lysine residue at position 9 of the third helix was mutated in glutamine while in the Xotx2 K→E construct the same lysine was replaced by glutamic acid.

Combined results from 10 experiments are shown. n, number of scored embryos; d, dorsal; v, ventral.
†Embryos with secondary axis were pooled and scored by whole-mount staining with the 2G9 antibody and XCG-1 antisense RNA. The histological analysis showed that 100% (19/19) of the embryos presented ectopic neural tissue stained by 2G9 often arranged as spinal cord, 58% (11/19) exhibited a secondary notochord; 53% (10/19) revealed ectopic clusters of cement gland cells positive to XCG-1.
‡Embryos with secondary cement gland were pooled and scored as above mentioned: 100% (21/21) showed ectopic neural tissue stained by 2G9, 52% (11/21) presented a secondary notochord.
*Embryos showing various degrees of microcephaly and delayed development (see text).
secondary cement glands, we decided to hybridize microinjected albino embryos with a cement gland specific probe, namely XCG-1 (Sive et al., 1989) (Fig. 7F). This experiment shows that XCG-1-expressing cells are present in ectopic position in most of microinjected embryos; therefore the numbers presented in Table 1 set a lower limit for the percentage of extra cement glands caused by Xotx2 injection.

We also analysed the expression of a neural marker in microinjected embryos, using the monoclonal antibody 2G9 (Jones and Woodland, 1989) (Fig. 7G). This experiment showed the presence of neural tissue in ectopic position in several embryos belonging to different phenotypic classes.

Fig. 8 shows histological sections of some selected microinjected embryos of the various classes. Embryos showing posterior defects (Fig. 8A) exhibit a limited reduction in number and size of the somites and other posterior axial structures. The notochord appeared slightly larger in diameter but shorter than in controls (not shown).

Histological examination of embryos with additional cement glands (Fig. 8B) revealed the presence of ectopic tissues associated (arrow) with the secondary cement gland. These ectopic tissues resemble a partial secondary axis due to the presence of neural tissue stained by 2G9, muscle and, in about half of the examined cases, notochord (Fig. 8C,D).

We also examined, by RNase protection assay, the effect of microinjecting Xotx2 on the expression of other regulatory genes. We analyzed mesodermal markers such as goosecoid (Cho et al., 1991), Xhox3 (Ruiz i Altaba and Melton, 1989), muscle actin (Mohun et al., 1988) and Xwnt-8 (Smith and Harland, 1991) and neuroectodermal markers such as N-CAM (Kintner and
Melton, 1987), XhoxB3 (Xhox2.7, Dekker et al., 1992), XhoxB9 (XIHbox6, Sharpe et al., 1987). No significant differences were observed for the expression of these genes in microinjected embryos as compared to control embryos. Conversely, an overall 5- to 6-fold increase of goosecoid expression was observed (Fig. 9). We also studied these genes in animal caps of microinjected embryos (Fig. 9 and Table 2). Again, among these only goosecoid appears to be induced in animal caps of microinjected embryos. This induction is not caused by a general mesodermal induction because no expression of muscle actin is detectable. Animal caps explanted from embryos injected with Xotx2 RNA into the animal pole at the 2-cell stage were also hybridized in whole-mount with XCG-1 (not shown). Most of these showed an hybridization signal, whereas uninjected control caps did not show any hybridization.

Overexpression of Xotx2 in UV-treated embryos

In order to understand better the effect of Xotx2 mRNA, we microinjected it into UV-treated embryos. We failed to observe a complete rescue of these ventralized phenotypes (Table 3). Nevertheless, even if they were not easily assigned to conventional DAI figures, a certain degree of anterior-posterior polarity was evident in most of these embryos, very often associated with the presence of muscle, notochord and neural tissue. About 50% of them also developed a cement gland (Table 3), in keeping with what was observed in microinjected normal embryos. The presence of a cement gland is never observed in ventralized embryos of comparable severity (average DAI 0.5). These data were confirmed by whole-mount in situ hybridization of these embryos with XCG-1 (not shown). The localization of the cement gland most often correlated with the site of microinjection, suggesting a direct connection between the presence of Xotx2 transcripts and the appearance of a cement gland.

Table 2. Markers scored in animal caps injected with Xotx2 RNA

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expression</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesodermal markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>goosecoid</td>
<td>+</td>
<td>RNase protection</td>
</tr>
<tr>
<td>Xhox3</td>
<td>-</td>
<td>&quot;</td>
</tr>
<tr>
<td>Xwnt-8</td>
<td>-</td>
<td>&quot;</td>
</tr>
<tr>
<td>muscle actin</td>
<td>-</td>
<td>&quot;</td>
</tr>
<tr>
<td>Neuroectodermal markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-CAM</td>
<td>-</td>
<td>&quot;</td>
</tr>
<tr>
<td>XHbox6</td>
<td>-</td>
<td>&quot;</td>
</tr>
<tr>
<td>XCG-1</td>
<td>+</td>
<td>Whole-mount in situ hybridization</td>
</tr>
</tbody>
</table>

Markers have been scored when sibling controls have reached stage 12.5, in the case of goosecoid, Xhox3 and Xwnt-8, and stage 24 in all the other cases. goosecoid and Xhox3 are markers of dorsoanterior and posterior mesoderm respectively; Xwnt-8 is a ventral mesoderm specific marker while muscle actin is an axial mesodermal marker. Neural markers include N-CAM, which is a general neural marker, Xhox2.7, which is expressed in the hindbrain with maximal intensity in the branchial area at the level of rhombomere 5 and XIHbox6, which is a marker of the spinal cord. XCG-1 is a cement gland-specific marker.

Table 3. Presence of cement gland upon microinjection of UV-treated embryos

<table>
<thead>
<tr>
<th>RNA injected (ng)</th>
<th>n</th>
<th>% embryos with cement gland</th>
<th>Frequency of neural tissue in embryos with cement gland</th>
<th>Frequency of notochordal tissue in embryos with cement gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xotx2</td>
<td>3.5</td>
<td>112</td>
<td>34</td>
<td>4/6 (67%)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>193</td>
<td>50</td>
<td>11/11 (100%)</td>
</tr>
<tr>
<td>△Xotx2</td>
<td>3.5</td>
<td>65</td>
<td>9</td>
<td>n. d.</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>72</td>
<td>8</td>
<td>n. d.</td>
</tr>
<tr>
<td>Uninjected</td>
<td>341</td>
<td>7</td>
<td>1/5 (20%)</td>
<td>1/5 (20%)</td>
</tr>
</tbody>
</table>

Effect of microinjected Xotx2 and △Xotx2 RNA on UV-treated embryos. n, number of scored embryos. Frequencies shown in the table were based on clearly recognizable cement glands; in situ hybridization analysis with the XCG-1 probe revealed a higher frequency of cement gland cells in Xotx2-injected embryos. Samples of embryos were treated with 2G9 and analyzed histologically.

*Embryos with single (67%, 4/6) and double (16%, 1/6) notochord.
†Embryos with single (36%, 4/11), double (54%, 6/11) and triple (9%, 1/11) notochord.

The average DAI of UV-treated controls was 0.5.

DISCUSSION

We studied Xotx2, a Xenopus homeobox gene of the bicoid class homologous to Otx2 (Simeone et al., 1992, 1993). In the mouse this gene is expressed in the epiblast of prestreak embryos and subsequently in very anterior regions of late-gastrulae, including the neuroectoderm of presumptive fore- and mid-brain.

Xotx2 is expressed in prechordal mesendoderm and anterior neuroectoderm

We first analyzed its expression in normal Xenopus embryos. RNase protection analysis (Fig. 2) shows that maternal tran-
scripts of this gene are already detectable in unfertilized eggs. Right after the midblastula transition, when zygotic transcription starts, \textit{Xotx2} expression increases. Whole-mount in situ hybridization experiments revealed an early localized expression in dorsal internal regions of marginal zone at stage 9.5 (Fig. 3A). At stage 10.25 (Fig. 3B) the major expression site of \textit{Xotx2} is in migratory deep zone cells that crawl along the blastocoel roof and are fated to give rise to prechordal mesendoderm (Keller et al., 1992). At stage 10.5 \textit{Xotx2} expression extends to cells of presumptive anterior neuroectoderm (Fig. 3C), where it persists throughout embryogenesis. At stage 14 (Fig. 4), \textit{Xotx2} expression appears to be confined...
to mesendoderm and ectoderm cells of anterior embryonic regions. At this stage the expression domain is spatially restricted in a sort of anterior stripe extending across all three germ layers. This observation suggests that Xotx2 might be more directly implicated in regionalization of cell fates than in regulating tissue-specific gene expression. Intriguingly, this is highly reminiscent of the orthodenticle expression domain in Drosophila blastoderm (Finkelstein and Perrimon, 1990; Finkelstein and Boncinelli, 1994). It is also of interest to point out that the regions of early embryos where Xotx2 is expressed specifically contain those cells that never undergo convergence and extension movements during gastrulation and neurulation that are characteristic of more posterior regions of the axis (Keller et al., 1992).

This expression pattern bears some resemblance (Fig. 10) to that exhibited by goosecoid, a Xenopus homeobox gene (Cho et al., 1991) that has been suggested to play a role in executing Spemann’s organizer phenomenon. Goosecoid is expressed on the dorsal side of the marginal zone of the embryo. At the start of gastrulation goosecoid expression is most intense in an arc of about 60° just above the dorsal lip. This corresponds to the region where Spemann’s organizer is located (Gerhart et al., 1989). In sagittal sections, goosecoid expression is detectable in the internal layer of the dorsal lip zone. The normal fate of these cells is to become pharyngeal endoderm, head endoderm and notochord (De Robertis et al., 1992). Expression of goosecoid in these tissues is transient as no expression is detectable by the time of neurulation. Microinjection of goosecoid mRNA into the ventral side of the embryo is sufficient to start formation of a secondary body axis at relatively high frequency (Blumberg et al., 1991; Cho et al., 1991; Niehrs et al., 1993).

In late blastulae and early gastrulae the two genes are expressed in similar localizations. The two expression domains are similar but not identical (Fig. 10). In fact, comparison of the expression of the two genes at stage 10.5 revealed that Xotx2 transcripts are present in a more narrow domain included in that of goosecoid, but they extend further anteriorly. Furthermore, goosecoid expression never extends to neuroectoderm, decreases by midgastrula and disappears during neurulation (Cho et al., 1991). Microinjection of Xotx2 significantly
increases *goosecoid* expression in both total embryos and isolated animal caps (Fig. 9). Taken together, these data suggest that the regulation of *goosecoid* expression is among the functions of *Otx2* regulatory action during early development.

Comparison of the expression domains of *Otx2* (Simeone et al., 1992, 1993; Boncinelli et al., 1993) and *Otx2* in early embryos shows several similarities and some dissimilarities. Before the onset of neurulation this gene is expressed in presumptive rostral brain in both mouse and *Xenopus*. In both cases the expression of this gene demarcates presumptive fore- and mid-brain. This localized neuroectodermal expression appears in *Xenopus* after a phase during which *Otx2* is expressed in migrating anterior mesendoderm. It is very difficult to detect expression of *Otx2* in the corresponding cells of extending head process in mouse embryos, but this cannot be excluded. Major differences between the number of cells contained in these structures in the two species and a different relative expression intensity might explain this discrepancy. The early presence of *Otx2* transcripts in the entire epiblast of pre-streak mouse embryos raises the question of the corresponding structure in early *Xenopus* embryos. Data of *Otx2* expression in *Xenopus* blastulae suggest an equivalence of mouse epiblast and *Xenopus* animal cap. A third question concerns the relationship between early expression in the entire mouse epiblast and later expression in anterior neuroectoderm exhibited by *Otx2*. From the observation of mouse embryos it is difficult to assess whether *Otx2* later expression derives directly from the first wave of expression in epiblast, simply because some posterior cells no longer express *Otx2* while anterior cells still contain these transcripts. Comparison of mouse and *Xenopus* expression data rather suggest that restricted expression in anterior neuroectoderm is a new event, regulated independently from the previous extended expression, possibly through different control regions. Anagous conclusions have been drawn by the analysis of the chick homologue of *Otx2*, c-otx2 (Bally-Cuif et al., in press).

Moreover in *Xenopus* it has been possible to show that the restricted neuroectodermal expression depends on the presence of an underlying mesendoderm; in fact complete exogastrulae express *Otx2* exclusively in anterior regions of mesendoderm.

**RA treatment abolishes *Otx2* expression in anterior neuroectoderm**

Treatment of embryos with UV light or RA inhibits *Otx2* expression, while treatment with LiCl deeply reorganizes it (Fig. 5). They also suggest a role of *Otx2* as an important intermediary between the first positional specification mediated by the cortical rotation originated by sperm entry (Gerhart et al., 1989) and the establishment of the anterior-posterior axis. *Otx2* expression in RA-treated embryos is of particular interest in view of the specific malformations caused in embryos by this treatment. RA essentially abolished *Otx2* expression in neuroectoderm of gastrulating embryos (Fig. 5). The most relevant observation is that starting from stage 10.5, no expression is detectable in neuroectoderm of these embryos. This lack of expression in neuroectoderm may in turn be due to the reorganization of *Otx2* expression in prechordal mesendoderm induced by RA or, alternatively, could be an independent effect of this treatment.

RA-treated embryos lack most of the rostral brain including fore- and mid-brain, whereas hindbrain and spinal cord regions are not reduced (Durston et al., 1989; see also Fig. 5C). In normal *Xenopus* embryos *Otx2* is specifically expressed in rostral brain. It is tempting to speculate that there is a direct correlation between the deletion of these regions and the lack of *Otx2* expression in neuroectoderm of embryos which had been subjected to RA treatment. It is known that RA affects expression of many other genes including homeobox genes of the *Hox* family (McGinnis and Krumlauf, 1992 for a review). All these genes contribute to the actual phenotype of RA-treated embryos, but *Otx2* is the only gene found so far to be expressed in the presumptive rostral brain whose expression is downregulated by RA, both in cultured cells (Simeone et al., 1993) and in embryos. In RA-treated embryos, inhibition of *Otx2* expression in specific structures correlates with deletion of the same structures. It is probably worth remembering that a role as a developmental gap gene has been proposed for the *Drosophila* homologue of *Otx2*, namely *orthodenticle* (Finkelstein and Perrimon, 1990). In fact, null alleles of this fly gene cause abnormalities that can be best explained as deletions of head regions, particularly in the antennal and pre-antennal segments. Finally, lack of anterior structures in embryos derived from UV-treated zygotes, where *Otx2* expression is also significantly reduced, is consistent with the notion of a role of *Otx2* in the development of the head region.

**Microinjection of *Xotx2* RNA causes phenotypes with major posterior defects**

Results of microinjection experiments lend further support to the hypothesis of a role of *Otx2* in patterning the anterior-posterior axis of the embryo. We observed a major class of shortened embryos with reduced trunk and tail structures and an expansion of internal head structures (Figs 7C, 8A).

These phenotypes can be viewed in the light of at least two interpretative schemes. According to the first scheme, the relative sizes of the body regions allocated in early mouse embryos (Figs 7C, 8A) of mouse embryos is difficult to assess whether *Otx2* later expression derives from the observation of mouse embryos it is among the functions of extending head process in mouse embryos, but this cannot be excluded. Major differences between the number of cells contained in these structures in the two species and a different relative expression intensity might explain this discrepancy. The early presence of *Otx2* transcripts in the entire epiblast of pre-streak mouse embryos raises the question of the corresponding structure in early *Xenopus* embryos. Data of *Otx2* expression in *Xenopus* blastulae suggest an equivalence of mouse epiblast and *Xenopus* animal cap. A third question concerns the relationship between early expression in the entire mouse epiblast and later expression in anterior neuroectoderm exhibited by *Otx2*. From the observation of mouse embryos it is difficult to assess whether *Otx2* later expression derives directly from the first wave of expression in epiblast, simply because some posterior cells no longer express *Otx2* while anterior cells still contain these transcripts. Comparison of mouse and *Xenopus* expression data rather suggest that restricted expression in anterior neuroectoderm is a new event, regulated independently from the previous extended expression, possibly through different control regions. Analogous conclusions have been drawn by the analysis of the chick homologue of *Otx2*, c-otx2 (Bally-Cuif et al., in press).

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result from interferences with the movements of convergence and extension taking place during gastrulation and neurulation and giving rise to more posterior regions. We already noticed that endogenous Xotx2 is expressed in presumptive tissues which are not involved in these movements. An extensive misexpression of Xotx2 transcripts might interfere with the fate of posterior regions of the axis. Of course, the two interpretative schemes need not to be mutually exclusive.

A second major class of microinjected embryos exhibit, in addition to an anteriorized phenotype, the presence of an additional cement gland, both in anterior and posterior localizations (Fig. 7D). Hybridization with a cement gland specific probe, XCG-1, actually reveals that the vast majority of embryos obtained by microinjecting Xotx2 contain additional groups of cells fated to become cement gland (Fig. 7F). Ectopic tissues are often present in these embryos in the vicinity of the secondary cement gland. Microinjection of Xotx2 into UV-treated embryos causes a partial rescue of their ventralized phenotypes including the production of cement gland, muscle, notochord and neural tissue (Table 3). In this respect, we can hypothesize that Xotx2 is not sufficient in itself to induce an axis and may require the cooperation of dorsalizing genes to produce complete head structures.

Cement gland is one of the most anterior structures of the developing Xenopus body (Sive et al., 1989). The ectodermal area in which the cement gland will eventually form is unique because there the ectoderm remains in close contact with the endoderm after gastrulation with no intervening mesoderm (Hausen and Riebesell, 1991). This arrangement is considered to be important for the proper positioning of the cement gland anlage and suggests an homology between the cement gland anlage in Xenopus and buccopharyngeal membrane in amniota. Induction of a secondary cement gland by ectopic expression of Xotx2 strongly suggests for this gene a role in specifying very anterior head regions.

We scored other classes of phenotypes in microinjected embryos. Among them a certain number of embryos show (Fig. 7E) a partial secondary axis originating from the trunk region and extending further anteriorly. Staining with the 2G9 antibody (Fig. 7G) as well as hybridization with XCG-1 (Fig. 7F) reveal, however, that clusters of cells exhibiting features typical of cement gland and neural tissue are ectopically present in most microinjected embryos, independently of their assignment to one of the four phenotypic classes. Histological analysis also reveals that the percentage of embryos with a partial secondary axis is as relevant as that of embryos with secondary cement glands.

The presence of both secondary axes and secondary cement glands is characteristic of Xotx2 microinjected embryos. These two phenotypes may be caused independently or may result from a common mechanism. Among the analyzed markers we observed a significant effect on goosecoid and XCG-1 whose expression increases both in injected embryos and isolated animal caps. We can hypothesize that cement gland induction is a direct effect of Xotx2 overexpression, whereas the appearance of secondary axes might be at least in part mediated by goosecoid activation.

Microinjected Xotx2 RNA requires its DNA binding specificity to cause all these phenotypes. Constructs lacking a relevant portion of the homeodomain do not cause abnormal phenotypes. Similarly, constructs containing a homeodomain where the relevant lysine residue has been substituted either do not cause abnormal phenotypes, as is the case for the substitution K →E, or cause a certain percentage of abnormal phenotypes, but these are substantially different from those observed for the intact Xotx2. In fact, the construct Xotx2 K →Q, containing a glutamine residue at position 9 of the recognition helix, cause microcephaly and delayed development. Some of these abnormalities are reminiscent of those observed upon treatment with low doses of RA. It is possible that the products of this construct interfere with the action of other homeodomain proteins containing a glutamine, including the Hox proteins. None of the control constructs causes the appearance of secondary cement glands.

Otx2 in the mouse demarcates anterior structures of the

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**Fig. 10.** Diagrammatic comparison of Xotx2 and goosecoid expression, indicated by stippling, in early Xenopus embryos: top, dorsal-posterior view; bottom, sagittal view. Areas 1 to 6 represent: 1, prechordal plate; 2, bottle cells; 3, chordamesoderm; 4, presumptive spinal cord; 5, presumptive rostral brain; 6, presumptive epidermis.
embryo during gastrulation, even before headfold formation. Expression pattern of Xotx2 in Xenopus embryos suggests that this gene plays a role in events leading to preparation and execution of gastrulation, especially in connection with the specification and possibly patterning of anterior regions. Results of microinjection experiments suggest that Xotx2 gene products are able to respectify the subdivision of the body along the anterior-posterior axis. In conclusion, both in mice and Xenopus the Otx2 gene might play a central role in the initial events of axis formation and in particular in specifying anterior head regions and their spatial relationship with trunk structures. Both genes encode a transcription factor and must act by regulating the expression of other genes. The number and nature of downstream target genes remain to be explored.

We wish to thank Igor Dawid for the cDNA library, Eddy De Robertis for the goosecoid and XHbox6 probes, Hazel Sive for the XCG-1 probe, Jim Smith for the Brachury, N-CAM probes and the 2G9 antibody. Richard M. Harland for the Xiwt-8, Tim Mouhnn for pSpt1 cardiac actin probe and Antonello Mallamaci, Igor Dawid, Erika-Jan Dekker, Eddy De Robertis, Tony Durston and Jim Smith for comments and helpful suggestions. This work was supported by grants from EC BIOTECH Programme, Progetti Finalizzati CNR ‘Ingegneria Genetica’ and ACRO, the VII AIDS Project of the Ministero della Sanita’ and the Italian Association for Cancer Research (AIRC). M. A. is recipient of an AIRC fellowship.

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Xotx2 and the fate of anterior regions