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Mechanisms of Development 61 (1997) 187–198

Xrx1, a novel *Xenopus* homeobox gene expressed during eye and pineal gland development

Simona Casarosa[a], Massimiliano Andreazzoli[a], Antonio Simeone[b], Giuseppina Barsacchi[a,*]

[a]*Laboratorio di Biologia Cellulare e dello Sviluppo, Università di Pisa, Via Carducci 13, 56010 Ghezzano-Pisa, Italy*
[b]*Istituto Internazionale di Genetica e Biofisica, Via Marconi 12, 80125 Napoli, Italy*

Received 7 October 1996; revised version received 20 November 1996; accepted 21 November 1996

Abstract

We have isolated a novel *Xenopus* homeobox gene, *Xrx1*, belonging to the *paired*-like class of homeobox genes. *Xrx1* is expressed in the anterior neural plate, and subsequently in the neural structures of the developing eye (neural retina and pigmented epithelium), and in other forebrain structures deriving from the anterior neural plate: in the pineal gland, throughout its development, in the diencephalon floor and in the hypophysis. Its rostral limit of expression corresponds to the chiasmatic ridge, which some authors consider as the anteriormost limit of the neural tube: thus, *Xrx1* may represent one of the most anteriorly expressed homeobox genes reported to date. Moreover, its expression in organs implicated in the establishment of circadian rhythms, may suggest for *Xrx1* a role in the genetic control of this function. Finally, analysis of *Xrx1* expression in embryos subjected to various treatments, or microinjected with different dorsalizing agents (*noggin*, *Xwnt-8*), suggests that vertical inductive signals leading to head morphogenesis are required to activate *Xrx1*.

Keywords: *Xenopus*; Homeobox gene; Eye and pineal gland development; Circadian rhythms

1. Introduction

Ever since Spemann's first experiments (Spemann, 1901) on the inductive effect of one tissue (optic vesicle) over another (ectoderm), the development of the eye has become a paradigm for the study of inductive events in vertebrate embryos. The first morphological evidence of eye development occurs during neurulation, when a region of the anterior neural plate evaginates from the lateral walls of the forebrain, giving rise to the bilaterally paired optic vesicles. All the neural structures of the eye, the retina, the pigmented epithelium and the optic stalks, will originate from the eye vesicles. Following the cooperative induction by a planar signal from the neural plate and a vertical signal from the eye vesicle, the ectoderm will form the cornea and the lens (Grainger, 1992; Saha et al., 1992). Once formed, the lens placode will in turn induce the optic vesicle to invaginate into a cup, made of an inner layer and an outer layer, that will give rise to the retina and to the pigmented epithelium, respectively. The pigmented epithelium thins and differentiates along the back of the eye, whereas the retinal epithelial cells will continue to proliferate to eventually give rise to the photoreceptors, interneurons, ganglion cells and glia, that make up the mature eye (reviewed in Harris and Holt, 1990).

After the initial inductive events, eye development in vertebrates can be divided into two phases: an early phase involving the morphogenetic processes through which the basic elements of the eye are set in place and shaped, and a late phase consisting of differentiation events, in which the cells positioned in the first phase differentiate into the appropriate fate according to their position (Holt et al., 1988). In lower vertebrates, growth is maintained in a region at the periphery of the eye, the ciliary margin, even after differentiation in the central regions of the retina is complete (Hollyfield, 1971). In response to signals coming from the retina (Reh, 1987), the cells of the ciliary margin, that can be viewed as stem cells, are able to generate all the cellular types of the mature retina.

In recent years, several genes that appear to be impli-

* Corresponding author. Tel.: +39 50 878356; fax: +39 50 878486.

0925-4773/97/$17.00
PII S0925-4773(96)00640-5

cated in eye development have been characterized. A major role is played by *Pax6*, a *paired* box-containing transcription factor belonging to the *Pax* gene family, expressed in discrete regions of the developing neural tube and in the developing eye of the mouse (Walther and Gruss, 1991). It has recently been shown that the targeted expression of either *Pax6* or of its *Drosophila* homologue, *eyeless*, is able to induce ectopic eye structures in *Drosophila* (Halder et al., 1995), thus suggesting for *Pax6* an evolutionary conserved role as a master control gene in eye morphogenesis.

Other vertebrate genes have been isolated that could play relevant roles in eye development. *Six3* is the murine homologue of the *sine oculis* gene, implicated in *Drosophila* eye development. *Six3* demarcates the most anterior border of the neural plate and is expressed in the developing eye (Oliver et al., 1995). Other transcription factor genes, that are thought to have a role in the patterning of the forebrain, are expressed in the developing eye vesicles. This is the case of the murine *Otx2* (Simeone et al., 1993) and of its *Xenopus* homologue, *Xotx2* (Kablar et al., 1996); and also of some of the genes of the *Distal-less* class, both in the mouse (Dollé et al., 1992), and in *Xenopus* (Papalopulu and Kintner, 1993). Neither the precise role of these genes in eye development, nor their possible relationships within a molecular network, are presently known.

During the late phase in eye development, in which differentiation takes place, the newborn retinal ganglion cells send their axons to the optic tectum, where they make topographically ordered connections. Over 30 years have passed since the formulation of a model, in which the ordered set up of retinotectal connections is accomplished by orthogonal gradients of molecules in the retina that give each cell a positional identity (Sperry, 1963). The occurrence of soluble or cell-surface molecules that are asymmetrically distributed along the A-P (anterior-posterior) and D-V (dorso-ventral) axis of the retina (reviewed in Kaprielian and Patterson, 1993) appears to support this model. A few transcription factors with an asymmetrical distribution have also been found, and they could direct the expression of these Sperry-type molecules. A member of the murine *Pax* gene family, *Pax2*, is expressed in the ventral portion of the retina (Nornes et al., 1990). The chick homeobox gene *SOHo-1* is expressed at high levels in the nasal retina, and at low levels in the temporal retina, suggesting a role in the patterning of the retina A-P axis (Deichter et al., 1994). Two *winged-helix* transcription factors, *BF-1* and *BF-2*, have adjacent domains of expression within the developing mouse retina (Hatini et al., 1994). The recently isolated *Xenopus* homeobox gene, *Xbr1*, is expressed in a small dorsal region of the ciliary margin (Papalopulu and Kintner, 1996). Interestingly, *X-Notch-1*, the *Xenopus* homologue of the *Drosophila Notch*, implicated in retinal development, is expressed in the ciliary margin, but seems to be absent from a zone of cells located at the very edge of the eye, that are those expressing *Xbr1* (Papalopulu and Kintner, 1996). Thus, different subpopulations of cells can be identified in the proliferating ciliary margin.

In this paper we report the isolation and the spatio-temporal pattern of expression of a novel *Xenopus* homeobox gene, *Xrx1*, which may play a role in eye development. In fact, *Xrx1* starts being expressed at the end of gastrulation in the anteriormost region of the neural plate and its subsequent expression accompanies the optic vesicles all throughout their development, being found at later stages in neural structures only, such as the retina and the pigmented epithelium. Notably, *Xrx1* is expressed not only in the eye, but also in other specific forebrain structures developing from the most anterior regions of the neural plate, such as the ventral diencephalon (chiasmatic ridge and posterior hypothalamus), pineal gland, and hypophysis. Thus, *Xrx1* pattern of expression is suggestive of possible roles in the establishment of positional information at the anterior boundary of the developing embryo, in the morphogenesis of anterior structures, and perhaps in some aspects of cell determination and/or differentiation. In addition, *Xrx1* expression in embryos subjected to various treatments, or microinjected with different dorsalizing agents (*noggin*, *Xwnt-8*), suggests that vertical inductive signals leading to head morphogenesis are required to activate *Xrx1*. Finally, the coincidental expression of *Xrx1* in those areas of the central nervous system where the components of the biological clock are physically located, may suggest a role for *Xrx1* in the genetic control of their development, eventually resulting in the establishment of such a fundamental function.

2. Results

2.1. Isolation of a novel Xenopus homeobox gene

A stage 24/25 *Xenopus* cDNA library was screened at low stringency using as probes two fragments of the murine homeobox gene *Orthopedia* (Simeone et al., 1994). This screening brought about the isolation of a novel homeobox gene. The identified cDNA clone contains the whole coding region, and Fig. 1A shows that two putative translation initiation codons are present in the same open reading frame as the homeodomain. We cannot be certain as to which of the two initiation codons is the functional translational start site. Using the first ATG, the predicted protein comprises 322 amino acids, with the homeodomain spanning from amino acid 130 to 189. Another feature of the predicted protein is a proline-rich (16.4%) C-terminal region, which is a potential activation domain (Mitchell and Tijan, 1989).

2.2. Homology to other homeobox-containing genes

The isolated homeobox gene is not closely related to any other homeobox genes from *Drosophila* or vertebrates

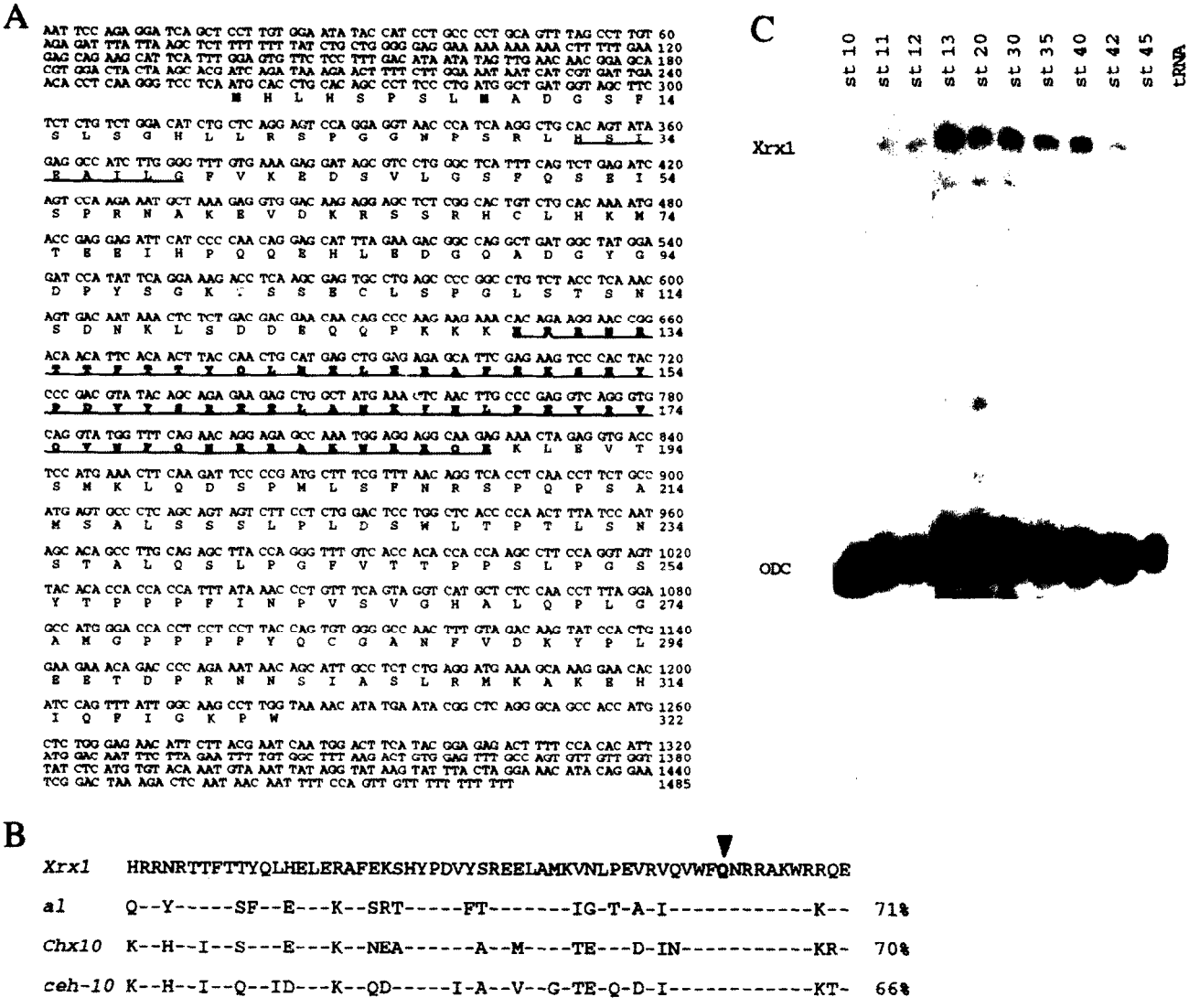

Fig. 1. Molecular characterization of *Xrx1*. (A) Nucleotide sequence of *Xrx1* cDNA and deduced amino acid sequence. The two putative initiation methionines are in bold. The octapeptide is underlined and the homeodomain is both underlined and in bold. (B) Sequence comparison of *Xrx1* homeodomain and other homeodomains. The glutamine residue at the ninth position of the recognition helix (arrowhead) is conserved in all the proteins shown. Dashes are conserved amino acids. The percentages of homologies to *Xrx1* is shown on the right. (C) *Xrx1* expression during development. RNase protection: embryonal stages are indicated in the upper part of the figure. The *X. laevis* gene for ornithine decarboxylase (ODC) was used as an internal control. *Xrx1* protected band is 153 nt, ODC protected band is 91 nt. Radiolabeled DNA fragments generated from Sau 3AI digestion of pBR 322 plasmid (ranging in size from 665 bp to 36 bp) were used as standard molecular marker (not shown).

characterized to date. In particular, the homeobox in the isolated cDNA does not appear to be closely related to the homeobox of the *Drosophila Antennapedia* class, which defines the *Hox* genes in vertebrates, or to other known homeobox sequences, although some homologies can be found (see below). Since a major domain of expression of the isolated gene is the retina (see below), we propose the name *Xrx1*, for *Xenopus r*etinal homeobo*x* 1.

Sequence comparisons with other homeobox-containing genes present in databases show that the deduced *Xrx1* homeodomain shares some homologies, at the amino acid level, with other genes. In particular, *Xrx1* homeodomain is 70% homologous to the homeodomain of the murine gene *Chx10* (Liu et al., 1994); and 66% to the *Caenorhabditis elegans* homologue of *Chx10*, *ceh-10* (Svendsen and McGhee, 1995) (Fig. 1B). However, *Xrx1* should not represent the *Xenopus* homologue of *Chx10* and *ceh-10*, since the homology it shares with them is not extended to regions outside the homeodomain, while *Chx10*, *ceh-10* and their goldfish homologue *Vsx-1* (Levine et al., 1994) share another highly conserved region, called the CVC domain (Svendsen and McGhee, 1995). Interestingly, even though these genes do not seem to be homologous to *Xrx1*, they show related patterns of expression with the *Xenopus* gene (see Section 3). The homeodomain of *Xrx1* is also 71% homologous to the homeodomain of the *Drosophila* gene *aristaless* (Fig. 1B), which is expressed in patches of cells in the embryo,

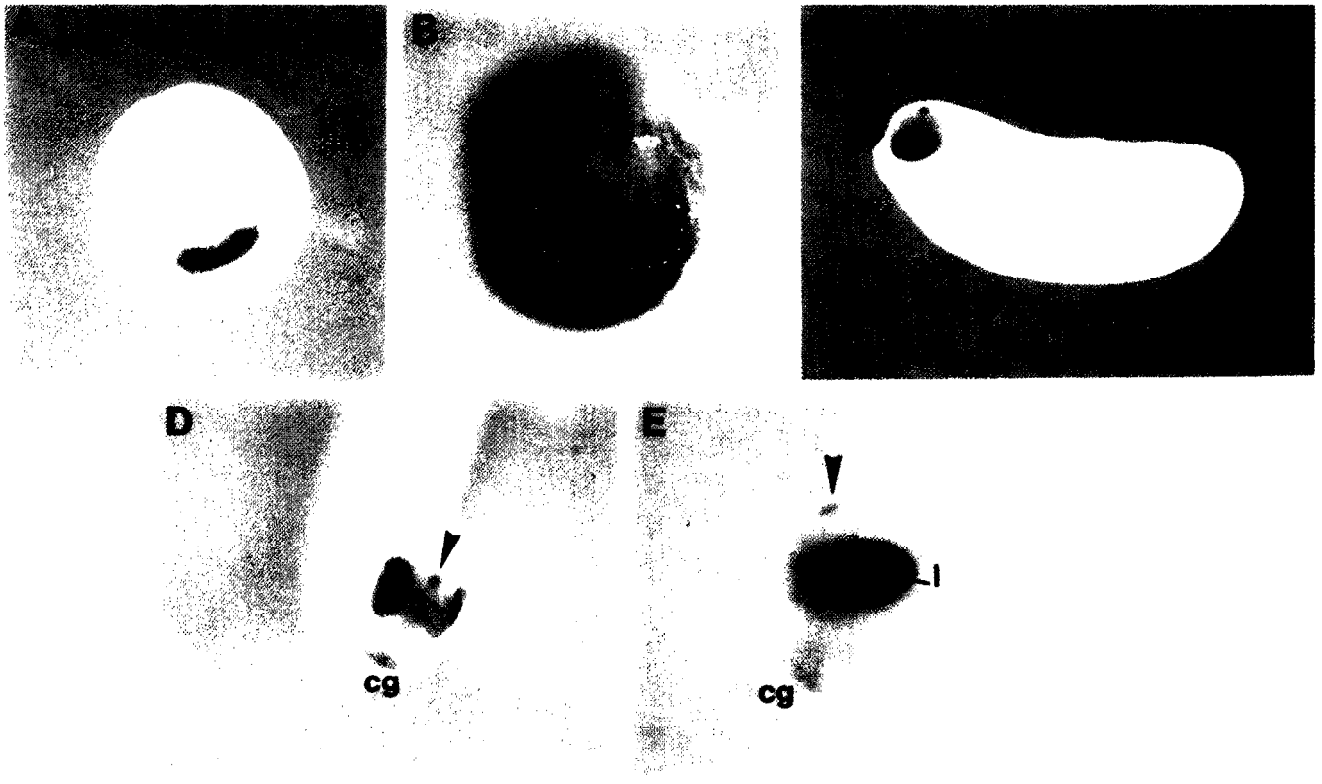

Fig. 2. Developmental expression of *Xrx1* by whole-mount in situ hybridization. In panels (C,E) anterior is to the left and dorsal to the top. In panels (C–E) *Xrx1* expression in the pineal gland is marked with an arrowhead. (A,B) *Xrx1* expression of stage 12.5 and stage 15 neurulae. Frontal views show the expression in the anterior neural plate and ridge. In (B) the dotted line defines the anterior limit of the neural ridge. (C) Lateral view of a stage 24 embryo showing *Xrx1* expression in the optic vesicle and pineal gland. (D) Dorsal view of a stage 28 embryo. Anterior is to the bottom. The fainter staining between the eyes is the diencephalon floor. (E) Lateral view of a stage 34 embryo showing that the lens (l) does not express *Xrx1*. Abbreviations: cg, cement gland; l, lens.

some of which will become part of sense organs (Schneitz et al., 1993), and also in this case the homology does not extend to regions outside the homeodomain.

According to the amino acid composition of its homeodomain, *Xrx1* belongs to the *paired*-like class, related to the *paired* (*prd*) class of homeobox genes (Bürglin, 1994). The genes mentioned above have also been assigned to this class, because they all share, together with *Xrx1*,

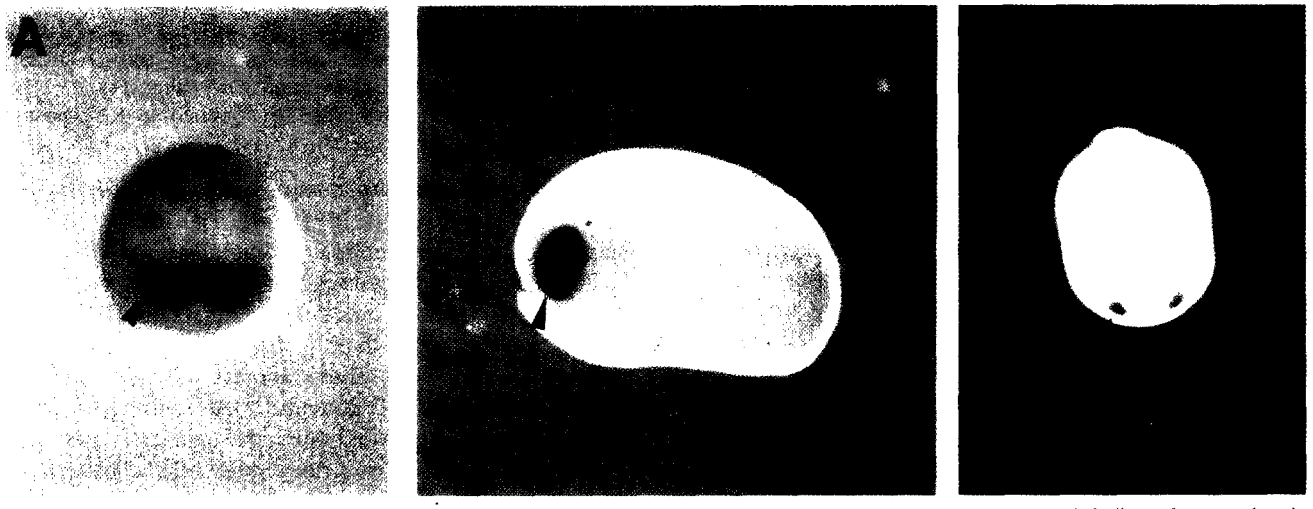

Fig. 3. Whole-mount in situ hybridization showing *Xrx1* asymmetrical expression in the eye. In panels (A–C) the arrowheads indicate the ventral region of stronger *Xrx1* expression. (A) Frontal view of a stage 17/18 embryo. The optic vesicles are still frontal and they show a stronger *Xrx1* expression in the ventral region. (B) At stage 22 the vesicles have rotated laterally, and the region of stronger *Xrx1* expression is ventro-nasal. (C) Dorsal view of (B), showing the gradient of *Xrx1* expression along the D-V axis of the eye.

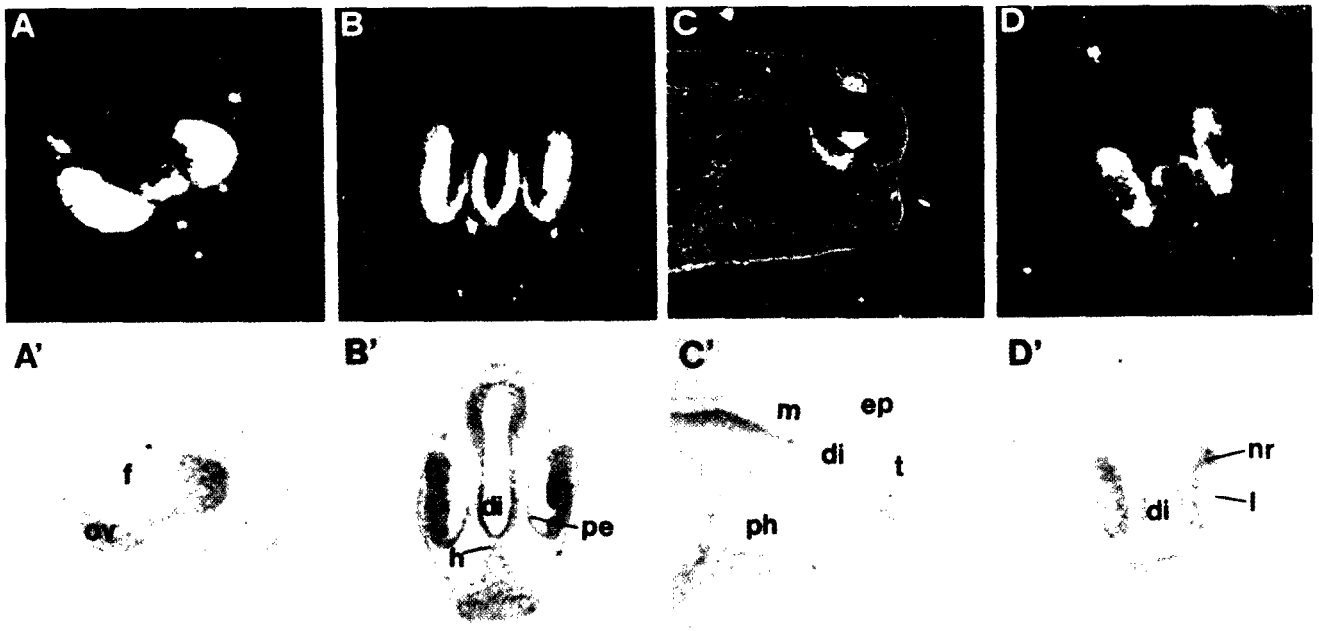

Fig. 4. Developmental expression of *Xrx1* by radioactive in situ hybridization on sections. Panels (A–D), dark fields; (A′–D′), respective bright fields. (A) Transversal section of a stage 20 embryo, showing *Xrx1* expression in the optic vesicles and in the floor of the forebrain. (B) Transversal section of a stage 28 embryo. The expression in the optic cups covers both the neural retina (nr) and the pigmented epithelium (pe). The arrow indicates the expression in the hypophysis. (C) Longitudinal section of a stage 34 embryo. The arrow shows the rostral limit of *Xrx1* expression in the chiasmatic ridge. (D) Horizontal section of a stage 35/36 embryo, showing the lack of *Xrx1* expression in the lens (l). Abbreviations: di, diencephalon; ep, epiphysis (pineal gland); f, forebrain; h, hypophysis; l, lens; m, mesencephalon; nr, neural retina; ov, optic vesicle; pe, pigmented epithelium; ph, pharynx; t, telencephalon.

some features that make it impossible for them to fit in the *prd* class: (i) they have no paired-box domain; (ii) the residue at position 50 of the homeodomain (residue 179 of *Xrx1* protein) is a glutamine and not a serine as in the *prd* class; (iii) a highly conserved region N-terminal to the homeodomain, found in many *prd* class genes, is not present. Moreover, the *Xrx1* deduced protein, like several other transcription factors with *prd*-like homeodomains, contains an HSIDGILG octapeptide (Noll, 1993) between residues 32 and 39: HSIEAILG (Fig. 1A).

2.3. Early expression of Xrx1 in Xenopus embryos

We have investigated the developmental expression of *Xrx1* by RNase protection assay and by whole-mount in situ hybridization. By RNase protection, expression is first detected at stage 11 and then appears to be maintained at fairly stable levels up to stage 45, when it declines (Fig. 1C).

By whole-mount in situ hybridization the first localized transcripts are detected at the end of gastrulation (stage

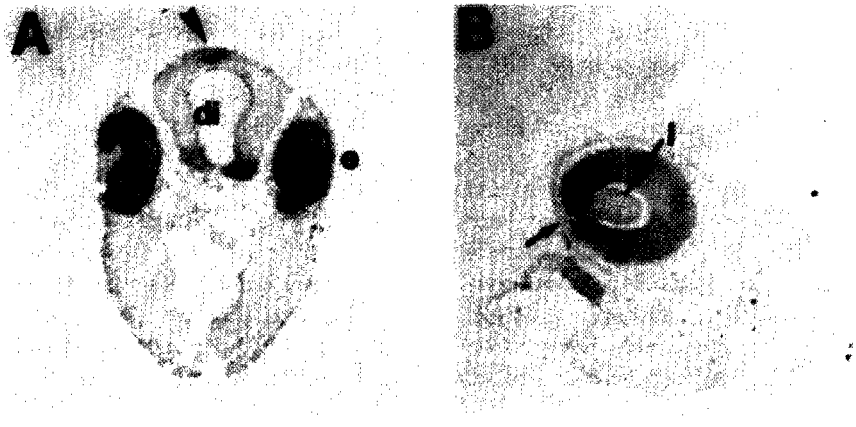

Fig. 5. Expression pattern of *Xrx1* in embryo sections, after whole-mount in situ hybridization. (A) Transversal section of a stage 32 embryo. Expression in the pineal gland is indicated by the arrowhead. (B) Longitudinal section of a stage 35/36 embryo, showing *Xrx1* stronger expression in the regions that border the optic fissure (arrow). Abbreviations: as in Fig. 4; e, eye.

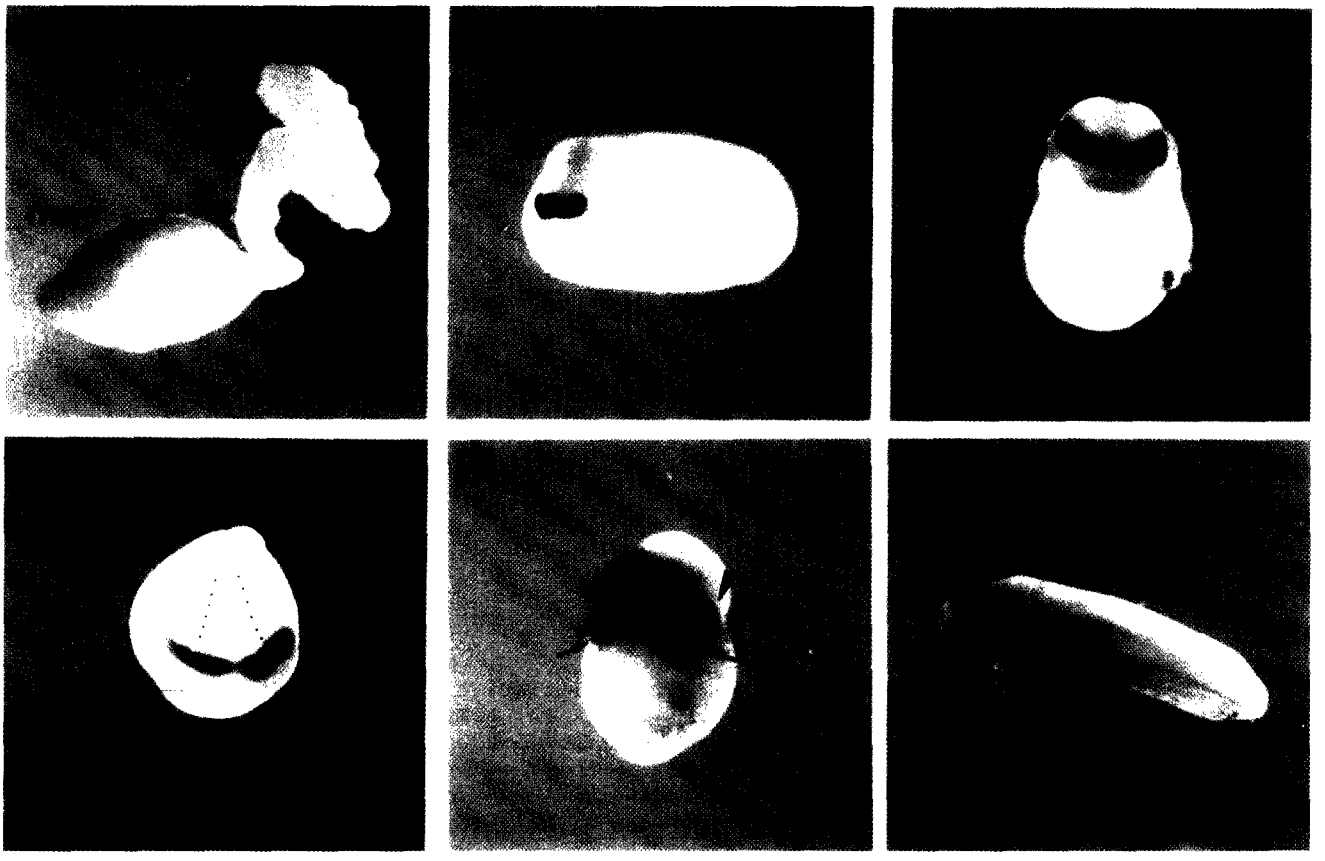

Fig. 6. Expression of *Xrx1* by whole-mount in situ hybridization in embryos subjected to various treatments. (A) Albino exogastrula at stage 24 showing the lack of *Xrx1* expression. (B) Stage 23 albino embryo treated with a 30 min pulse of 10^{-6} M RA at stage 10^{+}. (C) Stage 34 pigmented embryo treated with 0.3 M LiCl for 10 min at 32-cell stage. The circular cement gland is indicated by the arrowheads. (D) Dorsal view of a stage 17 albino embryo that was injected with 100 pg of *Xwnt-8* synthetic RNA in a vegetal position at two cell stage. Dotted lines indicate the dorsal midlines of the two axes; hybridization signals are detected in the anterior neural plate of both axes. (E) Frontal view of a stage 23 pigmented embryo that was injected with 100 pg of *Xwnt-8* synthetic RNA in a ventral-vegetal position at 4 cell stage, showing three eyes with *Xrx1* hybridization signal (arrowheads) and two cement glands (arrows). (F) Dorsal view of a stage 23 pigmented embryo that was injected with 100 pg of *noggin* synthetic RNA in a ventral-vegetal position at 4 cell stage. *Xrx1* expression is detected in the primary but not in the secondary axis (indicated by arrowheads).

12.5), when expression appears to be restricted to the most anterior region of the neural plate (Fig. 2A). At stage 15 the signal is much stronger and encompasses the whole mid-anterior and mid-lateral anterior neural ridge and the antero-lateral neural plate (see Eagleson et al., 1995) (Fig. 2B).

According to the fate map of the *Xenopus* neural plate, these regions will give rise to the retina and to the whole floor of the diencephalon, including the hypothalamus and the chiasmatic ridge, where *Xrx1* is expressed at later stages (Eagleson et al., 1995). The pineal gland, which is another site of expression of *Xrx1* (see below), also originates from the lateral anterior ridge (Eagleson et al., 1995). Thus, *Xrx1* is one of the few homeobox genes to be expressed in the most anterior regions of the neural plate.

2.4. *Xrx1* expression during eye development

In *Xenopus*, the optic vesicles begin to form and protrude from the walls of the diencephalon at stage 16/17 (Nieuwkoop and Faber, 1967). From the beginning, their formation is accompanied by a strong expression of *Xrx1* (see Fig. 3A). As the vesicles move away from the brain, they continue to express *Xrx1*, all throughout their development (Fig. 2C,D).

At all stages tested, *Xrx1* is found to be expressed only in eye structures of neural origin, that is, in the neural retina and the pigmented epithelium, while ectoderm derived structures, such as the lens and the cornea, never show *Xrx1* expression. Whole-mount in situ hybridization of later stage embryos show that the forming lens does not express *Xrx1* (Fig. 2E). This can be better seen by radioactive in situ hybridization of embryo sections, both at early and late stages of development (Fig. 4A,B,D), and by sectioning the whole-mount embryos (Fig. 5). *Xrx1* expression in the eye begins to decline at around stage 42 and is at very low levels, barely detectable by both RNase protection and whole-mount procedures, when

the embryos reach the swimming tadpole stage (stage 45) (Fig. 1C). In the period comprised from stage 39 to 42 the photoreceptors, which are the last cells to differentiate in the neural retina, would begin their differentiation, while from stage 43 onwards, the onset of visual function occurs (Grant et al., 1980).

Interestingly, when the staining reaction at the end of the whole-mount procedure was brought to an early stop, before the enzymatic reaction could reach a plateau level, a stronger *Xrx1* expression was evident in a portion of the eye vesicle only. A frontal view of a neural folds stage embryo (stage 16/17), shows this stronger staining in the anterior region of the eye vesicles (Fig. 3A). As the vesicles rotate laterally and begin to approximate the surface of the head, the region of stronger *Xrx1* expression becomes ventro-nasal (Fig. 3B). As the development of the eye proceeds, the region identified by the higher *Xrx1* level of expression is seen to surround the forming optic fissure, that is, an infolding of the eye cup that will serve as the route both for the axons of the ganglion cells to enter the optic stalks and to form the optic nerve, and for the blood vessels that will vascularize the eye (Fig. 5B).

2.5. *Xrx1 expression in the forebrain*

Besides the eyes, other forebrain structures appear to express *Xrx1* during neural development. These structures originate from the presumptive territories covered by *Xrx1* expression at early neurula stages. First, *Xrx1* expression marks the developing pineal gland. The gene expression precedes any morphological differentiation of this structure, since the pineal gland begins to evaginate from the roof of the diencephalon at around stage 26 (Nieuwkoop and Faber, 1967), while *Xrx1* expression in this territory begins at around stage 24 (Fig. 2C). Subsequently, *Xrx1* expression is maintained as the pineal gland vesicle pinches off from the diencephalon, and thereafter (Fig. 2D,E; Fig. 4C). *Xrx1* expression begins to be turned off approximately at the same time as in the eye, around the swimming tadpole stage.

Xrx1 is also expressed in the ventral diencephalon: from the moment the optic vesicles start to evaginate from the forebrain, it can be seen that the region that connects them expresses *Xrx1* (Fig. 4A). This region includes the stomodeal-hypophyseal anlage (data not shown); at later stages only the dorsal part of the anlage, the hypophyseal portion, can be seen to maintain *Xrx1* expression (Fig. 4B). At stage 34, when the diencephalon is differentiating, *Xrx1* expression in the forebrain comprises the whole diencephalon floor, from the chiasmatic ridge to the posterior hypothalamus (Fig. 4C).

2.6. *Xrx1 is not expressed in exogastrulae*

In order to determine whether *Xrx1* is expressed in the neural plate in the absence of the underlying mesoderm, we examined its expression in complete exogastrulae by whole-mount in situ hybridization (Ruiz i Altaba, 1992). In all of the complete exogastrulae examined either at stage 24 or at stage 37, we were not able to detect any expression of *Xrx1* (Fig. 6A). As a positive control, we also studied *en2* expression in exogastrulae of these stages. As expected, *en2* transcripts are present in the ectodermal region of the exogastrulae (Ruiz i Altaba, 1992) (not shown). We can conclude from these data that the establishment and/or maintenance of *Xrx1* expression in normal embryos may depend on inductive signals from the underlying mesoderm.

2.7. *Xrx1 expression demarcates the modified eye territories in retinoic acid and lithium chloride treated embryos*

RA and LiCl treatments are known to modify in different ways eye development, so we decided to analyse how *Xrx1* expression responds to these treatments. It has been shown that RA administration during *Xenopus* gastrulation leads to suppression of anterior structures, due to transformation of anterior tissue towards a more posterior fate (Durston et al., 1989). The anterior structure that is more sensitive to RA treatment is the eye, being already affected with the lowest doses of RA at the shortest time of exposure used (Sive et al., 1990). Embryos treated with a 30 min pulse of 10^{-6} M RA at stage 10^{+} and hybridized with *Xrx1* at stage 23, show a restriction of *Xrx1* expression domain. In particular, the expression in the eye vesicles, that in control embryos is bilaterally paired, appears as a single anterior spot as if the two eye vesicles were fused medially (Fig. 6B). This is in accordance with the observation that this treatment results in microcephalic, cyclopic embryos. Notably, within the area of expression, the intensity of the signal, as judged by whole-mount in situ hybridization, does not appear to be diminished compared to control embryos, suggesting that RA does not directly affect *Xrx1* expression.

LiCl treatment, on the other hand, has an opposite effect on eye development. Application of LiCl at early blastula stages (from 32 to 128 cell stages) causes all the mesoderm to be specified as dorsal, resulting in an embryo with enhanced dorso-anterior structures. Extreme phenotypes (Dorso-Anterior Index 10) obtained with this treatment display a radial symmetry showing a circular 'retinal band' and a circular cement gland that occupies the circumference of the head (Kao et al., 1986). When stage 34 LiCl-treated embryos showing the extreme phenotype were subjected to whole-mount in situ hybridization with a *Xrx1* antisense probe, we detected a specific signal in an almost continuous band, probably corresponding to the described 'retinal band', above the circular cement gland (Fig. 6C). Thus, *Xrx1* seems to respond positively to dorsalizing signals as its expression domain

extends, becoming circular in dorsalized, radially symmetrical embryos.

2.8. Analysis of Xrx1 expression in secondary axes generated by noggin and Xwnt-8

Since extended dorsalizing signals elicited by LiCl treatment are able to induce ectopic expression of *Xrx1*, we asked what molecules could represent these signals. Recently, various secreted factors with dorsalizing activity have been identified (Kessler and Melton, 1994). Among them we analyzed *noggin* (Smith and Harland, 1993) and *Xwnt-8* (Smith and Harland, 1991), using their ability to induce a secondary axis as a dorsalization assay, and analyzing whether *Xrx1* is expressed in these secondary axes. To this purpose we injected pigmented embryos at the 4-cell stage with 100 pg of either *noggin* or *Xwnt-8* mRNA in a ventral-vegetal position. Embryos injected with *Xwnt-8* RNA displayed complete secondary axes while *noggin* RNA injections resulted in the formation of incomplete secondary axes, in agreement with published data (Lemaire et al., 1995). Whole-mount in situ hybridization of these embryos showed that *Xrx1* is expressed in *Xwnt-8* secondary axes, already at stage 17 (Fig. 6D,E), but not in *noggin* secondary axes (Fig. 6F); in these experiments *Xrx1* expression in the primary axes serves as an internal control. *Xotx2* is a gene involved in the specification of anterior regions, and its overexpression induces anterior body regions which do not show morphologically distinguishable eyes (Pannese et al., 1995); nonetheless, the eye is a strong site of *Xotx2* expression, indicating that this gene could play a role in eye formation (Kablar et al., 1996). To test whether *Xotx2* is able to activate *Xrx1*, we hybridized *Xotx2*-injected embryos with *Xrx1*. No *Xrx1* ectopic expression was observed (data not shown), thus suggesting that *Xrx1* is activated only by signals able to induce eye morphogenesis, like those mimicked by *Xwnt-8* injection.

3. Discussion

3.1. Xrx1 and Chx10 may have been ancestrally related

We have described the isolation and expression pattern of a novel *Xenopus* homeobox gene, that could be a representative of a new family of homeobox genes. After its pattern of expression we propose to name it *Xrx1*, for *Xenopus* retinal homeobox 1. The amino acid composition of its homeodomain leads us to propose that it may belong to the *paired*-like class of homeobox genes (Bürglin, 1994). It is to this class that the genes to which *Xrx1* shows the highest degree of homology actually belong. In fact, the homeodomain of *Xrx1* shows a fairly high degree of homology (66–70%) with a gene family comprising three members, as identified to date. One is the murine gene *Chx10*, which, as *Xrx1*, is expressed in the optic vesicle and neural retina (Liu et al., 1994); the second is its goldfish homologue *Vsx1*, expressed in the adult and regenerating retina (Levine et al., 1994); and the third is their *C. elegans* homologue *ceh-10*, which is expressed in an interneuron potentially implicated in photoreception (Svendsen and McGhee, 1995). Thus, these genes not only have in common with *Xrx1* homologies in the homeodomain, but also their patterns of expression are related to that of *Xrx1*. However, the assignment of *Xrx1* to the same gene family does not seem obvious, since its homology with the three genes does not extend to regions outside the homeodomain; on the contrary, *Chx10*, *ceh-10* and *Vsx-1* share another highly conserved region, the CVC domain, whose functional significance is not known (Svendsen and McGhee, 1995). We may then think that *Xrx1* and the family to which *Chx10*, *ceh-10* and*Vsx-1* belong could have been ancestrally related, and then have diverged during evolution.

3.2. Xrx1 is expressed during eye development

In situ hybridization experiments have revealed a very specific spatio-temporal pattern of *Xrx1* expression during *Xenopus* embryogenesis. *Xrx1* mRNA is first detected at the end of gastrulation in the anteriormost portion of the neural plate, and then its expression comes to coincide with a few forming forebrain structures: eyes, pineal gland, hypophysis and the diencephalon floor. Thus, *Xrx1* expression precedes any morphological appearance of these structures and then accompanies their development.

In the eyes, *Xrx1* mRNA is localized in the neural structures of the developing eye, that is the retina and the pigmented epithelium, while the lens, cornea and the other non-neural structures never show any *Xrx1* expression. The expression in the eye appears to be drastically reduced at around swimming tadpole stages, when differentiation of specific cell types in the maturing retina is almost complete, and the onset of visual function occurs. The last cells to differentiate are the photoreceptors, and among them the rods, which are not yet mature at swimming tadpole stages (Grant et al., 1980). Thus, it would be interesting to verify whether the reduced *Xrx1* expression observed at these stages is restricted to a specific retinal layer, and/or to specific cell types.

An interesting feature of *Xrx1* expression in the developing eye is seen when the staining reaction, at the end of the whole-mount procedure, is brought to an early stop, before staining could reach a plateau level: in these instances a stronger expression of *Xrx1* is observed in the ventral portion of the eye vesicle. The recent finding of asymmetrically distributed transcription factors along the A-P or D-V axis of the retina (see Section 1) supports models after which each cell in the retina comes to possess a unique array of molecules, that would give it a specific positional information along the two axes (Sperry, 1963).

This information is thought to be essential for the establishment of ordered connections between the retina and the optic tectum (reviewed in Holt and Harris, 1993). Due to its asymmetrical distribution, *Xrx1* could also be part of this positional information signalling network. Alternatively, or in addition, *Xrx1* stronger domain of expression could be related to the morphogenesis of the optic fissure: in fact, this structure originates from the ventral region of the optic cup marked by the stronger *Xrx1* expression domain, which, at later stages, appears to surround the optic fissure itself (see Fig. 5B).

The asymmetrical distribution of *Xrx1* in the eye has some similarities with the expression pattern of the murine gene *Pax2*, whose early domain of expression is in the ventral half of the developing optic cup only, to coincide later with the regions of invagination forming the optic fissure (Nornes et al., 1990). Thus, both *Pax2* and *Xrx1* are in a position to be involved in the formation of the optic fissure and/or migration of the ganglion cell axons through the fissure, into the optic stalks; and/or to regulate the expression of target genes in a localized manner, i.e. in the ventral portion of the developing eye. The possible relationships between the *Xenopus Pax2* homologue and *Xrx1* remain to be established.

3.3. Xrx1 demarcates the most anterior border of the neural plate

At early neural plate stages *Xrx1* is expressed in the most anterior regions of the neural plate. According to the fate map of the *Xenopus* neural plate, from these regions will originate the retina, optic stalks, and the structures of the ventral diencephalon, that is, the chiasmatic ridge and the hypothalamus (Eagleson et al., 1995): all these structures will express *Xrx1* at later stages.

One of the most controversial topics of neural development is what structures demarcate the rostralmost end of the neural tube. Several models have been proposed, and one of them places this boundary in the middle of the chiasmatic ridge (Puelles and Rubenstein, 1993; reviewed in Shimamura et al., 1995; but see also Kuhlenbeck, 1973). From this model, *Xrx1* can be considered one of the few homeobox genes expressed in the most anterior regions of the neural plate, and later of the body axis. In fact, many of the homeobox genes that are thought to be implicated in the development of anterior body regions, are excluded from this rostralmost region of the neuroectoderm. This is the case of *Xotx2*, whose expression at neurula stages is localized to the most anterior portion of all three germ layers, excluding the most anterior region of the neural plate (Pannese et al., 1995). In fact, as development proceeds, it can be seen that *Xotx2* expression is absent from the chiasmatic region (Kablar et al., 1996), that instead expresses *Xrx1*. In *Xenopus*, genes expressed in this anteriormost region are the homologues of the *Drosophila Distal-less* gene, *X-dll3* and *X-dll4*, (Papalopulu and Kintner, 1993); in mouse, in addition to genes of the *Distal-less* class (Price et al., 1991), the newly found *Six3* homeobox gene also has an expression that covers the chiasmatic region (Oliver et al., 1995). Thus, *Xrx1* can be considered as an early marker for the most anterior regions of the neural tube, and it may possibly be involved in the processes that establish positional information at the anterior boundary of the developing embryo.

3.4. Planar and vertical signals in Xrx1 expression

Classical studies have shown that contact with the underlying head mesoderm (hypomandibular pockets) has an essential action for a correct development of the eye (Lehmann, 1938). This has been confirmed by recent studies: in fact, anterior structures, including eyes, are not formed in complete exogastrulae, possibly because of the requirement of vertical inductive signals (Ruiz i Altaba, 1992). We were not able to detect any expression of *Xrx1* in complete exogastrulae, in agreement with the hypothesis that planar signals are not sufficient to activate the genetic pathway leading to eye formation. The necessity of vertical signals to induce eye development is also confirmed by our analysis of LiCl-treated embryos, where the *Xrx1* expression domain is expanded. This suggests that the extra dorsal mesoderm induced in these embryos is able to activate ectopically *Xrx1* through a vertical route. In the attempt to characterize the nature of the dorsalizing signals able to induce *Xrx1* expression, we tested two factors with known dorsalizing activity, Xwnt-8 and noggin: specifically, we tested whether *Xrx1* is expressed in secondary axes induced by microinjection of *Xwnt-8* or *noggin* mRNA. It has previously been shown that *Xwnt-8* overexpression can induce complete secondary axes (Smith and Harland, 1991), while *noggin* microinjections produce secondary axes that lack differentiated anterior structures (Lemaire et al., 1995). Nonetheless, the expression of antero-posterior markers to determine the anterior extension of *noggin* partial secondary axes, has not been described. Using *Xrx1* as a marker of the anterior neural plate and of the eye, we asked whether the anteriormost regions of the embryo are specified in *noggin*-induced secondary axes, regardless of the absence of morphologically recognizable structures. Whole-mount in situ hybridization of embryos injected with either *Xwnt-8* or *noggin* mRNA showed that only secondary axes generated by *Xwnt-8* display expression of *Xrx1*. Moreover, overexpression of *Xotx2*, a gene strongly expressed in the eye and able to induce anterior regions but not eye structures, does not activate *Xrx1*. Taken together these data suggest that the dorsal signal(s) required for *Xrx1* activation, and mimicked by LiCl treatment and *Xwnt-8* overexpression, could be part of a complex signalling pathway including a cascade of inductions that refine positional information laid during gastrulation and leading to eye morphogenesis.

3.5. Is Xrx1 related to the establishment of circadian rhythms?

The nervous system of all vertebrates contains pacemaker tissues, possessing intrinsic oscillatory functions, that are directly light sensitive, as the retina and the pineal gland, or that receive input from the visual system, as the suprachiasmatic nuclei of the hypothalamus (Underwood, 1990; Cahill and Besharse, 1993). In general, the endogenous pacemaker, or biological clock, is conceived as an autoregulatory feedback loop, that can be influenced by the day-night physical cycle, to regulate the rhythmicity of most vertebrate physiological functions (Sassone-Corsi, 1994; Kay and Millar, 1995). In mammals, environmental lighting conditions are transduced by the pineal gland from a neuronal to an endocrine message, the rhythmic secretion of melatonin, which, in turn, controls the function of the hypothalamic-hypophyseal-gonadal axis (Underwood, 1990). Presently, the genetic and molecular mechanisms that underlie circadian rhythms are being elucidated, and efforts are also devoted to understanding the respective role of the clock components in lower vertebrates (Stehle et al., 1993; Borjigin et al., 1995; Minutini et al., 1995). Interestingly, *Xrx1* is expressed in structures where a rhythmic mode of function will be physically located, and these structures include components of the biological clock. Thus, the isolation of *Xrx1* may provide a first clue as to a genetic control, acting through development, eventually resulting in the establishment of the biological rhythms network. How *Xrx1* may exert this putative control is a matter of speculation, but we may hypothesise that it could act by playing a role in the determination and/or differentiation of specific cell types. Since both the retina and pineal gland, and possibly the hypothalamus as well (Foster et al., 1993), contain cell types implicated in photoreception, *Xrx1* could act by participating in the genetic control of photoreceptor formation. On the other hand, *Xrx1* expression in the hypophysis – which lacks photoreceptors, but is endowed with a rhythmic activity – makes the possible involvement of *Xrx1* in the molecular architecture underlying the establishment of a rhythmic function, such as the pulsatile hormone secretion, an attractive working hypothesis.

4. Experimental procedures

4.1. Library screening and cDNA sequence analysis

Approximately 1.2×10^6 p.f.u. of a λgt11 cDNA library prepared from stage 24/25 *Xenopus* embryos (kindly provided by I. Dawid) were screened at low stringency (50% formamide, 5 × SSC, 0.1% SDS, 0.3 mg/ml denatured yeast RNA, 37°C) by hybridization with two murine *Otp* cDNA probes (Simeone et al., 1994), one containing the whole homeobox region, the other spanning the 3′ region of the homeobox and 340nt downstream. Washing was carried out in 2 × SSC, 0.1% SDS at 48°C. Purified phage DNA was subcloned in pGEM3 (Promega Biotec) and sequenced on both strands using a Sequenase sequencing kit (US Biochemical) according to the supplier's instructions.

4.2. Xenopus laevis embryos

Induction of ovulation of females, in vitro fertilization and embryo culture were carried out as described by Newport and Kirschner (1982). Embryos were staged according to Nieuwkoop and Faber (1967).

4.3. RNA extraction and RNase protection

RNA from embryos was extracted according to Maniatis et al. (1989). For RNase protection the following ^{32}P-labelled antisense probes were synthesized using T7 or SP6 RNA polymerase: *Xrx1* (194 bp *AluI* fragment from the coding region), ODC (381 bp *BglII* fragment) (Isaacs et al., 1992). Total RNA (30 μg) was used for each sample. RNase digestion and electrophoresis were carried out according to Maniatis et al. (1989).

4.4. In situ hybridization

In situ hybridization on sections of paraffin embedded embryos was performed as described by Kablar et al. (1996). Synthetic RNA probes were transcribed from a cloned *AccI* fragment 400 nt long, containing the 3′ region of the homeobox.

Whole-mount in situ hybridization was performed on staged pigmented and albino embryos according to Harland (1991), as described by Kablar et al. (1996). The antisense or control sense-strand RNA probes were generated from linearized plasmids containing full-length cDNA inserts.

4.5. Exogastrulae

Complete exogastrulae were obtained as described by Kablar et al. (1996) and subsequently processed for whole-mount in situ hybridization when they reached stage 24 or stage 37. Ten exogastrulae have been assayed for each stage.

4.6. Retinoic acid, lithium chloride treatments and microinjection of embryos

Embryos were treated with a 30 min pulse of 10^{-6} M retinoic acid (RA) (Sigma) at stage 10^+. Lithium chloride (LiCl, 0.3 M) was applied for 10 min to 32-cell embryos. The dorso-anterior index (DAI), a measure of the effectiveness of some treatments, ranged between 8 and 10 in LiCl-treated embryos. Capped synthetic RNAs were generated by in vitro transcription of a full coding sequence of

Xwnt-8 or *noggin*, RNAs were resuspended in 88 mM NaCl, 5 mM Tris (pH 7.5) and injected in the vegetal pole region of one ventral blastomere at 4 cell stage for the pigmented embryos or in the vegetal pole region of one cell of two cell stage albino embryos, using a Drummond 'Nanoject' apparatus. During injection, embryos were cultured in 0.1 × MMR, 5% Ficoll 400. The medium was gradually replaced with 0.1 × MMR when embryos reached stage 20. Injected embryos were allowed to develop until uninjected control embryos reached stage 17, 23 or 34, and then processed for whole-mount in situ hybridization.

Acknowledgements

We wish to thank Igor Dawid for the *Xenopus* cDNA library, Richard Harland for the *Xenopus en2* probe and for the *Xwnt-8* and *noggin* cDNAs, and Jonathan Slack for the ODC probe. We also would like to thank Robert Grainger, Nick Foulkes and Paolo Sassone-Corsi for helpful comments, Yuri Bozzi and Robert Vignali for reading the manuscript, and Marzia Fabbri and Donatella De Matienzo for their invaluable technical assistance. This work was supported by grants from M.U.R.S.T., Progetti Finalizzati C.N.R. 'Ingegneria Genetica' and 'Progetto Strategico Oligonucleotidi Antisenso'. S.C. is a recipient of a PhD fellowship from the Italian government.

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