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Role of *Xrx1* in *Xenopus* eye and anterior brain development

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SUMMARY

The anteriormost part of the neural plate is fated to give rise to the retina and anterior brain regions. In *Xenopus*, this territory is initially included within the expression domain of the *bicoid*-class homeobox gene *Xotx2* but very soon, at the beginning of neurulation, it becomes devoid of *Xotx2* transcripts in spatiotemporal concomitance with the transcriptional activation of the *paired*-like homeobox gene *Xrx1*. By use of gain- and loss-of-function approaches, we have studied the role played by *Xrx1* in the anterior neural plate and its interactions with other anterior homeobox genes. We find that, at early neurula stage *Xrx1* is able to repress *Xotx2* expression, thus first defining the retina-diencephalon territory in the anterior neural plate. Overexpression studies indicate that *Xrx1* possesses a proliferative activity that is coupled with the specification of anterior fate. Expression of a *Xrx1* dominant repressor construct (*Xrx1-EnR*) results in a severe impairment of eye and anterior brain development. Analysis of several brain markers in early *Xrx1-EnR*-injected embryos reveals that anterior deletions are preceded by a reduction of anterior gene expression domains in the neural plate. Accordingly, expression of anterior markers is abolished or decreased in animal caps coinjected with the neural inducer *chordin* and the *Xrx1-EnR* construct. The lack of expansion of mid-hindbrain markers, and the increase of apoptosis in the anterior neural plate after *Xrx1-EnR* injection, indicate that anterior deletions result from an early loss of anterior neural plate territories rather than posteriorization of the neuroectoderm. Altogether, these data suggest that *Xrx1* plays a role in assigning anterior and proliferative properties to the rostralmost part of the neural plate, thus being required for eye and anterior brain development.

Key words: *Xrx1*, *Rx1*, *Otx2*, *Pax6*, *Six3*, *engrailed* repressor, *Xenopus laevis*, Anterior neural plate patterning, Eye, Forebrain

INTRODUCTION

Eye development is a multistep process that requires specific inductive signals and precise morphogenetic movements. Although classical experimental embryology studies have been fundamental to our current knowledge of eye formation (see Spemann, 1938), it is only recently that the genetic bases underlying this complex phenomenon have begun to be unraveled. Integration of genetic and developmental biology studies performed in *Drosophila* and vertebrates suggested that key regulatory genes in eye development have been conserved during evolution. Among these genes *eyeless* (*ey*), *sine oculis* (*so*), *eyes absent* (*eya*) and *dachshund* (*dac*) appear to play important roles in *Drosophila* as they have been shown to be necessary for eye formation and sufficient, when overexpressed, to induce ectopic eyes. Vertebrate homologues of *ey* (*Pax6*), *so* (*six* gene family) and *eya* (*Eya* genes) have been described and, at least in the case of *Pax6* and *Six3*, a functional role in vertebrate eye development has also been shown (reviewed in Oliver and Gruss, 1997). In vertebrates, the initial function of master regulators of eye development has probably to take place at early neurula stage, when the presumptive eye territories are first clearly determined. In fact, lineage tracing studies performed in *Xenopus* have identified the anterior neural plate as the region fated to give rise to the retina and the anterior brain (Eagleson and Harris, 1990; Eagleson et al., 1995). The expression domains of some homeobox genes appear to pattern the *Xenopus* anterior neural plate already at early neurula stage. This is the case of *Xotx* genes, expressed in presumptive forebrain and midbrain regions (Kablar et al., 1996) and *XBF-1*, *Xdll-3* and *Xemx* genes (Papalopulu and Kintner, 1993, 1996; Pannese et al., 1998), expressed in different presumptive forebrain areas. Another restricted class of homeobox genes is expressed in the most anterior part of the neural plate mainly confined to the eye prospective territories. Members of this class are the *Xenopus* homologues of *Pax6* (*Xpax6*, Hirsch and Harris, 1997; Li et al., 1997) and *Six3* (*Xsix3*, this work) as well as the recently isolated *paired*-like homeobox gene *Xrx1* (Casarosa et al., 1997; Mathers et al., 1997). Identifying the network of interactions occurring between these genes, represents a primary goal toward the understanding of eye and anterior brain patterning mechanisms.

We previously showed that *Xrx1* expression is first activated at the early neurula stage in the anterior neural plate by vertical signals from the dorsal mesoendoderm. Later on, *Xrx1* transcripts are detected in the neural structures of the developing eye and other anterior neural plate derivatives such as the pineal gland, the diencephalon floor and the hypophysis (Casarosa et al., 1997). Functional studies on this gene have shown that its overexpression in *Xenopus* results in ectopic retinal and neural tissue formation, while mouse embryos carrying a null allele of the *Xrx1* murine homologue, lack optic cups and display a reduction of brain structures (Mathers et al., 1997). Nevertheless, until now the functional relationship between *Xrx1* and other homeobox genes in patterning the anterior neural plate has not been investigated.

In the present work, we address the early role of *Xrx1* and regulatory interactions occurring between *Xrx1* and other anterior homeobox genes, making use of gain- and loss-of-function approaches available in the *Xenopus* system. Overexpression experiments, including analysis of anterior markers, indicate that *Xrx1* proliferative activity is linked to the specification of anterior fate. Inactivation of *Xrx1* function, performed microinjecting RNA encoding a Xrx1 engrailed repressor fusion protein (*Xrx1-EnR*), leads to a remarkable reduction of anterior neural plate territories, as judged by the expression of several anterior markers, causing a failure in eye and anterior brain formation. Analysis of apoptosis and hindbrain markers expression indicate that these effects are due to an early loss of anterior territories rather than a posteriorization of the neuroectoderm. Finally, we propose that *Xrx1* plays an early role defining prosencephalic territories in combination with *Xotx2*, *Xpax6*, *Xsix3* and *XBF-1* in the anterior neural plate.

MATERIALS AND METHODS

Embryos and histology

Induction of ovulation of females, in vitro fertilisation and embryo culture were carried out as described by Newport and Kirschner (1982). Staging was according to Nieuwkoop and Faber (1967). Histological examination was performed according to Casarosa et al. (1997).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed on staged embryos, as well as on animal caps, essentially as described by Harland (1991). The antisense or control sense-strand RNA probes from *Xotx2* (Pannese et al., 1995), *XAG-1* (Sive et al., 1989), *XBF-1* (Papalopulu and Kintner, 1996), *Krox20* (Bradley et al., 1993), *En2* (Hemmati-Brivanlou et al., 1991), *gsc* (Cho et al., 1991) and *Xotx-b* (kindly provided by Dr R. Harland) were generated from linearized plasmids using either digoxigenin or fluorescein RNA labelling mix (Boehringer).

For double whole-mount in situ hybridization, the embryos were hybridized with both probes at the same time under standard conditions. After detection of the first probe with BM purple (Boehringer), the alkaline phosphatase was inactivated in 100 mM glycine pH 2.2, 0.1% Tween-20 and the embryos blocked in MAB (100 mM maleic acid, 150 mM NaCl), 2% Boehringer blocking reagent and 20% heat-inactivated sheep serum. Following incubation with the second antibody, the alkaline phosphatase reaction was performed with Magenta-Phos (Sigma). In some cases (Fig. 1M-O), RNA encoding for β-galactosidase containing a nuclear localization signal was used as a tracer.

Preparation of constructs and PCR cloning

Capped sense *Xrx1* RNA was generated from T7TS-*Xrx1* clone consisting of the full-length *Xrx1* cDNA cloned in the expression vector T7TS. Capped antisense RNA for *Xrx1* was prepared from CS2-AS*Xrx1* obtained subcloning the full-length cDNA into CS2+ vector (Rupp et al., 1994).

The *Xrx1* construct lacking the OAR domain (ΔOAR) was prepared subcloning the *Ava*II fragment from T7TS-*Xrx1* into CS2+ plasmid. The *Xrx1-EnR* construct was prepared subcloning the *Ava*II fragment from T7TS-*Xrx1* in frame with the *Drosophila engrailed* repressor sequence (amino acids 1-296) contained in ENG-N vector (a kind gift of Dr Dan Kessler). *Xsix3*, *Xpax2* and *Xpax6* were cloned by RT-PCR from *Xenopus* stage 19 RNA. PCR products were subcloned using a pGEM-T vector system (Promega). Degenerate primers used for *Xsix3* amplification were designed based upon the amino acid sequences WPPGACEA and AMWLEAHYQ, which are specifically found only in mouse Six3 and not in other members of the Six family. Over the amplified region, Xsix3 predicted amino acid sequence was 100% identical to zebrafish Six3 (Seo et al., 1998) and 97% to mouse (Oliver et al., 1995) and chick (Bovolenta et al., 1998) Six3. For *Xpax2* amplification, degenerate primers based upon the evolutionary conserved Pax2 amino acid sequences IIRTKVQQ and YPTSTLAG were used. *Xpax2* amplified region was identical to the *Xpax-2a* (3) nucleotide sequence described by Heller and Brandli (1997). Similarly, *Xpax6* was amplified using degenerate primers corresponding to the *Pax6* evolutionary conserved amino acid sequences NLASEKQQ and QIEALEKE. *Xpax6* amplified region was found to be identical to *Xenopus Pax6* nucleotide sequence deposited in GenBank by Hollemann, Bellefroid and Pieler, GenBank accession number U67887.

Embryo microinjections and animal cap assay

Capped synthetic RNAs were generated by in vitro transcription of CS2-AS*Xrx1*, T7TS-*Xrx1*, ΔOAR, *Xrx1-EnR*, T7TS-*Xotx2* (Pannese et al., 1995) and *chordin* (Sasai et al., 1995). *Xpax6* capped RNA was transcribed from P6mycS (Hirsch and Harris, 1997), a generous gift of Dr William Harris, and its activity was tested by the ability to induce ectopic β-B1 crystallin expression (not shown, see Altman et al., 1997). Embryo microinjections were performed as described in Andreazzoli et al. (1997). Animal caps were dissected out of stage 8-9 embryos in 1× MBS and, after healing, they were cultured in 0.5× MBS. When sibling control embryos reached stage 12.5, animal caps were fixed and stored in ethanol at −20°C.

TUNEL staining

Whole-mount TUNEL staining was performed as described in Hensey and Gautier (1998).

RESULTS

Effects of *Xrx1* RNA microinjection on the expression of anterior genes

Xrx1 is expressed in the anterior neural plate at the end of gastrulation and subsequently in the neural structures of the developing eye and other forebrain structures derived from the anterior neural plate (Casarosa et al., 1997; Mathers et al., 1997). This expression pattern raises the question whether *Xrx1* plays a role in the specification of anterior neural plate regions and structures that derive from it. As a first approach to study the role of *Xrx1* during development, we overexpressed this gene by microinjection of its capped RNA into single blastomeres of 2-, 4- and 8-cell stage *Xenopus* embryos. We found that tadpoles developed from embryos injected, at 8-cell stage, into a dorsal animal blastomere, which

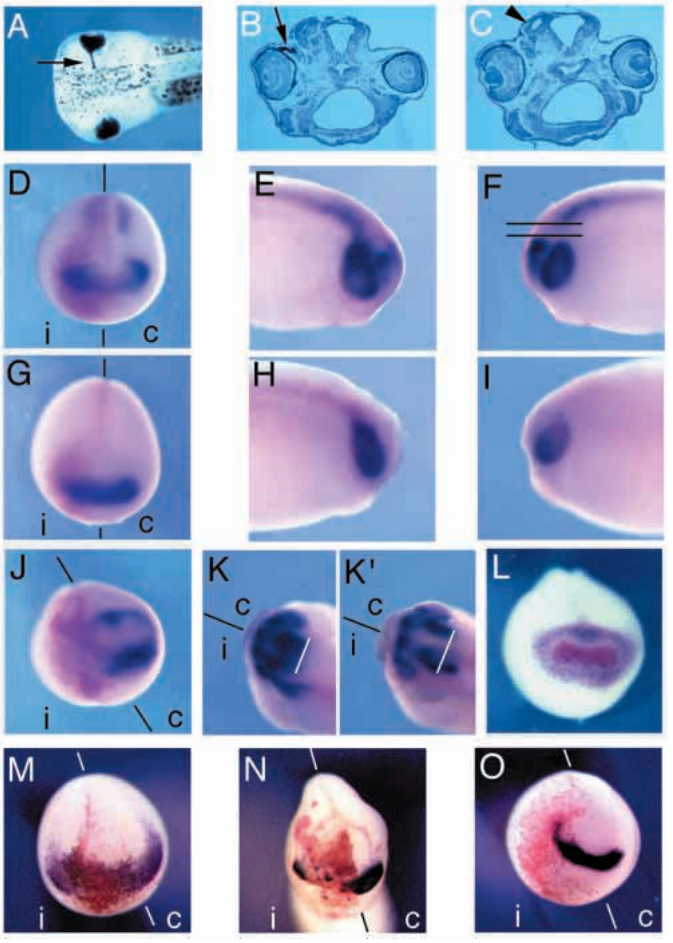

Fig. 1. Effects of *Xrx1* overexpression. (A-C) Embryos microinjected with *Xrx1* RNA in the right dorsoanimal blastomere at 8-cell stage. The arrows point to ectopic pigmented retina located between eye and diencephalon. (B,C) Transverse sections of an embryo similar to the one shown in A; (B) a section where the ectopic pigmented retina is visible (arrow); (C) a more posterior section where the duplication of the neural tube becomes evident (arrowhead). (D-O) Whole-mount in situ hybridization analysis of embryos microinjected with *Xrx1* RNA at 8-cell stage in one dorsoanimal blastomere. (D-K′) The staining pattern of the gene of interest (blue) on the injected side (i) should be compared with that on the uninjected control side (c). In the same panels, the distribution of *Xrx1*-injected RNA is visualized by cohybridization with *Xrx1* antisense RNA revealed by magenta staining. (M-O) Nuclear β-gal RNA has been used as a tracer and the β-galactosidase activity is represented by the red staining. (D-F) Expression of *Xpax6* (blue) in *Xrx1*-injected embryos. (D) *Xpax6* expression at stage 13 is not significantly affected by *Xrx1* overexpression; (E,F) *Xpax6* expression in the injected and control side of a stage 23 embryo, respectively. Note that the normal *Xpax6* gap of expression in the midbrain present in the control side (area between lines) has been filled by ectopic *Xpax6* expression in the injected side of the embryo. (G-I) Expression of *Xsix3* (blue) in *Xrx1*-injected embryos. (G) Expression of *Xsix3* at stage 13 is not affected by *Xrx1* RNA injection. (H,I) *Xsix3* expression in the injected and control side of a stage 23 embryo, respectively. The eye expression of *Xsix3* in the injected side appears expanded dorsoposteriorly when compared to the control side. (J,K,K′) Expression of *Xotx2* (blue) in *Xrx1*-injected embryos. (J) Expression of *Xotx2* at stage 13 is repressed by *Xrx1* overexpression. (K,K′) Examples of *Xotx2* expression in stage 23 *Xrx1*-injected embryos where *Xotx2* expression is extended laterally (K) or posteriorly (K′) in the injected side. The white line marks the posterior boundary of *Xotx2* expression in the control side. (L) Double whole-mount in situ hybridization performed on a stage 13 normal embryo showing the complementarity of *Xrx1* (magenta) and *Xotx2* (blue) expression domains. (M,N) Expression of *XAG-1* (blue) in *Xrx1*-injected embryos. (M) Expression of *XAG-1* is repressed at stage 13 by *Xrx1* overexpression. (N) *XAG-1* is ectopically activated in the injected side of a stage 23 embryo. (O) Expression of *XBF-1* (blue) in *Xrx1*-injected embryos at stage 13. *XBF-1* expression is expanded laterally on the injected side.

is fated to give rise to dorsoanterior regions of the embryo (Huang and Moody, 1993), very frequently show the presence of ectopic pigmented epithelium (Fig. 1A,B; Table 1) often associated with an overproliferating neural retina and neural tube (Fig. 1C and data not shown), in agreement with the work done by Mathers et al. (1997). Despite of a broader distribution of the injected RNA, the ectopic pigmented epithelium and neural tissue are always localized in a region comprising tissue between the eye and the brain. This area derives from a region of the anterior neural plate that is initially competent to become retina but this fate is later suppressed by signals coming from the prechordal plate (Li et al., 1997). These observations raise the possibility that *Xrx1* may be able to exert its function only in a spatially restricted area because it needs to interact with other eye-brain-specific transcription factors. For this reason, we analyzed the expression of several anterior genes in *Xrx1*-injected embryos at early neurula (stage 13) and tailbud (stage 24) stages.

Embryos injected with *Xrx1* RNA in a dorsal-animal blastomere at 8-cell stage were analyzed at later stages by double whole-mount in situ hybridization using as probes digoxigenin-labelled antisense RNA for the gene of interest, together with fluorescein-labelled antisense *Xrx1* RNA. This allowed us to detect both the distribution of injected *Xrx1* RNA (magenta staining) and the expression of the gene that we wanted to test (blue staining). Since the signal given by hybridization of *Xrx1* antisense probe with the injected RNA was stronger and appeared much earlier than endogenous *Xrx1* signal, the staining reaction could be stopped when only injected *Xrx1* RNA was detected.

Analysis of *Xpax6* expression in *Xrx1*-injected embryos showed that, while at stage 13 the expression of this gene does not appear to be affected (77% normal expression, 23% slightly reduced expression, *n*=93; Fig. 1D), an effect is observed at stage 24. At this later stage, *Xpax6* normal expression, which can be seen in the uninjected control side of the embryos, is detected in the optic vesicle, forebrain, hindbrain and spinal cord with a characteristic gap of expression in the midbrain (Fig. 1F). Interestingly, inspection of the injected side of the embryos revealed that *Xpax6* expression had expanded dorsally to the eye vesicle, thus filling the gap (67%, *n*=30; Fig. 1E).

A similar response to *Xrx1* overexpression was observed analyzing *Xsix3* expression. As in the case of *Xpax6*, *Xsix3* expression did not appear to be affected at early stage (100%, *n*=35; Fig. 1G) while it was expanded at tailbud stage (54%, *n*=28; Fig. 1H). *Xsix3* ectopic expression is observed as a dorsal expansion of the eye expression domain of this gene.

Xotx2 displays a dynamic pattern of expression during early development. At the end of gastrulation (stage 12), *Xotx2* is

Table 1. Effects of microinjection of *Xrx1* constructs and rescue experiments

RNA inject. (pg)	*n*	Stage	% ectopic pigmented epithelium	% reduced eyes	% anterior reductions	% minor defects	% normal embryos
Xrx1 350	242	1DA/8C	60	0	0	3	37
Xrx1 antisense RNA 1100	70	1DA/8C	0	17	6	0	77
ΔOAR 400	72	2DA/8C	0	29	3	10	58
Xrx1-EnR 400	118	2DA/8C	0	52	28	0	20
Xrx1-EnR+Xrx1 400+600	151	2DA/8C	28*	12	2	4	54
Xrx1EnR+Xpax6 400+150	220	2DA/8C	0	60‡	36‡	0	4
Xrx1-EnR+Xotx2 400+600	121	2DA/8C	0	50‡	40‡	0	10
Uninjected	520		0	0	0	3	97

RNAs were injected into one or two dorsal-animal blastomeres at 8-cell stage, indicated as 1DA/8C and 2DA/8C, respectively.
*These embryos were composed by 33% embryos with eyes of regular size and 67% embryos displaying slight eye reduction.
‡These embryos also displayed posterior deficiencies.

expressed in all the presumptive anterior neuroectoderm but subsequently (stages 12.5-13) its expression appears to be repressed in the most anterior part of the neural plate (Pannese et al., 1995; Kablar et al., 1996). In order to define the relationship between territories expressing *Xotx2* and *Xrx1* at early neurula stage, we performed a double in situ hybridization. As can be seen in Fig. 1L, the two genes show complementary expression domains with *Xrx1* being activated in the place and time corresponding to *Xotx2* repression. *Xrx1* and *Xotx2* expression domains remain almost completely mutually exclusive for most of neurulation but at tailbud stage, because of *Xotx2* activation in the eye vesicles and in the diencephalon, the two expression domains largely overlap. In agreement with the observed concomitance between appearance of *Xrx1* transcripts and downregulation of *Xotx2* at early neurula stage, we found that *Xrx1* overexpression is able to repress *Xotx2* at this same stage (95%, *n*=60; Fig. 1J). However, at tailbud stage, *Xotx2* shows a different response to *Xrx1* overexpression, being ectopically activated (53%, *n*=68) laterally (Fig. 1K) or dorsally (Fig. 1K′). A similar effect is observed for the cement-gland-specific marker *XAG-1*, which is repressed by *Xrx1* overexpression at early neurula stage (78%, *n*=45; Fig. 1M) but ectopically activated at tailbud stage (67%, *n*=42; Fig. 1N). The early repressive effects of *Xrx1* on *Xotx2* and *XAG-1* find a correlation with the spatial relationship between the expression domains of these three genes in the early neurula. On the contrary, the later ectopic expression of *Xpax6*, *Xsix3* and *Xotx2* coincides with the area where the hyperproliferative activity of injected *Xrx1* takes place (see Fig. 1A-C and data not shown) and should be considered an attribute of the proliferated tissue. The anterior character of the proliferating tissue is also confirmed by the late ectopic activation of *XAG-1*. This gene, which is known to be inducible by *Xotx2* (Andreazzoli et al., 1997; Gammill and Sive, 1997), has only been found ectopically expressed in the anterior ventrolateral ectoderm, in keeping with the observation that dorsal ectoderm is not competent to form cement gland (Gammill and Sive, 1997). We also analyzed the expression of *XBF-1*, an early marker of the presumptive telencephalon, in *Xrx1*-injected embryos. Ectopic activation of *XBF-1* at early neurula stage is observed to expand laterally, seemingly along the border of the neural plate (65%, *n*=20; Fig. 1O). Another gene that we analyzed, because of its interesting expression pattern, was *Xpax2*. At stage 24, *Xpax2* major expression sites in the head region are the ventral optic vesicles, otic vesicles and midbrain-hindbrain boundary. While *Xrx1* overexpression does not affect the ventral optic vesicle domain and, in some cases, only slightly reduces *Xpax2* expression in the otic vesicle, a strong repressive effect is observed in the midbrain-hindbrain expression domain (83%, *n*=24; Fig. 2A,B). In order to see if this effect was restricted to *Xpax2*, we analysed the expression of *En2*, which is also specifically expressed in the midbrain-hindbrain boundary. As shown in Fig. 2C, *En2* expression is also repressed by *Xrx1* overexpression (85%, *n*=26). To extend this analysis to the hindbrain, we used *Krox20* as a marker and found that, in *Xrx1*-injected embryos, *Krox20* expression in rhombomere 3 was almost totally suppressed while expression in rhombomere 5 was only reduced (100%, *n*=20; Fig. 2D). No significant change in spinal cord expression of *Xpax6* and *Xpax2* was observed (Fig. 1D,E and data not shown) suggesting that the effects of *Xrx1* overexpression do not extend to the posterior nervous system.

Inactivation of *Xrx1* function

As a complementary study to analyse *Xrx1* function during embryogenesis, we used different approaches with the aim of generating a functional inactivation of this gene. To this purpose, we first used a method that utilizes the microinjection of capped antisense RNA (Steinbeisser et al., 1995; Epstein et al., 1997). We observed an effect microinjecting *Xrx1* antisense RNA in a dorsal-animal blastomere at 8-cell stage, only when the dose of injected RNA reached 1100 pg. In this case, about a quarter of injected embryos displayed anterior deficiencies that ranged from reduced eyes to more severe suppression of anterior head development (Table 1). Further increase in the amount of microinjected RNA only led to various aspecific defects in embryo development.

As a second approach, we microinjected the embryos with

RNA generated from a construct (ΔOAR) lacking the putative transactivation domain of *Xrx1* (Fig. 3A). Expression of similar truncated proteins has been shown to cause a passive repression of target genes (Jaynes and O'Farrell, 1991; Epstein et al., 1997). Although transactivation domains have not been functionally mapped in any of the homologues of *Xrx1*, sequence comparison analysis revealed the presence of an amino acid motif, located at the carboxy-terminus, conserved between proteins of the Rx family and other paired-like homeodomain proteins (Furukawa et al., 1997). Since this motif, which was named OAR domain, acts as a transcriptional activation domain in Orthopedia protein (Simeone et al., 1994), we reasoned that it could play a similar role in Xrx1 as well. Microinjection of ΔOAR RNA did not produce any of the effects described for wild-type *Xrx1* overexpression, while about one third of the injected embryos showed the same anterior defects observed in *Xrx1* antisense injection experiments (Table 1).

Looking for a more efficient way of inactivating *Xrx1* function, we prepared a construct encoding a chimeric protein in which the truncated form of *Xrx1* lacking the OAR domain was fused in frame with the repressor domain of *Drosophila engrailed* (*Xrx1-EnR*, Fig. 3A). Embryos obtained after microinjection of RNA generated from this construct, showed strongly underdeveloped or, in extreme cases, absent eyes often associated with a reduction of anterior brain (Fig. 3B). These phenotypes closely resemble those obtained after microinjection of *Xrx1* antisense or ΔOAR RNAs with the difference that the frequency of affected embryos in the case of *Xrx1-EnR* injection was much higher (Table 1). To test if *Xrx1-EnR* specifically antagonises *Xrx1* function, we coinjected both transcripts in dorsoanimal blastomeres at 8-cell stage. About 70 % of coinjected embryos showed a complete or partial rescue of eye and anterior head structures (Fig. 3C; Table 1). As already described for this kind of rescue experiments (Conlon et al., 1996; Isaacs et al., 1998), in many cases the effects of *Xrx1* overexpression became evident before the *Xrx1-EnR* anterior defects were completely rescued. This resulted in embryos displaying slightly reduced eyes together with ectopic pigmented retina (Fig. 3C). We also tested if *Xotx2* or *Xpax6*, two genes involved in eye and anterior brain development, were able to rescue *Xrx1-EnR* phenotype. We found that neither *Xotx2* nor *Xpax6* could substitute for *Xrx1* and rescue *Xrx1-EnR* defects (data not shown). As a matter of fact, embryos coinjected with *Xrx1-EnR* and *Xotx2* or *Xpax6* showed both anterior deficiencies and posterior defects, the latter being typical of *Xotx2* and *Xpax6* overexpression (Pannese et al., 1995; Hirsch and Harris, 1997). Therefore these data suggest that *Xrx1-EnR* specifically interferes with endogenous Xrx1 binding to target genes.

Both inspection of external morphology and histological examination (data not shown) suggested that the inactivation of *Xrx1* leads not only to the lack of optic vesicles, but also to deletion of telencephalic regions where the gene is not expressed at late stages. This prompted us to check if at early stages *Xrx1* was expressed in telencephalic presumptive regions. Overlapping of *Xrx1* and *XBF-1* expression domain at stage 12.5 revealed that, indeed, *Xrx1* expression encompasses also presumptive telencephalic territories at early neurula stage (Fig. 3D,E).

Xrx1-EnR RNA microinjection leads to reduced expression domains of anterior neural plate markers

In order to better define these phenotypes, embryos injected with *Xrx1-EnR* RNA in both dorsoanimal blastomeres at 8-cell stage were examined for alterations in the expression of several anterior markers (Fig. 4). At stage 13, the anterior expression domains of *Xotx2*, *Xpax6* and *Xsix3* appeared to be remarkably reduced in size and, in some instances, also in intensity (*Xotx2*, 100%, *n*=26; *Xpax6*, 100%, *n*=24; *Xsix3*, 100%, *n*=28; Fig. 4B,F,J) compared to control uninjected embryos (Fig. 4A,E,I). *XBF-1* expression, which at stage 13 labels the presumptive telencephalon (Papalopulu and Kintner, 1996; Fig. 4M), is either strongly repressed or completely suppressed (50% with reduced expression, 50% with no expression, *n*=18; Fig. 4N and data not shown). Our interpretation of these data (see Discussion) is that *Xrx1* is required for proper formation of anterior neural plate territories and that this has a bearing on the phenotypes and gene expression patterns observed at tailbud stage for *Xrx1-EnR*-injected embryos. Analysis of *Xsix3* expression at stage 24 showed that optic vesicles expression was absent, while a residual forebrain expression probably corresponding to the diencephalon was still detected (100%, *n*=32; Fig. 4L). Similarly, at this later stage, *Xpax2* optic vesicles expression was repressed while expression domains corresponding to midbrain-hindbrain boundary, otic vesicles, hindbrain and spinal cord were still present (100%, *n*=18; Fig. 4P). In the case of *Xpax6*, besides expression in the spinal cord, only a residual signal is found in the most anterior dorsal region of the embryo, while no obvious optic vesicles expression is detectable (100%, *n*=18; Fig. 4H). Stage 24 *Xotx2* expression domain also appeared to be spatially reduced in the anterior part (100%, *n*=16; Fig. 4D) compared to control embryo (Fig. 4C). Nevertheless, the posterior boundary of expression, corresponding to the posterior end of the midbrain, appeared to be sharply defined as it is in normal embryos. A very similar pattern was also observed in *Xrx1-EnR*-injected embryos hybridized with *Xotx1*, a gene that shares with *Xotx2* the same posterior boundary of expression (data not shown). Since expression analysis of *Xpax6*, *Xsix3*, *Xotx2* and *Xotx1* in *Xrx1-EnR*-injected embryos suggests that some region anterior to the rhomboencephalon-mesencephalon boundary are present in these embryos, we tested the presence of a diencephalic marker. We used *Xotx-b* (gift of Dr Harland) which at stage 24 is expressed primarily in the pineal gland (Fig. 4S). As shown in Fig. 4T, *Xotx-b* is expressed in *Xrx1-EnR*-injected embryos, although at lower level compared to control embryos (100%, *n*=15), thus indicating that at least part of dorsal diencephalon is present. We then asked whether the anterior deletion observed upon inactivation of *Xrx1* function may reflect a reduction of the anterior mesoendoderm. Using *goosecoid* (*gsc*) as a marker of this region (Cho et al., 1991), we did not observe any significant difference in *gsc* expression comparing *Xrx1-EnR*-injected and control embryos (Fig. 4Q,R; *Xrx1-EnR*-injected embryos, 84% normal expression, 16% slightly reduced expression; *n*=51).

Xrx1-EnR activity in *chordin*-injected animal caps

In order to study the activity of *Xrx1-EnR* in a simplified system that could mimic the anterior neural plate, we analyzed the effects of *Xrx1-EnR* in animal caps neuralized by *chordin* (Sasai et al., 1995) RNA microinjection. For this analysis we

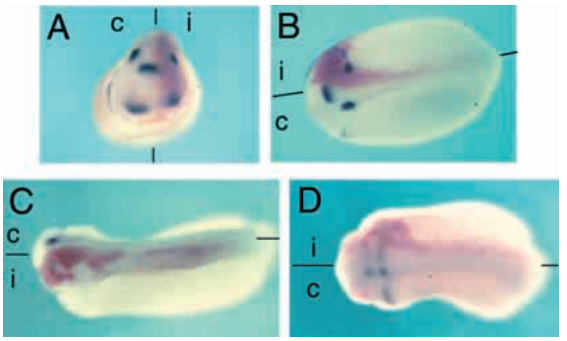

Fig. 2. Effects of *Xrx1* RNA microinjection in one blastomere at 8-cell stage on *Xpax2*, *En2* and *Krox20* expression. The staining pattern of the gene of interest (blue) on the injected side (i) should be compared with that on the uninjected control side (c). The distribution of *Xrx1*-injected RNA is visualized by cohybridization with *Xrx1* antisense RNA (magenta staining). (A,B) Expression of *Xpax2* in stage 23 *Xrx1*-injected embryos. (A) Frontal view showing repression of *Xpax2* expression in the midbrain-hindbrain boundary but not in ventral optic vesicles; the dorsal side of the embryo is on the top. (B) Dorsal view of another embryo showing strong repression in the *Xpax2* midbrain-hindbrain expression domain and a weak reduction of the otic vesicle domain. (C) Repression of *En2* expression in a stage 24 *Xrx1*-injected embryo. (D) Expression of *Krox20* in a stage 24 *Xrx1*-injected embryo. *Krox20* expression at the level of rhombomere 5 appears to be reduced while rhombomere 3 expression domain is almost completely abolished. (B-D) The anterior part of the embryo is oriented to the left.

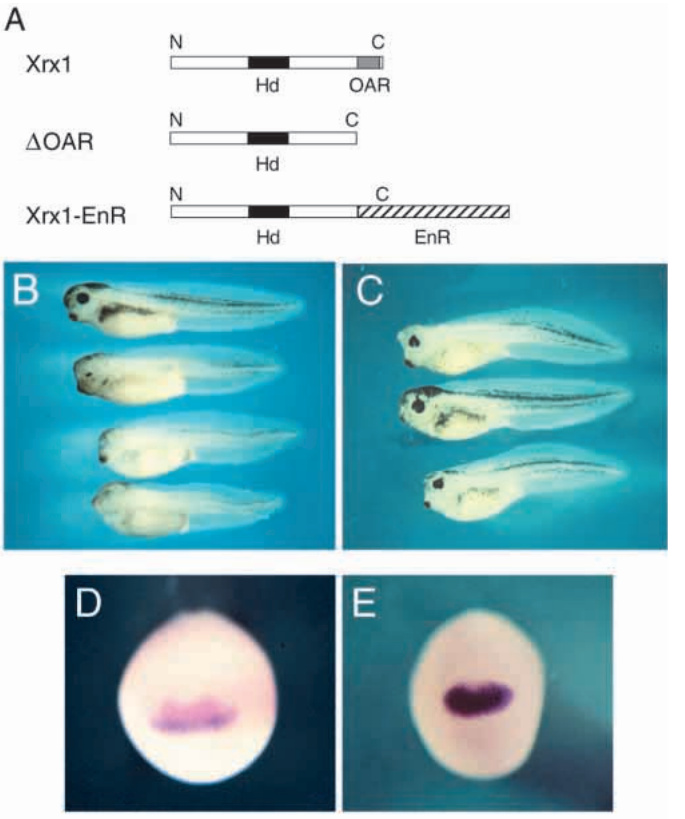

Fig. 3. Effects of *Xrx1-EnR* RNA microinjection on eye and anterior head development. (A) Schematic diagrams of *Xrx1* constructs used in microinjection experiments. The homeodomain (Hd), OAR domain (OAR) and *engrailed* repressor domain (EnR) are indicated as well as the amino- and carboxy-termini of the proteins (N and C, respectively). (B) Stage 41 embryos resulting from microinjection of 400 pg *Xrx1-EnR* RNA in both dorsoanimal blastomeres at 8-cell stage. The top embryo is an uninjected control. (C) *Xrx1* rescues the *Xrx1-EnR* phenotypes. Embryos were coinjected with 400 pg of *Xrx1-EnR* RNA and 600 pg of *Xrx1* RNA per blastomere in both dorsoanimal blastomeres at 8-cell stage and analyzed at stage 41. (D,E) Expression of *Xrx1* in presumptive forebrain regions of stage 12.5 normal embryos. (D) Double whole-mount in situ hybridization with *Xrx1* (magenta) and the forebrain-specific marker *XBF-1* (blue). *Xrx1* expression partially overlaps with that of *XBF-1*. (E) Control embryo hybridized with *Xrx1* alone (blue).

focused on the expression of *XBF-1*, *Xpax6* and *Xotx2*, which are among the genes whose expression domain is reduced by *Xrx1-EnR* injection already at early neurula stage. As expected from the described forebrain-like neuralization induced by *chordin*, we found that all these anterior genes are activated in *chordin*-injected caps (Fig. 5A-C; 100% positive in all cases, *XBF-1*, *n*=26; *Xpax6*, *n*=24; *Xotx2*, *n*=27), while no expression was detected in uninjected control caps (0% in all cases, *XBF-1*, *n*=18; *Xpax6*, *n*=15; *Xotx2*, *n*=15; not shown). Coinjection of *chordin* and *Xrx1-EnR* leads to a strong suppression of *XBF-1* (Fig. 5D; 88% negative, 12% weak positive; *n*=42) and *Xpax6* (Fig. 5E; 75% negative, 25% weak positive; *n*=36) expression but only to a moderate inhibition of *Xotx2* activation (Fig. 5F; 76% positive, 18% weak positive, 6% negative; *n*=38). It is worth noting that *XBF-1*, which is the most affected gene by *Xrx1-EnR* in animal caps, is also the only gene whose expression is completely abolished in 50% of *Xrx1-EnR*-injected embryos.

Causes for anterior truncations in *Xrx1-EnR*-injected embryos

The anterior truncations observed in *Xrx1-EnR*-injected embryos could be due to a posteriorization of the anterior neuroectoderm or, alternatively, to an early loss of anterior territories. To distinguish between these two possibilities, we performed two series of experiments. In the first set of experiments, we asked whether hindbrain territories were enlarged as a consequence of posteriorization in *Xrx1-EnR*-injected embryos comparing the expression domains of *En2* and *Krox20* in injected and control embryos at tailbud stage. As shown in Fig. 6, the expression domains of *En2* and *Krox20* in *Xrx1-EnR*-injected embryos (Fig. 6B,D, respectively) do not appear to differ significantly in size and shape (*En2*, 88% normal expression, 12% slightly reduced expression; *n*=42; *Krox20*, 87% normal expression, 13% slightly reduced expression; *n*=40) from those of uninjected control embryos (Fig. 6A,C, respectively). In the second set of experiments, we looked at the rate of apoptosis occurring in injected and control embryos making use of the TUNEL technique. For best comparison, embryos were analyzed at stage 12 when apoptosis, normally occurring during development, is at low levels especially in the prospective anterior neural plate (Hensey and Gautier, 1998). At this stage, *Xrx1-EnR*-injected embryos showed an accumulation of apoptotic nuclei in the anteriormost part of the embryo mainly corresponding to the presumptive anterior neuroectoderm (87% with exclusively anterior signal, 13% with also some dorsally sparse signal;

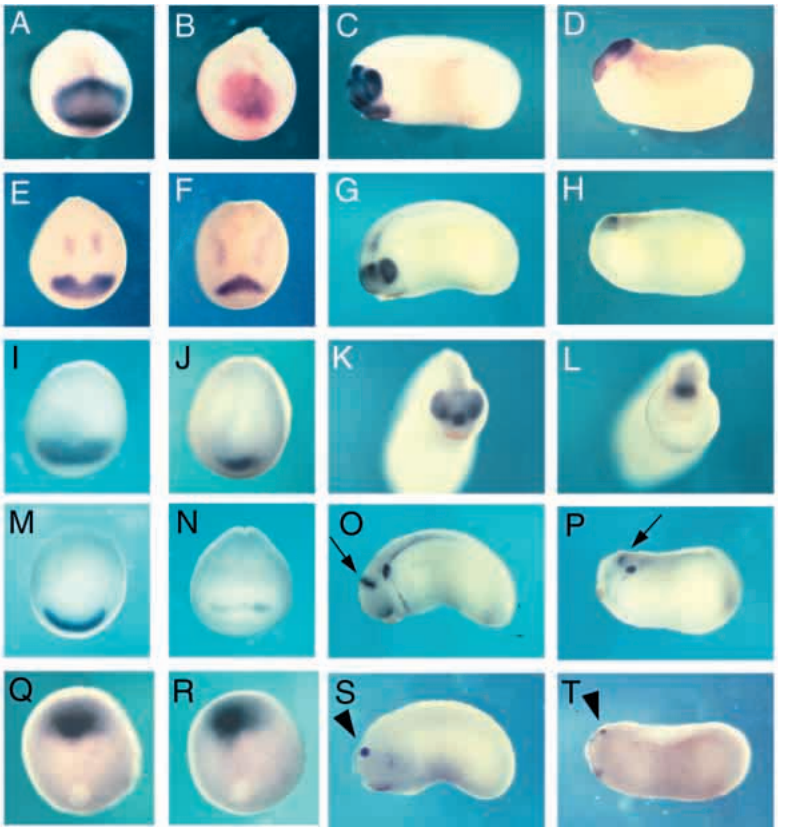

Fig. 4. Effects of *Xrx1-EnR* RNA microinjection in both dorsoanterior blastomeres at 8-cell stage on the expression of anterior genes. (A-D) Embryos analyzed for *Xotx2* expression. (A,C) Dorsoanterior and lateral views of the normal expression in stage 13 and stage 24 embryos, respectively. (B,D) Dorsoanterior and lateral views of embryos at stage 13 and 24, respectively, injected with *Xrx1-EnR* RNA. (E-H) Embryos analyzed for *Xpax6* expression. (E,G) Dorsoanterior and lateral views of the normal expression in stage 13 and stage 24 embryos, respectively. (F,H) Dorsoanterior and lateral views of embryos at stage 13 and 24, respectively, injected with *Xrx1-EnR* RNA. (I-L) Embryos analyzed for *Xsix3* expression. (I,K) Dorsoanterior and frontal views of the normal expression in stage 13 and stage 24 embryos, respectively. (J,L) Dorsoanterior and frontal views of embryos at stage 13 and 24, respectively, injected with *Xrx1-EnR* RNA. (M,N) Embryos analyzed at stage 13 for *XBF-1* expression (dorsoanterior views). (M) Control uninjected embryo. (N) Embryo injected with *Xrx1-EnR* RNA. (O,P) Embryos analyzed at stage 24 for *Xpax2* expression (lateral views). (O) Control uninjected embryo. (P) Embryo injected with *Xrx1-EnR* RNA. Arrows indicate *Xpax2* expression domain corresponding to the midbrain-hindbrain boundary. (Q,R) Embryos analyzed at stage 12.5 for *gsc* expression (dorsal views). (Q) Control uninjected embryo. (R) Embryo injected with *Xrx1-EnR* RNA. (S,T) Embryos analyzed at stage 24 for *Xotx-b* expression (lateral views). (S) Control uninjected embryo. (T) Embryo injected with *Xrx1-EnR* RNA. Arrowheads indicate *Xotx-b* expression in the pineal gland.

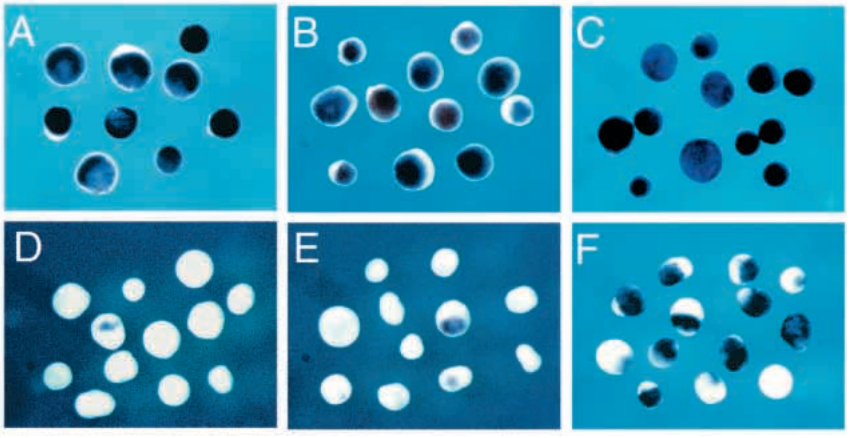

Fig. 5. Coinjection of *chordin* and *Xrx1-EnR* in animal caps. (A-C) Animal caps dissected from embryos microinjected with *chordin* RNA. (D-F) Animal caps dissected from embryos coinjected with *chordin* and *Xrx1-EnR* RNAs. The probes used were *XBF-1* (A,D), *Xpax6* (B,E) and *Xotx2* (C,F).

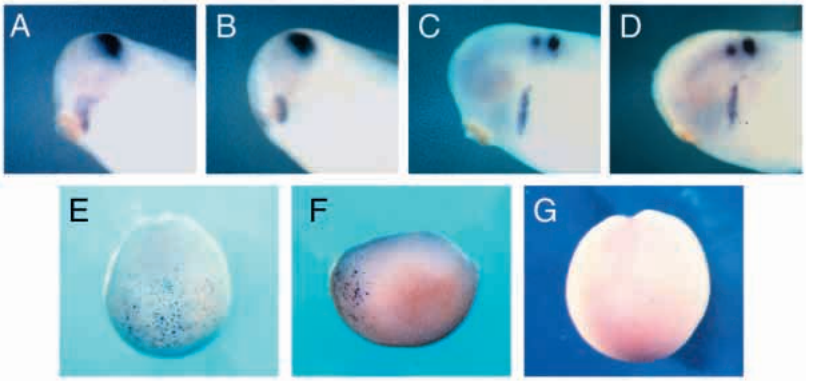

Fig. 6. (A-D) *En2* (A,B) and *Krox20* (C,D) expression in uninjected (A,C) and *Xrx1-EnR*-injected embryos (B,D), as observed at stage 28. (E-G) TUNEL staining in stage 12 embryos. Dorsal-anterior (E) and lateral (F) views of *Xrx1-EnR*-injected embryos are shown. (G) Dorsal-anterior view of a control uninjected embryo. In E and G, posterior is to the top and anterior to the bottom. In F, dorsal is to the top and anterior to the left.

n=39; Fig. 6E,F). No significant signal was observed in control uninjected embryos (Fig. 6G). Together with the reduction of expression of anterior markers (Fig. 4), these data suggest that anterior deficiencies of *Xrx1-EnR*-injected embryos are due to an early loss of anterior neural plate territories rather than a transformation of anterior neuroectoderm into more posterior neural areas.

DISCUSSION

In this study, making use of gain- and loss-of-function approaches, we aimed at better understanding the role of the *paired*-like homeobox gene *Xrx1* and its relationships with other homeobox genes in the establishment and patterning of the anterior neural plate.

Homeobox genes in early anterior neural plate patterning

One of the earliest genes to be expressed in the presumptive anterior neuroectoderm already at the end of gastrulation is the *bicoid*-class homeobox gene *Xotx2*. This gene shows a dynamic expression in this region being repressed in the anteriormost part of the neural plate at the beginning of neurulation. We noticed that this anterior repression of *Xotx2* coincides spatially and temporally with the first appearance of *Xrx1* transcripts and, in fact, a double whole-mount in situ hybridization showed an almost complete complementarity between the expression pattern of these two genes at stage 12.5. At this time, *Xrx1* is also expressed in presumptive telencephalon where it overlaps with the expression of *XBF-1* and partially with that of *Xotx2*. Thus, different combinations of gene expression appear to pattern the anterior neural plate and define specific territories. The area expressing *Xrx1* but neither *Xotx2* nor *XBF-1* is fated to give rise to retina and diencephalon territories (Eagleson and Harris, 1990; Eagleson et al., 1995). Even if not perfectly overlapping with *Xrx1*, *Xpax6* and *Xsix3* are also expressed in this region. The neural plate area where *Xrx1*, *XBF-1* and *Xotx2* are coexpressed corresponds to the presumptive telencephalon while, ventrally to *Xrx1* expression domain, the cement gland presumptive region is marked by the expression of *XAG-1* and, in part, *Xotx2*. The lack of apparent activation of *Xpax6* and *Xsix3* by *Xrx1* overexpression in stage 13 embryos may suggest that these two genes are not downstream of *Xrx1* at least at this stage, although other explanations cannot be formally excluded (see Discussion in the following section). Moreover, the inability of injected *Xpax6* RNA both to rescue the *Xrx1-EnR* phenotypes and to modify *Xrx1* expression (data not shown) seems to indicate that *Xpax6* and *Xrx1* play non redundant functions in early head development even if occurrence of such interactions at later stages cannot be rigorously ruled out (see next section).

In our overexpression experiments, we found that *Xrx1* is able to repress *Xotx2* and *XAG-1* at early neurula stage. This suggests that *Xrx1* plays an active role in repressing *Xotx2* expression in the anteriormost region of the neural plate and, possibly through this activity, in setting the dorsal border of *XAG-1* expression. There is also evidence that *Xrx1*might be important to the *XBF-1* activation. This is suggested by the strong inhibition of *XBF-1* expression carried out by *Xrx1-EnR* in both *chordin*-injected caps and in early neurula embryos, as well as by the ectopic activation of *XBF-1* in *Xrx1*-injected embryos. Such ectopic activation seems to coincide with the lateral-anterior neural plate border. The inability of *Xrx1* to ectopically activate *XBF-1* elsewhere in the neural plate may indicate that other factors are required to promote *XBF-1* expression during normal development and/or that inhibiting factors prevent the expansion of *XBF-1* expression in other regions of the neural plate.

Effects of *Xrx1* overexpression suggest a linkage between proliferation and anterior fate specification

Regions of the anterior neural plate, where *Xrx1* is expressed, are also characterized by a prolonged proliferative period, undergoing neurogenesis with a remarkable delay compared to the posterior neural plate (Papalopulu and Kintner, 1996). Overexpression experiments have shown that *Xrx1* is able to induce hyperproliferation of the neural tube, neural retina and retinal pigmented epithelium (Fig. 1A-C; Mathers et al., 1997), suggesting that *Xrx1* may be responsible for some of the proliferative properties of the anterior neural plate. When the expression of various neuroectodermal markers in *Xrx1*-injected embryos was analyzed at tailbud stage, the anterior genes *Xpax6*, *Xsix3* and *Xotx2* were found to be ectopically activated in the proliferating area. This ectopic activation is not appreciable at early neural stage, suggesting the existence of stage-dependent differences in *Xrx1* activity. For example, in a very speculative scheme, the concentration of Xrx1 at early neurula might be above a threshold level required to support the intensive neural plate proliferation (Eagleson et al., 1995), thus rendering *Xrx1* overexpression partly ineffective; on the contrary, the subsequent decrease in the proliferation rate (Eagleson et al., 1995) could be counteracted by *Xrx1* overexpression, as observed at tailbud stage. A particular case is represented by *Xotx2* and its indirect target gene *XAG-1*, which are both repressed at early neurula and ectopically activated at tailbud stage. These effects might reflect *Xrx1* activity in anterior neural plate patterning, as discussed in the previous section. Accordingly, the abnormal *XAG-1* expression, which is restricted to the ventral-anterior region in keeping with the lack of competence of the dorsal side to form cement gland (Gammill and Sive, 1997), could be seen as a consequence of an early *XAG-1* repression by the microinjected *Xrx1*, which would lead to a split cement gland field; however, this hypothesis would not explain the occurrence of ectopic spots of *XAG-1* expression located outside the cement gland field. Alternatively, the abnormal expression of *XAG-1* might be explained as promoted by the anterior overproliferating tissue, which also expresses the cement gland inducer *Xotx2*, thus reflecting the *Xrx1* function in anterior proliferation. This hypothetical scheme is in accordance with results from the gene functional ablation (see next section).

Expression in midbrain-hindbrain boundary of both *Xpax2* and *En2* as well as rhomboencephalic expression of *Krox20* are found to be repressed in *Xrx1*-injected tailbud embryos. These data suggest that the anteriorizing activity of *Xrx1* antagonizes with posteriorizing signals acting in caudal brain regions. This leads to speculate that, during normal development, *Xrx1* might contribute to exclude the most anterior regions of the neural plate, where it is expressed, from the range of action of

posteriorizing signals. Since posteriorizing signals have also been shown to trigger neuronal differentiation (Papalopulu and Kintner, 1996), their repression in the anterior neural plate could represent a basic requirement to allow cell proliferation. Altogether these results indicate that the proliferative activity of *Xrx1* is linked to the promotion of the anterior fate, in agreement with other lines of evidence suggesting that mechanisms regulating cell proliferation-neuronal differentiation interact with those that control the anterior-posterior patterning (Papalopulu and Kintner, 1996; Bourguignon et al., 1998).

Anterior deletions in *Xrx1-EnR*-injected embryos are due to early loss of the anterior neural plate territories

In order to perform a loss-of-function analysis of *Xrx1*, we used three different approaches. In distinct experiments, we microinjected *Xrx1* antisense RNA, ΔOAR RNA, coding for a truncated form of *Xrx1* lacking the putative transactivation domain (OAR motif) and RNA generated from a fusion construct where the OAR domain was substituted by the *engrailed* repressor domain. It is noteworthy that all these approaches produced similar phenotypes, namely embryos with anterior deficiencies and reduced or absent eyes, although with different efficiencies. The fact that ΔOAR RNA injection does not generate any of the effects described for full-length *Xrx1*, suggests that the OAR motif might be a transactivation domain as it has been shown to be the case for the Orthopedia protein (Simeone et al., 1994). Accordingly, the effects observed after ΔOAR microinjection may be caused by a passive repression of *Xrx1* target genes. Microinjection of *Xrx1-EnR*, which is supposed to actively repress *Xrx1* targets, produced very similar phenotypes but with the highest frequency and penetrance. Since antisense RNA and *Xrx1-EnR* injections are supposed to block *Xrx1* function at different independent levels, the convergence of phenotypes generated by the two different approaches is a first indication that the observed effects are specific. The antisense RNA approach has been shown to work for genes expressed during gastrulation or earlier and, because of this, the low efficiency that we observed with this method can be attributed to the relatively late activation of *Xrx1* expression. Further support for the specificity of *Xrx1-EnR* effects comes from the similarity between *Xrx1-EnR* and mouse *Rx1* knockout phenotypes (Mathers et al., 1997) and from the rescue effected by *Xrx1* RNA.

To better characterize the affected regions of *Xrx1-EnR* embryos, we examined the expression of several anterior markers, an analysis that was not performed in mouse *Rx1* null mutants. This study indicates that, at tailbud stage, the telencephalon, ventral diencephalon and eye vesicles have not formed. Moreover, the strongly reduced expression domains of *Xotx2*, *Xpax6*, *Xsix3* and *XBF-1* in *Xrx1-EnR*-injected embryos already at early neurula stage suggest that the unsuccessful formation of telencephalic and diencephalic regions occurs early, when these regions are first specified. Another clue indicating that *Xrx1-EnR* is blocking the early function of *Xrx1* is provided by the absence of telencephalon in *Xrx1-EnR* phenotypes. In fact, *Xrx1* is expressed in presumptive telencephalon at early neurula stage, as shown by cohybridization with *XBF-1* (Fig. 3D), but not at later stages (Casarosa et al., 1997).

We also noticed that the expression of *gsc*, an anterior mesoendodermal marker, is not altered in *Xrx1-EnR*-injected embryos indicating that the lack of anterior brain regions is not caused by the absence of the anterior mesoendoderm. Thus, the inactivation of *Xrx1* function specifically leads to a failure in the formation of those neuroectodermal regions that normally express this gene. One exception is perhaps represented by the pineal gland. Persistence of *Xotx-b* pineal-gland-specific expression in *Xrx1-EnR*-injected embryos, although reduced in intensity, suggests that *Xrx1* may not be required for the formation of this structure. Alternatively, *Xotx-b* and *Xrx1* might be expressed in different cells within the pineal gland and only the subset expressing *Xrx1* could be affected by *Xrx1-EnR* action.

Analysis of *Xrx1-EnR* activity in animal caps injected with *chordin*, which induces a forebrain-like neuralization, basically confirmed the data obtained in whole embryos. Perhaps more clearly, animal caps experiments showed that *Xrx1-EnR* represses *XBF-1* better than *Xpax6* while it is not efficient in blocking *Xotx2* transcription. It is interesting to note that, in the early neurula, while *Xrx1* and *Xotx2* expression domains overlap only in a very restricted region, *Xrx1* expression domain shows a good overlapping with that of *Xpax6* and includes the one of *XBF-1*. Therefore, a possible explanation for the different responses shown by these genes is that, if *Xrx1* plays a role in the specification and/or proliferation of the neurula territories where it is normally expressed, then the genes more affected by *Xrx1-EnR* activity will be the ones expressed in the same regions.

The nature of the anterior deletions described in *Xrx1-EnR-injected* embryos were further analyzed to understand if they were caused by anterior neuroectoderm posteriorization or by an early loss of anterior territories. While *Xrx1-EnR*-injected embryos did not show any significant change in *En2* and *Krox20* expression, suggesting that hindbrain territories are normally specified, a TUNEL staining revealed that early neurulae displayed accumulation of apoptotic cells restricted to the anterior part of the embryo. Although these data indicate that the inhibition of *Xrx1* activity generates an early loss of anterior regions, at the moment it is not clear whether anterior cells die because of a lack of specification or proliferation, or both. In any instance, these data suggest that *Xrx1* could play a role in preventing programmed cell death. This hypothesis would not be in contrast with the previously proposed proliferating activity of *Xrx1* since anterior cells might need to escape programmed cell death in order to enter a proliferative phase.

In conclusion, we would like to propose that *Xrx1* plays an early role defining forebrain territories in combination with other homeobox genes expressed in the anterior neural plate. The function played by *Xrx1* in this context is essential for the development of these regions, as shown by the early loss of anterior territories in embryos injected with a *Xrx1* dominant repressor construct, and it seems to involve anterior specification, cell survival and cell proliferation. How these processes may be linked and temporally regulated remains a relevant question. Furthermore, investigations aimed at identifying *Xrx1* cofactors and direct targets will be important to elucidate the molecular mechanisms of anterior neural patterning.

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