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Homeobox genes in the genetic control of eye development

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ABSTRACT Vertebrate eye formation is a complex process which involves early specification of the prospective eye territory, induction events, patterning along the polarity axes and regional specification, to bring about the proper morphogenetic movements, cell proliferation, cell differentiation and neural connections allowing visual function. The molecular machinery underlying such complex developmental events is presently under an intense research scrutiny and many associated genetic factors have been isolated and characterized. These studies produced striking knowledge in the field, especially with respect to uncovering the role of key genes and their possible evolutionary conservation. Presently, a major task is to define the complex interactions connecting the multiplicity of molecular players that regulate eye development. We recently identified two homeobox genes, *Xrx1* and *Xvax2*, and studied their function by using the *Xenopus* embryo as a developmental model system. *Xrx1* and *Xvax2* control key aspects of eye development. In particular, *Xrx1* appears to play a role in the early specification of anterior neural regions fated to give rise to retina and forebrain structures, and in promoting cell proliferation within these territories. On the other hand, *Xvax2* is involved in regulating the eye proximo-distal and/or dorso-ventral polarity, and the morphogenetic movements taking place during formation of the optic stalk and cup. Here we review the experimental results addressing the roles of *Xrx1* and *Xvax2* and their vertebrate orthologues, and discuss their relationship with other molecules also playing a related function in eye development.

KEY WORDS: *eye field, eye polarity, rx1, vax2, homeobox genes.*

Introduction

Eye morphogenesis occurs with similar sequential steps in all vertebrate embryos (Land and Fernald, 1992). It begins at the end of neurulation, when the optic vesicle, a bulge-like structure that will originate the neural structures of the eye, evaginates from the ventro-lateral wall of the forebrain. Subsequently, the lens primordium becomes evident in the ectoderm overlaying the distal-most part of the optic vesicle. At the same time, the distal vesicle invaginates around the lens primordium forming the optic cup, a two-layered structure, whose external layer will differentiate into the pigmented epithelium, while the internal layer, closer to the lens, will give rise to the neural retina. On the other hand, the proximal part of the optic vesicle narrows and elongates forming the optic stalk, a transient tubular structure that collects the axons of the retinal ganglion cells into the optic nerve and allows the blood vessels to reach the eye. Invagination of the optic vesicle around the lens begins in its ventral part, and, at the end of the invagination process, a narrow opening, known as optic fissure, remains in the ventral-most part of the cup. The invagination process and formation of a ventral fissure occur not only in the optic vesicle, but also in the optic stalk. The optic fissure allows both the retinal axons to enter the optic stalk and the blood vessels to reach the retina. Both the optic stalk and the optic fissure are only transient structures; during late developmental stages, the optic fissure is closed by fusion of its retinal lips, while the optic stalk disappears and its cells differentiate into the astrocytes that will populate the body of the mature optic nerve.

Molecular processes that regulate this complex and coordinated sequence of morphogenetic events start much earlier, around late gastrula/early neurula stages of development, than the first morphological appearance of the eye bud at the end of neurulation. It is now established that molecular control of the very first events of eye formation involves two different and temporally distinct steps. In a first phase, a wide presumptive eye-forming region is identified as a continuous crescent shaped region in the anterior neural plate. In a second phase, this initially single eye field is subdivided in two distinct bilateral eye anlagen by prechordal

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plate signals which induce ventral diencephalic developmental fates into the antero-medial neural plate (Li *et al.,* 1997). At the same time, the eye anlage begins to be patterned so that the prospective ocular structures become regionally specified within this territory (McDonald *et al.*, 1995; Chiang *et al.*, 1996).

We recently isolated and characterized in *Xenopus* two homeobox genes, *Xrx1* and *Xvax2*, that play important roles both in the early specification of the eye field and in the patterning events of the eye, respectively (Casarosa *et al.*, 1997; Mathers *et al.*, 1997; Andreazzoli *et al.*, 1999; Barbieri *et al.*, 1999; Ohsaki *et al.*, 1999; Schulte *et al.*, 1999). We summarize below the experimental results supporting such roles, and we discuss the possible functions of these two genes with respect to the genetic hierarchies governing development of the vertebrate eye.

Genetic control of the eye field

Recent lineage tracing experiments in *Xenopus*, refining classical embryology observations, have identified the earliest region fated to become retina as a continuous area in the anterior-most part of the early neural plate (Eagleson *et al.*, 1995). Genes involved in early decisions in eye development would be expected to be expressed in the eye field when this is first defined. This is indeed the case for *pax6* (Fig. 1A), a *paired* class homeobox gene expressed in the eye field at early neurula stage that has been proposed to be at the top of the genetic cascade governing eye development. In fact, in *Drosophila*, targeted misexpression of

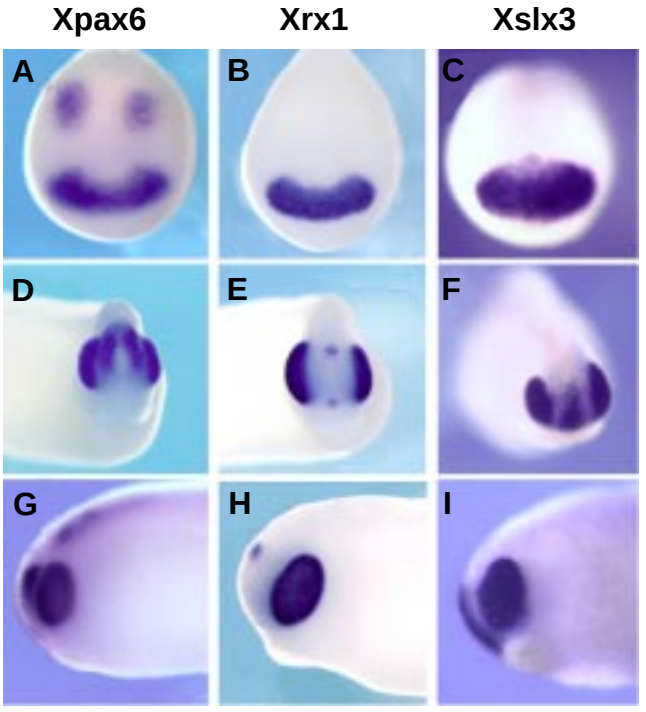

Fig. 1. Expression of *Xpax6, Xrx1* and *Xsix3* in the prospective eye field and in the optic vesicle. *Whole mount in situ hybridizations showing expression of Xpax6* **(A,D,G)**, *Xrx1* **(B,E,H)** *and Xsix3* **(C,F,I)**, *at early neurula (A-C) or tailbud (D-I) stage (st.) of Xenopus development. (A-F) Frontal view. (G-I) Lateral view. All three genes are expressed in a crescent shaped region of the anterior neural plate, including the prospective eye field (A-C) and later on, throughout the developing optic vesicle (D-I). At tailbud stage, specific expression domains within the forebrain are also evident (D-I).*

murine *Pax6* or its fly homologue *eyeless* (*ey*) leads to initiation of a complete eye developmental program in ectopic imaginal discs, while mutations in *ey*/*pax6* cause malformation or lack of eyes in *Drosophila*, mouse and humans (Hill *et al.*, 1991; Ton *et al.*, 1991; Quiring *et al.*, 1994). However, the identification in *Drosophila* of other genes whose mutations cause a failure in eye development has led to the idea that more than one gene is required to achieve the formation of a normal eye. In particular, functional studies on *sine oculis* (*so*), *dachshund* (*dac*) and *eyes absent* (*eya*) have shown that eye development is due to the interactions between these genes and *ey* rather than to the action of *ey* alone (see Desplan, 1997). Moreover, the ability to induce ectopic eye development does not appear to be a prerogative of *ey*, since both *eya* and *dac* can elicit eye formation, although with a much lower efficiency than *ey*. Furthermore, different combinations of *ey*, *eya*, *so* and *dac* induce eye development with a higher frequency and in a wider range of tissues than *ey*, *eya* or *dac* alone. Nonetheless, the recent isolation of *twin of eyeless* (*toy*), a second *pax6*-like gene in *Drosophila*, has restored the idea of the existence of a single master gene controlling eye development (Czerny *et al.*, 1999). In fact *toy*, whose structure and expression pattern are more closely related to vertebrate *pax6* than *ey*, acts upstream of *ey*, does not require feedback by *so*, *eya* and *dac* and its targeted expression induces ectopic eyes. The idea of a master eye gene has recently been strengthened by the observation that *pax6* misexpression is able to induce ectopic eyes also in a vertebrate, as shown by microinjection experiments in *Xenopus* (Chow *et al.*, 1999). This remarkable conservation of *pax6* function across species, has also led to propose a monophyletic origin for the eye (Gehring and Ikeo, 1999). The role of *pax6* in vertebrates has also been assessed by analysis of mice carrying a *Pax6* loss-of-function mutation (*Small eye*: *Sey*; see Callaerts *et al.*, 1997, and Treisman, 1999, and references therein). *Sey* mice, beside displaying defects in brain and nose development, show abnormally shaped optic vesicles and a complete absence of the lens. Interestingly, while in these mice a lens placode never forms, the optic vesicles initially undergo a normal evagination and make contact with the overlaying ectoderm. Although this contact is not maintained, probably due to the lack of a lens placode, and a proper optic cup does not form, *Sey* optic vesicles have been shown to display at least some aspects of proximo-distal patterning. Thus, even though *pax6* plays a crucial role in eye development, being sufficient to induce a complete eye under overexpression conditions, and being necessary for lens and late retina development, its function does not seem to be absolutely required for the initial steps of optic vesicle formation. This process is therefore likely to involve other genes during embryo development. The possibility that a second vertebrate *pax6* gene might play a specific (or redundant) function in these early events seems to be ruled out by the results of a search for *pax6* genes in various species. These results may suggest that a duplication of the *pax6* gene has only occurred in the genomes of holometabolous insects (Czerny *et al.*, 1999).

Obvious candidate partners for *pax6* are the vertebrate homologues of *so*, *eya* and *dac*. The search for the *so* homologue has revealed the existence of several vertebrate *so*-related genes, the *six* gene family. Paradoxically, the members more closely related to *so*, *Six1* and *Six2* are not expressed in the eye primordium (Oliver *et al.*, 1995b). On the other hand, two other members of this gene family, namely *Six3* (Oliver *et al.*, 1995a) and *Optx2* (Toy *et al.*, 1998), both of which are expressed early in eye territories, have

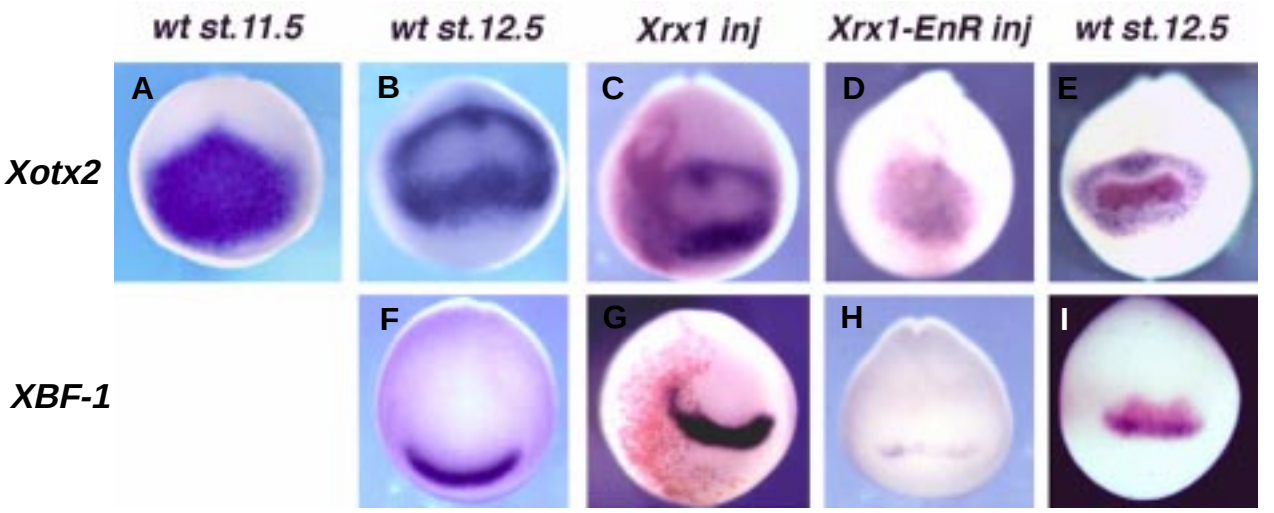

Fig. 2. Interactions between *Xrx1, Xotx2* and *XBF-1* within the early *Xenopus* neural plate. (A,B) *Expression of Xotx2 in the anterior neuroectoderm in wild-type embryos at midgastrula (st. 11.5, (A)) and late gastrula (st. 12.5, (B)) stages. At st. 12.5 (B) Xotx2 is downregulated in the prospective eye field.* **(F)** *Expression of XBF-1 in the presumptive telencephalon of a wild type embryo at late gastrula (st. 12.5) stage.* **(C,G)** *Expression of Xotx2 (C) and XBF-1 (G) at early neurula stage (st. 13) in embryos unilaterally injected with full-length Xrx1 RNA. Distribution of the injected RNA is detectable as magenta staining, while expression of the gene of interest is detectable as blue staining. Xotx2 expression is repressed, while the XBF-1 domain is expanded on the injected side.* **(D,H)** *Expression of Xotx2 (D) and XBF-1 (H) at early neurula stage (st. 13) in embryos bilaterally injected with Xrx1-EnR chimeric RNA. Both genes appear to be strongly downregulated.* **(E,I)** *Double in situ hybridizations on late gastrula (st. 12.5) wild type embryos with Xrx1 (magenta staining in (E,I)) and either Xotx2 (blue staining in (E)) or XBF-1 (blue staining in (I)), showing that at this stage, Xrx1 expression is complementary to that of Xotx2 and overlaps with the XBF-1 positive domain. st. = stage.*

now been shown to be the orthologues of another *Drosophila* gene, *optics*, whose function in the fly has not yet been assessed. (*Optx2* is also known as *Six6* in mouse and *Six9* in chick: Jean *et al.*, 1999; Lopez-Rios *et al.*, 1999). However, in *Xenopus*, *Xsix3* is co-expressed with *Xpax6* in the early eye field (Andreazzoli *et al.*, 1999; Fig. 1C), and microinjection experiments in fish have shown that *six3* is able to induce ectopic retina and lens, as well as an enlargement of the forebrain (Kobayashi *et al.*, 1998; Loosli *et al.*, 1999). *six3* function in brain and eye formation has also recently been underscored by the finding that mutations in human *SIX3* are the cause of a form of holoprosencephaly (Wallis *et al.*, 1999). *Xoptx2* is also expressed in the *Xenopus* eye field, even though at a later stage than *Xsix3*, and it has been shown to control cell proliferation thus determining the final size of the eye (Zuber *et al.*, 1999). Vertebrate homologues of *eya* and *dac* expressed in different eye regions have also been described (Xu *et al.*, 1997; Hammond *et al.*, 1998); however at the moment their role in eye development is unclear and awaits functional studies.

Besides looking for the vertebrate homologues of known *Drosophila* genes, other approaches have also led to the isolation of novel vertebrate transcription factors expressed in the eye field. In particular, this was the case for *Xrx1* (Casarosa *et al.*, 1997), a *paired*-like homeobox gene whose expression in *Xenopus* is activated in the eye field in concomitance with that of *Xpax6* and *Xsix3* (Fig. 1B) and that plays a role in retinal and anterior brain proliferation (see below).

Since the establishment of the eye field seems to involve several players, in order to understand the mechanisms of eye development in vertebrates it becomes now of primary interest to identify the interactions occurring between all these genes. Recent overexpression experiments have begun to address this issue. Thus, misexpression studies using *Xpax6* and *Xrx1* in *Xenopus* and in medaka, have shown that each of these genes is able to activate the expression of the other two, although with a different timing (Andreazzoli *et al.*, 1999; Chow *et al.*, 1999; Loosli *et al.*, 1999). *Xoptx2*, when overexpressed, can also activate *Xpax6* and *Xrx1*, while co-injection with *Xpax6* has been shown to potentiate the effects of *Xoptx2* on eye and brain enlargement (Zuber *et al.*, 1999). Altogether, the present data point to the existence of a complex set of regulatory interactions between the genes involved in vertebrate eye development.

Xrx1 and the eye-brain field

The *paired*-like homeobox gene *Xrx1* was isolated in our laboratory during a screening designed to look for homologues of the *Orthopedia* gene (Simeone *et al.*, 1994; Casarosa *et al.*, 1997). *Xrx1* shares indeed sequence similarity with *Orthopedia* both in the encoded homeodomain and in a novel motif denominated OAR (Furukawa *et al.*, 1997) which in Orthopedia has been shown to work as a transactivation domain. *Xrx1* was also independently isolated by Jamrich and coworkers during a screening for genes induced in *Xenopus* animal caps by ammonium chloride treatment, which is known to trigger anterior ectodermal fate (Mathers *et al.*, 1997). *Xrx1* transcripts are first detected at early neurula stage in the anteriormost part of neural plate including presumptive retina, diencephalic and telencephalic territories (Fig. 1B). Later on, *Xrx1* expression becomes restricted to the evaginating eye vesicles, diencephalon floor, pituitary and pineal gland (Fig. 1 E,H). When optic cups are formed, both presumptive neural retina and pigmented epithelium express *Xrx1*, while no expression is detected in the forming lens at any stage. Interestingly, *Xotx2*, a homeobox gene of the *bicoid* class, is expressed in the whole prospective anterior neuroectoderm already at the end of gastrulation, before the onset of *Xrx1* transcription (Pannese *et al.*, 1995; Fig. 2A). However, less than two hours later in development, *Xotx2* expression becomes

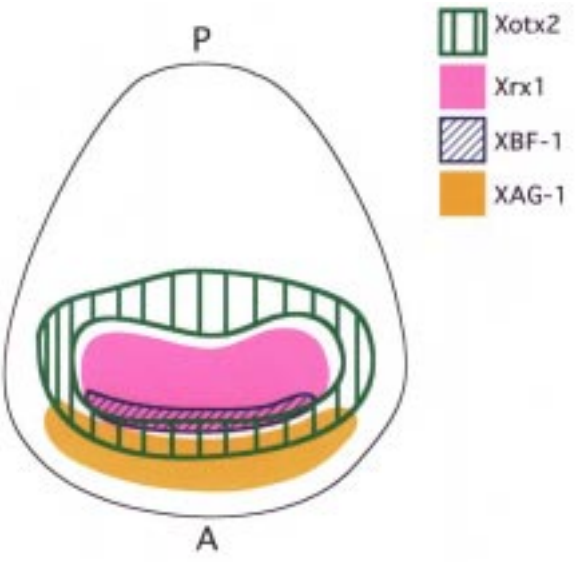

Fig. 3. Schematic representation of *Xotx2, Xrx1, XBF-1* and *XAG-1* expression in the early *Xenopus* neural plate. *Xrx1 (magenta) overlaps with both Xotx2 (green) and XBF-1 (blue) in the presumptive telencephalon, while it is complementary to Xotx2 in the presumptive diencephalon and retina territories, where it identifies the prospective eye field. XAG-1 expression (orange) identifies the presumptive cement gland and delimits the anterior border of the neural plate. A, anterior; P, posterior.*

repressed in most of the area where *Xrx1* is activated (Fig. 2 B,E). This is one of the earliest described events in anterior neural plate patterning, and comparison between the expression domain of *Xrx1* and those of other anterior genes indicates that the neural plate is indeed regionalized in specific territories already at the very beginning of neurulation (Andreazzole *et al.,* 1999; Fig. 3). Early *Xrx1* expression was found to overlap, although not perfectly, with that of *Xpax6* and *Xsix3*. *Xotx2* expression becomes almost completely complementary to *Xrx1*, except ventrally where *Xrx1*, *Xotx2* and *XBF-1* expressions overlap (Fig. 2 E,I; Fig. 3). Ventrally *Xrx1* borders, without overlapping, to the adjacent cement gland territory, marked by *XAG-1* expression. Thus, the combination of expression of different homeobox genes seems to correlate with a very early patterning of the neural plate (Fig. 3). The region expressing *Xrx1*, *Xpax6* and *Xsix3*, but not *XBF-1* and *Xotx2*, may correspond to retina and diencephalic territories, while the area coexpressing *Xrx1*, *XBF-1* and *Xotx2* may correspond to presumptive telencephalic areas. Consistent with the idea of an involvement of *Xrx1* in these early patterning events are the results from *Xrx1* overexpression studies. In fact, misexpression of *Xrx1* in anterior-dorsal territories results in the repression of *Xotx2* and *XAG1* and in the ectopic activation of *XBF-1* at early neurula stage (Fig. 2 C,G). It is tempting to think that the same interactions might occur during normal development, thus explaining how the different expression domains, and therefore the early neural plate patterning, are established.

The main feature of the *Xrx1* overexpression phenotype is the occurrence of hyperproliferation in the neural tube, neural retina and retinal pigmented epithelium (Mathers *et al.*, 1997; Andreazzoli *et al.*, 1999; Fig. 4 A,B). Expression analysis of eye-brain markers in tailbud injected embryos has shown that *Xpax6*, *Xotx2* and *Xsix3* are activated in the over-proliferating tissue (Fig. 4 C,D). On the other hand, *Xrx1* overexpression strongly represses *En2* and *Xpax2* expression in the midbrain-hindbrain boundary and reduces *Krox20* expression in the hindbrain. Thus, *Xrx1* appears to possess a proliferating activity which is linked to the promotion of anterior fate. Interestingly, the anterior neural plate and the optic vesicles, where *Xrx1* is expressed, are characterized by a high rate of proliferation and by a delay in neuronal differentiation when compared to the rest of the neuroectoderm (Eagleson *et al.*, 1995; Papalopulu and Kintner, 1996). Given its activity, *Xrx1* might be one of the genes that confer anterior proliferative properties to this region of the neuroectoderm. Other candidate genes for this function are transcription factors similarly expressed in the anterior neural plate, like *Six3* and *Xoptx2,* whose overexpression triggers over-proliferation in eyes and brain (Kobayashi *et al.*, 1998; Loosli *et al.*, 1999; Zuber *et al.*, 1999). *Xrx1* and other anteriorizing-proliferative genes in the early eye-brain field might also antagonize posteriorizing signals involved in promoting neuronal differentiation (cf. Bourguignon *et al.*, 1998; Papalopulu and Kintner, 1996). Future experiments will specifically address the role of *Xrx1* in counteracting the range of action of posteriorizing signals.

Loss-of-function experiments performed both in mouse and in *Xenopus* have stressed the necessity of *Xrx1* function for normal eye and brain development. Targeted disruption of the mouse *Rx1* gene leads to the absence of optic cups and forebrain as determined by histological analysis (Mathers *et al.*, 1997). A very similar effect is observed expressing in *Xenopus* embryos *Xrx1-EnR*, a *Xrx1* dominant repressor construct where the putative transactivation OAR domain was substituted by the *engrailed* repressor domain (Andreazzoli *et al.*, 1999). Expression analysis of anterior genes in *Xrx1-EnR* injected embryos showed a remarkable reduction of eye-brain presumptive territories already at early neurula stage (Fig. 2 D,H), which resulted in the absence of telencephalon, eye vesicles and ventral diencephalon at tailbud stage. Further analysis of *Xrx1-EnR* injected embryos has shown that early anterior neural plate cells undergo apoptosis. Together with the unchanged expression domains of hindbrain markers, these data suggest that the anterior deletions described in these embryos are due to an early loss of cells constituting the eye-brain field regions rather than to a posteriorization of the anterior CNS. Thus, *Xrx1* seems to play roles in cell survival, cell proliferation and anterior specification. All these functions might actually be strictly related and could be required by cells of the eye-brain field. In fact, these cells, which are specified as anterior, might have as first thing to avoid programmed cell death in order to enter a proliferative phase.

The function played by *Xrx1* is likely to be conserved through evolution, as suggested by the similarity between mouse and *Xenopus* loss-of-function phenotypes and also by the isolation of *Xrx1* homologues in several species, which share a similar expression pattern. In mouse only one homologue has been isolated (Furukawa *et al.*, 1997; Mathers *et al.*, 1997), while in zebrafish three genes of the *rx* family - *Zrx1*, *Zrx2* and *Zrx3* -, have been described (Mathers *et al.*, 1997; Chuang *et al.*, 1999). The three zebrafish genes are initially all expressed in the anterior neural plate but later *Zrx1* and *Zrx2*, which are closely related to each other, are only expressed in the optic vesicles, while *Zrx3* becomes expressed predominantly in the ventral forebrain and, at a low level, in the optic vesicles. The presence of a higher number of

homologues and the splitting of expression patterns between them seems to be a general characteristic of the zebrafish genome. Nevertheless, the recent isolation in chick of two *rx* homologues, namely *cRaxL*, which is closer to *Zrx1* and *Zrx2*, and *cRax*, which shares higher homology with *Xrx1* (Ohuci *et al*., 1999), suggests that the *rx* gene family may be larger than previously thought and that some members have probably not been identified yet. A common characteristic to all the vertebrate *rx* genes is their expression in the anterior neural plate first and optic vesicles later. Interestingly, this does not completely hold true for the only identified *Drosophila* homologue which is expressed in the protocerebrum, the anteriormost part of the fly brain, but neither in the eye primordia nor in the larval eye imaginal discs (Eggert *et al*., 1998). Unless a second *rx* homologue with an eye specific expression exists in *Drosophila*, these data seem to indicate that the most evolutionary conserved function of *rx* genes is in brain development, while *rx* expression in the eye could have been a recent acquisition of vertebrates. On this issue, it is worth to notice that while in *Drosophila ey* is required for eye development, its vertebrate homologue *Pax6* seems to be necessary mostly for lens development. Since both the vertebrate lens and the whole *Drosophila* eye derive from an ectodermal sensory placode it has been proposed that the lens could be the only vertebrate eye region sharing a common origin with the *Drosophila* eye (Treisman, 1999). The lack of *rx1* expression both in the vertebrate lens and in *Drosophila* eyes gives further support to this hypothesis.

Regulation of eye polarity

The vertebrate eye is a complex polarized structure where the developmental fate of its cells is highly dependent on their position within the prospective eye territory. Eye polarity is commonly described in terms of proximo-distal (P-D), dorso-ventral (D-V), and nasal-temporal axes of the ocular structure, that extend parallel to the latero-medial, dorso-ventral and antero-posterior axes of the body, respectively. P-D polarity of the eye becomes evident during the transition from the optic vesicle to the optic cup, when cells nearer to the brain contribute to the optic stalk, while cells located in distal positions with respect to the neuraxis give rise to the retina. At the same time, D-V polarity of the eye also becomes apparent, since cells located in different positions with respect to the dorso-ventral axis of the eye undertake different morphogenetic movements and different programs of proliferation, differentiation and axonal projections. The optic fissure is the most evident morphological marker of the D-V polarity of the developing eye: its specific occurrence in the ventral retina and optic stalk reflects the ventro-dorsal progression of the invagination movements of the optic vesicle around the lens to form the optic cup. Furthermore, a spatio-temporal gradient of cell differentiation has been detected in the retina, with cells in ventral positions proliferating longer and differentiating later with respect to dorsal retinal cells (Zuber *et al*., 1999). Regional differentiation of specific cell types also occurs along the retinal D-V axis, since, for example, middle- and shortwave cone photoreceptors dominate dorsal and ventral retinal regions, respectively (Szel *et al*., 1992). Finally, D-V polarity of the neural retina is also represented by the neuritic organization of the visual system, since ventral and dorsal retinal ganglion cells project their axons specifically to the dorsal and ventral tectum, respectively (Crossland *et al*., 1974). A similar reversed organization array in retinotectal projections has also been detected along the antero-posterior axis of the retina and optic tectum, and constitutes the main feature of the nasal-temporal polarity of the eye (Crossland *et al*., 1974).

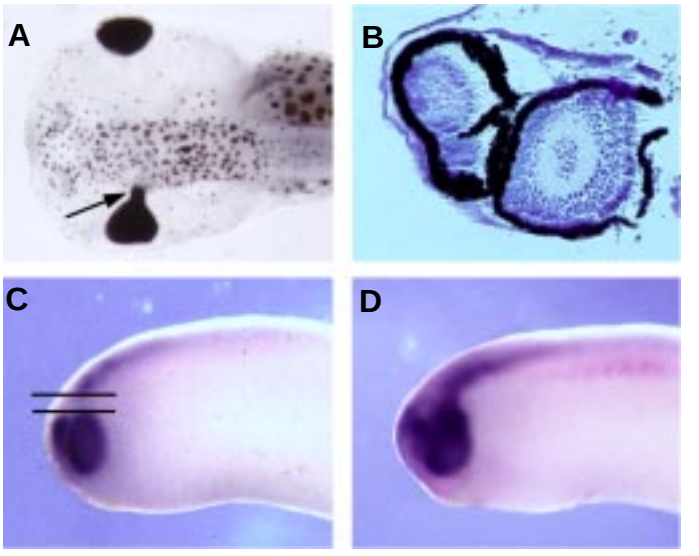

Fig. 4. Effects of *Xrx1* overexpression on eye development. (A) *Dorsal view of a swimming tadpole Xenopus embryo unilaterally injected on the left side with Xrx1 full-length RNA, showing the presence of ectopic pigmented retina extending from the eye to the diencephalon (arrow).* **(B)** *Transverse section through the eye of an Xrx1-injected embryo at the swimming tadpole stage, showing complete duplication of the optic cup. (C,D) Expression of Xpax6 in a tailbud stage Xenopus embryo unilaterally injected with Xrx1 RNA.* **(C)** *Lateral view from the control side. A gap of Xpax6 expression is detectable in the midbrain (area between lines).* **(D)** *Lateral view from the injected side. Xpax6 expression is ectopically expanded in the midbrain region. Distribution of the injected RNA is detectable as magenta staining in the embryo, while blue staining indicates Xpax6 expression.*

In the last few years our understanding of the molecular machinery regulating eye polarity has been greatly improved thanks to the use of animal models apt to both genetic and embryological studies. In particular, the zebrafish embryo has allowed the identification of several secreted or trasmembrane molecules involved in P-D and D-V patterning of the eye. The genes *cyclops (*Rebagliati *et al*., 1998; Sampath *et al*., 1998), coding for a nodal-related factor of the TGF-ß superfamily of secreted proteins, and *one-eyed pinhead* (Zhang *et al*., 1998), encoding a putative EGF receptor protein apparently working as an essential extracellular cofactor for *nodal* signaling molecules (Gritsman *et al*., 1999), have recently been identified. *cyclops* and *one-eyed pinhead* are expressed in anterior midline tissues and are responsible for the homonym zebrafish mutations, which are characterized by the striking occurrence of a single cyclopic eye and the lack of the ventral forebrain. The absence of the optic stalk, as assayed by detailed morphological and molecular analyses of these mutants, suggests the existence of a molecular link between the subdivision of the initially continuous eye field into two separate bilateral eye anlagen and the proximo-distal patterning of the eye (McDonald *et al*., 1995; Hammerschmidt *et al*., 1996). In particular, in the well characterized *cyclops* mutant, the *paired*-like homeobox gene *pax2* - a specific marker of the optic stalk and the ventral-most part of the

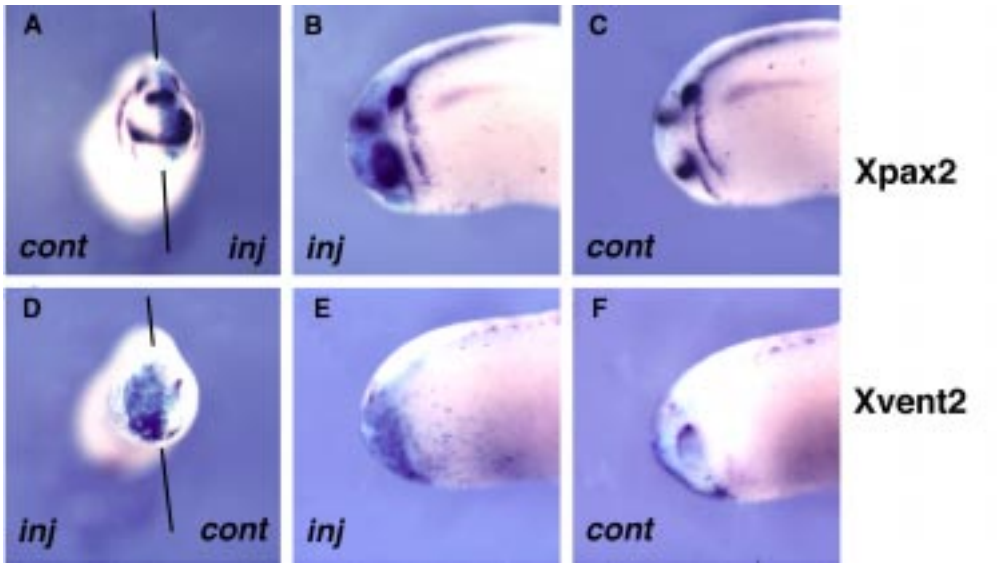

Fig. 5. *Xvax2* overexpression affects D-V patterning of the eye. *Expression of Xpax2 (A-C) and Xvent2 (D-F) genes in tailbud Xenopus embryos, unilaterally coinjected with Xvax2 and ß-galactosidase RNAs. The gene of interest is detectable as a dark blue staining, while the distribution of the injected RNA is visualized by the ß-galactosidase enzymatic reaction (light blue staining).* **(A,D)** *St. 23 injected embryos, frontal view. On the injected side (inj, right in A, left in D), the Xpax2 expression domain, normally confined to the ventral developing eye, is expanded dorsally (A), while Xvent2 expression, which is dorsal in the control side (cont), is strongly reduced (D).* **(B,E)** *St. 23 injected embryos laterally viewed from the injected side.* **(C,F)** *The same embryos as in (B,E) laterally viewed from the control side.*

retina - is almost completely suppressed in the mutant cyclopic eye. Reciprocally, expression of *pax6*, normally confined to the prospective retinal territory, is ubiquitous within the cyclopic eye (McDonald *et al.*, 1995).

The *sonic hedgehog* (*shh*) gene, isolated in several vertebrate species by its homology to *Drosophila hedgehog*, codes for a secreted signaling molecule that is involved in the developmental patterning of various structures, such as the neural tube, the limb bud, the somitic mesoderm, the tooth and the gut (Echelard *et al.*, 1993; Riddle *et al.*, 1993; Hebrok *et al.*, 1998; Borycki *et al.*, 1999; Hardcastle *et al.*, 1999). Early in development, *shh* is expressed in midline tissues. Within the nervous system, it plays a pivotal role in regulating development of the ventral neural tube, being both necessary and sufficient for differentiation of ventral neuronal cell types all along the antero-posterior axis of the embryo (Echelard *et al.*, 1993; Roelink *et al.*, 1994; Ericson *et al.*, 1995; Chiang *et al.*, 1996). *shh* also appears to play a role in establishing the ventral forebrain and proximal eye identity, since its expression in *cyclops* and *one-eyed pinhead* mutant embryos is strongly downregulated (McDonald *et al.*, 1995; Hammerschmidt *et al.*, 1996). Indeed, *shh* knockout in the mouse results in cyclopia and deletion of proximal eye and ventral forebrain structures (Chiang *et al.*, 1996). On the other hand, *sonic hedgehog* misexpression both in fish and mouse embryos activates ectopic expression of ventral forebrain and optic stalk markers, such as *nkx2.1*, *nk2.2* and *pax2*, while repressing expression of the retinal marker *pax6*. Simultaneously, an expansion of the optic stalk and a concomitant reduction of the optic cup are induced (Barth and Wilson, 1995; McDonald *et al.*, 1995; Shimamura and Rubenstein, 1997). These data suggest a role for *shh* as a morphogen, controlling specification of the latero-medial polarity of the anterior neural plate, which results in subsequent P-D and D-V regionalization of the eye and brain.

Retinoic acid (RA), a signaling molecule crucial in a number of developmental processes (Maden, 1999), also plays a role in establishing eye polarity. Studies performed in zebrafish have in fact pointed out a major role for RA in D-V patterning of the eye. RA synthesis is higher in the ventral than in the dorsal retina (McCaffery *et al.*, 1992, 1999; McCaffery and Drager, 1993). Moreover, RA treatments of zebrafish embryos induce an enlargement of the optic stalk and an expansion of the *pax2* expression domain in the eye. On the other hand, transcription of the homeobox gene *msh[c]*, a marker of the dorsal retina, is strongly downregulated following exposure of the embryos to RA. Furthermore, localized treatments by implantation of beads soaked in RA result in formation of supernumerary optic fissures near the site of implantation (Hyatt *et al.*, 1996). Finally, treatments of zebrafish embryos with inhibitors of RA synthesis result in ablation of the ventral retina, thus indicating that RA is both necessary and sufficient for development of ventral ocular structures (Marsh-Armstrong *et al.*, 1994).

The relationships among the *nodal*, *shh* and RA pathways in eye patterning are presently unclear and represent a central topic for further investigation. Nevertheless, as shown by either gain- or loss- of-function approaches, all these pathways appear to converge, directly or indirectly, on the *pax2* gene, that may therefore work as an intracellular determinant of ventro-proximal ocular fates. However, knockout studies of *pax2* in both mouse and fish brought to a reassessment of the relevance of the *pax2* role (McDonald *et al.*, 1995, 1997). The knock-out mutants in fact display an ectopic extension of the pigmented epithelium in the optic stalk territory and non-closure of the optic fissure, thus suggesting that, while *pax2* is important for correct development of proximal and ventral eye structures, it is not absolutely required for their initial specification.

Recently, a novel homeobox gene, *vax1*, was found to be specifically expressed in the ventral forebrain and in ventro-proximal structures of the developing eye, namely the optic stalk and the optic disk (Hallonet *et al.*, 1998). Functional studies have addressed the *vax1* role in eye development in both frog and mouse (Hallonet *et al.*, 1999). Knock-out of *Vax1* in the mouse results in an ocular phenotype very close to that observed in *Pax2-/-* mutant mice, i.e. non-closure of the optic fissure and ectopic extension of the pigmented epithelium into the optic stalk. Furthermore, both *vax1* and *pax2* appear to be necessary for glial differentiation of the optic stalk cells, which is similarly deficient in both *vax1-/-* and *pax2-/-* mutants (McDonald *et al.*, 1997; Bertuzzi *et al.*, 1999). Interestingly, both *Pax6* and *Rx* are ectopically expressed in the optic stalk of *Vax1-/-* mutant mice. Conversely, *Xvax1* overexpression in *Xenopus* embryos leads to a strong

repression of *Xrx1* expression. Moreover, overexpression in *Xenopus* embryos of either *shh* or *banded hedgehog*, another member of the *hedgehog* family, induces a striking expansion of *Xvax1* and a concomitant repression of *Xpax6* in the retinal territory. On these notions, it is possible to speculate that *pax2* and *vax1* could play a permissive role, downstream of *shh*, in allowing proper development of proximal eye structures, by repressing *pax6* and *rx* expression in such territories (Hallonet *et al*., 1999; Fig. 7B). However, the possibility that *Pax6* expression in the *Vax1-/-* optic stalk could be due to the abnormally open optic fissure allowing for the unimpeded migration of retinal cells into the nerve, cannot be ruled out (Bertuzzi *et al*., 1999). Surprisingly, *Pax2* expression is maintained in the optic stalk of *Vax1-/-* mutant mice, and *Vax1* transcription is still detectable in *Pax2-/-* mutant mice (Hallonet *et al*., 1999), thus suggesting that the two genes may work in parallel pathways, being necessary for closure of the optic fissure and glial differentiation in the optic nerve. Nevertheless, it is clear that neither *pax2* nor *vax1* are absolutely required for the initial formation of the optic stalk and the ventral retina. While asking for a *Pax2/Vax1* double mutant mouse, the present knowledge also leaves the question open of whether other genes may exist that play a role in the early specification of proximo-ventral eye territories.

The role of the *vax2* gene

Starting from an Expressed Sequence Tag (EST) database screening, aimed at the identification of novel homologues of *Drosophila* mutant genes, we recently isolated in mouse, human and *Xenopus* a gene closely related to *vax1*, that we accordingly named *vax2* (Barbieri *et al*., 1999). The same gene was independently identified by other groups through RT-PCR based screenings for novel ocular genes (Ohsaki *et al*., 1999; Schulte *et al*., 1999). The vax1 and vax2 proteins share an almost identical homeodomain, which is closely related to that found in the emx and not transcription factors. Remarkably, mapping studies in mouse and human assigned a close chromosomal location to *vax2* and *emx1* and to *vax1* and *emx2*, respectively, thus suggesting a possible common origin for these genes by tandem duplication events (Hallonet *et al*., 1998; Barbieri *et al*., 1999; Ohsaki *et al*., 1999; Schulte *et al*., 1999). However, up to now only one *vax* gene, named *cVax*, was identified in the chick (Schulte *et al*., 1999). While more similar to *vax1* in terms of protein sequence, *cVax* shows a spatial expression pattern covering both *vax1* and *vax2* expression domains in other species, thus suggesting that it may encompass the functions of both *vax1* and *vax2* in the chick. Mouse *Vax2* is strongly transcribed in the ventral developing retina, while only faint expression levels were detected in the optic stalk and the ventral forebrain, where *Vax1* is instead highly expressed (Hallonet *et al*., 1998; Barbieri *et al*., 1999). On the other hand, both *cVax* and *Xenopus Xvax2* are significantly expressed in the ventral retina, in the optic stalk and in brain regions, particularly in the ventral telencephalon, suggesting that *vax1* and *vax2* gene functions may have been segregated to distinct regional domains during evolution (Barbieri *et al*., 1999; Schulte *et al*., 1999; our unpublished results; Fig. 6D). Also noteworthy are the spatio-temporal relationships between *vax2* and *pax2* expression in the eye. At early developmental stages, soon after evagination of the optic vesicle, *pax2* and *vax2* are coexpressed in the proximo-ventral part of the vesicle, fated to give rise to the optic stalk and the ventral retina. However, after formation of the optic cup, while *pax2* becomes almost exclusively

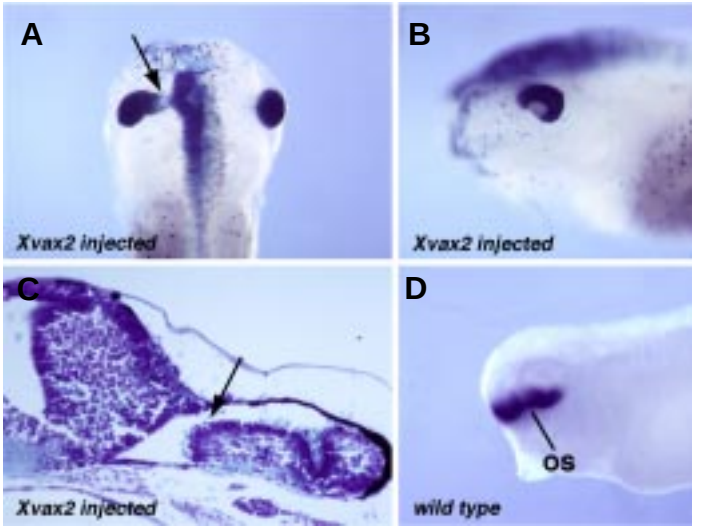

Fig. 6. *Xvax2* is expressed in the ventral retina and induces an aberrant morphogenesis of the eye. *(A-C) Effects of Xvax2 overexpression on eye development.* **(A)** *Dorsal view of a swimming tadpole Xenopus embryo unilaterally coinjected with Xvax2 and ß-galactosidase RNAs, showing that, on the injected side (left), the optic cup and the forebrain are connected by a giant optic stalk (arrow).* **(B)** *The same embryo shown in (A), laterally viewed from the injected side. The optic fissure remains widely open.* **(C)** *Transverse section of a swimming tadpole embryo injected with Xvax2 RNA. A wide optic stalk-like structure (arrow) abnormally joins the optic cup to the brain.* **(D)** *Lateral view of a wild type tadpole stage embryo hybridized with an Xvax2 probe, showing Xvax2 expression in the ventral part of the retina, in the optic stalk (os) and in the ventral forebrain.*

restricted to the optic stalk (with the exception of few retinal cells bordering the optic fissure), *vax2* is strongly maintained in the ventral half of the retina throughout development (Nornes *et al*., 1990; Barbieri *et al*., 1999; our unpublished results). Remarkably, prominent *VAX2* expression was also found in the developing human ventral retina, thus suggesting a *vax2* conserved role in regulating the retina D-V asymmetry (Barbieri *et al*., 1999).

The question concerning the role played by *vax2* in eye development was independently addressed by Barbieri *et al.* (1999) and Schulte *et al.* (1999) through overexpression studies performed in frog and chick, respectively. In either species *vax2* overexpression causes ventralization of the retina, as detected by the ectopic activation in the dorsal retina of ventral retinal markers, such as *pax2*, *vax2* itself and *EphB* receptors *EphB2* and *EphB3*, and by the concomitant down-regulation of dorsal retinal markers, namely *Xvent2*, *ET*, *Tbx5*, *ephrinB1* and *ephrinB2* (Barbieri *et al*., 1999; Schulte *et al*., 1999; our unpublished results; Fig. 5). Remarkably, this ventralizing effect was obtained after overexpression of any of the frog, mouse and human *vax2* genes and chick *cVax*, thus again pointing to a strong evolutionary conservation of *vax2* function in controlling the retinal D-V patterning. Notably, *vax2* overexpression in the frog resulted in a wide *pax2* ectopic expression in the retina even at the optic cup stage, when *pax2* is normally excluded from the retina and restricted to the optic stalk. This result raised the question of whether *vax2* overexpression could affect proper specification of the optic stalk and the optic cup territories. Strikingly, morphological and molecular analysis of the injected *Xenopus* embryos at late developmental stages revealed the presence of a giant optic stalk abnormally connecting the eye and the

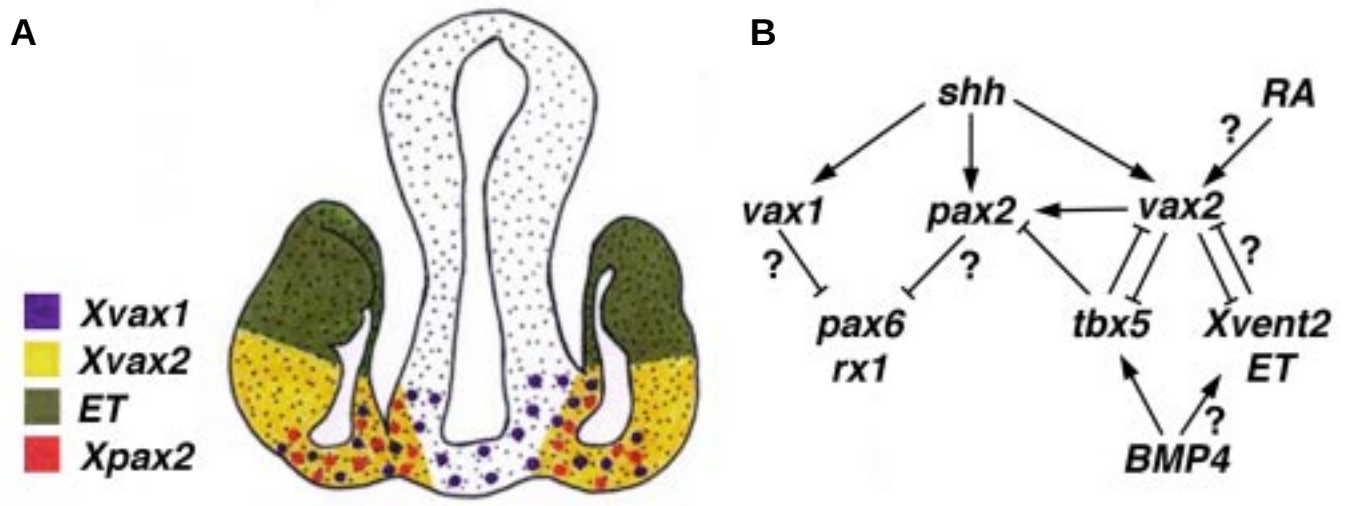

Fig. 7. Genetic regulation of eye polarity. (A) *Schematic representation of Xvax2 (yellow), ET (brown), Xvax1 (blue spots) and Xpax2 (red spots) expression in the eye and diencephalon of a late tailbud/early tadpole Xenopus embryo. At this stage, ET is expressed in the dorsal retina, Xvax2 is expressed in the ventral retina and in the optic stalk, Xvax1 expression covers the optic stalk and the ventral diencephalon, while Xpax2 is transcribed exclusively in the optic stalk.* **(B)** *Model for a molecular cascade involved in the regulation of proximo-distal and dorso-ventral polarity of the eye. vax1 and pax2 are induced by sonic hedgehog (shh) and may determine repression of the retinal genes pax6 and rx1 in the optic stalk. vax2, also regulated by the hedgehog pathway, is implicated in activation and/or maintenance of pax2 in proximo-ventral eye structures. A mutual antagonism between vax2 and tbx5, activated by BMP4 in the dorsal retina, is involved in regulating dorso-ventral polarity of the retina. Xvent2, ET and pax2 may also participate in this process. A possible regulation of vax2 by the retinoic acid (RA) pathway is proposed. Question marks indicate epistatic relationships still to be proved (see text for explanations).*

forebrain (Fig. 6 A,C). At the same time, morphogenesis of the optic cup was strongly affected, since the ventral retina did not encircle the lens correctly, but instead extended medially, thus resulting in a widely enlarged optic fissure (Fig. 6 B,C). Remarkably, both these morphological abnormalities correlated with ectopic *Xpax2* expression in the retina, that was detected both in dorsal retinal regions abnormally contiguous with the enlarged optic stalk and in the ventral part of the retina undertaking abnormal morphogenesis (Barbieri *et al.*, 1999). These data suggest that *vax2* overexpression may cause the observed morphological alterations by affecting the patterning of the eye along its P-D and/or D-V axis, thus supporting a *vax2* role in the regulation of eye polarity. On the other hand, proper regulation of *vax2* expression might be important in controlling the execution of the correct morphogenetic movements required for invagination of the optic vesicle and proper formation of the optic cup and stalk. Experiments performed in the chick also highlighted a pivotal role for *cVax* in regulating the establishment of a correct topographic map of retino-tectal projections along the D-V axis of the retina. In fact, following *cVax* misexpression, axonal trajectories of dorsal retinal cells appeared to be ventralized, as they never encompassed the ventral-most tectum, the area where they should normally focus, but grew in much more dorsal tectal positions (Schulte *et al.*, 1999). On the whole, these studies indicate that *vax2* may be involved in regulating eye polarity at different levels, such as regional specification of ocular structures, execution of correct morphogenetic movements and proper establishment of the D-V retinotectal projection map. Loss of function studies will help to elucidate for which of these processes *vax2* is indeed effectively required.

The possible relationships between *vax2* and other known genes involved in regulating eye polarity represent another crucial, open question. The striking effect on optic stalk development seen after *vax2* overexpression suggests that *sonic hedgehog* might represent a putative upstream regulator of *vax2*, in that it is known to play a pivotal role in the specification of proximal eye structures and to activate *vax1* expression in the eye. Indeed, we recently found that overexpression of *banded hedgehog* in *Xenopus* is able to strongly activate *Xvax2* expression throughout the optic cup (unpublished results), thus suggesting that *vax2*, *vax1* and *pax2* all lay on the *hedgehog* pathway. The partially overlapping expression domains of these three genes in the eye and ventral forebrain at the optic cup stage of frog development (Heller and Brändli, 1997; Hallonet *et al.*, 1998; Barbieri *et al.*, 1999; our unpublished results; Fig. 7A), may lead to further speculation about the existence of a morphogenetic gradient of *hedgehog* receptor stimulation. According to this view, *vax1* would be activated by high and intermediate *shh* levels in the ventral diencephalon and in the optic stalk, respectively; *vax2* would be turned on by intermediate and low *shh* levels in the optic stalk and in the ventral retina, respectively; and *pax2* would be activated by intermediate levels of *shh* in the optic stalk only. The activation of the *vax2*, *vax1* and *pax2* genes by the *hedgehog* pathway, as well as their co-expression in some territories, raises the question of their possible epistatic relationships in eye development. Results of knock-out experiments in the mouse, where *Vax1* and *Pax2* expression is maintained in *Pax2-/-* and *Vax1-/-* mutant mice, respectively, suggest that these two genes may lay on different hierarchies, independently turned on by the *hedgehog* pathway (Hallonet *et al.*, 1999). On the other hand, the strong upregulation of *pax2* following *vax2* overexpression (Barbieri *et al.*, 1999; Schulte *et al.*, 1999), may indicate that *vax2* is an upstream regulator of *pax2* in the eye. However, the occurrence of a direct activation of *pax2* by the *hedgehog* pathway, independently from *vax2*, cannot be ruled out, since transcription of *pax2* precedes *cVax* activation within the

presumptive chick ventral retina (Schulte *et al.*, 1999). Finally, the possible relationships occurring between the two *vax* genes themselves still remain to be addressed.

Besides *shh*, other signaling molecules may play a role in regulating *vax* gene expression. RA is a likely candidate to control *vax2* expression in the ventral optic vesicle. Indeed, RA treatment of fish embryos brings about phenotypic effects very close to those induced by *vax2* overexpression in the frog, namely enlargement of the optic stalk and *pax2* upregulation in the eye (Hyatt *et al.*, 1996). The signaling molecules cyclops (Rebagliati *et al.*, 1998; Sampath *et al.*, 1998) and one-eyed pinhead (Zhang *et al.*, 1998), which are involved in patterning the zebrafish eye and forebrain, could also be required for proper transcriptional regulation of the *vax* genes.

While positively acting on other genes expressed in the ventro-proximal part of the eye, as indicated by overexpression experiments *vax2* might also negatively interact with genes regulating dorsal retinal fates, thus restricting them to their appropriate functional domains (Barbieri *et al.*, 1999; Schulte *et al.*, 1999). Interestingly, *Tbx5*, a putative target of *vax2* repression (Schulte *et al.*, 1999), has recently been shown to play a somewhat complementary role with respect to *vax2* on retinal development (Koshiba-Takeuchi *et al.*, 2000). In fact, *Tbx5* overexpression in the chick is able to repress ventral retinal genes (including *cVax*), upregulate dorsal retinal genes and dorsalize the retinotectal projection pattern. Remarkably, the TGF-ß secreted protein BMP4 is expressed in the dorsal retina and is able to activate *Tbx5* and repress *cVax* transcription (Koshiba-Takeuchi *et al.*, 2000). It is tempting to speculate that the dorso-ventral patterning of the retina may involve the antagonistic action of the *hedgehog* and *BMP* pathways, intracellularly mediated by *vax2* and *Tbx5* in the ventral and dorsal retina, respectively (Fig. 7B). *shh* and *BMPs* are also involved in controlling dorso-ventral patterning of the brain and the spinal chord (Lee and Jessel, 1999): thus the developing eye, notwithstanding its unique features, would make use of the same molecular mechanisms that regulate the dorso-ventral polarity of the neural tube.

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