Vax1, a novel homeobox-containing gene, directs development of the basal forebrain and visual system

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The novel homeobox-containing gene *Vax1*, a member of the *Emx/Not* gene family, is specifically expressed in the developing basal forebrain and optic nerve. Here, we show that *Vax1* is essential for normal development of these structures. Mice carrying a targeted mutation of *Vax1* show dysgenesis of the optic nerve, coloboma, defects in the basal telencephalon, and lobar holoprosencephaly. With the help of molecular markers we determined that in the developing visual system, the absence of *Vax1* results in a proximal expansion of the activity of *Pax6* and *Rx*. This observation suggests that *Vax1* may interfere negatively with the expression of *Pax6* and *Rx*. In reciprocal gain-of-function experiments, injection of *Xvax1* mRNA or *Shh* into *Xenopus* embryos primarily affects the brain at the level of the eye primordium. Consistent with the loss-of-function results, the injection of *Xvax1* results in a down-regulation of *Rx*. Similarly, *Shh* injection expands the *Vax1* and *Pax2* territory at the expense of the *Pax6* and *Rx* region. On the basis of these results, we propose a model for a molecular cascade involved in the establishment of structures of the visual system.

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The forebrain is a complex structure originating from the anterior neural plate. At neural plate stages, inductive signals act on the neuroepithelium, resulting in the formation of the various regions of the future brain in which specific cell determination subsequently occurs generating the elaborate cellular diversity of the adult brain. In particular, Fgf8, which is expressed in the anterior neural ridge (ANR) (Crossley and Martin 1995), is able to induce the expression of *BF1* (Shimamura and Rubenstein 1997), whose function is necessary for growth of the telencephalon, and for expression of the ventral telencephalic marker *Dlx2* (Xuan et al. 1995). Likewise, the secreted molecule Shh, which is expressed in the medial ventral neural plate and in the dorsal foregut underlying the forebrain (Echelard et al. 1993), is required for the development of the prosencephalon (Belloni et al. 1996; Chiang et al. 1996). Shh induces the expression of *Nkx2.1* (Ericson et al. 1995; Dale et al. 1997; Shimamura and Rubenstein 1997) whose function is required for the formation of the ventral forebrain (Kimura et al. 1996; Sussel et al. 1999).

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The anterior ventral forebrain, including the septum, preoptic area, optic chiasm, and ganglionic eminences, originates from a rostral and medial region of the neural plate in contact with, or itself expressing, inducing signals such as FGF8, SHH, and members of the BMP family (see Rubenstein et al. 1998). The formation of these structures is particularly sensitive to mutations of *Otx2* (Acampora et al. 1995; Matsuo et al. 1995; Ang et al. 1996) or *Shh* (Belloni et al. 1996; Chiang et al. 1996), as malformations of the basal forebrain occur in heterozygous mutants for these genes. Despite recent advances, the complex genetic mechanisms regulating the specification of the various telencephalic subregions and neural cell-specific differentiation remain obscure.

The recently identified *Vax1* gene (Hallonet et al. 1998) is a close relative of the *Emx* and *Not* genes, which are required for the formation of structures where they are expressed (Talbot et al. 1995; Pellegrini et al. 1996; Qiu et al. 1996; Masai et al. 1997; Yoshida et al. 1997). *Vax1* expression is detected at 7.5 dpc in the most anterior medial neural plate, the ANR, and in the adjacent ectoderm. At 10.5 dpc, it is observed in the developing septum, preoptic area, anterior hypothalamus, basal ganglia, and optic stalks. Later at 13.5 dpc, *Vax1* expression in the basal ganglia is restricted in the ventricular and subventricular zones. *Vax1* could thus function early in

cell differentiation in the basal forebrain and optic stalk. A corresponding expression pattern was observed for *Xvax1* in *Xenopus* embryos (Hallonet et al. 1998).

Results and Discussion

To investigate the function of *Vax1*, we have generated a targeted mutation of *Vax1* by homologous recombination using embryonic stem (ES) cell technology. The mutation replaces the amino terminus of the *Vax1* protein including the exon coding for the two first helices and part of the third helix of the Vax1 homeobox, with the β-galactosidase reporter gene (Fig. 1a; Le Mouellic et al. 1992).

Mice heterozygous for the *Vax1* mutation were obtained from two independently mutated ES cell lines and grown in the mixed genetic backgrounds 129/NMRI and 129/C57Bl6; they were viable, fertile, and appeared normal. Genotyping analysis of embryos obtained from heterozygous matings showed no significant deviation from the expected Mendelian ratio between 7.5 and 18.5 dpc. After birth, however, only 6% of the recovered pups were homozygous in the 129/NMRI genetic background, indicating that most homozygous mutants died perinatally. Most surviving homozygous animals died at weaning, but some were able to live up to several months when reared in isolation and fed soft food. Homozygous survivors were sterile. No homozygous pups were recovered from the 129/C57Bl6 background, suggesting that the mutation is more penetrant in this genetic context.

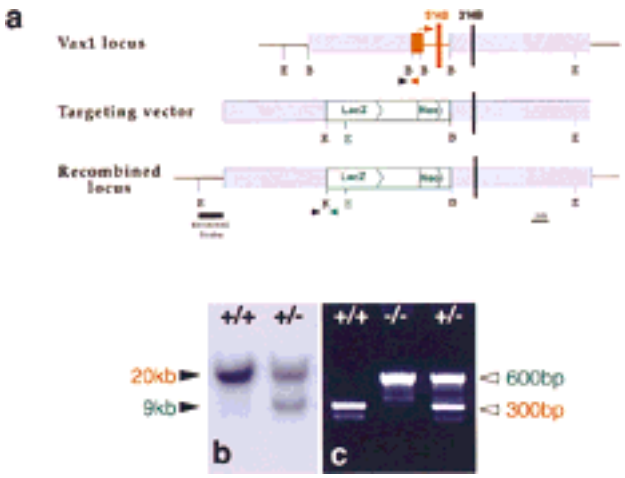

Figure 1. Disruption of the *Vax1* gene by targeted recombination. (*a*) Maps of the wild-type *Vax1* locus, the targeting vector, and the recombined allele. Relative positions of the exons coding for the two first helices (5′HB) and last helix (3′HB) of the homeobox are indicated by vertical bars. The map of the wild-type locus shows the deleted region in red, consisting of the start codon together with the exon coding for the first two helixes and amino-terminal part of the third helix of the homeobox and flanking intronic sequences. The map of the targeting vector shows the replacement of the deleted region by the β-galactosidase–neomycin cassette pGNA (green) (Le Mouellic et al. 1990). Arrowheads indicate the positions of the primers used for PCR genotyping. The dark line below the recombined locus denote the position of the genomic probe, external to the targeting vector, used to distinguish the *Eco*RV-generated 20- and 9-kb hybridizing bands on Southern gel of the wild type and recombined allele, respectively (*b*). Arrowheads in *a* denote the relative position of the primers used for PCR analysis and generating 300- and 600-bp amplification products for wild-type and recombined DNA, respectively (*c*). (E) *Eco*RV; (B) *Bam*HI; (K) *Kpn*I.

All homozygous mutants exhibited craniofacial malformations, including cleft palate, coloboma in the visual system, and growth defects in the basal telencephalon, regions where *Vax1* is normally expressed (Hallonet et al. 1998) and where *lacZ* expression was observed (data not shown). Identical defects were observed in F_2–F_4 generations, in either of the mixed genetic backgrounds. The phenotype observed in these animals is thus the consequence of the targeted *Vax1* mutation.

The Vax1 *mutation affects the development of the basal forebrain*

Vax1 homozygous embryos displayed brain and craniofacial malformations from 10.5 dpc. Homozygous embryos showed variable deficient growth of structures expressing *Vax1* or the *lacZ* reporter gene (see Hallonet et al. 1998; data not shown) in the medial anterior forebrain, namely the medial ganglionic eminence, preoptic area, and septum (Fig. 2b,d,f). Structures located medially were more affected than lateral ones. The optic chiasm and preoptic area were systematically absent so that the mutant optic nerves entered the brain at a lateral hypothalamic level (Fig. 2b, arrowheads). The telencephalic phenotype of *Vax1* homozygous mutants varied from a total absence of growth of medioventral structures (Fig. 2d) to a growth recovery of dorsolateral structures fusing medially (Fig. 2f). Only in this later case were fibers crossing the midline observed at the anterior commissure level (Fig. 2f) and at the level of the corpus callosum (data not shown). The medioventral defects typically included a defective cleavage of the dorsal forebrain into bilateral vesicles resulting in holoprosencephaly (Demyer 1987) (Fig. 2d,f).

Reduced growth and/or absence of midline structures was also observed at the craniofacial level in homozygous *Vax1* mutants. In particular, the maxillary incisors were severely abnormal or fused (data not shown). Interestingly, lobar holoprosencephaly associated with optic coloboma and fused maxillary incisors have been reported in humans (Hattori et al. 1987; Lieberfarb et al. 1987). Therefore, the *Vax1* mutant mice may provide an experimental model for a specific form of human holoprosencephaly.

To study how the loss of *Vax1* affects brain morphogenesis, we first tested whether the mutation impaired inducing signals known to act on the telencephalic neuroepithelium. We did not observe any significant differences in the expression pattern of *Fgf8* (Crossley and Martin 1995) (data not shown) and *shh* (Echelard et al. 1993) in wild-type and homozygous mutant embryos (Fig. 3a–d) before 12.5 dpc. These inducing signals, which

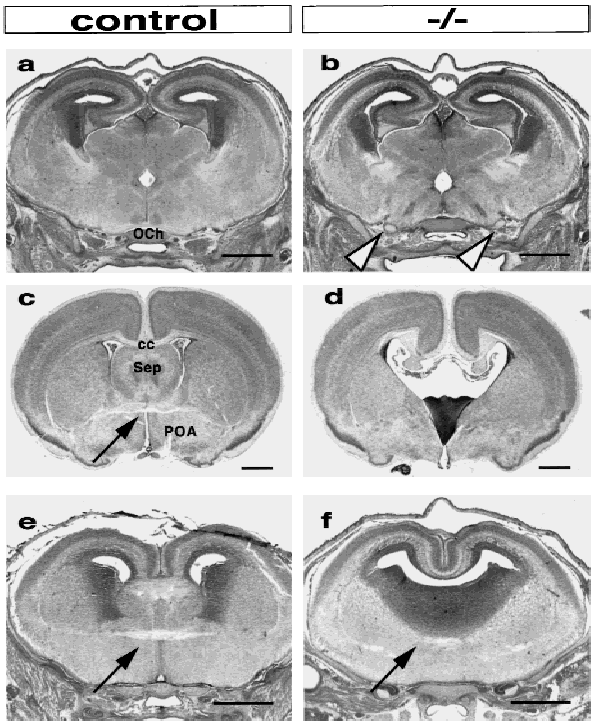

Figure 2. Growth and cell differentiation are deficient in the medioventral forebrain of homozygous *Vax1* mutants. (*a*,*b*,*e*,*f*) 16.5-dpc embryos, hematoxylin–eosin staining; (*c*,*d*) P15 brain, cresyl violet staining. The optic chiasm (*a*, OCh) was systematically absent from homozygous animals so that the mutant optic nerves (arrowheads in *b*) entered the brain at a lateral hypothalamic level. The telencephalic phenotype of *Vax1* homozygous mutants ranged from a total absence of growth of medioventral structures, including the septum (Sep) and preoptic area (POA) (*c*,*d*) to a growth–recovery of dorsolateral structures fusing medially (*e*,*f*). In this latter case only, fibers crossing the midline may be observed at the anterior commissure level (arrows in *c*,*e*,*f*). The medioventral growth defects resulted in lobar holoprosencephaly (*d*,*f*). Bar, 0.5 mm.

pattern the prosencephalic neural plate, are thus present in the neuroepithelium of *Vax1* homozygous mutants. To test further whether the *Vax1* mutation affects regionalization of the telencephalic neuroepithelium, we examined the expression of several markers with restricted expression patterns in the forebrain. Between 11.5 and 14.5 dpc in the telencephalon of wild-type embryos, *Nkx2.1* expression is restricted to the medial ganglionic eminence and the septum (Lazzaro et al. 1991), and *Dlx1* is expressed in both the medial and lateral basal ganglia and the septum (Bulfone et al. 1993). Despite the morphological alterations in the ventral telencephalon described above, *Nkx2.1* and *Dlx1* are expressed in *Vax1* homozygous mutants in restricted territories corresponding to those of wild-type embryos (Fig. 3e–h and i–l, respectively). The patterning of the ventral and anterior telencephalon would, therefore, occur independently of *Vax1*. Morphological analysis of dorsal structures and studies of the pattern of expression of the dorsal markers *Pax6* (Walther and Gruss 1991) (Fig. 3m–p), *Emx2* and *Emx1* (Simeone et al. 1992; data not shown) indicated that the development of the cerebral cortex (see Fig. 2) and olfactory bulbs (data not shown), adjacent to regions where *Vax1* is normally expressed (see Hallonet et al. 1998), was apparently unaffected in *Vax1* mutants. The effects of the mutation were, therefore, restricted to the basal forebrain. Nevertheless, the expression of *Nkx2.1* (Fig. 3q–t) in presumed differentiated or differentiating cells in the mantle layer of the wild-type

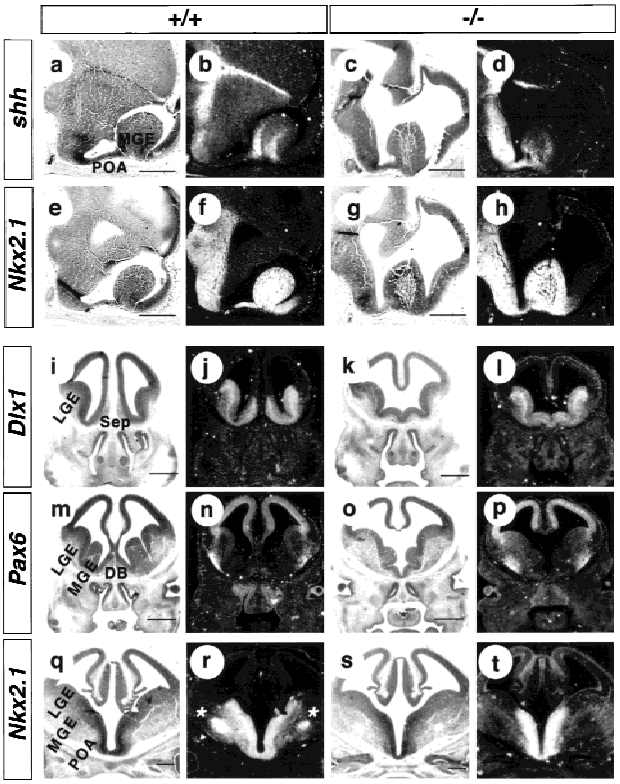

Figure 3. Despite morphological alterations, the patterning of the ventral telencephalon is apparently normal in *Vax1* mutants. Successive pictures are bright field and dark field of the same view to illustrate the morphological defects observed in the mutants. (*a–h*,*i–t*) In situ hybridization on sagittal sections of 11.5-dpc embryos, and on transverse sections of 13.5-dpc embryos, respectively. At 11.5 dpc, the anterior preoptic area (POA) is underdeveloped in the mutant (cf. *a* and *e* to *c* and *g*). At 13.5 dpc, deficient growth of the mutant ventral forebrain is observed in the septum (Sep) and the medial ganglionic eminence (MGE) (cf. *i* to *k* and *m* to *o*) and results in holoprosencephaly (see *k* and *o*). The pattern of expression of *Shh* (*b*,*d*) and *Nkx2.1* (*f*,*h*) at 11.5 dpc and that of *Dlx1* (*j*,*l*) or of *Pax6* (*n*,*p*) at 13.5 dpc are apparently not modified in homozygous mutants, suggesting that the patterning of the forebrain is not affected by the *Vax1* mutation. In addition, the *Nkx2.1* signal observed in differentiated or differentiating cells in wild-type embryos in the mantle layer over the subventricular zone of the MGE (asterisks in *r*) was greatly diminished or absent in *Vax1* homozygous animals (*t*), suggesting that the *Vax1* mutation perturbes the regulation of the cell proliferation or differentiation at least at that level. (DB) Diagonal band; (LGE) lateral ganglionic eminence; (MGE) medial ganglionic eminence; (POA) preoptic area; (Sep) septum. Bar, 0.5 mm.

MGE (asterisks in Fig. 3r) was greatly diminished or absent in *Vax1* mutant embryos (Fig. 3t). The reduction of the expression of *Nkx2.1* in absence of functional *Vax1* suggests that *Vax1* could be involved directly or indirectly in the regulation of *Nkx2.1* expression.

Mutations of both *shh* and *Vax1* cause holoprosencephaly associated with craniofacial abnormalities (Belloni et al. 1996; Chiang et al. 1996; Roessler et al. 1996), raising the question as to whether these genes act in the same developmental pathway in the developing forebrain. Both mutations affect the formation of the basal forebrain, however, the effects of the targeted mutations of *Vax1* and *shh* are significantly different because two well-separated eyes were always present in *Vax1* homozygous mutants. In contrast, cyclopia was observed in *shh* homozygous mutants (Chiang et al. 1996). Therefore, *Vax1* could act downstream of *shh* or in an independent developmental pathway in the differentiation of the basal forebrain.

The Vax1 *mutation affects development of the optic nerve*

To study the effect of the targeted mutation on the development of the optic stalk where *Vax1* is expressed in wild-type embryos (Hallonet et al. 1998), the morphology and the expression of *Pax6* (Walther and Gruss 1991), *Pax2* (Nornes et al. 1990), *Rx* (Furukawa et al. 1997; Mathers et al. 1997), and of *lacZ* were examined in *Vax1* mutant optic nerves.

The morphogenesis of the optic cup and optic stalk of homozygous *Vax1* mutants occurred between 9 and 12 dpc as in control animals. However, the crest of the choroid fissure, formed by the ventral extension of the optic cup, never fused in homozygous *Vax1* mutants leading to the formation of a so-called coloboma (Fig. 4a,b). The differentiation of the optic nerve was also severely affected in homozygous animals. From 12.5 dpc, the optic recess remained along the length of the mutant optic nerve (arrow in Fig. 4d). It was surrounded by a thickened epithelium ventrally (Fig. 4d) and a thin pigmented epithelium most dorsally (see Fig. 5j). The fibers of the optic nerve were located ventrally to the thickened epithelium (Fig. 4d).

Pax6 and *Rx* are essential for eye development in vertebrates (Hogan et al. 1986; Walther and Gruss 1991; Grindley et al. 1995; Furukawa et al. 1997; Mathers et al. 1997). Their expression is maintained in the retina but disappears from the developing optic nerve after 12 dpc in wild-type animals (Fig. 5a; data not shown). In homozygous *Vax1* mutants, *Pax6* and *Rx* are expressed ectopically in the mutant optic nerve after 12 dpc (Figs. 5b–d). Therefore, *Vax1* could be involved in regulating the expression of *Pax6* and *Rx*.

Pax2 is expressed specifically in the developing optic nerve and in the optic disk in wild-type animals (Fig. 5e) (Nornes et al. 1990), and is essential for the development of the optic nerve (Torres et al. 1996). In homozygous *Vax1* mutants, expression of *Pax2* occurs in the optic stalk and optic disk (Fig. 5f,g). Similarly to *Pax2*, *Vax1* transcription is detected in control optic disk and optic stalk (Fig. 5h) (Hallonet et al. 1998). In homozygous *Vax1* mutants, the expression of the *lacZ* reporter gene (Fig. 5i,j) was detected in the same structures as *Pax2*. Therefore, *Pax2* remained expressed in the mutant optic nerve despite the alteration indicated by the ectopic expression of *Pax6* and *Rx*. For this reason, *Vax1* does not seem to be involved in regulating *Pax2*. In contrast, it seems to act negatively on *Pax6* and *Rx*.

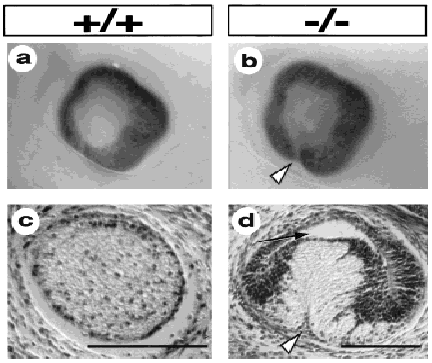

Figure 4. *Vax1* homozygous mutants show coloboma and dysgenesis of the optic nerve. (*a*,*b*) External view of 12.5-dpc eyes. (*c*,*d*) Hematoxylin and eosin staining of transverse section of the optic nerve of 16.5-dpc albino embryos. At 12.5 dpc, the optic fissure is closed in the wild-type eye (*a*) but remains opened in the mutant eye (*b*, white arrowhead). At 16.5 dpc, in contrast to the wild-type optic nerve (*c*), a thick epithelium is observed over the mutant optic nerve fibers (*d*) running over the open optic fissure (white arrowhead). The arrow in *d* points to the optic recess. Bar, 0.125 mm.

Like the induction of the optic stalk (Chiang et al. 1996), partitioning of the optic primordia into optic stalks and retinal tissue depends on *shh*, which induces *Pax2* expression and inhibits that of *Pax6* (Macdonald et al. 1995). This complementary regulation of the expression of *Pax2* and *Pax6* is apparently perturbed in *Vax1* mutants as expression of both genes is observed in the mutant optic nerve. However, Pax2 and Pax6 protein expression is largely exclusive in the *Vax1* mutant optic nerve at the cellular level (Fig. 5k,l); coexpression of the two proteins is only detected in few cells expressing both molecules at low level (white arrowheads in Fig. 5k and l) suggesting a *Vax1*-independent reciprocal inhibition between Pax2 and Pax6. Together, these observations suggest that *Vax1* participates in the regionalization of the visual system where it might function downstream of Shh in the down-regulation of *Pax6* expression.

The ectopic expression of *Pax6* and *Rx* and mixing of *Pax2*- and *Pax6*-positive cells in the mutant optic nerve could result from ectopic induction of these genes de novo or from cell migration in the developing nerve. Alternatively and more likely, the ectopic expression of *Pax6* and *Rx* could result from a failure of repression of *Pax6* and *Rx* in absence of functional *Vax1*, leading to the maintenance of the normal earlier expression in the optic stalk. The dysgenesis of the mutant optic nerve and the ectopic differentiation of retinal pigmented epithe-

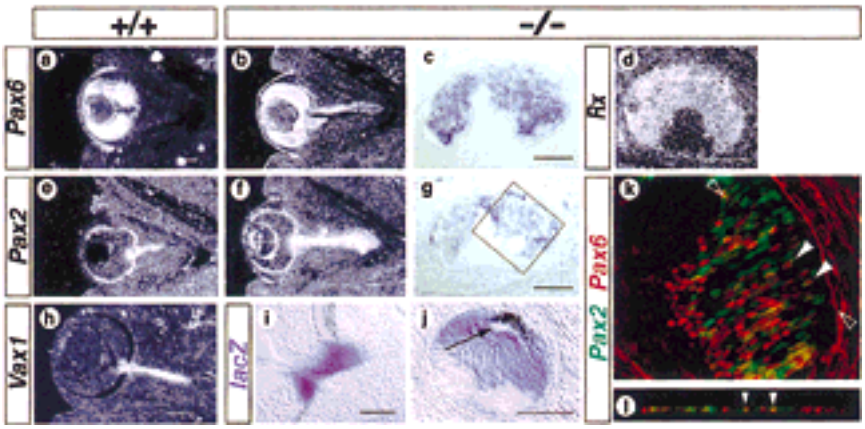

Figure 5. The *Vax1* mutation affects the development of the optic nerve in homozygous mutants. (*a–h*) In situ hybridization with antisense probes as indicated on the figure, 13.5-dpc embryos; (*i,j*) β-galactosidase staining, 12.5-dpc embryos; (*k,l*) Pax2 (green) and Pax6 (red) immunocytochemistry, homozygous *Vax1* mutant optic nerve at the temporal level, 13.5-dpc embryo; (*l*) confocal optical section through the *z*-axis of the section shown in *k* between the empty arrowheads. (*c,d,g,j,k*) Transverse sections of the mutant optic nerve. At 13.5 dpc, *Pax6* expression is confined to the retina of the wild-type animal (*a*), whereas it remains expressed in the mutant optic nerve (*b,c*). Similarly, *Rx* is expressed ectopically in the mutant optic nerve (*d*). At 13.5 dpc, *Pax2* expression is confined in the optic disk and optic nerve of wild-type animals (*e*) and remains expressed in these structures in *Vax1* mutants (*f,g*). Similarly, *Vax1* is expressed in the optic disk and optic nerve of wild-type animals (*h*) and the *lacZ* reporter gene is observed in these structures in *Vax1* mutants (*i,j*). The expression of *Pax2* and of *lacZ* indicates that the induction of the optic stalk occurred in *Vax1* mutants. The thick epithelium observed ventrally (*c,g,j*) and the pigmented epithelium observed dorsally (*j*) in the mutant optic nerve therefore, could result from an abnormal development of the optic stalk rather than from an elongation of the retina. *Pax2* and *Pax6* are both expressed in the mutant optic nerve (*k*). However, protein expression is mostly exclusive at the cellular level. Coexpression is only detected in few cells expressing both proteins at low level (white arrowheads in *k* and *l*) suggesting a reciprocal inhibition of Pax6 and Pax2 and a participation of *Vax1* in the down-regulation of *Pax6*. Dorsal is *up* in all pictures; rostral is *left* in *c,d,g,j,k*. The arrow in *j* points to the optic recess. Bar, 0.125 mm.

lium therefore, could result from an abnormal ectopic maintenance of the expression of genes normally expressed in the optic stalk only before 12 dpc. Thus, the dysgenesis of the *Vax1* mutant optic nerve could be an indirect consequence of the ectopic expression of *Pax6* or *Rx*.

Similar to mutation of *Vax1*, mutation of *Pax2* results in coloboma, ectopic extension of the pigmented retina into the optic nerve, and abnormal differentiation of the optic nerve (Torres et al. 1996), raising the possibility that these genes interact in the same developmental pathway. Although the expression of both genes in the optic stalk and disk indicates that they respond to similar regulatory mechanisms, the presence of *Pax2* transcripts in the *Vax1* mutant nerve (see Fig. 5f,g,k) and of *Vax1* transcripts in the *Pax2* mutant nerve (data not shown) suggests, however, that the two genes are regulated independently from one another. The genes may thus participate in parallel developmental pathways, both required for the closure of the optic fissure and for the formation of the optic nerve. Alternatively, similar phenotypes in the individual mutants could indicate that both gene products are necessary for the regulation of each other or of a common downstream target. Such a mechanism could be used by *Pax2* and *Vax1* to down-regulate *Pax6* expression.

Xvax1 *overexpression inhibits* Xrx *expression in* Xenopus *neurula stage embryos*

To investigate the function of *Vax1* in more detail, we performed *Vax1* misexpression experiments in *Xenopus* embryos and focused on the visual system. Markers for eye and forebrain development, such as *Pax6*, *Pax2*, *Otx2*, *Six3*, and *Xtll* were reduced but never absent (Table 1; data not shown). Complete inhibition of the transcription of the *Xrx* gene was observed in extreme cases with *Xvax1* mRNA injection in neurula stage embryos (Fig. 6c,d,f; Table 1). *Xrx* is normally expressed in the eye primordia, optic stalk, and ventral forebrain. In tadpole stage embryos, *Xrx* transcripts are only found in the ciliary margin of the neuroretina, in the hypophysis and the epiphysis (Casarosa et al. 1997; Mathers et al. 1997). Thus, during neurulation, *Xrx* and *Xvax1* are expressed in overlapping domains, which become progressively exclusive within the eye and optic nerve of normal embryos. The down-regulation of *Xrx* transcription as observed upon *Xvax1* overexpression, and, conversely, the ectopic expression of *Rx* transcripts in the optic nerve of *Vax1*-deficient mice suggest that *Vax1* might specifically participate in the regulation of *Rx* transcription.

Microinjection of *Xvax1* mRNA resulted in a general impairment of head development at tadpole stages, whereas trunk and tail development remained unaffected. The extent of the developmental defects was dose dependent. Low doses (up to 100 pg of RNA) resulted in only a slight reduction in eye diameter, whereas increasing doses produced cyclopic and microcephalic embryos and could inhibit head and eye formation completely (Fig. 6h,i; Table 1). Overexpression of *Xvax1* also generated malformations in head regions, which normally do not express the gene. In these areas, ectopic *Xvax1* might interact with or alter the function of other closely related homeobox-containing molecules, such as those of the Not and Emx families (von Dassow et al. 1993; Stein and Kessel 1995; Gont et al. 1996; Pannese et al. 1998).

Table 1. *Phenotypic effects resulting from* Xvax1 *microinjection in* Xenopus embryos

RNA injected (pg)	In situ probe used	Stage analyzed	Strong reduction (%)	Weak reduction (%)	Normal expression (%)	No. of embryos analyzed
Xvax1						
500	*Rx*	N	26	40	34	42
100		T	1	9	90	85
250		T	0	14	86	52
500		T	(19)	34	47	119
500	*Xpax2*	N	7	43	50	30
500		T	(16)	31	54	70
500	*Xpax6*	N	5	48	47	42
500		T	(16)	34	50	95
1acZ 500					100	57

Embryos were injected at the two-cell stage in both blastomeres, collected at neurula (N) or tailbud (T) stages and probed with *Xrx, Xpax2,* or *Xpax6,* as indicated. Data represent the results of at least three independent experiments. *lacZ* injections alone served as a control and did not result in any obvious phenotypic alterations. Neurula and tailbud stage embryos were counted as a strong reduction when they show no expression or at least an 80% reduction in marker gene expression in the eye field or in the eye cup. A 25%–80% was considered as a weak reduction. Because the injection of high doses (500 pg) of *Xvax1* inhibits head development, the anterior expression of all markers is lost at tadpole stages, as indicated by parentheses.

Overexpression of Xenopus *Hedgehog leads to ectopic expression of* Xvax1 *in the optic vesicle*

The expression pattern of *Xvax1* and the results discussed above suggest that a signal emanating from the midline could be involved in the regulation of *Xvax1* expression in the ventral forebrain and in the optic stalk.

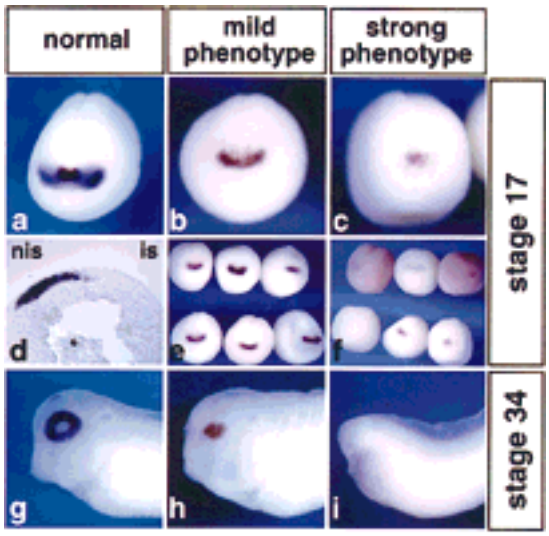

Figure 6. Effect of ectopic *Xvax1* overexpression on the transcription of *Xrx*. Normal expression pattern of *Xrx* at stages 17 (*a*) and stage 34 (*g*). A horizontal vibratome section of a stage 17 embryo is shown in *d* (anterior is *up*). Embryos were injected with 250 pg (*b,e,h*) or 500 pg [*c,d*(is),*f,i*] of *Xvax1* encoding mRNA into both blastomeres at the two-cell stage. The resulting effects were dose dependent. At stage 17, repression of *Xrx* is either moderate (*b,e*) or strong [*c,d*(is),*f*], resulting in a mild (*h*) or strong phenotype (*i*) at stage 34. (nis) Noninjected side; (is) injected side.

Candidates for such an activity are the members of the Hedgehog family of signaling molecules. To analyze whether Hedgehog (Hh) proteins can regulate *Xvax1* expression, we injected increasing amounts of synthetic mRNA encoding three different members of the *Xenopus* Hedgehog family (*X-bhh*, *X-chh*, *X-shh*; Ekker et al. 1995) into the animal pole of a single or of both cells of two-cell stage embryos (Table 2).

Xvax1 expression was examined at the tadpole stage in Hedgehog-injected embryos. All three Hedgehog signals were able to induce strong ectopic *Xvax1* expression in the entire optic vesicle (Table 2; Fig. 7d,e,g,h). Furthermore, Hedgehog injection resulted in an up-regulation of *Xvax1* in the ventral forebrain and in the optic stalk, areas normally expressing *Xvax1*. This effect could indicate hypertrophy of these tissues. In contrast to other

Table 2. *Ectopic expression of* Xvax1 *in the optic vesicle of* hedgehog-*injected* Xenopus *embryos*

	RNA injected (pg)	Cell injected	*n*	*Xvax1* induction (%)
X-*shh*	100	2/2	94	59
	500	2/2	48	67
	500	1/2	53	72
X-*bhh*	100	2/2	74	58
	500	2/2	67	83
	500	1/2	44	73
X-*chh*	100	2/2	69	36
	500	2/2	66	62
	500	1/2	102	52

Embryos were injected into one or both cells at the two-cell stage with the RNAs as indicated. At tadpole stage, embryos were scored for ectopic expression of *Xvax1* in the eye vesicle; (*n*) number of embryos examined.

experiments performed in zebrafish (Macdonald et al. 1995), the size of the optic vesicle itself was not reduced (Fig. 7d,e). On the other hand and consistent with what has been described in zebrafish (Macdonald et al. 1995), banded Hedgehog injection in *Xenopus* resulted in a severe reduction of *Pax6* expression in the eye, as well as in a loss of pigmented and neural retina (Fig. 7g,h). These effects are reminiscent with what we have observed as misexpression of *Xvax1* by mRNA injection (as detailed above). The loss of eye structure, as observed in the *Xvax1* mRNA injection experiments only, therefore may require the ectopic expression of *Xvax1* outside of the eye vesicle.

In mouse and *Xenopus*, *Vax1* transcripts are first detected in the anterior neural plate and anterior neural ridge at E7.5 dpc and stage 16, respectively (Hallonet et al. 1998), when the neural plate has already been influenced by surrounding structures (Rubenstein et al. 1998), suggesting that it will not participate in the early specification or determination of the neural plate but could function later in the specification or maintenance of ventral anterior identities in the forebrain. Our results indicate that *Vax1* is required downstream of inducing signals patterning the neural plate, including Shh, and support the idea that *Vax1* participates in the formation of the visual system (Fig. 8). In particular, our observations suggest that *Vax1* and *Pax2* inhibit the expression of *Pax6* and *Rx* in the developing optic nerve, and therefore, are involved in the partitioning of the developing visual system in eye and optic nerve.

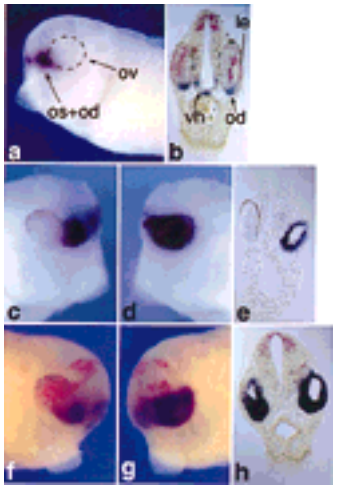

Figure 7. *Xvax1* expression is positively regulated by *X-bhh*. *Xenopus* embryos were injected into one cell (*c–g*) or into both cells (*h*) at the two-cell stage with synthetic mRNA encoding *X-bhh*. (*a*) Control embryos (stage 34) express *Xvax1* in the optic stalk and disc, as well as in the ventral forebrain. (*b*) Transverse section from a stage 34 control embryo at the level of the eye define differentially regulated *Xvax1* (purple) and *Xpax6* (red) expression domains. *Xvax1* is expressed in the ventral hypothalamus and in the eye disc, whereas *Xpax6* is strongly expressed in the neural retina and in the lens, as well as in the dorsal midbrain. (*c–h*) Injection of *X-bhh* leads to a strong induction of *Xvax1* (purple) expression (*d,e,g,h*) and to a severe inhibition of *Xpax6* (red) expression (*g,h*) in the optic primordium. (*c*) Uninjected side of the embryo shown in *d*. (*e*) Transverse section of the embryo in *c* and *d*. *Xvax1* (purple) is ectopically expressed in the whole remaining eye vesicle. (*f*) The uninjected side of the same embryo in *g* reveals almost normal *Xpax6* expression (red) in the eye. (*h*) Transverse section of an embryo injected into both cells at the two-cell stage. *Xvax1* is strongly expressed in both eye vesicles. (le) Lens; (od) optic disc; (os) optic stalk; (ov) optic vesicle; (vh) ventral hypothalamus.

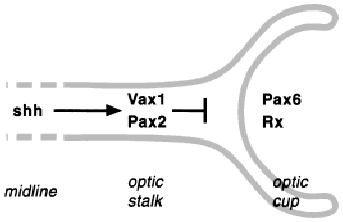

Figure 8. Molecular cascade involved in the partitioning of the visual system in eye and optic nerve and implicating *Vax1*. *Pax2* and *Vax1* are induced in the optic stalk by midline signals, such as shh, and confine *Pax6* and *Rx* expression in the optic cup.

Materials and methods

Animals

Vax1 mutant mice were generated by homologous recombination in the R1 ES cell line (Nagy et al. 1993) according to standard procedures (Nagy and Rossant 1993; Wurst and Joyner 1993) and positive mutant clones were used to produce chimeric animals by the aggregation technique. Chimeras were then mated to NMRI and C57/Bl6 mice for germ-line transmission.

Histology and in situ hybridization

Hematoxylin and eosin, cresyl violet, β-galactosidase staining, and in situ hybridization were done as described previously (Hallonet et al. 1998; Le Mouellic et al. 1992; Torres et al. 1996). Pax6 was detected with a mouse mAb raised against Pax6 (Developmental Studies Hybridoma Bank). Pax2 was detected with polyclonal antibody against Pax2 (BAbCO).

Xenopus *embryo microinjection procedures*

A full-length *Xvax1* expression plasmid, termed *Xvax1*-WT, was constructed in the expression vector pCS2+MT (Rupp et al. 1994): PCR was carried out using primers PCS-VAX-F (5-GCGGAATTCAATGTTTGAGAAGACAACAGAC) and PCS-VAX-R (5-CCGCTCGAGTCAGTCCAGGAGCTTTTTATC). The PCR product was digested with *Eco*RI and *Xho*I and inserted into the vector. Capped mRNA was transcribed using SP6 RNA polymerase as described (Kintner and Melton 1987). RNAs were injected in a volume of 5 nl at a concentration of 20–200 pg/nl into one or two blastomeres at the two-cell stage, as described previously (Coffman et al. 1990). *X-chh*, *X-bhh*, and *X-shh* RNAs were prepared as originally described (Ekker et al. 1995). In some experiments, *lacZ* mRNA was coinjected as a lineage marker. Injection of *lacZ* mRNA alone was used as a control. Whole mount in situ hybridization and vibratome sections were performed as described previously (Hollemann et al. 1998).

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Vax1, a novel homeobox-containing gene, directs development of the basal forebrain and visual system

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