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 BFI [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].

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; Matsumo 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 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
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 homzygous 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.

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 F2–F4 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...
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 Hallo net 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
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. 5i]. 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; Walthier 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.

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-
Vax1 overexpression inhibits Vax1 expression 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 Xtl1 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 Vax1 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).
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. 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 1. Phenotypic effects resulting from Xvax1 microinjection in Xenopus embryos

<table>
<thead>
<tr>
<th>RNA injected (pg)</th>
<th>In situ probe used</th>
<th>Stage analyzed</th>
<th>Strong reduction (%)</th>
<th>Weak reduction (%)</th>
<th>Normal expression (%)</th>
<th>No. of embryos analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xvax1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>Rx</td>
<td>N</td>
<td>26</td>
<td>40</td>
<td>34</td>
<td>42</td>
</tr>
<tr>
<td>100</td>
<td>T</td>
<td>T</td>
<td>1</td>
<td>9</td>
<td>90</td>
<td>85</td>
</tr>
<tr>
<td>250</td>
<td>T</td>
<td>T</td>
<td>0</td>
<td>14</td>
<td>86</td>
<td>52</td>
</tr>
<tr>
<td>500</td>
<td>Xpax2</td>
<td>T</td>
<td>(19)</td>
<td>34</td>
<td>47</td>
<td>119</td>
</tr>
<tr>
<td>500</td>
<td>T</td>
<td>(16)</td>
<td>7</td>
<td>43</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>500</td>
<td>Xpax6</td>
<td>T</td>
<td>5</td>
<td>48</td>
<td>47</td>
<td>42</td>
</tr>
<tr>
<td>500</td>
<td>T</td>
<td>(16)</td>
<td>34</td>
<td>50</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>lacZ</td>
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<td>500</td>
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</table>

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.

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

<table>
<thead>
<tr>
<th>RNA injected (pg)</th>
<th>Cell injected</th>
<th>Xvax1 induction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>injected</td>
<td></td>
</tr>
<tr>
<td>X-shh</td>
<td>100</td>
<td>2/2</td>
</tr>
<tr>
<td>500</td>
<td>2/2</td>
<td>48</td>
</tr>
<tr>
<td>500</td>
<td>1/2</td>
<td>53</td>
</tr>
<tr>
<td>X-bhh</td>
<td>100</td>
<td>2/2</td>
</tr>
<tr>
<td>500</td>
<td>2/2</td>
<td>67</td>
</tr>
<tr>
<td>500</td>
<td>1/2</td>
<td>44</td>
</tr>
<tr>
<td>X-chh</td>
<td>100</td>
<td>2/2</td>
</tr>
<tr>
<td>500</td>
<td>2/2</td>
<td>66</td>
</tr>
<tr>
<td>500</td>
<td>1/2</td>
<td>102</td>
</tr>
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</table>

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.

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’-GCC-GAATTCATGTTTGAGAAAGCAGACG) and PCS-VAX-R (5’-CCGCTCGAGTCAGTCCAGGACCTTATCC). The PCR product was digested with EcoRI and Xhol 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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