**Vax1 is a novel homebox-containing gene expressed in the developing anterior ventral forebrain**

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**SUMMARY**

The vertebrate forebrain is formed at the rostral end of the neural plate under the regulation of local and specific signals emanating from both the endomesoderm and neuroectoderm. The development of the rostral and ventral forebrain in particular was difficult to study, mainly because no specific markers are available to date. Here, we report the identification of Vax1, a novel homebox-containing gene identified in mouse, Xenopus and human. It is closely related to members of the Not and Emx gene families, all of which are required for the formation of structures where they are expressed. In mouse and Xenopus, Vax1 expression first occurs in the rostral neural plate, in the medial anterior neural ridge and adjacent ectoderm. Later, at midgestation in the mouse and tadpole stage in Xenopus, the expression remains confined in the derivatives of this territory which differentiate into rostromedial olfactory placode, optic nerve and disc, and anterior ventral forebrain. Together, these observations suggest that Vax1 could have an early evolutionary origin and could participate in the specification and formation of the rostral and ventral forebrain in vertebrates.

Comparison of the limits of the expression territory of Vax1 with that of Dlx1, Pax6 and Emx1 indicates that the corticostriatal ridge is a complex structure with distinct identifiable genetic compartments. Besides, the study of Vax1 expression in Pax6-deficient homozygous brains indicates that its regulation is independent of Pax6, although the expression patterns of these two genes appear complementary in wild-type animals. Vax1 chromosomal location is mapped at the distal end of the mouse chromosome 19, linked with that of Emx2. These two genes may have arisen by tandem duplication. The Vax1 gene is thus an interesting new tool to study the rostral ventral forebrain patterning, morphogenesis and evolution as well as the terminal differentiation of the forebrain in mouse and Xenopus.

Key words: Emx, Not1, Pax6, Dlx1, Brain patterning, Homeobox, Neural differentiation, Forebrain, Olfactory placode, Anterior neural ridge, Ganglionic eminence, Xenopus

**INTRODUCTION**

The function of the brain is based on its complex subdivision in regions and nuclei anatomically and histologically distinguishable. This elaborate organization is progressively built during ontogenesis at the rostral level of the developing central nervous system (CNS). Specific genetic mechanisms control the formation of the rostral part of the neural plate and, in particular, of the forebrain. For instance, the Emx and Otx homeobox-containing genes are only expressed rostrally to the hindbrain during early steps of neurulation whereas the Hox genes, absent from the forebrain and midbrain, are only expressed in the hindbrain and spinal cord (Simeone et al., 1992a,b, 1993; Finkelstein and Boncinelli, 1994; Krumlauf et al., 1994; see Thor, 1995; Luftkin, 1996). Moreover, mouse embryos homozygous for targeted null mutation of the Otx2 homebox-containing gene, or of the Lim1 zinc-finger-containing gene, lack head structures but have the remaining body axis normally developed (Acampora et al., 1995; Matsuo et al., 1995; Pannese et al., 1995; Shawlot and Behringer, 1995; Ang et al., 1996). Accordingly, the genetic mechanisms regulating the formation of the head and of the so-called trunkal structures are relatively distinct and independent. Besides, the rostral part of the neural plate is under the influence of specific inducing/organizing centers. In particular, the axial mesoderm in contact with the neural plate, namely the prechordal mesoderm at the most anterior level and the notochord at more caudal levels, have distinct neural inducing properties on the
adjacent developing CNS primordium (see Spemann and Mangold, 1924; Hamburger, 1988; Saha and Grainger, 1992; Doniach, 1993; Ruiz i Altaba, 1994; Ericson et al., 1996; Lumsden and Krumlauf, 1996; Dale et al., 1997). Moreover, specific and local neuroredotermal signals are necessary for the formation of forebrain structures (Ang et al., 1994; Thomas and Beddington, 1996). In particular, the anterior neural ridge or the FG8 molecule produced at this level regulates the expression of the BFI transcription factor essential for ventral forebrain and eye formation (Shimamura and Rubenstein, 1997). Similarly, the existence of an early anterior neural boundary organizer required for normal development of the forebrain has been demonstrated in zebrafish (Houart et al., 1998). Inhibition of both the Wnt and Bmp signalings, possibly by the cerberus protein, promotes head formation as opposed to trunk formation in Xenopus (Bouwmeester et al., 1996; Glinka et al., 1997). The developing forebrain is thus under the influence of local and specific signals emanating from the neuroectoderm and endomesoderm. However, despite recent advances, the genetic mechanisms regulating the induction and the early regionalization of the ventral forebrain remain largely obscure.

In order to study the genetic mechanisms of forebrain induction and specification, we identified a new homeobox-containing gene with high sequence homology with the members of the Emx and Not homeobox-containing gene families. The mouse and Xenopus genes share a high sequence homology and a similar pattern of expression during neurulation. These two genes could thus be truly orthologous. Expression is first detected in the rostral and medial neural plate and anterior neural ridge. Later, at midgestation in the mouse and at the tadpole stage in Xenopus, the expression remains confined in the ventral forebrain deriving from this territory, as predicted by fate maps, and could accordingly reveal a developmental unit. In addition, high sequence homology between the mouse and Xenopus genes suggests that it could function in the specification and formation of the rostral and ventral forebrain structures. Vax1 chromosomal location, sequence homology and pattern of expression in a phylogenetically ancient part of the forebrain lead us to conclude that Vax1 represents a new interesting tool to study the ventral forebrain induction, morphogenesis and evolution.

MATERIALS AND METHODS

Isolation of the Vax1 cDNA

A 180 bp DNA fragment containing the homeobox-coding sequence of the chicken Not1 gene (Stein and Kessel, 1995) was used to hybridize a mouse E14.5 brain library (see Oliver et al., 1995; Wijnholds et al., 1995) at 65°C in 10x Denhardt’s, 5x SSC, 0.1% SDS. Filters were rinsed three times at low stringency (20 minutes at 60°C in 1x SSC, 0.1% SDS). A partial cDNA clone, c5, containing the coding region of the mouse Vax1 homeobox was isolated and used to screen the same library under more stringent conditions (hybridization in the conditions described above, followed by three rinsings for 20 minutes at 65°C in 0.1x SSC, 0.1% SDS). We finally isolated a cDNA clone of 1.6 kb containing the mouse Vax1 putative full coding sequence.

Degenerated primers were used to amplify Xenopus Vax1-related sequences (primer P1 5’GGGAATTCCTGGAACCTGGAGCGCCCCAAG and primer P2 5’TCTCTAGAGCTTCACCTGCTCGGAGAG) from 30 aliquots of a stage 32 head cDNA library containing 1.5x10⁶ pfu in total (30x50,000 pfu) in a final volume of 25 μl as previously described (Hollemann et al., 1998). The PCR products were directly sequenced using the ABI cycle sequencing kit (USB). Screening of amplified cDNA libraries was performed as described with the degenerate primers specified above (Israel, 1993). Three rounds of PCR were performed to rescreen increasing dilutions of the stock library. Finally, 12 positive phage clones containing identical inserts with putative full coding sequence were isolated.

Quantitative RT-PCR analysis

RNA from oocytes and embryos and from tissues of adult frogs was extracted as described (Hollemann et al., 1998). RT-PCR analysis was carried out using the Gene Amp RT-PCR kit from PerkinElmer Cetus. First-strand cDNA was synthesized using 1 μg RNA from the various embryonic stages indicated and tissues with random hexanucleotides as primers. Initial denaturation was performed for 3 minutes at 95°C, followed by 40 amplification cycles (1 minute, 95°C; 1.5 minute, 52°C; 1.5 minute, 72°C) and a final polymerization step (10 minutes, 72°C).

For quantitative RT-PCR, first-strand cDNA was synthesized from 0.5 μg RNA as described above. Amplification was done in a 100 μl reaction containing the appropriate primer mix for Xvax1 (P3 corresponding to amino acid 161-177), 5AGAGACGCGCAGGAATTCACC and P4, corresponding to amino acid 275-281. Amplification was linear up to cycle number 30. Amplification of Xvax1 transcripts was done for 28 cycles and amplification of histone H4 transcripts for 23 cycles. One tenth of each PCR product was analyzed on a 6% polyacrylamide gel under denaturing conditions. As a size marker, α32P-end-labelled 1-kb ladder (Gibco) was used. Dried gels were analyzed using a PhosphorImager and the ImageQuant 2.0 program (Molecular Dynamics).

In situ analysis

In situ hybridization experiments, on paraffin sections with radiolabelled probes or whole embryos with DIG-labelled probes, were performed as previously described on NMRI mouse material (Wilkinson, 1992; Stoykova and Gruss, 1994). We used clone c5 cut with HindIII or XhoI and transcribed with SP6 or T7 RNA polymerase for Xvax1 sense or antisense probes, respectively. A Pax6b clone, spanning from the 3’ part of the homeobox to the 3’ untranslated region, cut with BamHI or HindIII and transcribed with T3 or T7 RNA polymerase was used for antisense or sense probes respectively. Dlx1, Emx1 and Emx2 probes were prepared as previously described (Price et al., 1991; Simeone et al., 1992b). In order to test possible regulation of Xvax1 expression by the Pax6 gene product, in situ hybridization experiments were also performed on Pax6-deficient embryos from either the classical see mutants (Roberts, 1967; Hogan et al., 1986), or from animals carrying an experimentally inactivated Pax6 gene (St-Onge et al., 1997). Homozygous embryos were selected on the basis of their eye size as previously described (Stoykova et al., 1996).

In situ hybridization of Xenopus embryos was performed as described by Harland (1991). Digoxigenin-UTP RNA-probes were used and developed with NBT/BCIP (Boehringer). For plastic sections, embryos were stained for 3 days at 6°C, embedded in Technovit 7100 (Kulzer), sectioned (6 μm) and mounted in Entellan (Merck). Photographs were taken on a Zeiss Axioskop using Nomarski interference optics.

Interspecific mouse backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6jXF. spretus)F1 females and C57BL/6j males as described (Copeland and Jenkins, 1991). A total of 205 N2 mice was used to map the vax1 locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described (Jenkins et al., 1982). The probe, a 460 bp BamHI 5’ cDNA fragment excluding
the homeobox coding region, was labelled with alpha-^32^P-dCTP using a random primed labeling kit (Stratagene); washing was done to a final stringency of 1.0 x SSC, 0.1% SDS, 65°C. A fragment of 13.5 kb was detected in SacI-digested C57BL/6J DNA and a fragment of 7.3 kb was detected in SacI-digested M. spretus DNA.

A description of the probes and RFLPs for two of the loci linked to Vax1 including Mxl1 and Aopl has been reported previously (Juan et al., 1997). These loci have not been reported previously for this interspecific backcross. The probe for Pax2 was an ~1.7 kb BamHI-EcoRI fragment of mouse cDNA that detected TaqI fragments of 4.6, 0.6 and 0.4 kb in C57BL/6J (B) DNA and 5.4, 4.6, 0.6 and 0.4 kb in M. spretus. (S) DNA. The probe for Fgfr8, an ~800 bp Small-Xhol fragment of mouse cDNA, detected PsrI fragments of 2.1, 1.3 and 1.1 (B) and 2.1, 1.1 and 0.9 (S) kb. This probe also detected HindIII fragments of 8.1 and 3.2 (B) and 20.5 and 3.2 (S) kb. The Psrl and HindIII data were combined. Finally, the probe for Emx2 was an ~1.1 kb Xhol fragment of mouse genomic DNA that detected BglII fragments of 8.2 and 3.2 (B) and 8.6 and 2.4 (S) kb. In each case, the inheritance of M. spretus-specific fragments was followed in backcross mice. Recombination distances were calculated using Map Manager, version 2.6.5.

RESULTS

Isolation and characterization of the mouse and Xenopus Vax1 sequence

In an attempt to identify new murine genes related to the Not sub-group of homeobox-containing genes (see Duboule, 1994), we used the chicken Not1 homeobox coding region to probe a mouse brain E14.5 cdna library at low stringency (Wijnholds et al., 1995). We finally isolated a cdna clone of approximately 1.6 kb containing a putative open reading frame coding for a new 338 amino acids long homeobox-containing gene (Fig. 1A).

Sequence comparison identified a human EST clone (GenBank accession number H92142) containing an unspliced intron inside the region coding for a homeodomain identical to that of Vax1 (see Fig. 1B).

The murine Vax1 gene being expressed in a phylogenetically ancient and evolutionarily conserved part of the ventral forebrain suggested that this gene could also exist in lower vertebrate. We thus used the mouse and human sequences in order to design primers to isolate the Xenopus homologous gene using PCR. The putative full-length Xvax1 cdnas has been isolated as detailed in Materials and Methods. The predicted amino acid sequence shares 71% homology with the mouse total sequence, and 95% at the level of the homeodomain (Fig. 1). In addition, both the mouse and Xenopus sequences share two putative translation initiation codons at identical positions (see Fig. 1A) (Kozak, 1986; Cavender and Ray, 1991; Boek and Kolakofsky, 1994).

The high degree of conservation in the homeodomain required for sequence-specific DNA binding suggests that the Vax1 genes binds to a highly conserved binding site. The predicted homeobox amino acid sequences share high homology with the Not1 genes and with the empty spiracles related genes (Fig. 1B). However, the YPW conserved amino acid residues present in all the Emx genes upstream of the homeobox (Simeone et al., 1992b) are absent from the mouse and Xenopus sequences (Fig. 1A).

Chromosomal location of Vax1

The mouse chromosomal location of Vax1 was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × Mus spretus)F1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 2000 loci that are well distributed among all autosomes as well as X chromosome (Copeland and Jenkins, 1991). C57BL/6J and M. spretus DNAs were digested with several enzymes and analyzed using Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a mouse cdna Vax1 probe. The 7.3 kb SacI M. spretus RFLP (see Materials and Methods) was used to follow
the segregation of the Vax1 locus in backcross mice. The mapping results indicated that Vax1 is located in the distal region of the mouse chromosome 19 linked to Pax2, Fgf8, Mxi1, Emx2 and Aop1. Although 113 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 2), up to 169 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere – Pax2 – (2/154) – Fgf8 – (8/159) – Mxi1 – (11/169) – Emx2 – (0/157) – Vax1 – (1/156) – Aop1. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± the standard error] are Pax2 – (1.3±0.9) – Fgf8 – (5.0±1.7) – Mxi1 – (6.5 – 1.9) – [Emx2, Vax1] – (0.6±0.6) – Aop1. No recombinants were detected between Emx2 and Vax1 in 157 animals typed in common suggesting that the two loci are within 1.9 cM of each other (upper 95% confident limit). The tight linkage and sequence relatedness between Vax1 and Emx2 suggest that the genes may have arisen by tandem duplication.

We have compared our interspecific map of chromosome 19 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (provided from Mouse Genome Database, a computerized database maintained at the Jackson Laboratory, Bar Harbor, ME). Vax1 mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus (data not shown).

The distal region of mouse chromosome 19 shares a region of homology with human chromosome 10q25-26 (summarized in Fig. 2) where the human EMX2 has been mapped (Kastury et al., 1994). This suggests that the human homolog of Vax1 is likely to map to this region as well. Apart from Brunelli and collaborators, who found that three out of eight patients with severe schizencephaly were heterozygous for different mutations in the EMX2 gene (Brunelli et al., 1996), no other human chromosomal alterations have been reported in this region (see Online Mendelian Inheritance in Man, John Hopkins University and National Center for Biotechnology Information, World Wide Web URL: http://www.ncbi.nlm.nih.gov./Omim/).

Vax1 expression pattern in the mouse

In the mouse, the expression of Vax1 is detectable at embryonic day 8 pc (E8), at the most rostral level of the medial neural plate, including the anterior neural ridge (ANR) and adjacent ectoderm (Fig. 3). After neural tube closure, during E9, expression is detected in the ectoderm in a transverse band located between the developing olfactory placodes, medially in contact with the neural tube (Fig. 4). Between embryonic days 10 and 12, the expression persists in the rostral oral ectoderm and in the rostral and medial part of the olfactory placodes. No clear expression was detected after E12 in the derivatives of these structures and in the vomeronasal organ in particular. From E9 to E14, the expression remains confined in the derivatives of the neural plate regions expressing the gene at E8 (Figs 4, 7 and 8) as predicted by fate maps (Coulby and Le Douarin, 1987; Eagleson and Harris, 1990; Eagleson et al., 1995). They are namely the optic disk, the optic stalk and later optic nerve, the optic chiasm, the suprachiasmatic area, the hypothalamic cell cord, the whole preoptic area, the septum, the entopeduncular area and the basal ganglia. Laterally, the expression is confined to the lateral and medial ganglionic eminences (LGE and MGE respectively). Caudally, the expression is restricted in the rostral part of the anterior hypothalamus (Fig. 7). The expression in the septum does not enter the roof of the cortical telencephalon (Fig. 8).

Vax1 expression pattern in the Xenopus

Quantitative RT-PCR demonstrates that zygotic XVax1 transcription, first detectable at early gastrula stages, gradually increases until stage 38. In adult frog tissues, XVax1 expression is restricted to neural derivatives and testes (Fig. 5). Whole-mount in situ hybridization experiments demonstrate
that, similarly to the situation in mouse, XVax1 is expressed in the anterior-most region of the open neural plate and midanterior and midlateral anterior ridge (stage 16, Fig. 6A). By late neurula stages (stage 19), XVax1 expression is detected in the derivatives of these regions as predicted by fate maps established in *Xenopus* (Eagleson and Harris, 1990; Eagleson et al., 1995), namely, in the anterior and rostral ventrolateral part of the forebrain neuroepithelium, the primordium striatum, the optic stalk, the chiasmatic ridge and the anterior hypothalamus (Fig. 6B). At tailbud stages (stage 26), the expression extends laterally and caudally with the enlargement of the forebrain ventricle (Fig. 6C). XVax1 expression in tadpole stage embryos (stage 34, Fig. 6D-H) is specific to the optic disc (Fig. 6C,D,G), the optic stalk (Fig. 6C,D,G,H), and the anterior hypothalamus (Fig. 6F).

In summary, the expression of Vax1 is strikingly similar in mouse and *Xenopus* embryos during early neurulation. As expression is mainly restricted to derivatives of the anterior neural plate during early vertebrate embryogenesis, we named the gene Vax1 for ventral anterior homeobox-containing gene.

**Expression of Vax1, Dlx1, Pax6 and Emx1 define distinct boundaries within the mouse forebrain**

In order to delineate and characterise with more precision the limits of the Vax1 expression domain, we compared Vax1
expression pattern with that of other markers of the mouse developing forebrain. We used the Dlx1 gene (Price et al., 1991; Bulfone et al., 1993) as a ventral marker and the Pax6 and Emx1 genes as dorsal markers (see Walther and Gruss, 1991; Simeone et al., 1992b; Stoykova and Gruss, 1994). The Dlx1 and Vax1 genes are both expressed in the septum, preoptic area and ganglionic eminences. However, Dlx1 gene expression extends more caudally and dorsally in the ventral thalamus and lateral hypothalamus in contrast to Vax1 (see Figs 7B,C,E,F, 8B,C) (see Price et al., 1991; Bulfone et al., 1993). Conversely, Vax1 is expressed in the optic chiasm and optic stalk regions where Dlx1 is absent (see Figs 7E,F, 8E,F). At the level of the basal ganglia, the expression of Vax1 is restricted to the ventricular zone and the deeper part of the subventricular zone (SVZ) (Boulder Committee, 1970). At this level, Dlx1 expression extends more distally outwards in the SVZ and is not as restricted to the deep SVZ as that of Vax1 (see Figs 7B,C, 8B,C). In addition, Vax1 expression is more restricted laterally in the LGE than Dlx1 expression. Dlx1 expression extends slightly more laterally in the lateral LGE than that of Vax1 toward the corticostriatal sulcus so that it nearly contacts the Emx1-expressing cortical region (Fig. 9).

Pax6 transcripts are detected as previously described (Walther and Gruss, 1991; Stoykova et al., 1996, 1997) (Figs 7A,D,G, 8A,D). As opposed to that of Emx1 (see Simeone et al., 1992b), the expression of the Pax6 gene transgresses the corticostriatal boundary and extends from the cortical compartment into the lateral LGE, where it apparently abuts that of Vax1 (Figs 7A-E, 8A,B, 9A,B). The pattern of expression of Pax6 and Vax1 genes are thus quite complementary and exclusive in the forebrain.

Vax1 gene expression in Pax6-deficient homozygous mutant mice

We therefore further tested the possibility that Pax6 could regulate the expression of the Vax1 gene in Pax6-deficient mice. Genetic lesions of Pax6 have already been extensively documented in the rodent small eye (sey) mutants, sharing ocular and brain malformations (for references, see: Stoykova et al., 1996; Caric et al., 1997).

Studies of rodent sey mutants demonstrate the Pax6 function in forebrain patterning and regionalization (Matsuo et al., 1993; Stoykova et al., 1996; Mastick et al., 1997). In particular, the corticostriatal boundary is impaired in Sey mice as evidenced by the Dlx1 gene expression transgressing this boundary in homozygous mutants, but not in wild-type animals (Stoykova et al., 1996; Mastick et al., 1997). Moreover, Pax6 has a probable role in embryonic cortical cell migration (Caric et al., 1997) and in diencephalic precursor proliferation (Warren and Price, 1997).

Fig. 5. Expression of Xvax1 during early development (stages as indicated, A) and in tissues of adult frogs (B) as detected by quantitative RT-PCR. Transcription of Xvax1 is activated in early gastrula and upregulated during neurulation. te, testis; ov, ovary; sc, spinal cord; br, brain; ey, eye; fa, fat body; bl, bladder; lu, lung; ki, kidney; li, liver; sp, spleen; in, intestine; st, stomach; gu, gut; mu, muscle; he, heart; sk, skin.

Fig. 6. Developmental expression of Xvax1 analyzed by whole-mount in situ hybridization. (A) Anterior view of an embryo at stage 16. Staining is detected in the anterior neural ridge. (B) Anterior view of Xvax1 expression at stage 19. Xvax1 signal is visible in the ventral forebrain. The optic vesicle (OV) shows no expression. (C) Ventral/anterior view of an embryo at stage 26. Xvax1 transcripts are detected in the ventral hypothalamus (vh), optic stalk (OS) and the optic disc (OD). (D) Lateral view at stage 34. Xvax1 transcripts are located the ventral and anterior forebrain. (E) To improve resolution, a cleared embryo at stage 34 is shown in a dorsal/lateral view. Strong Xvax1 expression is visible in the ventral anterior forebrain with a sharp dorsal border. (F-H) Sections of stained plastic embedded embryos at stage 34. F: In a sagittal section Xvax1 transcripts are detected in the chiasmatic ridge and the ventral hypothalamus. (G) Parasagittal section showing Xvax1 expression in the eye disc and in the optic stalk. (H) Transverse section at the level of the optic chiasm. ar, anterior ridge; ba, branchial arch; Ch, chiasmatic ridge; dl, diencephalon; fbv, forebrain vesicle; gu, gut; i, infundibulum; nc, notochord; OD, optic disk; OS, optic stalk; OV, optic vesicle; sep, septal/striatal region; vh, ventral hypothalamus; vtt, ventral tegmental tract.
We used both *sey* and *Pax6*-transgenic homozygous animals (St-Onge et al., 1997) to study *Vax1* expression pattern in the absence of functional *Pax6* and observed similar results. In homozygous animals, the restricted *Dlx1* expression is distorted at the level of the boundary between the LGE and the developing cortex, and so-called corticostriatal boundary, so that it extends ectopically in cortical areas (Fig. 10A) as previously reported (Stoykova et al., 1996). Similarly, *Vax1* expression is also shifted laterally at the level of the subventricular zone towards the cortical area. However, the lateral limit of expression of *Vax1* in these homozygous mutants (see arrows in Fig. 10B).

**Fig. 7.** *Vax1* expression at E13.5 pc compared with that of *Pax6* and *Dlx1* on horizontal sections. 
(A-H) In situ hybridization using radiolabelled probes, respectively, from dorsal to ventral. 
(A,D,G) *Pax6* probe; (B,E,H) *Vax1* probe; (C,F) *Dlx1* probe. *Vax1* is expressed in the basal forebrain in the ventricular and deep subventricular zones of the septum, LGE, anterior hypothalamus, optic chiasm, optic disk and optic nerve (B,E,H), but excluding the amygdala (A,B). The observed expression of *Pax6* corresponds with previous descriptions (Walther and Gruss, 1991) and confirms the presence of transcripts in the ventricular zone of the lateral LGE (A,D) and retrochiasmatic region (G). The expression domains of *Pax6* and *Vax1* are exclusive, apart from the septal area. Very low *Pax6* expression is also observed in the ventricular zone of the LGE (A,B; D-E). The observed expression of *Dlx1* corresponds to previous descriptions (Bulfone et al., 1993). *Dlx1* and *Vax1* are both expressed in the septum and ganglionic eminences (B,C,E,F). However, the expression of *Vax1* is more restricted in the deep subventricular zone. The dorsolateral limit of *Dlx1* expression is located slightly more laterally than that of *Vax1* in the lateral LGE. In addition, the *Dlx1* domain of expression extends more caudally than that of *Vax1* in the caudal hypothalamus and ventral thalamus. Inversely, *Vax1* is expressed in the posterior preoptic area as opposed to *Dlx1* (B,C). AHy, adenohypophysis; Ctx, developing cerebral cortex; HCC, hypothalamic cell cord; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Od, optic disk; On, optic nerve; POA, anterior preoptic area; POP, posterior preoptic area; RCH, retrochiasmatic area; SCH, suprachiasmatic area; Sep, Septum; VT, ventral thalamus. Scale bar: 1 mm.

**Fig. 8.** *Vax1* expression at E13.5 pc compared with that of *Pax6* and *Dlx1* on sagittal sections. 
(A-F) In situ hybridization using radiolabelled probes. (A,D) *Pax6* probe; (B,E) *Vax1* probe; (C,F) *Dlx1* probe. The expression domains of *Pax6* and *Vax1* are exclusive except in the septal area where *Pax6* is expressed in the ventricular zone and *Vax1* in the ventricular and deep subventricular zone (A,B). *Dlx1* and *Vax1* both express in the septum and LGE (B,C,E,F). However, the expression of *Vax1* is more restricted in the deep subventricular zone. The *Dlx1* domain of expression extends more caudally than that of *Vax1* in the caudal hypothalamus and ventral thalamus. In addition, *Vax1* is expressed in the posterior preoptic area as opposed to *Dlx1* (B,C). AHy, adenohypophysis; Ctx, developing cerebral cortex; HCC, hypothalamic cell cord; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Po, optic disk; On, optic nerve; POA, anterior preoptic area; POP, posterior preoptic area; RCH, retrochiasmatic area; SCH, suprachiasmatic area; Sep, Septum; VT, ventral thalamus. Scale bar: 1 mm.
DISCUSSION

The induction and later the regionalization of the rostral neural plate destined to become the brain depends on tissue interactions and genetic regulations distinct from those occurring in more caudal parts of the central nervous system during the early steps of neurulation (see Ruiz i Altaba, 1994; Lumsden and Krumlauf, 1996). In particular, signals from the endomesoderm and from the anterior neural ridge act together to induce and pattern the forebrain (Ang and Rossant, 1993; Bouwmeester et al., 1996; Shimamura and Rubinstein, 1997; Houart et al., 1998). However, the genetic mechanisms occurring within the developing forebrain during induction and specification remain largely obscure. In order to study these mechanisms, early forebrain markers with restricted pattern of expression are necessary. In this context, the Not1 subfamily of homeobox-containing genes (see Duboule, 1994) is of interest. High sequence homologies and similar patterns of expression of the Not1 genes identified in various species, and necessary for axial mesoderm formation (Talbot et al., 1995), attest a high sequence conservation during vertebrate evolution (von Dassow et al., 1993; Stein and Kessel, 1995; Talbot et al., 1995; Stein et al., 1996). No mouse members have been identified to date. Moreover, sequence homologies between the Not1 genes (von Dassow et al., 1993; Stein and Kessel, 1995; Talbot et al., 1995; Stein et al., 1996) and the Emx genes, both expressed in the forebrain (Simeone et al., 1992b), suggests that potential new Not- and Emx-related genes could also exist. Furthermore the mouse Emx1 and Emx2 genes are required during forebrain formation (Brunelli et al., 1996; Pellegrini et al., 1996; Qiu et al., 1996).

Characterisation of the Vax1 gene

In order to isolate novel Not- and Emx-related genes, we probed a E14.5 mouse brain cDNA library (Wijnholds et al., 1995) with the chicken Not1 homeobox-coding region (Stein and Kessel, 1995) at low stringency and isolated the Vax1 homeobox-containing gene. PCR-based screenings were performed to isolate the putative Xenopus counterpart, XVax1. The high degree of conservation of the mouse and Xenopus gene sequences (Fig. 1) and expression patterns (compare Figs 3 and 6 to 7) strongly suggests that both genes are orthologous. The Vax1 homeobox amino acid sequence shares high homology with the various members of the Not and Emx gene families (Fig. 1B). However, the YZW amino acid sequence which is present in ems and all Emx genes upstream of the homebox (Simeone et al., 1992b), is absent from Vax1 (Table 1). The Vax1 gene is thus a putative transcription factor which could accordingly reveal the existence of a new subgroup of homeobox-containing gene family.

The mouse Vax1 gene locus is localized at the very distal end of the mouse chromosome 19 (Fig. 2). Comparison of the position of this locus with that of Pax2, FGF8 and Emx2

Fig. 9. The corticostriatal boundary is a complex region, located between the developing cortex and LGE, where distinct limits of various gene expression are identifiable. (A-D) In situ hybridization on lateral sagittal sections with Vax1 (A), Pax6 (B), Dlx1 (C), and Emx1 (D) antisense probes. The lateral limit of Vax1 expression (A) apparently abuts the ventrolateral limit of Pax6 strong expression (B) in the lateral LGE. This limit does not correspond to that of the Dlx1 (C), which is located in the LGE, or to the ventrolateral limit of Emx1 expression (D), which does not extends into the LGE. Accordingly, the corticostriatal boundary region would comprise at least two zones confined between three distinct limits of gene expression. Ctx, developing cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. Scale bar, 1 mm.

Fig. 10. Pax6 function is necessary to maintain the normal Vax1 lateral limit of expression in the LGE subventricular zone. Transverse sections through a homozygous small eye (sey/sey) E13.5 mouse rostral forebrain. (A) Dlx1 antisense probe; (B) Vax1 antisense probe. In the s sey/sey brain, the Dlx1 expression is no longer confined into the septum and LGE, but extends in the developing cortex (A). As observed in wild-type brains (see Fig. 7B), the Vax1 expression is limited laterally in the ventricular zone of the s ey/sey LGE (B, see arrows). Though more limited than that of Dlx1, Vax1 ectopic expression towards the developing cortex also occurs in brains of Pax6-deficient homozygous animals at the level of the subventricular zone. Arrows indicate the lateral limit of Vax1 in the ventricular zone. Ctx, developing cerebral cortex; LGE, lateral ganglionic eminence; Sep, septum. Scale bar, 200 μm.
indicates a linkage of the \textit{Vax1} and \textit{Emx2} loci. The genes may therefore have arisen by a tandem duplication. However, apart from mutations in the \textit{EMX2} locus level (Simeone et al., 1992b; Brunelli et al., 1996), no human or mouse disorder consistent with a modification of \textit{Vax1} have been mapped at this level.

Similarly to the mouse \textit{Emx2} and \textit{Vax1} genes, the \textit{Drosophila} \textit{ems} and \textit{E5} (see Fig. 1B) genes share high sequence homology and identical cytological location in the region 88A of polytene chromosome (Dalton et al., 1989). This could indicate a highly conserved chromosomal organisation in vertebrate and arthropods at that level. However, further functional experiments in both genders are necessary to conclude about possible orthology between these genes.

Possible functions of \textit{Vax1}

Mutations of the \textit{Drosophila empty spiracles (ems)} gene affect embryonic head development and lead to loss of head structures (see Hirth et al., 1995). Therefore, \textit{ems} acts as both a gap gene, responsible for the formation of group of head segments, and a homeotic selector genes, specifying segment identity (Dalton et al., 1989; Walldorf and Gehring, 1992). Similarly, inactivation of \textit{Emx1} or \textit{Emx2} murine genes by homologous recombination in the mouse demonstrates that these genes are necessary for the formation of specific regions of the brain where they are expressed and for migration of some cortical neurons (Pellegrini et al., 1996; Qi et al., 1996; Yoshida et al., 1997). Mutations in the human \textit{EMX2} genes have also been identified in patients with holoprosencephaly (Brunelli et al., 1996). Likewise, \textit{Not1} inactivation in the zebrafish leads to absence of notochord where the gene is normally expressed (Talbot et al., 1995). \textit{Vax1} sharing high sequence homology with these genes could accordingly be necessary for ventral forebrain specification and patterning. Moreover, the stability of \textit{Vax1} pattern of expression during neurulation in mouse and \textit{Xenopus} suggests an important function of \textit{Vax1} in the specification and maintenance of basal forebrain identity. \textit{Vax1} is also expressed in the anterior neural ridge (ANR) (see Figs 3 and 6). This structure has inducing properties on the anterior neural plate mimicked by the FGF8 ridge (ANR) (see Figs 3 and 6). This structure has inducing forebrain identity.

\textbf{Vax1 expression in Pax6-deficient homozygous mutant mice}

\textit{Vax1}-expressing regions are apparently adjacent to the regions expressing \textit{Pax6} at high level. In particular, at the level of the corticostriatal-boundary of wild-type animals, \textit{Pax6} expression occurs at high level in the developing cortex and in the lateral part of the LGE. At this level, \textit{Vax1} expression occurs in the medial part LGE, and abuts that of \textit{Pax6} laterally. This observation suggests that some cross regulation may occur between these two genes or the regulatory cascades in which they are involved.

\textit{Vax1} pattern of expression is modified in \textit{Pax6}-deficient homozygous mice at the level of the corticostriatal boundary. As already described for \textit{Dlx1} (Stoykova et al., 1996) at the level of the subventricular zone, \textit{Vax1} expression is no longer confined in the LGE, or so-called striatal compartment, but extends towards the cortical area (Fig. 10A). However, the lateral limit of \textit{Vax1} expression is still clear-cut in the lateral LGE ventricular zone (see arrows in 10B) and remains more medial than that of \textit{Dlx1} (Fig. 10A,B). Accordingly, \textit{Pax6} is not necessary to restrict \textit{Vax1} expression pattern in the LGE ventricular zone. Nevertheless, the ectopic \textit{Vax1} expression in the cortical subventricular zone of \textit{Pax6}-deficient embryos suggests that the \textit{Pax6} gene product maintains and stabilizes the \textit{Vax1} expression pattern outside the ventricular zone. Besides, \textit{Pax6} regulates forebrain patterning in general by regulating cell adhesion properties in a specific territory (Stoykova et al., 1997) and thereby controlling boundary formation and, as a consequence, the neuromere identification (Mastick et al., 1997). Finally, the cells expressing \textit{Vax1} ectopically are no longer located over a ventricular zone also expressing the gene as in the normal situation. It is thus likely that the ectopic expression of \textit{Vax1} in these mutants is the consequence of an abnormal cell migration rather than a change of the identity of the lateralventral subventricular cortical compartment. Moreover, the possibility for striatal precursors to differentiate in the cortex has been already documented from grafting experiments (Fishell, 1997), and cell migration of \textit{Dlx}-dependent neuroblast from the LGE to the cortex have been characterized (see De Carlos et al., 1996;
Anderson et al., 1997a,b). These striatocortical routes could accordingly be followed by Vax1- and Dlx1-expressing cells in Pax6-deficient animals.

The question of the corticostriatal boundary
Comparison of the limits of the expression territory of Vax1 with that of Dlx1, Pax6 and Emx1 indicates that the morphological corticostriatal sulcus does not correspond to a general limit of gene expression (see Puelles and Rubenstein, 1993; Stoykova et al., 1997). Strong Pax6 gene expression occurs in the developing cortex, transgresses the anatomical sulcus and extends in the lateral part of the LGE, the so-called corticostriatal ridge or palliostriatal ventricular angle (Bayer and Altman, 1991). At this level, Pax6 expression is complementary with that of Vax1, which is confined to the LGE (Figs 7A,B, 9A,B). Additionally, Dlx1 expression is also confined to the LGE, but it however extents slightly more distally than Vax1 toward the cortical compartment (Figs 7B,C, 9B,C). Inversely, the Emx1 gene expression is strictly limited in the cortical compartment (Fig. 9D). Therefore, the corticostriatal sulcus is a simple anatomically identifiable structure, which does not correspond to a simple frontier for gene expression. Rather, the lateral LGE or palliostriatal ventricular angle is a complex structure with distinct genetic identifiable compartments expressing specific combinations of markers.

Moreover, tritiated thymidine dividing cell labelling demonstrated that endopiriform neurons originate in the palliostriatal ventricular angle (Bayer and Altman, 1991). Similarly, Dil cell labelling experiments demonstrated that the lateral LGE generate neurons of the primary olfactory cortex (De Carlos et al., 1996). Likewise, some cortical neurons producing GABA originate in the LGE, and their differentiation depends on the Dlx1 and Dlx2 genes (Anderson et al., 1997a). Vax1 could thus mark a strictly ventral part of the forebrain as opposed to more dorsal parts with cortical fate. Further experiments would be necessary to determine whether some aspects of the cortical development also depend on the activity of the Vax1 gene.

Vax1 transcripts are first present in the most anterior part of the neural plate in mouse and Xenopus. The expression is maintained during early embryonic development in the derivatives of this region. Vax1 therefore marks an embryological unit, being phylogenetically ancient and evolutionary conserved, and which differentiates in a functionally central part of the anterior ventral forebrain. The persistence of Vax1 expression during ontogenesis could suggest a role of Vax1 in maintaining the identity of this region. Further functional experiments are now in progress to determine the function of this new gene during early forebrain development and neural differentiation. Vax1 should be an interesting tool to study the specification, patterning and terminal differentiation of the forebrain.

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