

***Vax1* is a novel homeobox-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 homeobox-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; Lufkin, 1996). Moreover, mouse embryos homozygous for targeted null mutation of the *Otx2* homeobox-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 neuroectodermal 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 FGF8 molecule produced at this level regulates the expression of the BF1 transcription factor essential for ventral forebrain and eye formation (Shimamura and Rubinstein, 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 *Vax1* and *Not1* and *Emx* genes suggest 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 10× Denhardt's, 5× SSC, 0.1% SDS. Filters were rinsed three times at low stringency (20 minutes at 60°C in 1× SSC, 0.1% SDS). A partial cDNA clone, cf5, 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 0.1× 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'CGGAATCCGTGGACTGGACCGGCCAAAG and primer P2 5'TGCTCTAGAGCTTCACCTGGGTCTCGGAGAG) from 30 aliquots of a stage 32 head cDNA library containing 1.5×10⁶ pfu in total (30×50.000 pfu) in a final volume of 25 µl as previously described (Holleman 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 previously (Holleman et al., 1998). RT-PCR analysis was carried out using the Gene Amp RT-PCR kit from Perkin-Elmer 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 5'AGGAGCAGC-CGGGAACCTTAC and P4 corresponding to amino acid 275-281 5'TCTCTGCCGACCAATACTGTC resulting in a DNA product of 364 nucleotides), and Histone H4 as previously described. The linear range of amplification was determined by carrying out PCR including 0.5 µg of RNA of stage 32 embryos and 1 µCi of alpha-³²P-dCTP with increasing numbers of PCR cycles. 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 α-³²P-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 cf5 cut with *HindIII* or *XhoI* and transcribed with SP6 or T7 RNA polymerase for *Vax1* sense or antisense probes, respectively. A *Pax6* 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 *Vax1* expression by the *Pax6* gene product, in situ hybridization experiments were also performed on *Pax6*-deficient embryos from either the classical *sey* 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).

Whole-mount 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/6J×*M. 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-³²P-dCTP using a random primed labeling kit (Stratagene); washing was done to a final stringency of 1.0× SSCP, 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 *Mxi1* and *Aop1* has been reported previously (Juan et al., 1997) Three 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 *Fgf8*, an ~800 bp *SmaI-XhoI* fragment of mouse cDNA, detected *PstI* 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 *PstI* and *HindIII* data were combined. Finally, the probe for *Emx2* was an ~1.1 kb *XhoI* 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; Cavener 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

A

<i>mm Vax1</i>	1	MFGKPKDKMVDVRSCHSDTEAARVSKNAHKESEIREIKGAEGSLPAAFLKEPQGAFIGSGGASEDC
<i>xl Vax1</i>	1	M-E-TTD--I--NIE--NG-I--. .P-DNK---ETQAKM-STY---QP-TYPAP-S--L-
		NKSKSNSSADPDYCRRLVLRDAKGSIREIILPKGLDLDRPKRTRTSFTAEQLYRLEMEFQ
		A-N--S-AG--E-----S-----
		RCQYVVGRETELARQLNLSETQVKVWFQNRRTKQKKDQKDSSELRSVSVETAATCSVLR
		-----D-S-----
		LLEQGRLLSPPGLPALLPPCATGALGSALRGPSPALGAGAAAGSAAAAAAAAAATAPGP
		-----G-M--T--T-----R--NS
		AGAASQHPQAVGGAGPGPAGPGLHAGAPTASHGLFSLPVPVSLGGSVASRLSSAPLITMA
		S-PGTRSLAT-TST-PHQ-.PSP. .TG-NI-NM-----T--N--H-----
		GSLAGNLQELSARYLSSSAFEPYSRTNNKEGAEKKALD 338 ID
		-.H-----SIS-DSLDD--L-- 295 71%

B

<i>mm Vax1</i>	PKRTRTSFTAEOLYRLEMEFORCQYVVGRETELARQLNLSETQVKVWFQNRRTKQKKDQ	ID
<i>hs Vax1 EST</i>	-----	100%
<i>xl Vax1</i>	---S-----D-S---	95.0%
<i>dm ems</i>	---I--A-SPS--LK--HA-ESN----A--KA--QN-----H-RM-	66.7%
<i>dm E5</i>	---V--A-SPT--LK--HA-EGNH---A--KQ--QG-S-T-----H-RM-	61.7%
<i>mm Emx1</i>	---I--A-SPS--L--RA-EKNH---A--KQ--GS-S-----Y-RQK	63.3%
<i>mm Emx2</i>	---I--A-SPS--L--HA-EKNH---A--KQ--HS-S-T-----F-RQK	61.7%
<i>xl Not</i>	M--I--V--P---EK--K--LKQ--M--T--VD--ST---T-----I-W--QS	65.0%
<i>gg Not1</i>	M--V--V--KP---E---Q--LKQ--M--T--VD--AT-R-T-----WR-QS	63.3%

Fig. 1. (A) *Vax1* mouse and *Xenopus* amino acid sequence comparison. GenBank accession numbers for *Vax1* sequences are AF064554 and AF064601. (B) Mouse and *Xenopus Vax1* homeodomain amino acid sequence alignment in comparison to the homeodomain of *Drosophila (dm) ems*, *Homo sapiens (hs)*, mouse (*mm*) *Emx1* and *Emx2*, *Xenopus (xl)* and chicken (*gg*) *Not1*. Identical amino acids are represented by hyphens. Dots represent gaps introduced in the amino acid sequence in order to obtain optimal alignment. Identity (ID) values were calculated pairwise. The human and mouse *Emx* homeobox amino acid sequences are identical. Only the mouse sequences are thus presented. The underlined amino acid of the mouse *Vax1* sequence indicate the predicted positions of the three helices of the homeodomain. The intron sequence between position 44 (Q) and 45 (V) within the third helix of the homeobox of the human EST clone sequence has been removed from the sequence in this figure.

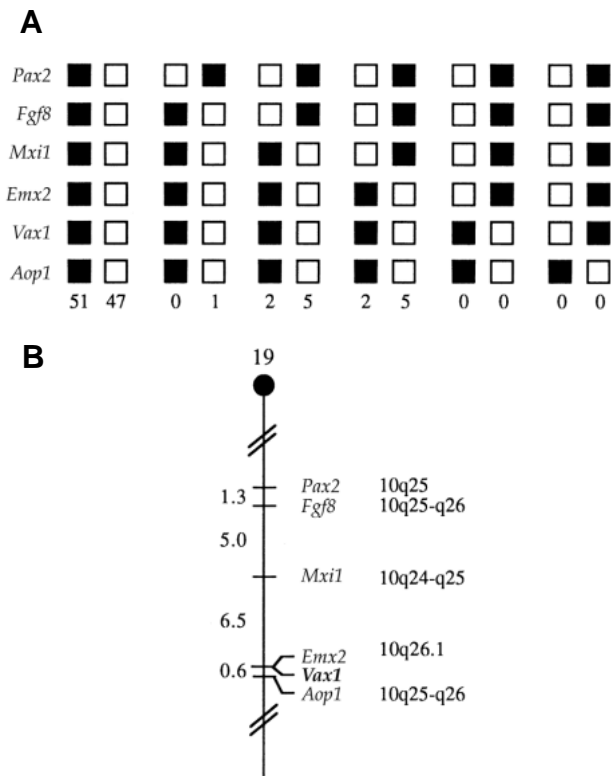


Fig. 2. *Vax1* is mapped in the distal region of mouse chromosome 19. (A) *Vax1* was placed on mouse chromosome 19 by interspecific backcross analysis. The segregation patterns of *Vax1* and flanking genes in 113 backcross animals that were typed for all loci are shown at the top of the figure. For an individual pair of loci, more than 113 animal were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*) F₁ parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. (B) A partial chromosome 19 linkage map showing the location of *Vax1* in relation to linked genes. Recombination distances between loci in centimorgans are shown to the left of the chromosome and the positions of the loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome DataBase), a computerized database of human linkage information maintained by the William H. Welsh Medical Library of The John Hopkins University (Baltimore, MD).

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 (Couly 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

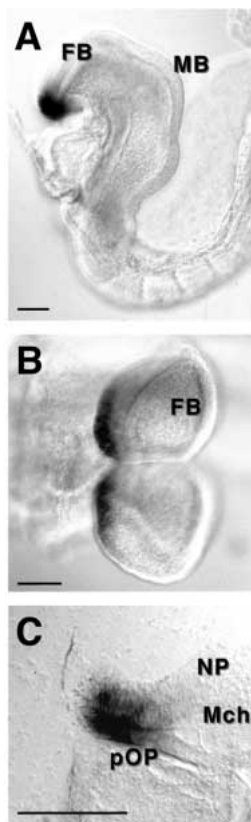


Fig. 3. Expression of *Vax1* in the telencephalic neural plate and adjacent presumptive olfactory placode ectoderm at E8. (A-C) Whole-mount in situ hybridization with a DIG-labelled *Vax1* probe. (A) Lateral view. The expression is restricted to the anterior end of the embryo. (B) Frontal view. The expression is restricted in the anterior border of the neural plate. (C) Lateral parasagittal vibratome section at the telencephalic level. *Vax1* transcription occurs in the anterior part of the telencephalic neural plate, anterior neural ridge, and in the adjacent prospective olfactory placode ectoderm. This neuroectodermal expression domain is very limited, and does not contact the cephalic mesenchyme. It apparently does not extend to the hypophysial placode located more posteriorly. MB, midbrain region; Mch, cephalic mesenchyme; FB, forebrain region; NP, neural plate epithelium; pOP, presumptive olfactory placode ectoderm. Scale bar, 100 μ m.

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*

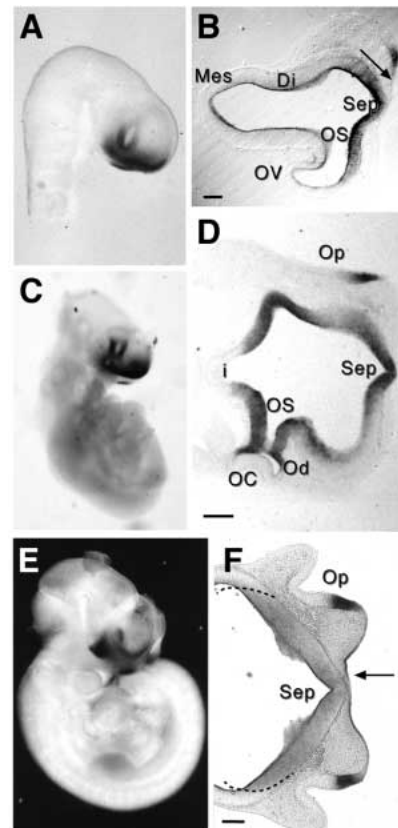


Fig. 4. *Vax1* expression between E9.5 and E12.5 in the basal forebrain, optic stalk, optic disk and medial olfactory placode. Whole-mount in situ hybridization with a DIG-labelled *Vax1* probe at different stages of development. (A) Lateral view at E9.5; (B) vibratome oblique coronal section through diencephalon and forebrain at E9.5. *Vax1* expression is restricted in the ventral forebrain and in a medial band of ectoderm between the two developing olfactory placodes, in contact with the ventral forebrain. At this stage, expression is restricted to the rostral and ventral part of the optic stalk. *Vax1* expression is also visible in the septum. (C) Lateroventral view of *Vax1* expression at E11; transcription is detected in the ventral forebrain and optic stalk. (D) Ventral coronal vibratome oblique section through the ventral forebrain at E11. The caudal and rostral part of the optic stalk express the gene. The expression is absent from the optic cup and is restricted to the optic disk and stalk. Caudally, this expression is limited at the border with the forming infundibulum (i), corresponding to the developing tuberal hypothalamus region. (E) Lateral view at E12.5; (F) coronal vibratome section showing the expression in the presumptive anterior oral ectoderm between the olfactory placodes also expressing the gene in their rostral and medial level. Dashed lines delineate the expression in the ventral forebrain. Di, diencephalon; i, developing infundibulum; Mes, mesencephalon; OC, optic cup; Od, optic disk; Op, developing olfactory placode; OS, optic stalk; OV, optic vesicle; Sep, Septal region. Scale bar, 100 μ m.

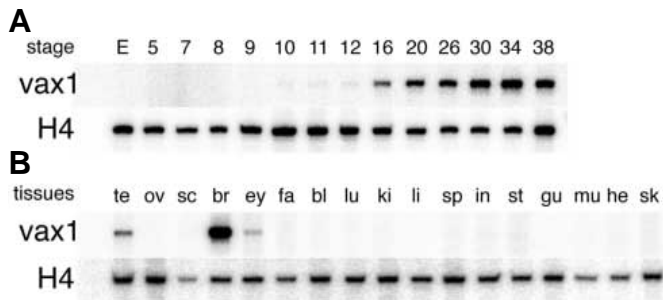


Fig. 5. Expression of *XVax1* during early development (stages as indicated, A) and in tissues of adult frogs (B) as detected by quantitative RT-PCR. Transcription of *Xvax1* is activated in early gastrula and upregulated during neurulation. te, testis; ov, ovary; sc, spinal cord; br, brain; ey, eye; 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.

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).

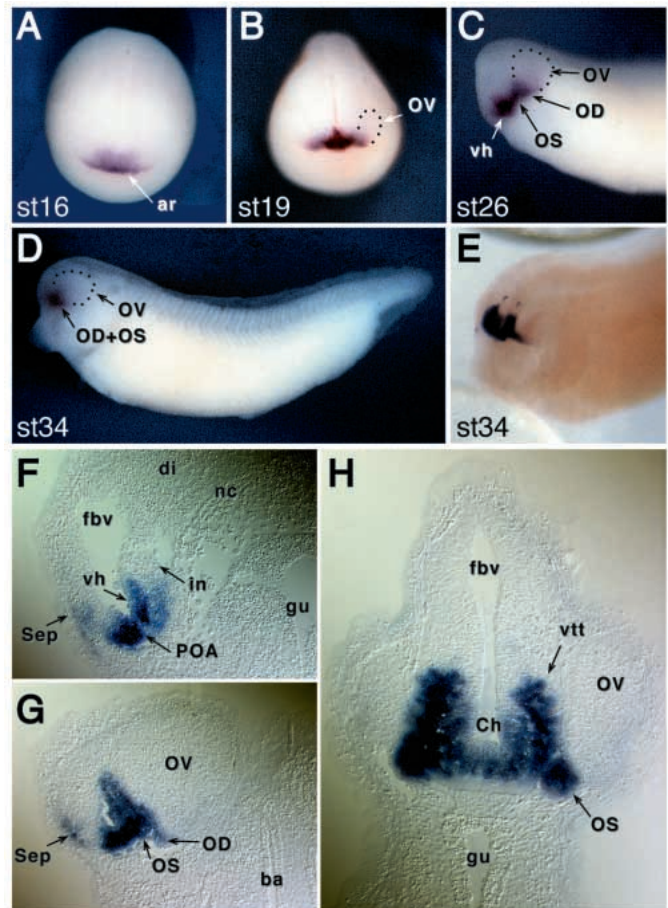
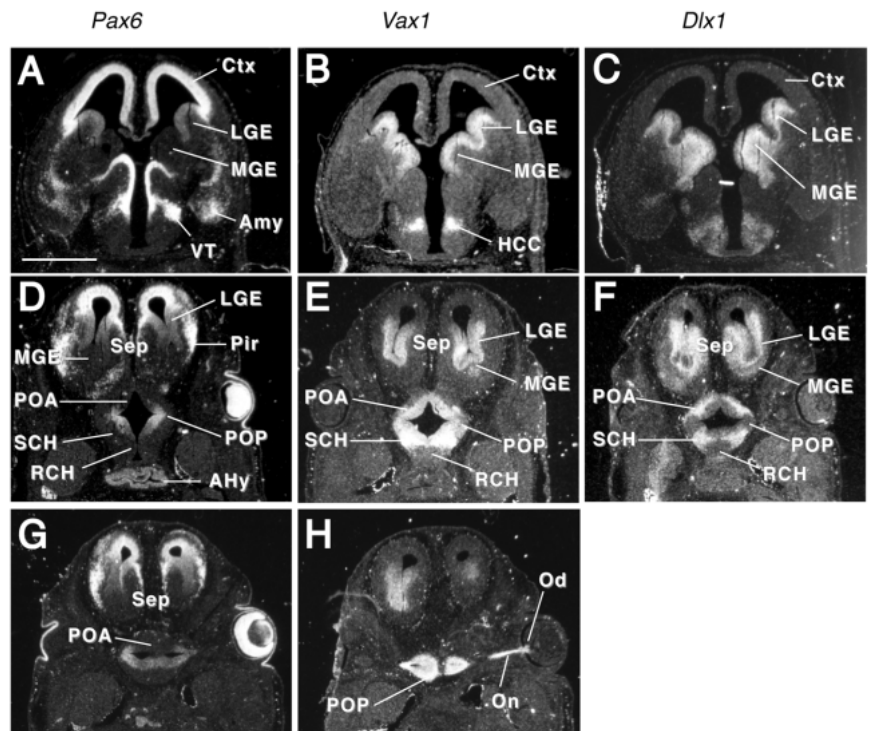


Fig. 6. Developmental expression of *XVax1* analyzed by whole-mount in situ hybridization. (A) Anterior view of an embryo at stage 16. Staining is detected in the anterior neural ridge. (B) Anterior view of *XVax1* expression at stage 19. *XVax1* signal is visible in the ventral forebrain. The optic 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 boarder. (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; di, diencephalon; fbv, forebrain vesicle; gu, gut; i, infundibulum; nc, notochord; OD, optic disc; OS, optic stalk; OV, optic vesicle; sep, septal/striatal region; vh, ventral hypothalamus; vtt, ventral tegmental tract.

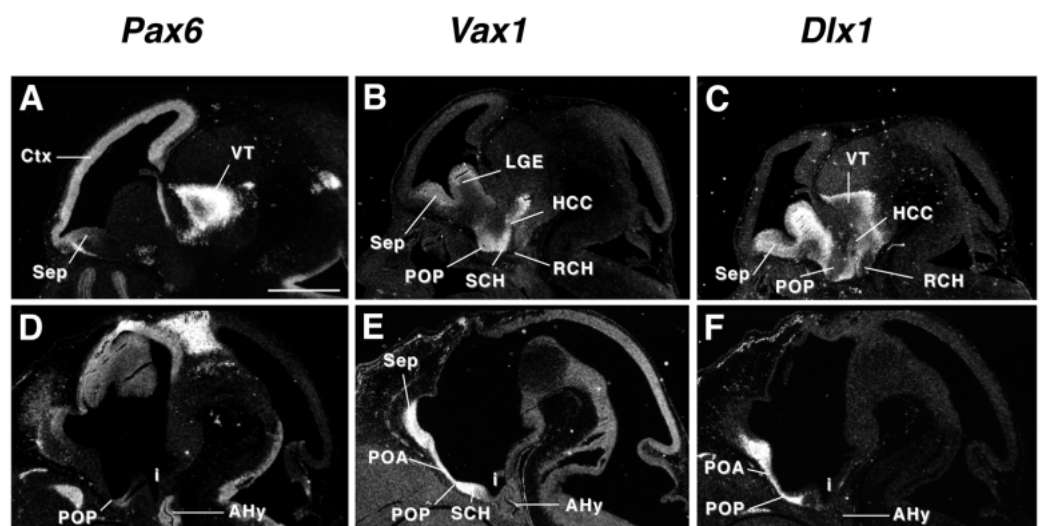
Fig. 7. *Vax1* expression at E13.5 pc compared with that of *Pax6* and *Dlx1* on horizontal sections. (A-H) In situ hybridization using radiolabelled probes, respectively, from dorsal to ventral. (A,D,G) *Pax6* probe; (B,E,H) *Vax1* probe; (C,F) *Dlx1* probe. *Vax1* is expressed in the basal forebrain 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.



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 the ventricular zone remains more medial than that of *Dlx1* in these homozygous mutants (see arrows in Fig. 10B).

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; POA, anterior preoptic area; POP, posterior preoptic area; RCH, retrochiasmatic area; SCH, suprachiasmatic area; Sep, Septum; VT, ventral thalamus. Scale bar: 1 mm.



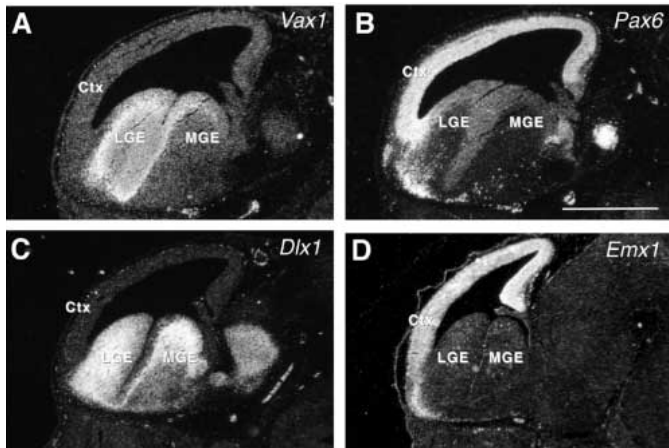


Fig. 9. The corticostriatal boundary is a complex region, located between the developing cortex and LGE, where distinct limits of various gene expression are identifiable. (A-D) In situ hybridization on lateral sagittal sections with *Vax1* (A), *Pax6* (B), *Dlx1* (C), and *Emx1* (D) antisense probes. 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 extend 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.

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 homeobox (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*

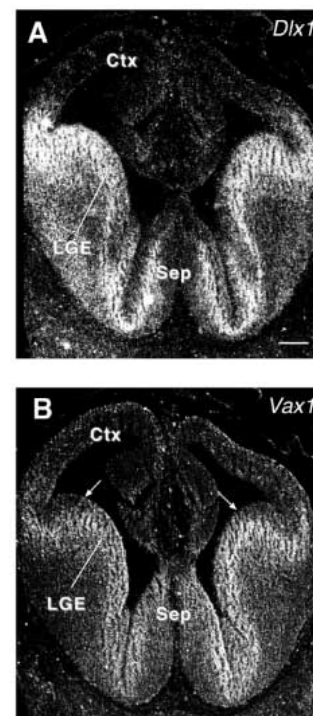


Fig. 10. *Pax6* function is necessary to maintain the normal *Vax1* lateral limit of expression in the LGE subventricular zone. Transverse sections through a homozygous *small eye* (*sey/sey*) E13.5 mouse rostral forebrain. (A) *Dlx1* antisense probe; (B) *Vax1*, antisense probe. In the *sey/sey* brain, 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 *sey/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 μ m.

indicates a linkage of the *Vax1* and *Emx2* loci. The genes may therefore have arisen by a tandem duplication. However, apart from mutations in the *EMX2* locus level (Simeone et al., 1992b; Brunelli et al., 1996), no human or mouse disorder consistent with a modification of *Vax1* have been mapped at this level.

Similarly to the mouse *Emx2* and *Vax1* genes, the *Drosophila ems* and *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 *Vax1*

Mutations of the *Drosophila empty spiracles (ems)* gene affect embryonic head development and lead to loss of head structures (see Hirth et al., 1995). Therefore, *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 *Emx1* or *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; Qiu et al., 1996; Yoshida et al., 1997). Mutations in the human *EMX2* genes have also been identified in patients with holoprosencephaly (Brunelli et al., 1996). Likewise, *Not1* inactivation in the zebrafish leads to absence of notochord where the gene is normally expressed (Talbot et al., 1995). *Vax1* sharing high sequence homology with these genes could accordingly be necessary for ventral forebrain specification and patterning. Moreover, the stability of *Vax1* pattern of expression during neurulation in mouse and *Xenopus* suggests an important function of *Vax1* in the specification and maintenance of basal forebrain identity. *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 expressed at this level (Shimamura and Rubinstein, 1997). *Vax1* could accordingly participate in early neural plate induction.

During early brain morphogenesis in mouse and *Xenopus*, *Vax1* transcripts are detected in regions that precisely correspond to the derivatives of the area where the gene is expressed at the neural plate stage (Figs 3, 4, 6-8) (Couly and Le Douarin, 1987; Eagleson and Harris, 1990; Eagleson et al., 1995) and indicate that the early *Vax1*-expressing region is an embryological unit. This region could correspond to the part of the brain induced in *Xenopus* by *Cerberus* and specified by signals from rostral endoderm (Bouwmeester et al., 1996; Thomas and Beddington, 1996).

Vax1 expression is simultaneous with neuronal progenitor proliferation and neural cell specification in the septum, preoptic area (Bayer and Altman, 1987a), and LGE (Fentress et al., 1981; Bayer and Altman, 1987b; Halliday and Cepko, 1992; De Carlos et al., 1996; Szele and Cepko, 1996). In addition, the subventricular zone where *Vax1* is expressed in the ganglionic eminences, is considered to be a transition zone that cells, originating in the ventricular zone, cross or where they can further proliferate and acquire information important

for their final fate (Boulder Committee, 1970). Furthermore, *Vax1* transcription occurring in the medial olfactory placode from E8 to E11 is no longer detected in the olfactory epithelium or in the vomeronasal organ at E13.5 (data not shown). The medial olfactory placode is the site of origin of the vomeronasal organ and of GnRH neurons that leave the placode from E11 onward, migrate out and settle in the septal and preoptic areas (Zheng et al., 1992; Pellier and Astic, 1994; De Carlos et al., 1995; Schwanzel-Fukuda et al., 1996). Accordingly, *Vax1* could function in early developing progenitors present in the medial olfactory placode before they migrate to the basal forebrain. In general, *Vax1* could thus function in the early steps of the neuronal differentiation.

Moreover, the derivatives of the embryonic brain region expressing *Vax1* are widely interconnected and may be considered as a functional unit (Carpenter, 1991) which appeared early in the evolution of vertebrates (Ariëns Kappers et al., 1967; Gans, 1989). *Vax1* is thus a marker of a phylogenetically ancient embryological unit, which differentiates in a functionally central part of the forebrain.

Vax1 expression in *Pax6*-deficient homozygous mutant mice

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

Vax1 pattern of expression is modified in *Pax6*-deficient homozygous mice at the level of the corticostriatal boundary. As already described for *Dlx1* (Stoykova et al., 1996) at the level of the subventricular zone, *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 *Vax1* expression is still clear-cut in the lateral LGE ventricular zone (see arrows in 10B) and remains more medial than that of *Dlx1* (Fig. 10A,B). Accordingly, *Pax6* is not necessary to restrict *Vax1* expression pattern in the LGE ventricular zone. Nevertheless, the ectopic *Vax1* expression in the cortical subventricular zone of *Pax6*-deficient embryos suggests that the *Pax6* gene product maintains and stabilizes the *Vax1* expression pattern outside the ventricular zone. Besides, *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 *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 *Vax1* in these mutants is the consequence of an abnormal cell migration rather than a change of the identity of the lateroventral 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 *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 extends 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, DiI 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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