

# Identification and expression of the lamprey *Pax6* gene: evolutionary origin of the segmented brain of vertebrates

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## SUMMARY

The *Pax6* gene plays a developmental role in various metazoans as the master regulatory gene for eye patterning. *Pax6* is also spatially regulated in particular regions of the neural tube. Because the amphioxus has no neuromeres, an understanding of *Pax6* expression in the agnathans is crucial for an insight into the origin of neuromerism in the vertebrates. We have isolated a single cognate cDNA of the *Pax6* gene, *LjPax6*, from a *Lampetra japonica* cDNA library and observed the pattern of its expression using in situ hybridization. Phylogenetic analysis revealed that *LjPax6* occurs as a sister group of gnathostome *Pax6*. In lamprey embryos, *LjPax6* is expressed in the eye, the nasohypophysial plate, the oral ectoderm and the brain. In the central nervous system, *LjPax6* is expressed in clearly delineated domains in the hindbrain, midbrain and

forebrain. We compared the pattern of *LjPax6* expression with that of other brain-specific regulatory genes, including *LjOtxA*, *LjPax2/5/8*, *LjDlx1/6*, *LjEmx* and *LjTTF1*. Most of the gene expression domains showed conserved pattern, which reflects the situation in the gnathostomes, conforming partly to the neuromeric patterns proposed for the gnathostomes. We conclude that most of the segmented domains of the vertebrate brain were already established in the ancestor common to all vertebrates. Major evolutionary changes in the vertebrate brain may have involved local restriction of cell lineages, leading to the establishment of neuromeres.

Key words: *Pax6*, Lamprey, Embryo, Neuromeres, Forebrain

## INTRODUCTION

Studies of families of regulatory genes are of particular interest in the field of evolutionary developmental biology, as they provide clues to the gene duplication events, as well as the functional diversity, associated with the evolution of the body plan. It has often been emphasized that patterns of regulatory gene expression, especially those of the homeobox genes, are surprisingly conserved in distantly related animals, indicating the early establishment of the morphological ground plan in metazoans. Among these regulatory genes, the Pax gene family encodes transcription factors that are characterized by paired-type homeodomains and paired domains (Goulding, 1992). Each member of the Pax family is involved in a wide range of developmental events, and the *Pax6* gene, a homolog of the *Drosophila* gene *eyeless*, has a recognized role in eye development in various metazoans (Callaerts et al., 1997).

*Pax6* homologs are also expressed in the olfactory receptors and in parts of the central nervous system (CNS) in most of the animal groups studied so far, including the flatworms, roundworms, nemertans, mollusks, arthropods, echinoderms

and chordates (reviewed by Callaerts et al., 1997; Gclardon et al., 1998). In the head surface ectoderm of jawed vertebrates (gnathostomes), *Pax6* expression is restricted to the olfactory placode, the eye placode and the anterior hypophysis (Walther and Gruss, 1991), indicating its function in the establishment of these cell types. Expression of *Pax6* in the CNS is restricted to the telencephalon, the diencephalon and the myelencephalon, the regions that constitute the developmental compartments of the brain. The gnathostome neural tube is assumed to consist of a series of segmental bulges or neuromeres along its anteroposterior axis, and those in the forebrain are specifically called prosomeres (reviewed by Rubenstein et al., 1998). Expression of the *Pax6* gene is regarded as a marker for some of the prosomeres and also for the forebrain alar plate (Stoykova et al., 1996).

Prosomeres have so far been observed in many gnathostomes, including teleosts (Wullimann and Puelles, 1999; Diaz-Regueira and Anadon, 2000), chicken (Redies et al., 2000), frog (Javier-Milan and Puelles, 2000) and mouse (Puelles and Rubenstein, 1993; Shimamura et al., 1995). As no neuromeric patterns are apparent in the amphioxus, the

development of the CNS in the lamprey, the sister group of the gnathostomes, is a crucial issue. Recent immunohistochemical analysis has implied that six prosomeres are identifiable in the adult lamprey, as in the amniotes (Pombal and Puellas, 1999; Pombal et al., 2001). The morphological pattern of the larval lamprey brain has also been described subsequent to the classical analyses of Bergquist, in which five prosomeres were counted (Bergquist and Källén, 1953). The relationship between the latter segments and the prosomeres has not been clarified. The development of the prosomeres is even more enigmatic, and only partly explained by Kuratani et al. (Kuratani et al., 1998) using immunohistochemical techniques. Although the embryonic expression patterns of several regulatory genes have been studied in lampreys (Tomsa and Langeland, 1999; Ueki et al., 1998; Myojin et al., 2001; reviewed by Kuratani et al., 2001), the developmental plan of the lamprey brain is still incompletely understood.

In the present study, we have screened a lamprey cDNA library and identified a cognate cDNA of *Pax6* (*LjPax6*). We have compared the expression patterns of *Pax6* in the embryonic brains of the lamprey and the gnathostomes using *in situ* hybridization, and have also studied the expression patterns of other regulatory genes and found that these genes are expressed in clearly delineated polygonal domains in the lamprey brain. Combining these data with immunostaining of the nerve tracts, we present a developmental plan for the lamprey brain, and conclude that the origin of the basic configuration of the vertebrate forebrain, which is largely similar to the amniote pattern, appears to date back to the ancestor common to all vertebrates.

## MATERIALS AND METHODS

### Embryos

Adult male and female *Lampetra japonica* were collected in a tributary of the Miomote River, Niigata, Japan, during the breeding season (early June) in 2000. The eggs were artificially fertilized and kept in 10% Steinberg solution (Steinberg, 1957) at 20°C. Embryonic stages were assessed morphologically according to the table of Tahara (Tahara, 1988) for *L. reissneri*, a brook lamprey species closely related to *L. japonica*. For *in situ* hybridization, embryos were fixed in 4% paraformaldehyde and 1% methanol in 0.1 M phosphate-buffered saline (PFAM/PBS).

### Isolation of cDNA clones of the lamprey *Pax6* gene

cDNA clones of the lamprey *Pax6* gene were isolated from the *L. japonica* larval head cDNA library. The pLjP1-PCRFR fragment which contains a *Pax1*-like paired domain (Ogasawara et al., 2000) was randomly labeled with [<sup>32</sup>P]-dCTP (Amersham Pharmacia Biotech), and 3.0 × 10<sup>5</sup> phages were screened under low-stringency conditions: 6 × SSPE, 0.1% sodium dodecyl sulfate (SDS), 1 × Denhardt's solution, 50% formamide at 37°C for 16 hours, and washed in 6 × saline sodium citrate (SSC), 0.1% SDS at 37°C for 20 minutes, in 2 × SSC, 0.1% SDS at 37°C for 30 minutes, and in 1 × SSC, 0.1% SDS at 37°C for 15 minutes. Isolated clones were sequenced using an ABI PRISM 377 DNA Sequencer (Perkin Elmer).

### Genomic Southern analysis

High molecular weight genomic DNA of *L. japonica* was extracted from a single adult liver using a standard procedure (Sambrook et al., 1989). After exhaustive digestion with *EcoRI*, the DNA fragments were separated electrophoretically on a 1% agarose gel and blotted onto Hybond-N+ nylon membranes (Amersham Pharmacia Biotech).

The blots were hybridized with randomly primed digoxigenin (DIG)-labeled DNA probes at 50°C for 16 hours, and washed under high-stringency conditions.

### Whole-mount *in situ* hybridization

Digoxigenin-labeled antisense and sense riboprobes were transcribed according to the manufacturer's instructions. Fixed embryos were dehydrated and stored in 100% methanol at -20°C. Specimens were treated with a mixture of hydrogen peroxide (one part) and methanol (five parts) overnight, and were rehydrated in PBS containing 0.1% Tween 20 (PBT). After treatment with 0.2 N HCl in PBT for 10 minutes at room temperature (RT), the samples were digested with 10 mg/ml proteinase K (Sigma). They were post-fixed for 20 minutes with PFA/PBT containing 0.2% glutaraldehyde, then washed with PBT, and prehybridized in hybridization buffer (50% formamide, 5 × SSC, 1% SDS, 0.05 mg/ml total yeast RNA, 50 mg/ml heparin sulfate, 5 mM ethylene diaminetetraacetic acid (EDTA)-Na<sub>2</sub>, 0.1% CHAPS) for 1 hour at 65°C. The specimens were then incubated in hybridization buffer with 0.1 mg/ml DIG-labeled RNA probe for 48 hours at 65°C. After hybridization, the specimens were washed twice in 50% formamide, 5 × SSC, 1% SDS for 30 minutes at 65°C, and the solution was substituted gradually with 10 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl and 0.1% Tween 20 (TST). RNaseA was added to a final concentration of 0.05 mg/ml and the specimens incubated for 30 minutes at RT. The samples were washed twice with 2 × SSC in 50% formamide for 30 minutes at 65°C, twice in 2 × SSC containing 0.3% CHAPS for 30 minutes at 65°C, and twice in 0.2 × SSC containing 0.3% CHAPS for 30 minutes at 65°C. For immunological detection, the embryos were blocked with TST containing 0.5% blocking reagent (Boehringer Mannheim) for 60 minutes, and incubated with alkaline phosphatase (AP)-conjugated anti-digoxigenin Fab fragments (diluted 1:4000; Boehringer Mannheim), at 4°C overnight. The specimens were washed five times for 60 minutes each in TST at RT. Alkaline phosphatase activity was detected with NBT/BCIP in NTMT (Boehringer Mannheim). Stained specimens were fixed in PFA/PBS, rehydrated, and clarified with BABB (1:2 mixture of benzyl alcohol and benzyl benzoate).

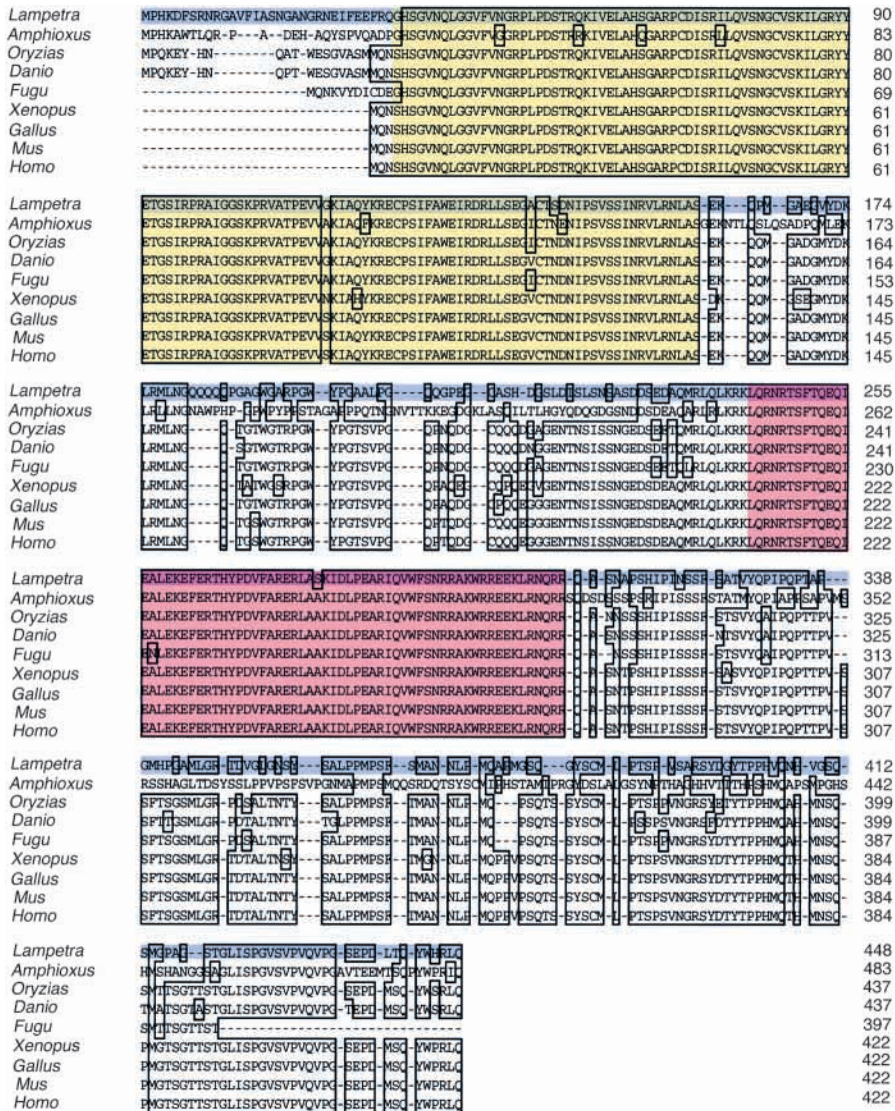
### Whole-mount immunostaining

Fixed embryos stored in methanol were placed in dimethylsulfoxide (DMSO) and methanol (1:1). After washing with TST containing 5% DMSO (TSTd), the embryos were blocked with aqueous 1% periodic acid and 5% nonfat dry milk in TSTd (TSTM). They were incubated in the primary antibody (acetylated tubulin (Sigma) diluted 1:1000 in TSTM) for 2-4 days at RT. After washing with TST, samples were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:200 in TSTM; Zymed). After a final wash in TSTd, the embryos were incubated with the peroxidase substrate 3,3'-diaminobenzidine (DAB, 100 mg/ml) in TST for 1 hour, and allowed to react in TST with the same concentration of DAB with 0.01% hydrogen peroxide.

## RESULTS

### *Pax6* cognate transcripts in *L. japonica*

To isolate cDNA clones for Pax-related genes from the lamprey, an *L. japonica* cDNA library was screened with a <sup>32</sup>P-labeled pLjP1-PCRFR fragment encoding the *Pax1*-like paired domain (Ogasawara et al., 2000) under low-stringency conditions. This yielded several Pax-related cDNA clones, and some cDNA clones encoded the *Pax6*-related paired domain. The insert contained in the largest clone was 2742 bp long, excluding the polyA tail. This clone had a single open reading frame (ORF) encoding 488 amino acids, a 376 bp 5' untranslated region



**Fig. 1.** Comparison of amino acid sequences of Pax6 genes. Predicted amino acid sequences of *L. japonica* Pax6 cDNA clone (LjPax6) and various Pax6 proteins of vertebrates including *Oryzias* (Loosli et al., 1998), *Danio* (Nornes et al., 1998), *Xenopus* (Jaworski et al., 1997), *Fugu* (Miles et al., 1998), *Gallus* (Kawakami et al., 1997), *Mus* (Walter and Gruss, 1991), *Homo* (Ton et al., 1991), and the amphioxus *Branchiostoma floridae* (Gardlon et al., 1998), are compared. LjPax6 amino acids are shown in blue, the paired domains in yellow and the homeodomains in red. Conserved amino acids are boxed.

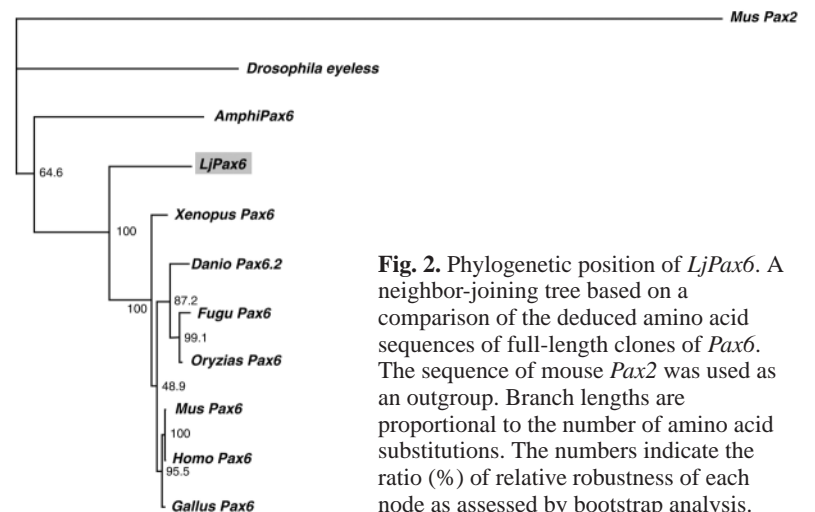
**Developmental expression of LjPax6 mRNA**

The expression of *LjPax6* was examined using whole-mount in situ hybridization. No *LjPax6* transcripts were detected during the cleavage, blastula or gastrula stages. At the late neurula stage (stage 19), weak expression was seen in the anterior neural tube (Fig. 3A). By stage 20, the neural fold had developed into a neural rod, and *LjPax6* mRNA was detected at high levels in the presumptive prosencephalon (Fig. 3B) and at low levels in the anterior hindbrain (Fig. 3B). No *LjPax6* transcripts were detected in the presumptive mesencephalon (midbrain) or the posterior hindbrain. At the head process stage (stage 21), strong expression of *LjPax6* was noted in the anterior hindbrain, whereas no transcripts were detected in the posterior hindbrain (Fig. 3C).

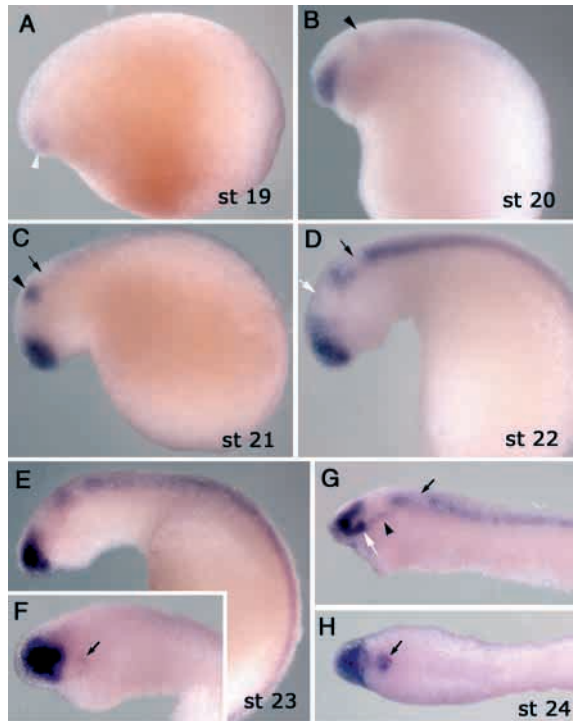
At stage 22, some brain compartments, including the forebrain, midbrain and hindbrain, were first observed. *LjPax6* was now clearly expressed in the forebrain and the entire hindbrain and the spinal cord except for rhombomere 4 (r4), which did not express the gene (Fig. 3D). From stage 23, *LjPax6* transcripts were always detected on the

(UTR) including an in-frame stop codon, and a 1019 bp 3' UTR. The predicted protein product contains a paired domain and a homeodomain, but not an octapeptide, exhibiting the structural features characteristic of the Pax6 protein. Therefore, we designated this gene *LjPax6* (*L. japonica* Pax6). The nucleotide and amino acid sequences have been assigned to the DDBJ/EMBL/GenBank Accession Number AB061220. Comparison of the predicted amino acid sequences of LjPax6 and gnathostome Pax6 proteins reveals that the paired domains and homeodomains are highly conserved between vertebrate species (Fig. 1).

Genomic Southern blot analysis using a DIG-labeled *LjPax6* fragment as probe detected a single band in the *EcoRI* digest of about 8 kb (not shown). The evolutionary relationships of *LjPax6* were examined by molecular phylogenetic analysis using the neighbor-joining method. As shown in Fig. 2, *LjPax6* constitutes a sister sequence to gnathostome Pax6. This group is further included in a larger clade that contains metazoan Pax6.

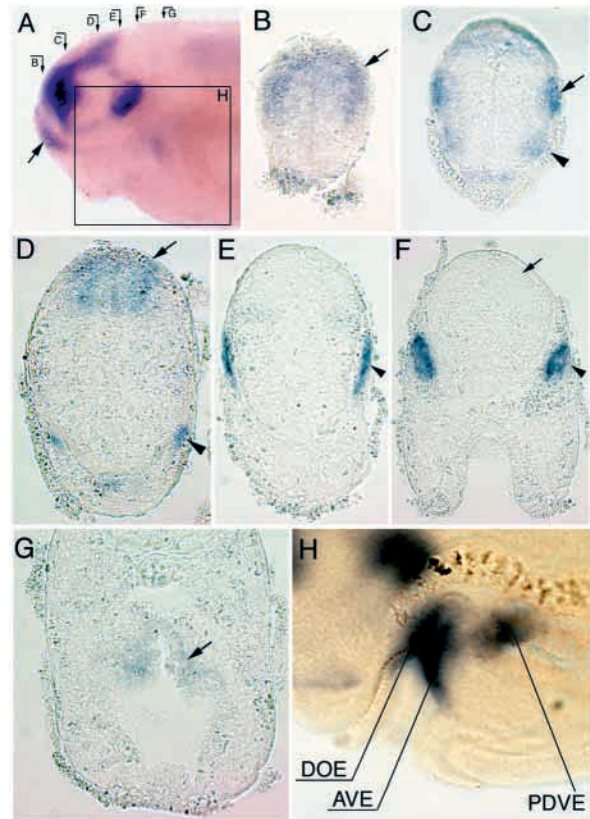


**Fig. 2.** Phylogenetic position of *LjPax6*. A neighbor-joining tree based on a comparison of the deduced amino acid sequences of full-length clones of Pax6. The sequence of mouse Pax2 was used as an outgroup. Branch lengths are proportional to the number of amino acid substitutions. The numbers indicate the ratio (%) of relative robustness of each node as assessed by bootstrap analysis.



**Fig. 3.** *LjPax6* expression in developing embryos of *L. japonica* using whole-mount in situ hybridization. Lateral (A-E,G) and ventral (F,H) views. Anterior is towards the left in all the embryos. (A) *LjPax6* expression at stage 19. The weak expression in the anterior neural tube is indicated by a white arrowhead. (B) Stage 20. Transcripts are detected in the anterior neural tube and hindbrain (black arrowhead). (C) Stage 21. Expression is restricted to the rostral hindbrain (black arrowhead) and no transcripts are seen in the posterior hindbrain (black arrow). (D) Stage 22. *LjPax6* expression is detected in the forebrain and the spinal cord. The part of the hindbrain that corresponds to r4 is devoid of *LjPax6* expression (black arrow). (E,F) Stage 23. Forebrain expression is intensified. Transcripts are also detected in the dorsal oral ectoderm (arrow in F). (G,H) Stage 24. *LjPax6* transcripts appear in the optic vesicle (white arrow), the ectoderm behind the optic vesicle (arrowhead) and the dorsal oral ectoderm (arrow in H). *LjPax6* is weakly expressed in r4 (arrow in G).

dorsal surface of the oral ectoderm (Fig. 3F,H). In the hindbrain of a stage 24 embryo, *LjPax6* expression intensified relative to the previous stage, except in r4 where the transcripts were only weakly expressed (Fig. 3G; also see Fig. 5A). From this stage, *LjPax6* transcripts were detectable in the optic vesicle (Fig. 3G), the optic stalk, and the ectoderm behind the optic vesicle (Fig. 3G). Later (stages 25-26), the basic brain segment was completely formed, and the boundary of *LjPax6* expression became clearer in the brain; transcripts were detected in the telencephalon (Fig. 4B), on the dorsolateral side of the anterior diencephalon (Fig. 4C) and on the dorsal side of the posterior diencephalon (Fig. 4D). The optic stalk and the eye itself also expressed *LjPax6* mRNA (Fig. 4C-E). No transcripts were detected in the midbrain (Fig. 4F). In the hindbrain, *LjPax6* transcripts were now expressed in all the rhombomeres including r4 (Fig. 6A). In the dorsal hindbrain where the gnathostome cerebellar primodium emerges, no *LjPax6* transcripts were detected (Fig. 6A). *LjPax6* transcripts were detected de novo in the



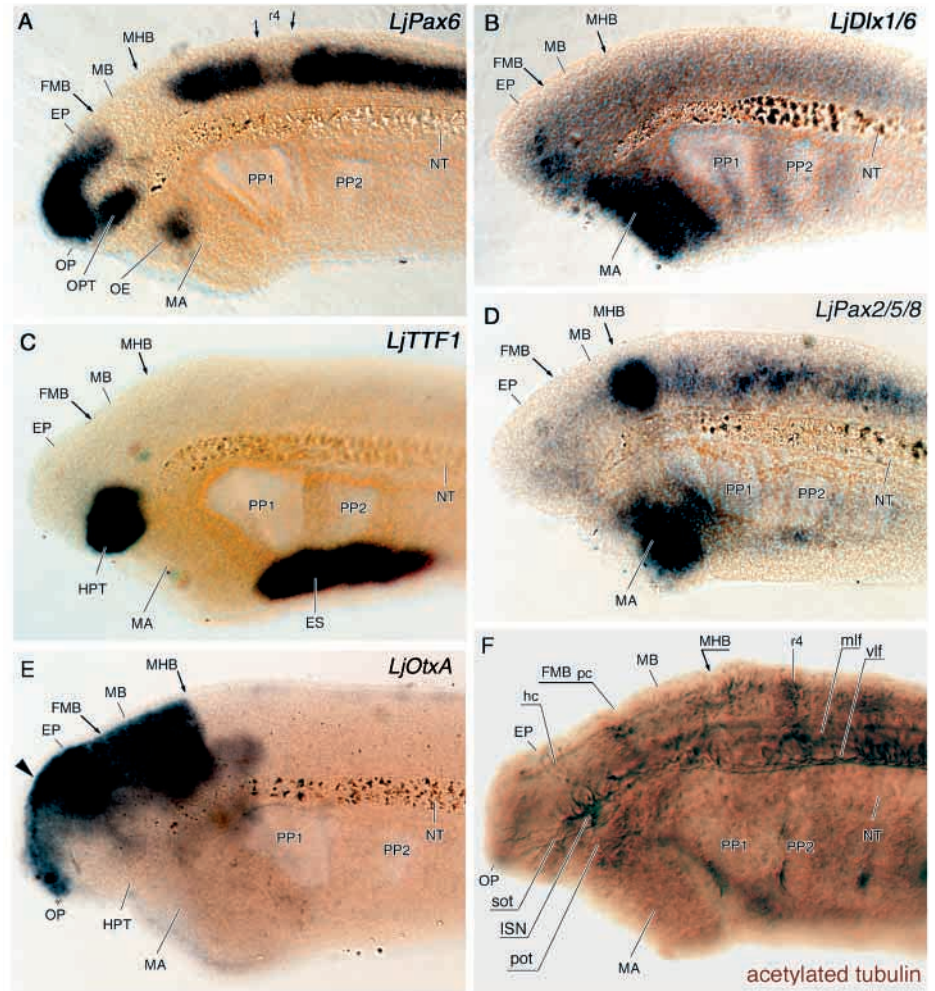
**Fig. 4.** Histological analysis of *LjPax6* expression. (A-G) Transverse sections of a stage 26 embryo for the whole-mount embryo in A, in which the levels of the sections (B-G) are shown by arrows. Box indicates the enlarged portion in H. It is clear from A, that *LjPax6* is expressed in the nasohypophysial plate (arrow). (B-G) High levels of *LjPax6* transcripts are found in the dorsal telencephalon (arrow in B), the diencephalon (arrows in C,D), the optic stalk (arrowheads in C-E), the eye (arrowhead in F) and the dorsal oral ectoderm (arrow in G), whereas no transcripts are seen in the midbrain (arrow in F). (H) *LjPax6* expression in the oral ectoderm. *LjPax6* expression is seen in the dorsal oral ectoderm (DOE), the anterior velum ectoderm (AVE) and the posteriodorsal velum ectoderm (PDVE).

nasohypophysial plate, a structure homologous to the gnathostome nasal and hypophysial placodes (Fig. 4A). *LjPax6* expression was also seen in subregions of the dorsal oral ectoderm (Fig. 4G). In a transparent whole-mount embryo, *LjPax6* expression was seen in the dorsal oral ectoderm, the anterior velum ectoderm and the posteriodorsal velum ectoderm (Fig. 4H).

#### Expression patterns of *LjPax6* and other marker genes in brain subdivisions

To determine the segmental organization of the lamprey forebrain, the expression of *LjPax6* was compared with the expression of other regulatory genes known to be restricted to particular compartments of the gnathostome brain. Comparisons were made at stage 24 when *LjPax6* is expressed in the dorsal forebrain, and no transcripts were seen in the ventral region (Fig. 5A). Expression of *LjDlx1/6*, which is related to the common ancestor of gnathostome *Dlx1* and *Dlx6*,

**Fig. 5.** Comparative expression of regulatory genes in the lamprey brain. Whole-mount in situ hybridization of stage 24 lamprey embryos. (A) Expression of *LjPax6*. (B) Expression of *LjDlx1/6*. Transcripts are seen in the anterior forebrain and mandibular arch (MA). (C) Expression of *LjTTF1*. Transcripts are restricted to the hypothalamus (HPT) and the endostyle (ES). (D) Expression of *LjPax2/5/8* at the mid-hindbrain boundary (MHB) and mandibular arch (MA). (E) Expression of *LjOtxA* is seen in the CNS rostral to the mid-hindbrain boundary (MHB) and in the olfactory placode (OP). (F) Immunohistochemical staining of nerve fibers. Several nerve tracts are observed including the habenular commissure (hc), the medial longitudinal fascicle (mlf), the posterior commissure (pc), the posterior optic tract (pot), the supraoptic tract (sot) and the ventral longitudinal fascicle (vlf). Abbreviations: EP, epiphysis; ES, endostyle; FMB, fore-midbrain boundary; hc, habenular commissure; HPT, hypothalamus; ISN, interstitial nucleus; MA, mandibular arch; MB, midbrain; mlf, medial longitudinal fascicle; MHB, mid-hindbrain boundary; NT, notochord; OE, olfactory epithelium; OP, olfactory placode; OPT, optic stalk; pc, posterior commissure; pot, posterior optic tract; PP, pharyngeal pouch; r4, rhombomere 4; sot, supraoptic tract; vlf, ventral longitudinal fascicle.

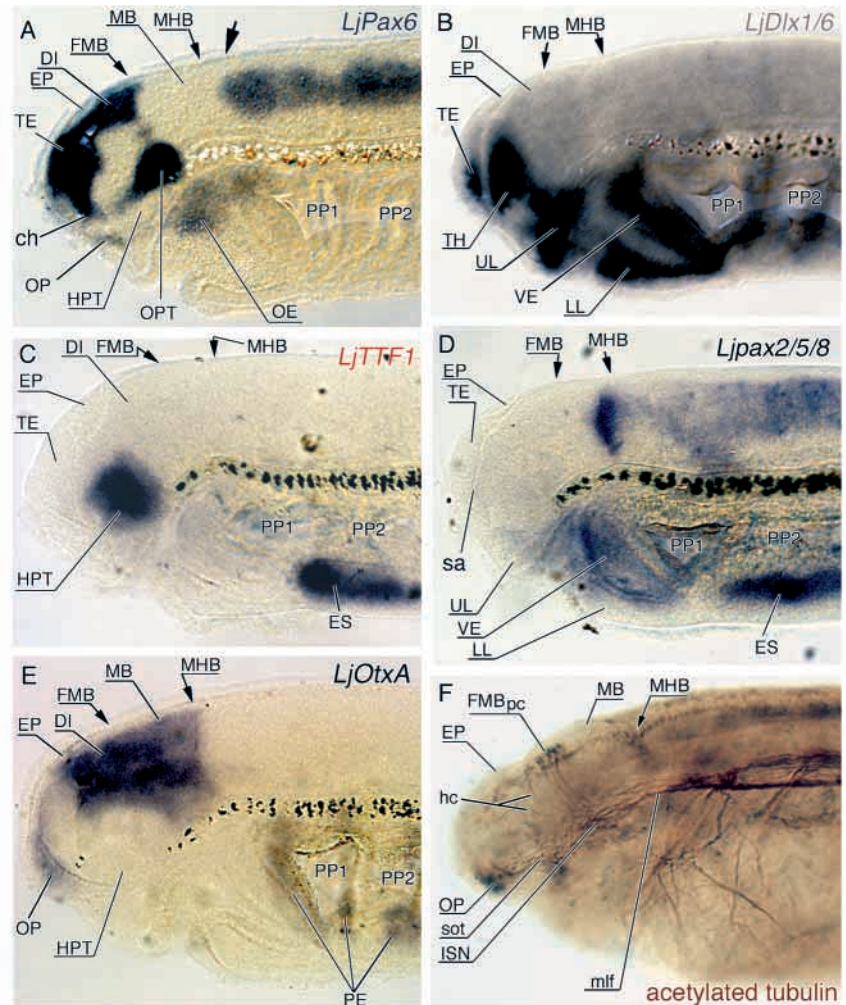


is expressed in the anterior forebrain (Fig. 5B). Gnathostome *Dlx1* has been shown to be expressed in the diencephalic roof, rostral to the zona limitans (see Fig. 8). Expression of *LjTTF1*, a cognate transcript of *TTF1* (*Nkx2.1*) that serves as a marker for the ventral forebrain, was similarly localized in the ventral forebrain, which corresponds to the hypothalamus of the lamprey (Fig. 5C). No *LjTTF1* transcripts were detected in any other region. The *Pax2/5/8* cognate transcript, a marker for the gnathostome isthmus (Asano and Gruss, 1992), was expressed at the morphologically detectable mid-hindbrain boundary in the lamprey brain (Fig. 5D). *LjOtxA*, the expression pattern of which is similar to that of the gnathostome gene *Otx2* (Ueki et al., 1998), was here similarly expressed in the anterior neural tube corresponding to the forebrain and midbrain of the lamprey (Fig. 5E).

In the stage 26 embryo, many of the brain components can be identified anatomically. The boundary between the midbrain and forebrain corresponds to the position of the posterior commissure, while the boundary between the diencephalon and the telencephalon is distinguished by the anterior intraencephalic sulcus (sa in Fig. 6D; von Kupffer, 1906). At this stage, no *LjPax6*, *LjDlx1/6* or *LjTTF1* transcripts were detected in the midbrain (Figs 6A-C). *LjPax2/5/8* mRNA was observed at the mid-hindbrain boundary (Fig. 6D). *LjOtxA* was detected throughout the midbrain (Fig. 6E).

In the diencephalon, *LjPax6* was expressed exclusively in the dorsal forebrain (Figs 6A, 7A). Expression of *LjDlx1/6*, on the other hand, was restricted to the anterior-dorsal thalamus, where it overlapped the region of *LjPax6* expression (Figs 6B, 7C). *LjTTF1* mRNA was restricted to the anterior-ventral diencephalon, complementary to the *LjPax6* and *LjDlx1/6* domains (Figs 6C, 7B). *LjOtxA* was expressed in the posterior diencephalon, adjacent to the anterior parts of the *LjDlx1/6*- and *LjTTF1*-expressing regions (Figs 6E, 7D). In the telencephalon, *LjPax6* was expressed in the dorsal subdivision (Fig. 7E), whereas the expression of *LjDlx1/6* was detected in the anterior subdivision (Fig. 7F). *LjEmx* was restricted to the posterior subdivision, overlapping the region of *LjPax6* expression (Fig. 7G).

Immunohistochemical staining of nerve fibers using the anti-acetylated tubulin antibody allowed us to identify some neuronal tracts, including the habenular commissure, the posterior commissure, the supraoptic tract, the ventral longitudinal fascicles, the medial longitudinal fascicle and the interstitial nucleus. The positions of these tracts correspond to the boundaries between the gene-expression domains described above (compare Figs 6F and 7A-D). For example, the posterior commissure develops in the pretektum or P1, and the habenular commissure in the dorsal thalamus or P2 (Figs 5F and 6F). The supra- and post-optic tracts develop dorsally



**Fig. 6.** Comparison of regulatory gene expression in the lamprey brain. Whole-mount in situ hybridization in stage 26 lamprey embryos. (A) Expression of *LjPax6*. An arrow indicates the dorsal hindbrain that does not express this gene. (B) Expression pattern of *LjDlx1/6*. Transcripts are detected in the telencephalon (TE), thalamus (TH), upper lip (UL), lower lip (LL) and velum (VE). (C) Expression of *LjTTF1* in the hypothalamus (HPT) and endostyle (ES). (D) *Ljpax2/5/8*. Transcripts are found at the mid-hindbrain boundary (MHB), velar epithelium and endostyle. (E) *LjOtxA* is expressed in the MHB, the midbrain (MB), the diencephalon (DI), the olfactory placode (OP) and the pharyngeal ectoderm (PE). (F) Immunohistochemical staining of nerve tracts. The habenular commissure (hc), medial longitudinal fascicle (mlf), posterior commissure (pc), posterior optic tract (pot), supraoptic tract (sot) and ventral longitudinal fascicle (vlf) are stained. Abbreviations: ch, optic chiasm; DI, diencephalon; EP, epiphysis; ES, endostyle; FMB, fore-midbrain boundary; hc, habenular commissure; HPT, hypothalamus; ISN, interstitial nucleus; LL, lower lip; MB, midbrain; mlf, medial longitudinal fascicle; MHB, mid-hindbrain boundary; NT, notochord; OE, oral ectoderm; OP, olfactory placode; OPT, optic stalk; pc, posterior commissure; pot, posterior optic tract; PP1 & PP2, pharyngeal pouches; sa, intraencephalic sulcus; sot, supraoptic tract; TE, telencephalon; UL, upper lip; vlf, ventral longitudinal fascicle.

and ventrally to the optic chiasm, respectively, both merging to form a single tract, the medial longitudinal fasciculus, that extends posteriorly along the basal plate.

## DISCUSSION

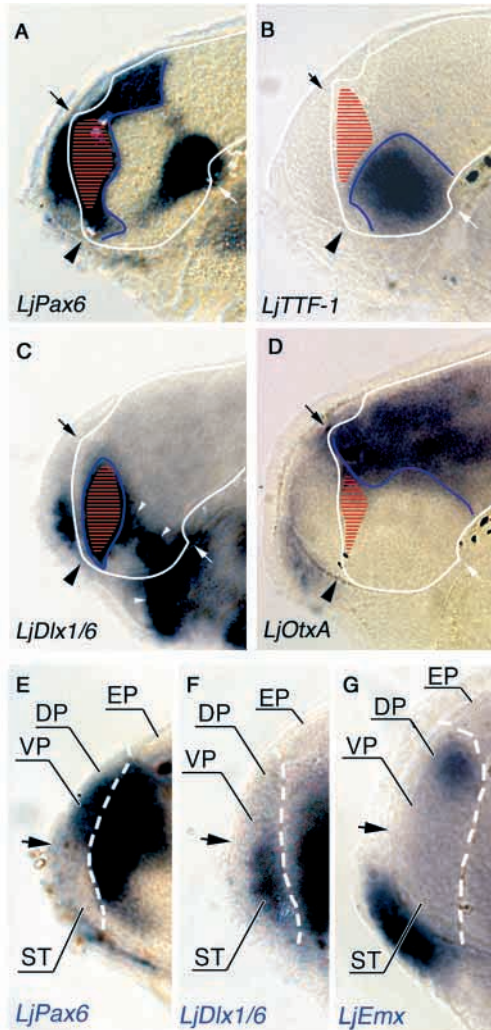
### Identification of lamprey *Pax6* and the evolution of the vertebrate *Pax* genes

The *Pax* gene family appears to have undergone sequential duplication events during the course of evolution. This is a curious topic in evolutionary developmental biology, as duplication events are tightly linked with the functionalization of the genes that determine the developmental program that dictates the body plan. By amino acid sequence analysis, the vertebrate *Pax* genes have been classified into four or five groups (*Pax1/9*, *Pax2/5/8*, *Pax3/7*, *Pax4/6* and *PaxA*; Miller et al., 2000; Breitling and Gerber, 2000). Of those, duplication of *Pax4* and *Pax6* appears to date back to the origin of metazoans (Breitling and Gerber, 2000). In the present study, a full-length cDNA (*LjPax6*) homologous to gnathostome *Pax6* was isolated from the cDNA library of *L. japonica*.

The deduced amino acid sequence of *LjPax6* shows a high level of homology with that of *Pax6* cognates of the gnathostomes. The phylogenetic position of *LjPax6* as the

outgroup of gnathostome *Pax6* is consistent with the taxonomic position of *Lampetra*. Genomic Southern blot analysis using both the paired and homeodomains as probes indicated that *L. japonica* possesses a single *Pax6*-related gene in the haploid genome. It therefore seems likely that *LjPax6* evolved from a single ancestral gene shared by the agnathans and the gnathostomes. A previous study of *Pax1/9* genes has indicated that *Pax1* and *Pax9* were already duplicated in the lamprey as pan-vertebrate orthologs (Ogasawara et al., 2000). Likewise, the *Pax6* gene seems to have arisen before the lamprey-gnathostome split. Some *Pax* genes in tunicates and amphioxus have been shown to represent ancestral forms before the duplication, as exemplified by *Pax1/9*, *Pax2/5/8* and *Pax3/7* (Holland and Holland, 1995; Wada et al., 1997; Wada et al., 1998; Ogasawara et al., 1999; Kozmik et al., 1999; Holland et al., 1999). Therefore, at least three subfamilies (*Pax1/9*, *Pax3/7*, and *Pax2/5/8*) duplicated in the lineage of vertebrates. Lampreys are thus crucial for the evolutionary sequence of the *Pax* gene evolution. Although one of *Pax2/5/8* genes has been isolated from the lamprey, the number of this gene family still remains unknown. *Pax3/7* are of particular interest not only in terms of the gene duplication, but also for the evolution of the midbrain and neural crest.

A sequential addition of expression repertoires of the *Pax1/9* genes has been proposed in the transition from the agnathans



**Fig. 7.** Expression boundaries of regulatory genes in the diencephalon and telencephalon. (A-D) Diencephalic expression domains for *LjPax6* (A), *LjTTF1* (B), *LjDlx1/6* (C) and *LjOtxA* (D). The outline of the diencephalon is shown by white lines. For each figure, the contours of the gene expression domain are shown by a blue line, which is identified anatomically by the relative positions of the telencephalon (arrowheads), the rostral end of the notochord (white arrows), and the anterior end of the epiphysis (black arrows). Red hatched lines indicate the domains where *LjPax6* and *LjDlx1/6* are both expressed. White arrowheads in C indicate the expression in the tissue lateral to the brain. (E-G) Regionalized expression patterns of regulatory genes in the stage 26 lamprey telencephalon showing the possible tripartite configuration. In all three embryos, broken white lines indicate the anterior intraencephalic sulcus and arrows indicate the hypothetical boundary between the dorsal and ventral telencephalon assumed in the present study. Expression patterns of *LjPax6*, *LjDlx1/6* and *LjEmx* are shown in E-G, respectively. (E) *LjPax6* is expressed in the dorsal part of the telencephalon (DP+VP). (F) *LjDlx1/6* is expressed in the ventral telencephalon. (G) Weak expression of *LjEmx* is restricted to the dorsal region of the telencephalon, possibly corresponding to the dorsal pallium. Abbreviations: DI, diencephalon; DT, dorsal telencephalon; EP, epiphysis; VT, ventral telencephalon.

to the gnathostomes (Ogasawara et al., 2000). However, comparison of *Pax6* expression in the agnathans and gnathostomes indicates that the field of expression of this gene

has not increased substantially during evolution, but has rather diversified in each lineage (see below).

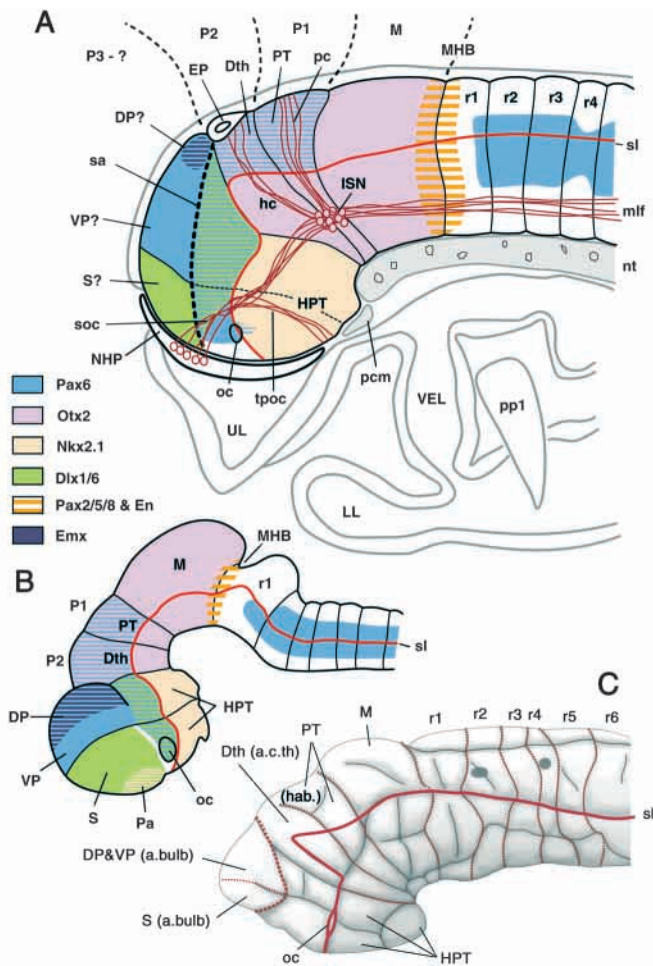
### *LjPax6* gene expression is developmentally and spatially regulated

*Pax6* expression has been analyzed in various vertebrate species, including the mouse (Grindley et al., 1995; Schubert et al., 1995; Stoykova and Gruss, 1994; Stoykova et al., 1996), zebrafish (Püschel et al., 1992; Hauptmann and Gerster, 2000), human (Gerard et al., 1995), chicken (Li et al., 1994) and rat (Matsuo et al., 1993). In every case, *Pax6* expression is developmentally regulated, and is first seen in the forebrain, hindbrain and the spinal cord. Later, *Pax6* transcripts appear in the telencephalon, the diencephalon, the eye and the myelencephalon in the gnathostomes. Similarly, in the lamprey, *LjPax6* transcripts appear in almost the same subset of neural regions, ultimately localizing in the same set of structures (Figs 3, 6). This shared sequence and pattern of expression indicates that the regions in which *Pax6* is expressed in the CNS had already been acquired in the ancestor common to all vertebrates. However, additional expression domains are evident in the gnathostomes, such as the cerebellar primordium. In the *Pax6*-null mutant (*small eye*) mouse, cell proliferation and initial differentiation seem unaffected, but cell migration and neurite extension are disrupted. Therefore, *Pax6* may be involved in the migration of cerebellar cells (Engelkamp et al., 1999). The lamprey cerebellum is rudimentary, and its homology to the corpus cerebellum of the jawed vertebrates is unresolved (Nieuwenhuys and Nicholson, 1998). We observed no *LjPax6* transcripts in the rostrordorsal myelencephalon, which corresponds to the cerebellar primordium. This suggests that the *Pax6*-regulated cerebellar patterning of the gnathostomes may have evolved independently after the divergence between the gnathostomes and agnathans.

*LjPax6* also exhibits some peculiar expression patterns associated only with the lamprey. In the early stages of development, *LjPax6* was not detected in r4, the developmental significance of which is not clear in the present study (Fig. 3C,D). No similar deficit has been observed in gnathostome embryos. Furthermore, *LjPax6* is strongly expressed in the dorsal oral ectoderm, the anterior velum ectoderm and the posteriodorsal velum ectoderm, in which gnathostome *Pax6* is not expressed. As the velum is unique to agnathans (reviewed by Mallatt, 1996; Kuratani et al., 2001; Ogasawara et al., 2000), *LjPax6* expression in the velar ectoderm may have been acquired independently in the lamprey lineage. *LjPax6* may be involved in the formation of the oral apparatus unique to the lamprey.

### Brain patterning in the lamprey

The pathway of evolution of the vertebrate brain is not completely resolved. Although the amphioxus has some neuronal components comparable with the vertebrate brain (Lacalli et al., 1994), it has no neuromeres identifiable either anatomically or developmentally (Hatscheck, 1881; Willey, 1891; Franz, 1927; Lacalli et al., 1994; Glardon et al., 1997; Glardon et al., 1998; Kozmik et al., 1999; reviewed by Wada and Satoh, 2001). Expression of these genes is regionalized but not delineated by clear boundaries by which developmental segments can be inferred.



In the vertebrate forebrain, four to six subdivisions called prosomeres (P1-P6) have been proposed that provide the developmental bases for its anatomical architecture (Puelles, 1995). In gnathostomes, at least some of the prosomeres have specific cell lineages, and the cells of one compartment do not easily mix with those of neighboring compartments (Figdor and Stern, 1993). Although there is general agreement on the presence of two posterior compartments representing the pretectum (P1) and the dorsal thalamus (P2), the number of more rostral compartments is still controversial.

The lamprey has a more advanced CNS than the amphioxus, and several compartments have been identified anatomically (Bergquist and Källén, 1953; Pombal and Puelles, 1999; Nieuwenhuys and Nicholson, 1998; Kuratani et al., 1998). In a series of review papers, Bergquist has shown that the larval lamprey brain consists of polygonal domains found in the brain wall, which are arranged serially along the dorsoventral and anteroposterior axes of the neural tube (Fig. 8C). These domains are actually discrete cell masses that Bergquist calls *Grundgebiete* or migration areas, which possibly represent local restrictions of cell lineages or, at least, regionalizations of neuroblasts. Bergquist's scheme is very similar, if not identical, to the prosomeric models postulated for the gnathostomes (Fig. 8B). In the adult lamprey brain, immunohistochemical analysis has also revealed that the common segmental and longitudinal organization of the neural tube still exists (Pombal and Puelles, 1999; Pombal et al.,

**Fig. 8.** The developmental plan of the lamprey brain.

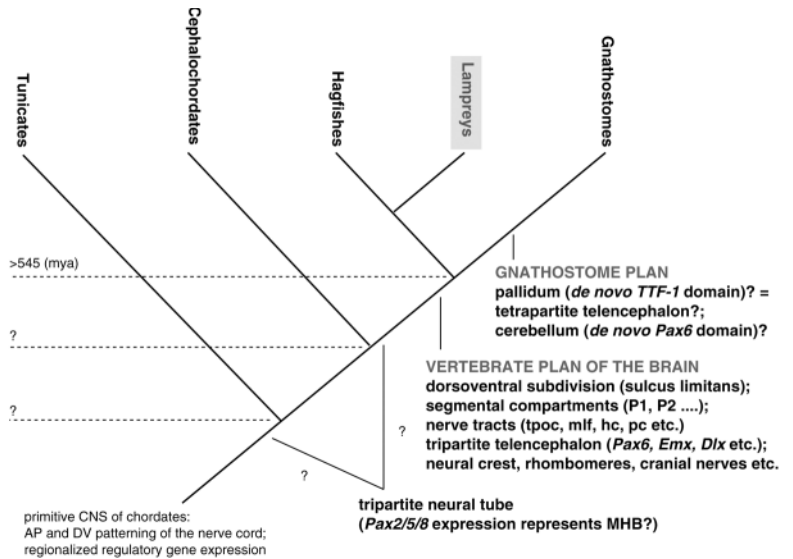
(A,B) Comparison of the developmental plans between lamprey and amniote brains. On the basis of the gene expression patterns and nerve tracts described in this study, expected segments such as P1 and P2, as well as the sulcus limitans, are drawn on the stage 26 lamprey brain as unbroken lines (A). Note that the ventral limit of *Pax6* expression corresponds to the sulcus limitans both in the lamprey and the mouse (B). Broken lines in A represent the hypothetical boundaries proposed in other studies but not confirmed in this study. Homologies between the domains are based on a comparison with the model proposed by Puelles and Rubenstein (Puelles and Rubenstein, 1993; B). No data were obtained in the present study to show more subdivisions rostral to the P2/P3 boundary. Boundaries of the rhombomeres are based on the reports of Kuratani et al. (Kuratani et al., 1997) and Horigome et al. (Horigome et al., 1999). The region rostral to the sulcus intraencephalicus anterior (sa) has here been tentatively termed the 'telencephalon' (T). Note that three gene expression domains are detected in this telencephalon, possibly corresponding to the dorsal and lateral pallium (DP and LP), and the striatum (S). Also note that *Lj/TTF1(Nkx2.1)* expression in the lamprey is restricted to the hypothalamus, and that this gene is not expressed in any region rostral to the optic chiasm. In the amniote brain, the rostral expression domain of *TTF1* corresponds to the pallidum (Pa in B) which is believed to be absent in the lamprey brain (Nieuwenhuys and Nicholson, 1998). (C) Polygonal configuration of the ammocoete brain postulated by Bergquist, with the segmental boundaries (broken red lines) and the sulcus limitans (longitudinal unbroken red line) proposed in the present study. Note that some of the boundaries were not defined in Bergquist's model. This polygonal model is not isomorphic with the pattern shown in B. Brain regions are named according to the postulated model of the larval lamprey brain in this study, and those of Bergquist are shown in parentheses. Redrawn from Bergquist and Källén (Bergquist and Källén, 1953). Abbreviations: a.bulb, olfactory bulb; a.c.th., area caudalis thalami of Bergquist and Källén (1953); DP, dorsal pallium; Dth, dorsal thalamus; EP, epiphysis; hab., habenula; hc, habenular commissure; HPT, hypothalamus; ISN, interstitial nucleus; LL, lower lip; M, midbrain; MHB, mid-hindbrain boundary; mlf, medial longitudinal fasciculus; NHP, nasohypophysial plate; nt, notochord; oc, optic chiasm; P1-P3, prosomeres; Pa, pallidum; pc, posterior commissure; pcm, prechordal mesoderm; pp1, pharyngeal pouch 1; r1-4, rhombomeres; S, striatum; sa, anterior intraencephalic sulcus; sl, sulcus limitans; soc, supraoptic commissure; tpoC, postoptic tract; UL, upper lip; VEL, velum; VP, ventral pallium.

2001). In the lamprey embryo, rhombomeric compartments and a possible P1/P2 boundary have been identified immunohistochemically (Kuratani et al., 1998; Horigome et al., 1999). Furthermore, lamprey *Otx* transcripts are expressed in the rostral neural tube, including the midbrain and forebrain, with the caudal limit of this expression possibly at the mid-hindbrain boundary (Ueki et al., 1998; Tomsa and Langeland, 1999). Lamprey *Dlx* cognate transcripts are expressed in the ventral diencephalon and telencephalon (Neidert et al., 2001; Myojin et al., 2001). In the evolutionary context, the crucial questions are, therefore, how many segments are arranged in which pattern in the lamprey brain, and which of these patterns are shared between the lamprey and the gnathostomes?

In gnathostomes, the positions of the nerve tracts are conserved between species (von Kupffer, 1896; Figdor and Stern, 1993; Kuratani and Horigome, 2000; Kuratani et al., 2000). Such anatomical conservation is known to be associated with compartmentalization of the neural tube: P1 is characterized by the posterior commissure and P2 by the



**Fig. 9.** Evolution of the vertebrate brain – a hypothesis. On the phylogenetic tree of the chordates, hypothetical evolutionary events are positioned according to the present findings in the lamprey. In the common ancestor of chordates, the dorsal nerve cord was patterned anteroposteriorly by some regulatory genes. Segmentation of the brain had not appeared even after the divergence of the amphioxus lineage. The present study suggests that the common ancestor of the vertebrates had already acquired rhombomeric segmentation and at least three longitudinal subdivisions in the forebrain. The most fundamental event in the establishment of the ancestral vertebrate brain is assumed here to have involved cell lineage restriction of neuroepithelial cells, both along the dorsoventral and the anteroposterior axes, to develop compartmentalized polygonal subdivisions. Note that lampreys and hagfishes are considered to form a monophyletic group in this figure, based on recent molecular data from Kuraku et al. (Kuraku et al., 1999) and Mallat and Sullivan (Mallat and Sullivan, 1998). After the divergence of the agnathans and gnathostomes, the evolution of the pallidum may have evolved specifically in the lineage of the latter.



habenular commissure (reviewed by Figdor and Stern, 1993). Caudal to the optic chiasm, tuberal and mammillary hypothalamic territories are clearly identifiable. In the present study, the posterior commissure, the habenular commissure and the optic chiasm were found to have homologous topography (Fig. 9), implying the presence of P1 and P2 in all vertebrate brains. This is also consistent with the expression of regulatory genes. In the stage 26 lamprey brain, the rostral domain of *LjOtxA* expression overlaps the caudal part of the *LjPax6*-expressing domain (compare Figs 6A,E, 8A). Considering the positions of the epiphysis, the posterior commissure and the habenular commissure, and comparing the pattern with that known for the embryonic mouse brain (Fig. 8B), the *LjOtxA-LjPax6* co-expressing domain most probably corresponds to the dorsal thalamus plus the pretectum (P1+P2, Fig. 8A). Gene expression patterns and anatomical structures are the only clue to the boundaries of the more rostral segments.

The *LjOtxA*-expressing domain terminates rostrally at the presumptive P2/P3 boundary (zona limitans), which is assumed from the position of the epiphysis. Rostral to this, *LjPax6* and *LjDlx1/6* are co-expressed in the dorsal diencephalon, and no expression is seen in the ventral diencephalon (hypothalamus; Figs 6A,B, 7A,C, 8A). The expression of *LjTTF1* appears to be complementary to the latter (in the ventral diencephalon or hypothalamus), and no transverse segmental boundaries in the forebrain can be detected for the telencephalon. Furthermore, the boundary between the *LjPax6-LjDlx1/6* co-expressing domain and the *LjTTF1*-expressing domain may correspond to the alar-basal plate boundary identified by Puelles and Rubenstein (Puelles and Rubenstein, 1993; Fig. 8A).

The morphology of the telencephalon is also problematic. Based on the expression patterns of the regulatory genes, the gnathostome telencephalon is assumed to be composed of three major components: the pallium (medial, dorsal, and lateral pallium; Puelles et al., 2000), the intermediate zone (ventral pallium; Puelles et al., 2000) and the subpallium (striatum). *Emx* and *Pax6* are expressed in the pallium, and *Dlx* in the subpallium (Puelles et al., 2000). In a previous study, we were

unable to detect the presence of such subdivisions in the lamprey telencephalon using transversely sectioned specimens (Myojin et al., 2001). However, after scrutinizing whole-mount embryos, we conclude that the lamprey telencephalon may be closer to the gnathostome plan than we originally believed. In the stage 26 forebrain, a transverse (morphologically horizontal) furrow is found, designated by von Kupffer (von Kupffer, 1906) as the sulcus intraencephalicus anterior (thick broken line (sa) in Fig. 8A). Characteristic gene expression is observed in the part of the brain that is rostral and dorsal to this sulcus. *LjPax6* is expressed in the dorsal part and *LjDlx1/6* in the ventral part, possibly corresponding to the pallium and striatum in the lamprey, respectively (Pombal and Puelles, 1999). Furthermore, in our new data, *LjEmx* is restricted to a small dorsal domain that expresses this gene plus *LjPax6*, and resembles the dorsal pallium of the gnathostomes (Figs 8A,B). These patterns of gene expression in this part of the lamprey brain are extremely reminiscent of the gnathostome telencephalon (Puelles et al., 2000; Fig. 8A). Although this may also imply the presence of the P3/P4 boundary (pallio-subpallial boundary) proposed by Puelles et al. (Puelles et al., 2000), it could not be followed into the dorsal diencephalic and hypothalamic regions (Fig. 8A). Finally, there is a region in the gnathostome telencephalon that includes the pallidum, in which *Dlx* and *TTF1* are both expressed (Fig. 8B). The loss of *TTF1* expression in the ventral telencephalic region of the lamprey forebrain (Figs 6C, 8A) may be related to the apparent absence of a pallidum in this animal (reviewed by Nieuwenhuys and Nicholson, 1998).

In conclusion, the present study of the lamprey brain primordium suggests the presence of the P1 and P2 segments, a longitudinally extending sulcus limitans that terminates rostrally close to the optic chiasm, a hypothalamus and a tripartite telencephalon-like domain. All these features are directly comparable with those in the model established in the mouse (Fig. 8B). Our results have not further clarified the number of segments in the rostralmost part of the brain (Fig. 8A). We may assume that the shared morphological patterns described above are very old in origin, possibly dating to the divergence of the lampreys and the gnathostomes (Fig. 9). As

recent analyses based on several molecules place hagfishes as the sister group of the lamprey (Mallatt and Sullivan, 1998; Kuraku et al., 1999), the segments listed above were already present in the common ancestor of all the vertebrates. The recent discovery of the earliest fossil vertebrates in the early Cambrian period (490-545 million years ago; Shu et al., 1999) suggests that the segmental plan underlying vertebrate brain development may have an even longer history (Fig. 9). The absence of compartments and the presence of similar anteroposterior regulation by various regulatory genes in cephalochordates imply that the vertebrate-specific compartments listed above were acquired by rough regionalization of the neurectoderm already present in the cephalochordates. It may have been proliferation of neurectodermal cells, as well as the restriction of local cell lineages to form boundaries, that facilitated this most curious evolutionary transition.

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