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Tripartite organization of the ancestral chordate brain and the antiquity of placodes: insights from ascidian *Pax-2/5/8*, *Hox* and *Otx* genes

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SUMMARY

Ascidians and vertebrates belong to the Phylum Chordata and both have dorsal tubular central nervous systems. The structure of the ascidian neural tube is extremely simple, containing less than 400 cells, among which less than 100 cells are neurons. Recent studies suggest that, despite its simple organization, the mechanisms patterning the ascidian neural tube are similar to those of the more complex vertebrate brain. Identification of homologous regions between vertebrate and ascidian nervous systems, however, remains to be resolved. Here we report the expression of *HrPax-258* gene: an ascidian homologue of vertebrate *Pax-2, Pax-5* and *Pax-8* genes. Molecular phylogenetic analyses indicate that *HrPax-258* is descendant from a single precursor gene that gave rise to the three vertebrate genes. The expression pattern of *HrPax-258* suggests that this subfamily of *Pax* genes has conserved roles in regional specification of the brain. Comparison with expression of ascidian *Otx* (*Hroth*) and a *Hox* gene (*HrHox1*) by double-staining in situ hybridizations indicate that the ascidian brain region can be subdivided into three regions; the anterior region marked by *Hroth* probably homologous to the vertebrate forebrain and midbrain, the middle region marked by *HrPax-258* probably homologous to the vertebrate anterior hindbrain (and maybe also midbrain) and the posterior region marked by *Hox* genes which is homologous to the vertebrate hindbrain and spinal cord. Later expression of *HrPax-258* in atrial primordia implies that basal chordates such as ascidians have already acquired a sensory organ that develops from epidermal thickenings (placodes) and expresses *HrPax-258*; we suggest it is homologous to the vertebrate ear. Therefore, placodes are not likely to be a newly acquired feature in vertebrates, but may have already been possessed by the earliest chordates.

Key words: Ascidian, *HrPax-258*, *Pax* gene, Midbrain, Hindbrain, Brain patterning, Placode, Ear, *Halocynthia roretzi*

INTRODUCTION

During vertebrate evolution, the nervous system has acquired significant complexity. The vertebrate brain contains an enormous variety of cell types that differentiate in specific positions. A first step in the differentiation of this complex brain is gross anatomical regionalization along the anteroposterior axis into three regions: forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (metencephalon-myelencephalon), accompanied by dorsoventral differentiation. During development, these three regions are further subdivided and give rise to specific neuronal cells according to the positions.

In this regionalizing process, one population of cells at the midbrain-hindbrain boundary (MHB) have a key function in organizing anteroposterior differentiation (reviewed by Joyner, 1996). When the cells from this region are transplanted into the diencephalon, the transplant cells themselves maintain their developmental fates and induce surrounding cells to form mesencephalic structures (Alvarado-Mallart, 1993; Marín and Puelles, 1994). In contrast, when transplanted into the hindbrain region, they induce surrounding cells into cerebellar structures (Martínez et al., 1995). Several genes, including *Wnt1*, *Fgf8*, *En1*, *2* and *Pax-2*, *Pax-5* and *Pax-8* genes, have been reported to be expressed in the MHB (Parr et al., 1993; Crossley and Martin, 1995; Davis et al., 1991; Nornes et al., 1990; Rowitch and McMahon, 1995; Joyner, 1996). Among them, *Pax-2* is the first gene to be expressed there (Rowitch and McMahon, 1995; Joyner, 1996). Together with the presence of Pax-binding sites in mouse *En-2* regulatory sequences (Song et al., 1996), this suggests that *Pax-2* is the most upstream regulatory gene controlling MHB development. Mice and zebrafish that have mutations in *Pax-2* locus (*noi* of zebrafish) show defects in MHB formation and subsequent defects in midbrain and cerebellum development (Torres et al., 1996; Favor et al., 1996; Brand et al., 1996).

The complex brain of vertebrates is not only a product of ontogenic process, but also a product of evolutionary history. Compared with the highly complex nervous system of vertebrates, the two most closely related animals, amphioxus and

ascidians, possess relatively simple nervous systems (Nicol and Meinertzhagen, 1991; Lacalli et al., 1994; Lacalli, 1996; Holland and Graham, 1995). Therefore the increased complexity of the brain evolved in the vertebrate lineage after divergence from these other groups of chordates. The life style of vertebrates has also changed from a filter-feeding (as in ascidians and amphioxus) to active predation; the more complex brain may have evolved for this new life style (Gans and Northcutt, 1983; Northcutt and Gans, 1983).

How has the complex vertebrate brain evolved? In both ascidians and amphioxus, homologues of *Otx* and *Hox* genes, which in vertebrates mark fore-midbrain and hindbrain respectively, are expressed in anteroposteriorly restricted regions of neural tube (Wada et al., 1996b; Katsuyama et al., 1995, 1996; Williams and Holland 1996; Holland et al., 1992). The regions marked by the expressions of these genes are likely to be homologous to vertebrate brain regions spanning from forebrain to hindbrain. Therefore, the vertebrate brain is not a newly added structure, but it has evolved by elaboration and expansion of preexisting anterior neural tube (Holland et al., 1992). More detailed comparisons between the vertebrate brain and those of ascidians and amphioxus are necessary to deduce how this elaboration and expansion occurred during evolutionary history.

The ascidian neural tube is extremely simple even compared with that of amphioxus. The ascidian (*Ciona intestinalis*) neural tube consists of only 370 cells, among which less than 100 cells are neurons (Nicol and Meinertzhagen; 1991). The ontogeny of these cells can be traced back to 16 cells at the 110-cell stage (just before gastrulation when most cells have acquired tissue-restricted fates; Nishida, 1987). Despite this small number, the ascidian neural tube is formed by neurulation processes similar to that of vertebrates, and is clearly a homologous structure (Satoh, 1978). Previous studies on *Hox1*, *Otx*, *Pax-3/7* and *HNF-3* homologues of ascidians suggested that the similarity in organization and patterning mechanisms between vertebrates and the simple neural tube of ascidians (Katsuyama et al., 1995, 1996; Wada et al., 1996a,b, 1997; Corbo et al., 1997). Ascidian neural tube development is expected to be simple in another sense; invertebrates, including ascidians and amphioxus, are thought to have approximately one-quarter as many genes as vertebrates (Bird, 1995; Holland et al., 1994). This means that the ascidian neural tube is likely to be patterned by a correspondingly smaller number of genes. For these reasons, ascidians offer a unique opportunity to study a simple and primitive neural tube patterning mechanism. Comparisons of organization and patterning mechanisms between ascidians and vertebrates should have implications for the evolutionary history of chordate neural tubes and the evolution of complexity in the vertebrate brain.

In order to study the patterning mechanisms of the simple neural tube of ascidians, and to draw evolutionary implications, an informative strategy is to compare the expression patterns of genes whose vertebrate homologues have already been shown to be involved in patterning of the brain. Here we focus on the expression of the *HrPax-258* gene: an ascidian homologue of vertebrate *Pax-2, Pax-5* and *Pax-8* genes, which have key roles in the development of the vertebrate MHB. We report cloning, molecular phylogenetic and expression studies of this gene. Double-labeling in situ hybridization shows that *HrPax-258* marks neural tube cells located between *Hroth*- and *HrHox1*-expressing cells. This observation suggests there is a common ground plan to chordate neural tubes based on division into three parts: fore-midbrain region marked by *Otx*, anterior hindbrain (and maybe also midbrain) region marked by *Pax-2/5/8* and a hindbrain region and spinal cord marked by *Hox* genes. Later in the ascidian tadpole larva stage, *HrPax-258* is expressed in the primordial pharynx and atrial primordia. This expression has implications for the evolution of the otic region and placodes in chordates.

MATERIALS AND METHODS

Ascidians

Adult ascidians, *Halocynthia roretzi*, were purchased from fishermen near Asamushi Marine Biological Station, Tohoku University, Aomori, or Ohtsuchi Marine Research Center of the Ocean Research Institute, University of Tokyo, Iwate, Japan. Naturally spawned eggs were fertilized with a suspension of non-self sperm and were cultured at 11-13°C.

PCR amplification

PCR was carried out using *H. roretzi* cDNA library from RNA of early tailbud embryos as a template DNA (Miya et al., 1996). In order to improve the specificity of PCR amplification, nested PCR was performed using two nested 3′ primers. The first round of PCR was done using primer Pax-uniF and Pax-258R2. After purification of DNA of expected size from an agarose gel, the DNA was used for the second round PCR in which primer Pax-uniF and Pax-258R1 were used. PCR conditions were 30 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute for both rounds. The primer sequences were as follows; Pax-uniF: 5′-GTNAA(CT)CA(AG)(CT)TNGGNGGNGTNTT-3′, Pax-258R1: 5′-CANGCGAACATNGTNGG(GA)TT(TC)TG-3′ and Pax-258R2: 5′-GGNACNGT(AG)TC(GA)TT(AG)TC(AG)CA-3′ (where N=A, C, G or T).

cDNA screening and nucleotide sequencing

The DNA fragment isolated by PCR was subcloned into the *Sma*I site of the plasmid pUC18 (Pharmacia Biotec). Using this as a probe, a cDNA library made from early tailbud stage embryos was screened (Miya et al., 1996). The clone isolated was subcloned into pBluescript II SK(+) and both strands were sequenced by the dideoxy chain-termination method (Sanger et al., 1977).

Sequence comparisons and molecular phylogenetic analyses

Sequences were aligned using SeqApp 1.9 manual aligner for Macintosh (Gilbert, 1993). Phylogenetic analyses were performed on the amino acid sequences of the paired domain. Estimation of molecular phylogeny was carried out by the neighbor-joining method (Saitou and Nei, 1987) using CLUSTAL V (Higgins et al., 1992) program. Confidence in the phylogeny was assessed by bootstrap resampling of the data (Felsenstein, 1985).

Whole-mount in situ hybridization

In situ hybridization of whole-mount specimens was carried out as described in Wada et al. (1997). For probe preparation, the pBluescript plasmid DNA containing the 2.1 kb *HrPax-258* cDNA was used as a template for in vitro RNA synthesis after digestion with *Bam*HI. Other probes were synthesized in the same way. Larval specimens were treated with higher concentrations of proteinase K (20-50 μg/ml) according to Okamura et al. (1994) and Wada et al. (1995) after the tunic was removed using needles. Double staining was performed by a modification of the methods of Jowett and Lettice (1994) and Hauptmann and Gerster (1994) as described in Wada et al. (1997). Specimens were simultaneously hybridized with two different riboprobes labeled with digoxigenin and fluorescein respectively.

After washing, specimens were incubated with phosphatase-conjugated anti-fluorescein antibody and stained blue with NBT-BCIP (Boehringer-Mannheim). After sufficient staining, specimens were treated with 0.1 M glycine-HCl (pH 2.2), 0.1% Tween 20 for 10 minutes at room temperature to inactivate the first phosphatase. After washing, specimens were incubated with phosphatase-conjugated anti-digoxigenin antibody (Boehringer-Mannheim) and stained with Fast-Red (Sigma).

RESULTS

Cloning of ascidian homologue of *Pax-2/5/8* subfamily genes

The vertebrate *Pax-2* gene is closely related to *Pax-5* and *Pax-8* (Gruss and Walther, 1992; Noll, 1993); together they may be considered as members of a subfamily of *Pax* genes (*Pax-2/5/8*). We reasoned that ascidians may not possess a direct homologue (orthologue) of *Pax-2*, but should possess one or more members of the subfamily. We therefore designed a PCR strategy to amplify any ascidian gene belonging to the *Pax-2/5/8* subfamily. cDNA prepared from RNA of *Halocynthia roretzi* early tailbud-stage embryos was used for PCR amplification. Nested PCR using primers Pax-uniF, Pax-258R1 and Pax-258R2 identified a fragment of a gene containing a paired box closely related to the vertebrate *Pax-2*, *Pax-5* and *Pax-8*. This fragment was used for screening a cDNA library made from early tailbud embryos, and a cDNA clone containing an open reading frame of 1296 nucleotides was isolated (Fig. 1). We designate this clone *HrPax-258*. This clone ends with 3′ poly(A) tract. Two in-frame termination codons in 5′ flanking region beyond the first ATG codon indicate that this cDNA clone contains the full protein coding sequence.

Sequence of *HrPax-258* and the molecular evolution of *Pax-2/5/8* subfamily genes

Conceptual translation of the cDNA shows that *HrPax-258* encodes a deduced protein of 432 amino acid residues (Fig. 1). The HrPax-258 paired domain shows 82.6%, 83.5%, 82.0% and 82.6% amino acid sequence identity to mouse Pax-2, Pax-5, Pax-8 and pax[zf-b] (a zebrafish Pax-2/5/8 subfamily gene), respectively; the vertebrate genes share more than 90% identity between them (Fig. 2). A characteristic octapeptide found in many *Pax* genes is also found in HrPax-258, from amino acid 198 to 205 (double underlined in Fig. 1). The *Pax-2/5/8* subfamily genes of vertebrates encode a 25 amino acid sequence showing similarity to the first helix of homeodomains and is thought to be a corroded remnant of a homeodomain. HrPax-258 has two stretches of sequence that have weak similarity to this region (underlined in Fig. 1), although neither of them match to the homeodomain consensus sequence (Bürglin, 1994). Dörfler and Busslinger (1996) demonstrated that C-terminal region of *Pax-2/5/8* genes contain a conserved transactivation and inhibitory domain. We found a sequence with significant similarity to this domain in the C terminus of HrPax-258 (Fig. 1).

In order to deduce the molecular evolutionary history of *HrPax-258*, we performed molecular phylogenetic analyses. *Pax* genes have been classified into four groups according to their sequence similarity, of which *Pax-2/5/8* is just one (others include *Pax-3/7* and *Pax-1/9*; Gruss and Walther, 1992; Noll, 1993); however, a recent report on cnidarian *PaxA* genes

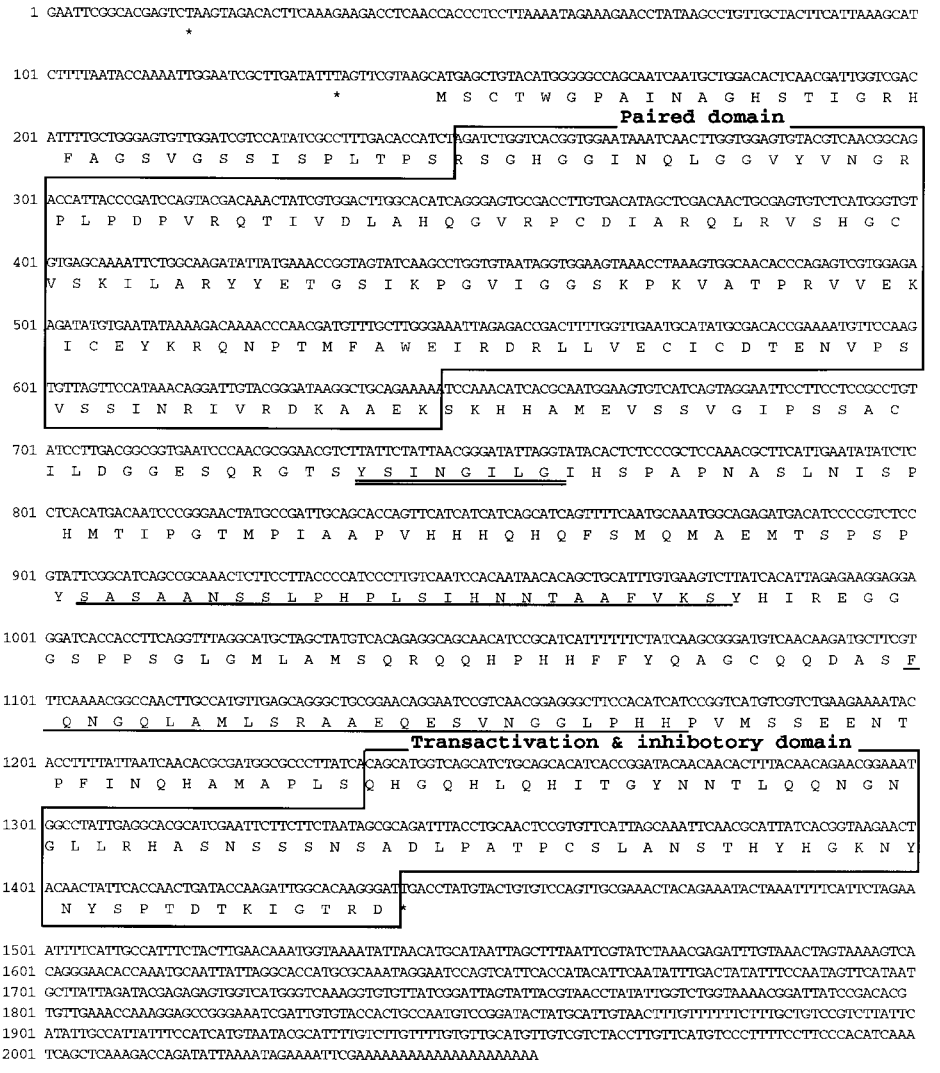

Fig. 1. Nucleotide and deduced amino acid sequences of *HrPax-258*. The paired domain and the conserved transactivation and inhibitory domain (Dörfler and Busslinger, 1996) are boxed. Octapeptide is double underlined and amino acid sequences that show weak similarity to the first helix of the homeodomain are single underlined. The stop codon at the termination site and those preceding the putative translation initiation sites are indicated by (*). The sequence has been deposited in the DDBJ data bank (Accession No. AB006675).

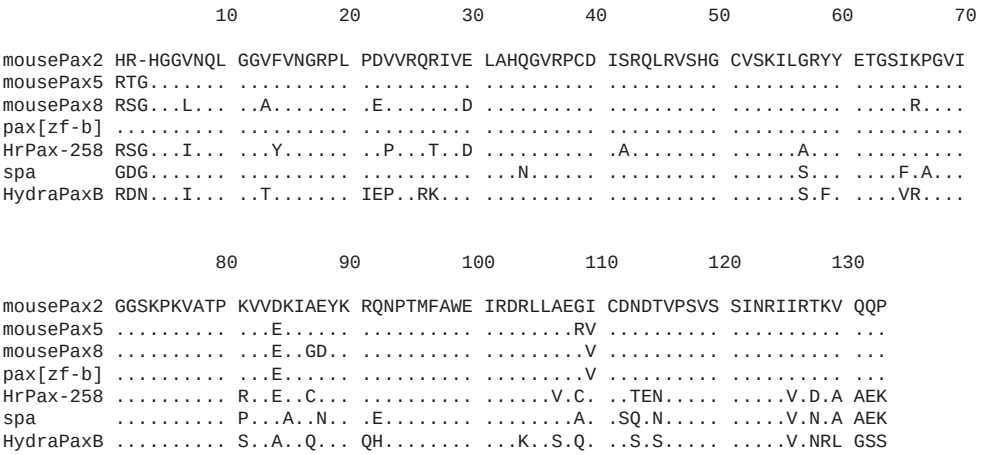

Fig. 2. Comparisons of amino acid sequences between the paired domains of *Pax-258* subfamily genes.

indicates that these genes form a fifth group with *Drosophila pox neuro* (Sun et al., 1997). First, in order to confirm that *HrPax-258* belongs to the *Pax-2/5/8* subfamily genes, a molecular phylogenetic tree of all *Pax* genes reported so far was constructed (Fig. 3A). In this analysis, only the paired domain sequence was alignable and used for calculations. The molecular phylogenetic tree clearly indicates that *HrPax-258* belongs to the *Pax-2/5/8* subfamily with *Drosophila sparkling* and cnidarian *PaxB* genes (bootstrap value of 91.7%; Fu and Noll, 1997; Sun et al., 1997). Interestingly, the *Pax-2/5/8* subfamily is more closely related to *Pax-4/6* group and *pox neuro* group of genes than to *Pax-3/7* or *Pax-1/9* (bootstrap value of 93.6%), a suggestion also supported by shared exon-intron boundaries (Glardon et al., 1997), and by other molecular phylogenetic analyses of *Pax* genes (Sun et al., 1997; Balczarek et al., 1997).

The molecular phylogenetic tree in Fig. 3A also suggests (with low confidence) that *HrPax-258* is descendent from the precursor of *Pax-2*, *Pax-5* and *Pax-8*. To investigate this possibility further, the analysis was rerun using only *Pax-2/5/8* subfamily genes, with *spa* and cnidarian *PaxB* genes as an outgroup. This confirmed the suggestion with a 80% bootstrap value (data not shown). This indicates that all the vertebrate *Pax-2/5/8* subfamily genes have evolved by gene duplication after divergence from the ascidian lineage and that *HrPax-258* is a direct descendent of the precursor gene.

Molecular phylogenetic analysis also allows us to resolve a question about the molecular nature of *pax[zf-b]*. The zebrafish gene *pax[zf-b]* clearly belongs to *Pax-2/5/8* subfamily, but how *pax[zfb]* relates to mouse genes has not been previously elucidated with confidence, because of the absence of a suitable outgroup for analyses. The above analyses indicate that *HrPax-258* is an ideal outgroup for addressing a question such as this. The molecular phylogenetic tree in Fig. 3B was constructed from 227 amino acid sites including conserved regions outside the paired domain. This tree clearly indicates that *pax[zf-b]* is an orthologue of mouse *Pax-2* (supported by 99.3% bootstrap value). This tree also strongly supports that the first gene duplication occurred between *Pax-8* and the precursor gene of *Pax-2* and *Pax-5* (bootstrap value of 83.8%).

HrPax-258 is expressed in a restricted region of the neural tube

The developmental expression of *HrPax-258* was examined by whole-mount in situ hybridization. The expression was first detected at the neurula stage in one bilateral pair of cells in the folding neural tube (Fig. 4A). Like other ascidian genes (e.g., Yasuo and Satoh, 1993; Satou et al. 1995; Wada et al., 1996a), this first sign of expression is detected in the nuclei. From this stage until the initiating-tailbud stage, weak signals are also detected in epidermal cells (Fig. 4B). This epidermal expression is only transient and is not detected after the mid-tailbud stage.

In initiating- and mid-tailbud embryos, the expression of *HrPax-258* is detected only in two bilateral pairs of cells in neural tube, which are probably daughters of those cells expressing *HrPax-258* at the neurula stage (Fig. 4C-F). Interestingly, the relative position of these cells to the anterior tip of the notochord shift anteriorly during development. Thus at the initiating tailbud stage, the *HrPax-258*-expressing cells are located well behind the anterior tip of the notochord (Fig. 4C), whilst later at the mid-tailbud stage, they are approximately at the same level of the anterior tip of the notochord (Fig. 4E). This positional shift between notochord and neural tube continues until the larval stage; in larvae, the anterior tip of the notochord is at the level of *HrHox1*-expressing cells (which are located posterior to *HrPax-258*-expressing cells as described below).

HrPax-258 is expressed in primordial pharynx and atrial primordia

In late-tailbud embryos, when just the otolith but not ocellus has melanized, expression in neural tube is no longer detected and new expression commences in the primordial pharynx (Fig. 4G). Later in swimming larvae, *HrPax-258* expression is also detected in atrial primordia (Fig. 4H-J). These organs are primordia of the adult oral and atrial siphons, which are formed by invaginations of ectoderm (Katz, 1983; see Fig. 4J).

Molecular map of ascidian neural tube

In order to precisely locate the *HrPax-258*-expressing neural tube cells of tailbud embryos, we compared the expression of *HrPax-258* with other genes by double-staining in situ hybridization. *Hroth* is the ascidian homologue of vertebrate *Otx* genes, and is reported to be expressed in both involuting mesendoderm and anterior ectoderm during gastrulation, and later in anterior neural cells and some epidermal cells (Wada et al., 1996b). Comparison of *HrPax-258* expression with *Hroth* is of special interest because vertebrate homologues of the two

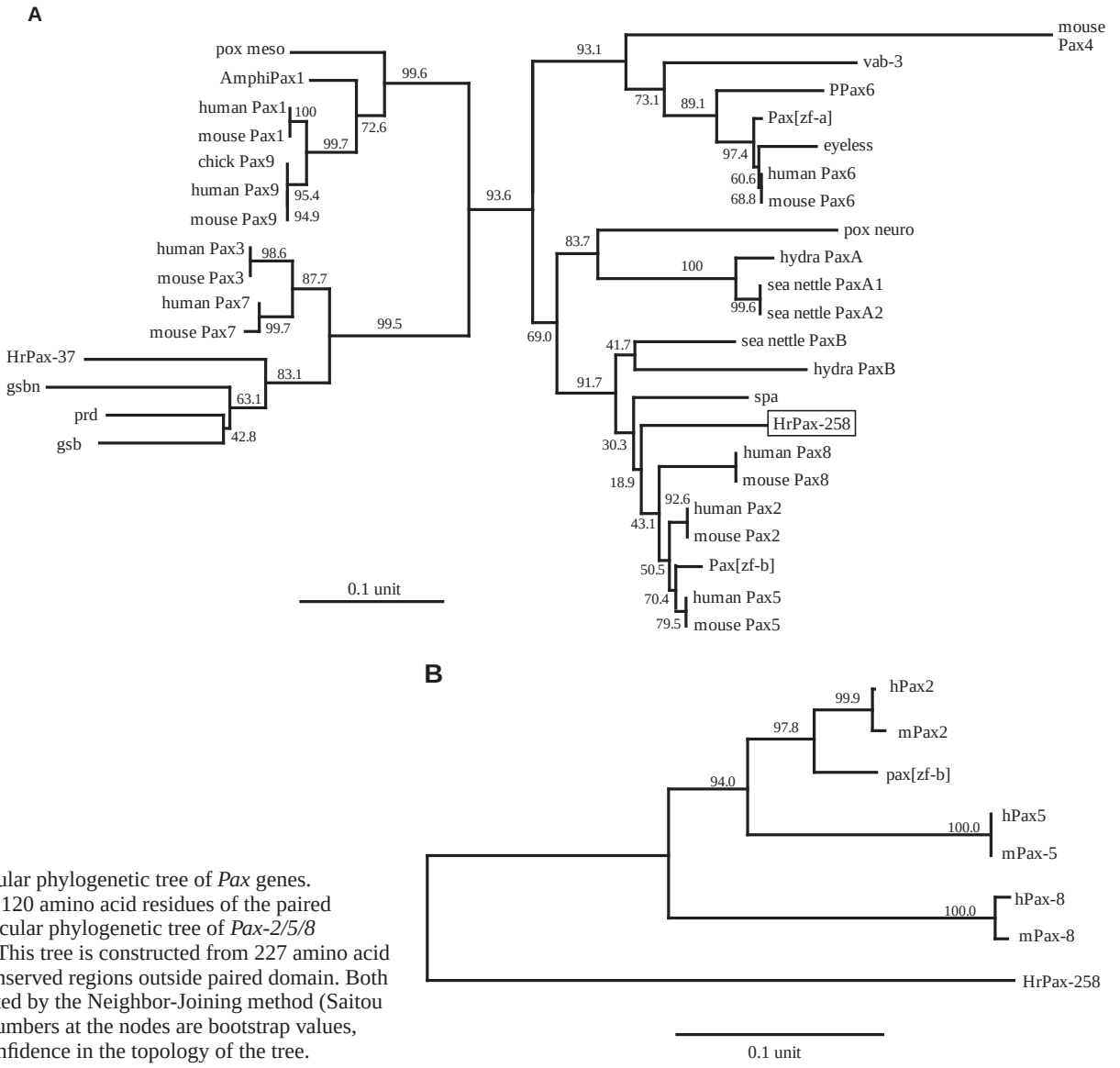

Fig. 3. (A) Molecular phylogenetic tree of *Pax* genes. Constructed from 120 amino acid residues of the paired domain. (B) Molecular phylogenetic tree of *Pax-2/5/8* subfamily genes. This tree is constructed from 227 amino acid sites including conserved regions outside paired domain. Both trees are constructed by the Neighbor-Joining method (Saitou and Nei, 1987); numbers at the nodes are bootstrap values, which indicate confidence in the topology of the tree.

genes abut each side of the MHB. Thus, the caudal limit of *Otx2* marks the boundary between midbrain and hindbrain, whilst *Pax-2* expression is on the hindbrain side of the MHB (Millet et al., 1996; Rowitch and McMahon, 1995). In both neurula and tailbud ascidian embryos, *HrPax-258* is expressed in cells exactly posterior to *Hroth*-expressing cells and no cells express both genes (Fig. 5A,B). This indicates that, from the earliest expression, *HrPax-258* does not overlap with *Hroth*.

HrHox1 is the ascidian homologue of group 1 *Hox* genes expressed with a clear anterior boundary in the neural tube, which is likely to mark part of the hindbrain-homologous region of the ascidian neural tube (Katsuyama et al., 1995). Double staining in situ hybridization indicates that *HrPax-258*-positive cells sit just anterior to the *HrHox1*-expressing cells and no cells express both genes (Fig. 5C). There is a gap of two cell widths between the expression of *HrHox1* and *Hroth*, precisely filled by expression of *HrPax-258* (Fig. 5D).

HrPax-37 is the ascidian homologue of *Pax-3* and *Pax-7* genes, and is expressed in three populations of cells in the neural tube (Wada et al., 1996a). The posterior expression site is contained within the domain marked by *HrHox1*, coinciding specifically with the most anterior *HrHox1*-expressing cells (Fig. 5E). The middle population of *HrPax-37*-expressing cells is contained with the *Hroth* domain and marks the posterior limit of *Hroth* expression (Fig. 5F). Thus these two populations of *HrPax-37*-positive cells are located either side of the *HrPax-258* expression domain (rostral and caudal); there is no overlapping expression of *HrPax-37* and *HrPax-258* (Fig. 5G). The anterior expression *HrPax-37* is one cell anterior to the most anterior *Hroth*-expressing cells (Fig. 5F), and probably marks the most anterior cells of the neural tube (Wada et al., 1996a).

Hrlim is an ascidian gene with a LIM class homeobox that shows highest identity to that of *Xlim-3*. At the tailbud stage, *Hrlim* is expressed in three anteroposteriorly restricted domains of the neural tube (the most posterior domain is fading at this stage) and in anterior ectodermal cells (Wada et al., 1995). Comparison of expression with *HrHox1* and *HrPax-37* indicates that the middle domain corresponds to just posterior to *HrHox1*-positive cells (Fig. 5H), whilst the anterior domain

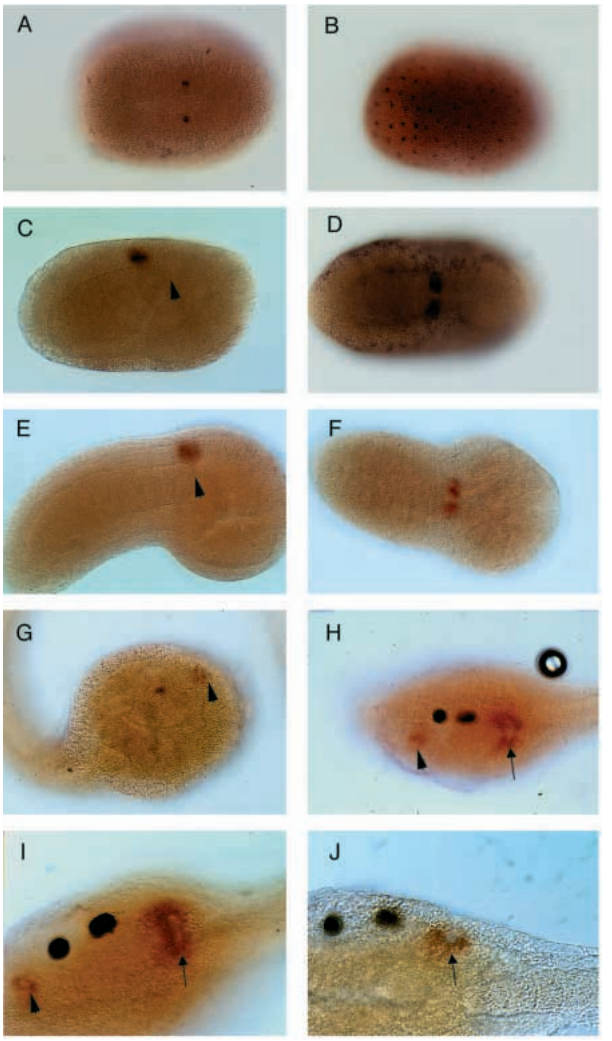

Fig. 4. Spatial and temporal expression of *HrPax-258* as deduced from in situ hybridization. (A) Dorsal view of the embryo at neurula stage. At this stage, the neural tube is folding up from the posterior and the *HrPax-258* signal is detected in one bilateral pair of presumptive neural tube cells. (B) Ventral view of the embryo at the neurula stage showing epidermal expression of *HrPax-258*. (C-F) Lateral (C,E) and dorsal (D,F) views of initiating- (C,D) and mid-tailbud (E,F) embryos. Two bilateral pairs of cells in the neural tube express *HrPax-258*. Arrowheads indicate the anterior tip of the notochord, which shifts anteriorly relative to the *HrPax-258*-expressing cells. (G) Dorsal view of late-tailbud embryo in which only the otolith is melanized. Expression of *HrPax-258* in the neural tube is no longer detected and new expression comes out in primordial pharynx (arrowhead). (H-J) Dorsal (H,I) and lateral (J) views of the larvae. *HrPax-258* is expressed in the primordial pharynx (arrowhead) and atrial primordia (arrow).

sits just posterior to the anterior expression of *HrPax-37* (Fig. 5I). The expressions of *HrPax-258*, *Hroth*, *HrHox1*, *HrPax-37* and *Hrlim* are summarized in Fig. 6.

In order to deduce the relationship between gene expression domains and the differentiation of cells in the brain, we also compared the gene expression patterns in larvae. Because double staining in situ hybridization is not feasible in larval specimens for unknown technical reasons, we compared the expression in terms of relative position to

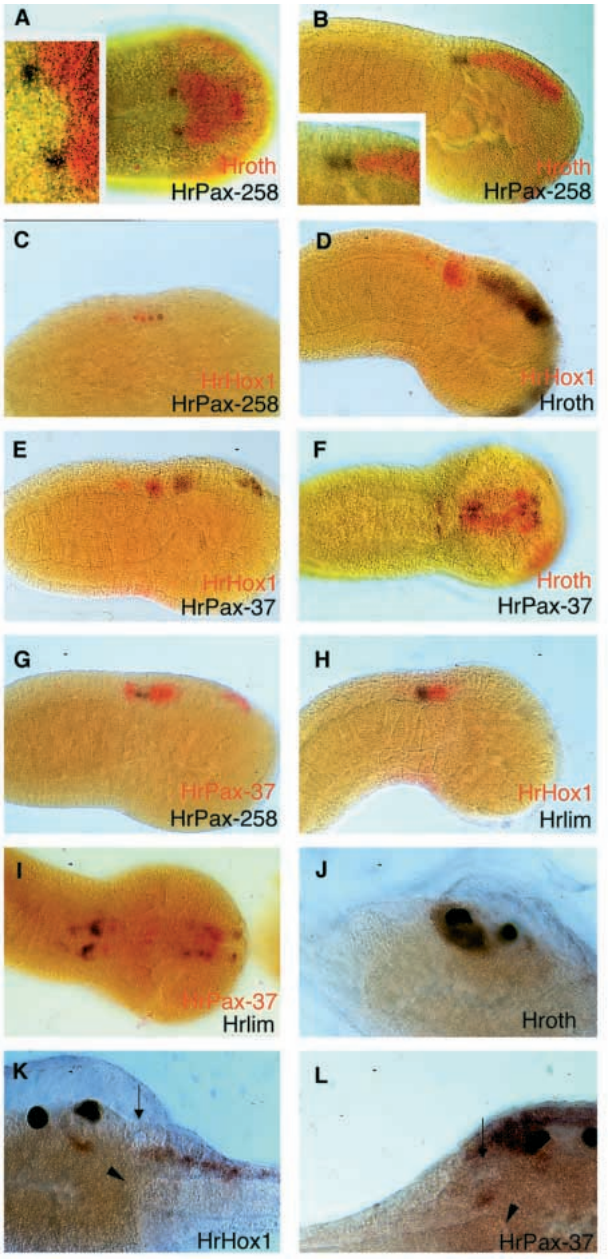

Fig. 5. Comparisons of genes expressed in the neural tube. (A,B) Comparison of expression between *HrPax-258* (red) and *Hroth* (blue). Dorsal view of neurula embryo (A) and lateral view (B) of mid-tailbud embryo. Close views of the expression boundaries clearly show that the expression of these two genes are not overlapped. (C) Comparison of expression between *HrPax-258* (blue) and *HrHox1* (red). Lateral view of early-tailbud embryo. (D) Comparison between *HrHox1* (red) and *Hroth* (blue). Lateral view of mid-tailbud embryo. (E) Comparison between *HrPax-37* (blue) and *HrHox1* (red). Lateral view of early-tailbud embryo. (F) Comparison between *HrPax-37* (blue) and *Hroth* (red). Dorsal view of mid-tailbud embryo. (G) Comparison between *HrPax-37* (red) and *HrPax-258* (blue). Lateral view of early tailbud embryo. (H) Comparison between *HrHox1* (red) and *Hrlim* (blue). Lateral view of mid-tailbud embryo. (I) Comparison between *HrPax-37* (red) and *Hrlim* (blue). Dorsal view of mid-tailbud embryo. (J-L) Expression of *Hroth* (J), *HrHox1* (K) and *HrPax-37* (L) in larvae. The atrial primordia are marked by arrows. The anterior tip of the notochord is marked by arrowheads. For convenience of comparisons, (B,E,H) are flipped horizontally.

the anterior tip of the notochord and the atrial primordia. Larval expression of *Hroth* in the neural tube is restricted to the sensory vesicle and the posterior limit clearly marks the posterior boundary of the sensory vesicle (Fig. 5J). The expression of *HrHox1* is observed in a small number of cells in the left side of the sensory vesicle (a larval-specific site; Katsuyama et al., 1995) and also in a neural tube domain posterior of the level of the atrial primordia and anterior tip of the notochord (Fig. 5K). In another species of ascidian *Ciona intestinalis*, the visceral ganglion is a morphologically distinct structure caudal to the sensory vesicle and on a level with the anterior end of the notochord. The visceral ganglion is not morphologically distinguishable in *Halocynthia roretzi*, although we suspect that its anterior border is marked by the anterior limit of *HrHox1* expression in the neural tube due to its positional relationship with the notochord. The middle and posterior domains of *HrPax-37* expression are still at the same level relative to the expression of *Hroth* and *HrHox1* (posterior end of *Hroth* and anterior end of *HrHox1*), suggesting there has been no substantial change in the expression sites of these genes between tailbud and larval stages (Fig. 5L).

DISCUSSION

Molecular evolution of Pax genes

In order to understand the evolution of gene functions, it is also important to know the evolutionary relationship of the genes themselves. This is especially pertinent in the case of *Pax-2*, *Pax-5* and *Pax-8* genes, since the gene regulatory network involving *Wnt1*, *En1*, *En2* and *Pax-2*, *Pax-5*, and *Pax-8* in vertebrate MHB development is often regarded as a homologous network to that between *engrailed*, *wingless* and *paired* in *Drosophila* segmentation. However, it is clear from the molecular phylogenetic tree in Fig. 3A that *Drosophila paired* is not a counterpart of *Pax-2*, *Pax-5* and *Pax-8*, but is closer to *Pax-3* and *Pax-7*. Therefore, although interaction between *En-1* and *Wnt-1* may be homologous to that between *engrailed* and *wingless*, their interaction with *paired* or *Pax-2*, *Pax-5*, *Pax-8* cannot be homologous.

Also from the present molecular phylogenetic analyses, the ascidian gene *HrPax-258* is confirmed to be descendent from the precursor gene of vertebrate *Pax-2, Pax-5* and *Pax-8* genes. This allows us to deduce the relationships between vertebrate (and evolutionary history of) *Pax-2, Pax-5* and *Pax-8* genes. We show that the first duplication in the gene subfamily gave rise to *Pax-8* and the precursor gene of *Pax-2* and *Pax-5*. There is an interesting implication of this evolutionary history. Since both *Pax-2* and *Pax-8* have functions in kidney development (Dressler et al., 1990; Plachov et al., 1990; Rothenpieler and Dressler, 1993; Torres et al., 1995; Favor et al., 1996; Dehbi and Pelletier, 1996), but *Pax-5* does not, it is most parsimonious to conclude that the function in kidney development is a primitive function of *Pax-2*, *Pax-5* and *Pax-8* genes, which has been lost by *Pax-5*.

Another point resolved by use of the *HrPax-258* sequence is the phylogenetic position of zebrafish *pax[zf-b]*. Although the expressions of *pax[zf-b]* in optic stalk, otic vesicle and nephritic primordium (Krauss et al., 1991) suggests its orthology to *Pax-2*, molecular phylogenetic analyses were not able to support this orthology because of the absence of a suitable outgroup. Present analysis using *HrPax-258* as an outgroup clearly show that *pax[zf-b]* is an orthologue of mouse *Pax-2* (Fig. 3B). This phylogenetic status of *pax[zf-b]* implies that the gene duplications that gave rise to the multiple vertebrate *Pax-2/5/8* subfamily genes predated the divergence of teleosts and tetrapods. This also suggests the existence of some other, as yet undiscovered, *Pax-2/5/8* subfamily genes in zebrafish.

Evolutionary history of chordate brain patterning

HrPax-258 show clearly restricted expression in the ascidian neural tube. Double staining in situ hybridization revealed that the *HrPax-258*-expressing cells locate precisely posterior to *Hroth*-expressing cells and precisely anterior to *HrHox1*-expressing cells. Therefore, from the expression domain of these genes, the anterior neural tube of ascidian can be subdivided into three domains. This subdivision can be easily reconciled with models of regionalization suggested from ascidian morphology. Nicol and Meinertzhagen (1991) subdivided the ascidian neural tube into four domains: sensory vesicle, neck, visceral ganglion and tail. Comparing the present study to these frameworks, we conclude that *Hroth* expression

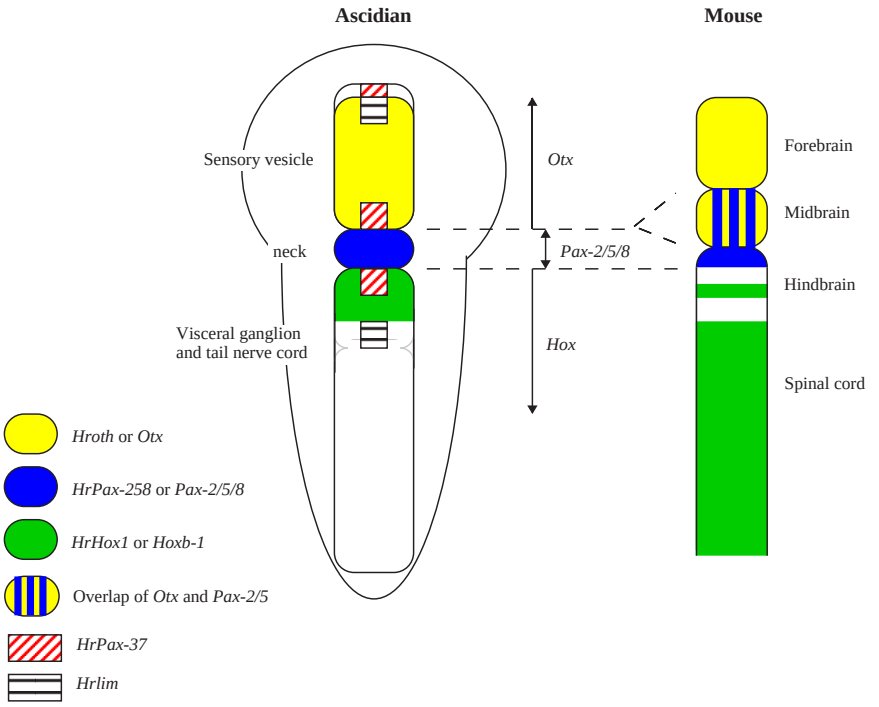

Fig. 6. Comparison of brain organization and gene expression between mouse and ascidian; indicating hypothesized homology of brain regions.

is limited to the sensory vesicle (Fig. 5J). The anterior limit of *HrHox1* is at the level of atrial primordia and the anterior tip of the notochord, which equates to the anterior limit of the visceral ganglion according to Nicol and Meinertzhagen (1991) (Fig. 5K). Therefore, we conclude that the expression of *HrPax-258* corresponds to the presumptive 'neck' region of the neural tube. To summarize, the ascidian anterior neural tube can be subdivided into three domains: sensory vesicle marked by *Hroth*, neck region marked by *HrPax-258* and visceral ganglion, the anterior limit of which is marked by *HrHox1* (Fig. 6).

Using both the anatomical subdivisions and regulatory gene expression, we can propose putative homologous regions between the vertebrate brain and the ascidian neural tube. The identification of homologous regions from gene expression data is prone to difficulties, but we suggest we are on safer ground in this instance since the particular genes examined here are concerned with regional differentiation. We must, however, be careful to compare correct genes. Abouheif et al. (1997) stress that orthologous genes should be compared whenever possible in the search of homology. This is not possible when comparing ascidians and vertebrates due to the widespread gene duplications that occurred early in vertebrate evolution. Thus, we must take into account subsequent evolutionary changes of gene functions after gene duplications. Both *Otx-1* and *Otx-2* are expressed in forebrain and midbrain, with the posterior expression boundary marking the posterior limit of midbrain (Simeone et al., 1992; Shimamura et al., 1995; Millet et al., 1996). Pariticularly with *Otx-2*, Millet et al. (1996) indicated that the posterior expression boundary tightly links to the posterior boundary of the midbrain. In 9.5 dpc mouse embryo, *Pax-2* and *Pax-8* are expressed on the opposite (hindbrain) side of MHB (Rowitch and McMahon, 1995; Asano and Gruss, 1992). Combining comparative expression between *Pax-2* and *Wnt-1* (Rowitch and McMahon, 1995) and that between *Wnt-1* and *Otx-2* (Bally-Cuif et al., 1995) suggests that the expression of *Otx-2* and *Pax-2* is likely to abutt at the boundary between midbrain and hindbrain at 9.5 dpc; direct comparison by double staining in situ hybridization is necessary to verify this conclusion. Their ascidian homologues, *Hroth* and *HrPax-258*, show clear abutting expressions domains, with this demarcation located at the posterior end of the sensory vesicle. We suggest, therefore, that the vertebrate forebrain plus midbrain is homologous to the ascidian sensory vesicle; and that the most anterior vertebrate hindbrain is homologous to the ascidian 'neck' region. However, in earlier stages (8.0 dpc), *Pax-2* is expressed more widely in both mesencephalon and metencephalon, and the expressions of *Pax-2* and *Otx* genes overlap in mesencephalon (Püschel et al., 1992; Rowitch and McMahon, 1995). In addition, *Pax-5* is expressed in the midbrain region (Asano and Gruss, 1992; Adams et al., 1992; Urbánek et al., 1994; Rowitch and McMahon, 1995). *Pax-2* and *Pax-5* mutant mouse show defects not only in the anterior hindbrain but also in midbrain (Urbánek et al., 1994; Favor et al., 1996; Torres et al., 1996), which may result from loss of organising activity in the MHB. Therefore, an alternative possibility is that the ascidian neck region, marked by *HrPax-258*, is homologous to a region including both anterior hindbrain and midbrain of mouse. Posterior to these regions, the posterior hindbrain and spinal cord of vertebrates (characterized by *Hox* gene expression) are homologous to the ascidian visceral ganglion and tail nerve cord (Fig. 6). Exact mapping of the boundary between the *Pax-2/5/8* region and *Hox* region must await close examination of posterior expression limit of *Pax-2*, *Pax-5* and *Pax-8* genes in relation to *Hox* gene expressions.

In addition to the overlapping expression of *Otx* and early *Pax-2* and *Pax-5*, there is another gene expression difference between ascidian and mouse. The expression domain of *HrHox1* reaches as far anterior as the posterior limit of *HrPax-258* expression. In contrast, mouse *Hoxa-1* and *Hoxb-1* are more posterior and are not even the most rostrally expressed *Hox* genes (Murphy and Hill, 1991). This difference has interesting implications for the evolution of *Hox* genes. It seems that in ascidians, the group 1 gene marks the anterior limit of the region regulated by *Hox* genes. In contrast, the most anterior expressing *Hox* gene in vertebrates is from group 2 (Krumlauf, 1993); the anterior limits of *Hox-a2* and *b2* are rhombomere 2 and 3, respectively, whilst *Hox-b1* is expressed only in rhombomere 4 and in posterior neural tube. We suggest that a simple colinear expression of *Hox* genes (i.e., 3′ genes express more anteriorly) is the primitive condition of chordates and that ascidian *HrHox1* reflects this primitive condition. Therefore, we suggest that the expression of vertebrate group 1 *Hox* genes has shifted posteriorly as a secondary (derived) condition in evolution.

Pax-2/5/8 and the mouth

At the larval stage, *HrPax-258* is expressed in the primordial pharynx and atrial primordia. These organs develop into the oral and atrial siphons of adult ascidians. Ascidians filter-feed by taking sea water (with small organic particles) into the oral siphon, capturing the small particles by filtration through a mucous filter supported by gill slits, and passing the water out through the atrial (exhalent)siphon. Therefore, the oral siphon of adult ascidians can be regarded as a mouth. The cell lineage of the ascidian pharynx is closely allied with that for the anterior neural tube: at the 110-cell stage when most cells acquire a tissue-restricted fate, the a8.19 cells develop into both anterior neural tube and pharynx. The pharynx then locates anteriorly to the neural tube in larvae (Nishida, 1987). This cell lineage of the ascidian pharynx is comparable to that of the vertebrate stomodeum, which develops from anterior neural ridge (Couly and Le Douarin, 1985). This similarity of cell lineage strongly supports the homology between the ascidian mouth and that of vertebrates. However, we note that no vertebrate *Pax-2/5/8* genes are expressed in the stomodeum. One possibility is that, in ascidians, the *Pax-258* gene has been co-opted to pharynx development, perhaps in connection with the evolution of similar cellular properties in pharynx and atria. To further investigate this hypothesis, we need to know whether the presence or absence of *Pax-2/5/8* expression in the mouth is primitive; expression of *Pax-2/5/8* subfamily genes in amphioxus may help in this regard.

Evolutionary antiquity of ears and placodes

The expression of *HrPax-258* in atrial primordia of ascidian larvae has interesting evolutionary implications. The atrial primordia develop as a pair of ectodermal invaginations which fuse to form one atrial siphon. This mode of formation is strikingly similar to that of vertebrate placodes. Bone and Ryan (1978) have shown that the atrium of adult ascidians develops

ciliated sensory cells in cupular organs resembling those of the vertebrate acoustico-lateralis system. Based on this observation they suggested that atrial primordia of ascidian larvae are homologous to the inner ear (otic system) of the vertebrates. *HrPax-258* expression in atrial primordia adds further support to this view because vertebrate *Pax-2* is also expressed in otic placodes (Nornes et al., 1990; Krauss et al., 1991) and has an essential function in inner ear development (Torres et al., 1996; Favor et al., 1996). Although vertebrate *Pax-2/5/8* subfamily genes are also expressed in a number of other cell type; e.g. *Pax-8* expression in thymus, two independent lines of evidence (morphological similarity and gene expression) support homology between the vertebrate ears and ascidian atrial primordia. This implies that the chordate ancestors had ears as a paired sensory organ.

Neural crest and placodes are often regarded as newly acquired features of vertebrates (Northcutt and Gans, 1983; Maisey, 1986; Schaeffer, 1987; Nielsen, 1995). As for neural crest, several recent studies suggest that vertebrate neural crest is 'regionally' homologous to lateral neural plate and/or epidermal cells adjacent to the neural tube of ascidians and amphioxus (Wada et al., 1996a, 1997; Holland et al., 1996). However, considering its migrating behavior and its potential to differentiate into skeletal tissues despite its ectodermal origin, the neural crest can still be regarded as a new feature of vertebrates. However, the ascidian atrial primordia clearly fit the definition of placodes: they form from thickenings of the epidermis and differentiate to form sensory organs (Northcutt and Gans, 1983). The ascidian atrial primordia develops similar sensory cells to those in vertebrate inner ear (Bone and Ryan, 1978), both of which express closely homologous genes: *HrPax-258* or *Pax-2*. We conclude that placodes are utilized in ascidian development, and are not a new feature of vertebrates.

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