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# Characterization of an amphioxus paired box gene, *AmphiPax2/5/8*: developmental expression patterns in optic support cells, nephridium, thyroid-like structures and pharyngeal gill slits, but not in the midbrain-hindbrain boundary region

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## SUMMARY

**On the basis of developmental gene expression, the vertebrate central nervous system comprises: a forebrain plus anterior midbrain, a midbrain-hindbrain boundary region (MHB) having organizer properties, and a rhombospinal domain. The vertebrate MHB is characterized by position, by organizer properties and by being the early site of action of *Wnt1* and *engrailed* genes, and of genes of the *Pax2/5/8* subfamily. Wada and others (Wada, H., Saiga, H., Satoh, N. and Holland, P. W. H. (1998) *Development* 125, 1113-1122) suggested that ascidian tunicates have a vertebrate-like MHB on the basis of ascidian *Pax258* expression there. In another invertebrate chordate, amphioxus, comparable gene expression evidence for a vertebrate-like MHB is lacking. We, therefore, isolated and characterized *AmphiPax2/5/8*, the sole member of this subfamily in amphioxus. *AmphiPax2/5/8* is initially expressed well back in the rhombospinal domain and not where a MHB would be expected. In contrast, most of the other expression domains of *AmphiPax2/5/8* correspond to expression domains of vertebrate *Pax2*, *Pax5* and *Pax8* in structures that are probably homologous – support cells of the eye, nephridium, thyroid-like structures and pharyngeal gill slits; although *AmphiPax2/5/8* is not transcribed in any structures that could be interpreted as homologues of vertebrate otic placodes or otic vesicles. In sum, the developmental expression of *AmphiPax2/5/8* indicates that the amphioxus central nervous system lacks a MHB resembling the vertebrate isthmic region. Additional gene expression data for the developing ascidian and amphioxus nervous systems would help determine whether a MHB is a basal chordate character secondarily lost in amphioxus. The alternative is that the MHB is a vertebrate innovation.**

Key words: Pax, Midbrain-hindbrain boundary, Mes-metencephalic region, Isthmic region, Gill slit, Nephridium, Pronephros, Thyroid gland, Otic placode

## INTRODUCTION

An emerging paradigm is that the central nervous system of developing vertebrates is divided rostrocaudally into three principal regions, each expressing characteristic families of developmental genes (Bally-Cuif and Wassef, 1995; Joyner, 1996; Boncinelli et al., 1998). Region 1, comprising forebrain and anterior midbrain, represents the site of action of the *Otx* and *Emx* gene families. Region 3, comprising the rhombospinal domain posterior to the first rhombomere of the hindbrain, is the site of action of the *Hox* genes. Interposed between regions 1 and 3, region 2 comprises the posterior midbrain plus the first rhombomere and is variously known as the midbrain-hindbrain boundary region (MHB), the mes-metencephalic region, the isthmus/cerebellum or simply the isthmic region.

In the vertebrates, the MHB region is the early site of action of *Wnt1* and *engrailed* genes, and of genes of the *Pax2/5/8* subfamily, interrelated in a network that has some parallels with the cascade establishing parasegmental boundaries in *Drosophila*. During vertebrate embryology, the MHB region differs strikingly from regions 1 and 3, because, after ectopic transplantation to other brain regions, it can develop independently and act as an organizer, recruiting nearby cells to form structures characteristic of the MHB region. The vertebrate MHB is thus defined by its morphology (comprising structures of the posterior midbrain plus the first rhombomere), its developmental genetics and its function as an organizer.

In protostome invertebrates, there is morphological and developmental genetic evidence that the central nervous system is divided into a head domain and a trunk domain (reviewed by Bruce and Shankland, 1998). However, there have been no suggestions that these two domains of protostomes are

separated by a third region comparable to the vertebrate MHB. Thus, a MHB appears to have originated in the deuterostome line of evolution; it is interesting to consider whether this structure first appeared in some invertebrate deuterostome or was a vertebrate innovation.

Recently, Wada et al. (1998) and Williams and Holland (1998) used developmental gene expression data to argue that the nervous system of ascidian tunicate larvae includes a MHB, although a simple one without organizer activity. In contrast, gene expression studies of another invertebrate chordate, amphioxus, do not indicate the presence of a MHB region. For example, whereas vertebrate *engrailed* and *Wnt1* genes are early markers for the MHB, they are not expressed where such a region would be expected to occur in the nerve cord of amphioxus (Holland et al., 1997, our unpublished data). In the present paper, we characterize amphioxus *AmphiPax2/5/8*, another potential marker for the MHB region, and find its early neural expression is distant from any region that might be interpreted as an amphioxus MHB.

The central nervous systems of ascidians and amphioxus are tripartite in the sense of having an anterior region of *Otx* expression, a posterior region of *Hox* expression and an intervening zone where neither of these genes is expressed. Even so, if one is to propose a useful homology between this intervening zone and the vertebrate MHB, additional characters are needed – at least a common developmental genetic program, even if not organizer activity. A vertebrate-like MHB in ascidians has been proposed on the basis of the developmental expression of ascidian *Pax258* there. In contrast, amphioxus homologues of *Wnt1* and *engrailed* genes, and genes of the *Pax2/5/8* subfamily are not involved in the development of the intervening brain region in amphioxus. Further work (especially on expression of ascidian homologues of *wingless* and *engrailed* genes) will be needed to determine whether the MHB is (1) a vertebrate innovation or (2) a basal chordate character that has been secondarily lost in amphioxus.

## MATERIALS AND METHODS

### Obtaining animals; isolation and sequencing of *AmphiPax2/5/8*

Adults and developmental stages *Branchiostoma floridae* were obtained by the methods of Holland and Holland (1993). A cDNA library in λZap II (Stratagene, La Jolla, CA) from mixed 8- to 18-hour embryos was used as a template in the polymerase chain reaction (PCR) with degenerate primers specific for the Pax2/5/8 subfamily paired box: 5′GCGGAATTCA(G/A)GGN(C/A)GNCCN(C/T)TNC-CNGA3′ and 5′GCGAGAAGCTT(T/C)TCCCANGC(G/A)AAC-ATNGT3′ where N=A/C/G/T. The resulting PCR fragment was subcloned into PUC18 and sequenced. This sequence information was used to design a primer for 5′RACE (5′GCGAAGCTTGGAG-ATGTCGCAGGGACGCAC3′) and a primer for 3′RACE (5′GCGAATTCGATTGCCGAGTACAAGCGACAG3′). RACE was performed with Taq polymerase and PCR additive (Taq EXTENDER, Stratagene, La Jolla, CA) with the mixed embryonic cDNA library as a template (35 PCR cycles: 1 minute at 92°C, 1 minute at 65°C and 3 minutes at 72°C). The 5′RACE products were subcloned into a T-vector pT7Blue (Novagen, Madison, WI) and the 3′RACE products were cut with *Eco*RI and subcloned into the pSP64 vector (Promega, Madison, WI). For five independent 5′RACE clones and five independent 3′RACE clones, both strands were sequenced, revealing an α and a β splice variant.

### Search for multiple amphioxus genes of the Pax2/5/8 subfamily; phylogenetic analysis

To help confirm that the cDNAs encoding the α and β isoforms of AmphiPax2/5/8 are derived from one gene by alternative splicing of the mRNA, we used PCR to amplify part of the paired box of genomic DNA pooled from 20 amphioxus adults (Holland et al., 1996). Degenerate primers, which corresponded to GSKPKVA and TMFAWEI (amino acid sequences invariably conserved in all Pax2, Pax5, and Pax8 genes), were 5′GCGGAATTCGGN-(T/A)(G/C)NAA(G/A)CCNAA(G/A)GTNGC3′ and 5′GCGAGAAG-CTT(T/C)TCCCANGC(G/A)AACATNGT3′, respectively, where N=A/C/G/T. The resulting 110-bp portion of the paired box was characterized by restriction digests and sequencing.

Accession numbers for the sequences used in the phylogenetic analysis were: hydra Pax-B, U96194; *Drosophila* dPax2/5/8 (sparkling), AF016888; sea urchin suPax258, AF016883; ascidian tunicate HrPax258, AB006675; zebrafish Pax2.1 (Pax[b]), Q90268; zebrafish Pax2.2, AF072547; zebrafish Pax5, AF072548; zebrafish Pax8, AF072549; *Xenopus* Pax2, Y10119; mouse Pax2, P32114; human Pax2, M89470; mouse Pax5, P32114; human Pax5, Q02548; mouse Pax8, Q00288; and human Pax8, Q06710. Full-length protein sequences, including conserved and non-conserved regions, were compared over 569 amino acid sites for AmphiPax2/5/8α and fifteen other proteins within the same Pax subfamily. The phylogenetic tree was constructed by an heuristic approach with random stepwise addition (PAUP 3.1.1.) with hydra Pax-B as the outgroup. Bootstrap values were based on 1,000 iterations of ten topology searches each. For comparison, the paired domains alone (as defined by Pfeffer et al., 1998) were analyzed for the same sixteen proteins (excluding the first and last amino acids, which were highly diverse from one sequence to the next). Each round of a 10-round, heuristic search yielded the same six trees of equal length, and a consensus tree was constructed from those.

### Reverse transcription-PCR analysis

To study expression of mRNA variants of *AmphiPax2/5/8*, 5 μg of total RNA from 7-, 9-, 12-, or 14-hour embryos (Chomczynski and Sacchi, 1987) was reverse-transcribed with random hexamers (SuperscriptII kit, Gibco-BRL, Gaithersburg, MD). The single-stranded cDNA was subjected to PCR (35 cycles: 30 seconds at 94°C, 30 seconds at 63°C and 1 minute at 72°C) with a common forward primer near the start of the homeodomain (5′GGTGACCCGG-AGCAGAAGC3′) mixed with two reverse primers, one α-specific (Fig. 1) (5′GCGAATTCTT-ACCAAGCATCGTTGTAGTGGCT3′) giving a 533-bp product, and the second β-specific (Fig. 2) (5′GCGGTGGG-GGAGGGAGAGGGTTCG3′) giving 336-bp product. For positive controls, the PCR templates comprised *AmphiPax2/5/8α* alone, *AmphiPax2/5/8β* alone or mixtures of both at ratios of 1:1, 3:1 or 1:3. The negative control was yeast RNA. PCR products, separated by electrophoresis on a 2% agarose gel, were stained with ethidium bromide.

### Riboprobes, in situ hybridization and histology

Embryos and larvae were fixed according to Holland et al. (1996). Fertilization envelopes were removed with pins to insure penetration of reagents. For antisense riboprobes, restriction fragments of the α cDNA were subcloned into pBluescript (Stratagene, La Jolla, CA). These were a 459-bp *Alu*I fragment common to both the α and β cDNAs (nucleotides 476-934) and a 1070-bp stretch of the cDNA encoding the α isoform between a *Sma*I site at nucleotide 628 and a 3′ *Eco*RI site in the polycloning site of the vector (both gave the same in situ hybridization results). An antisense riboprobe made against the distinctive 3′ end of the cDNA encoding the β isoform failed to demonstrate hybridization. Riboprobe synthesis, and in situ hybridization and histology were according to Holland et al. (1996).

## RESULTS

### Sequence analysis

Two splice variants (designated *AmphiPax2/5/8α* and *AmphiPax2/5/8β*) were isolated (Figs 1,2). The nucleotide and deduced amino acid sequences of the α and β variants upstream from nucleotide 1012 differ by only four polymorphic base substitutions, none affecting the sequences of the N-terminal 317 amino acids, which are identical in the two protein isoforms. Starting with nucleotide 1012, the 3′ third of the coding region and the 3′-UTR diverge between the two variants, apparently due to alternative splicing.

In the deduced proteins, the 22 amino acids immediately upstream from the paired domain include five methionines. Starting with the N-terminal ATG (arbitrarily considered to be the start codon), the agreement with the canonical initiation sequence is: 7/10, 6/10, 8/10, 6/10, 7/10 (Kozak, 1987). The N-terminal 317 amino acids of both isoforms include a paired domain, a conserved octapeptide and a 30-amino acid partial homeodomain (Fig. 1).

The amino acid sequences of the C-terminal third of the two protein isoforms are very divergent. In the canonical (α) isoform, amino acids 318-444 include an 84-amino acid transactivation domain plus a 32-amino acid inhibitory domain (Fig. 1), as defined by Dörfler and Busslinger (1996). In the non-canonical (β) isoform (Fig. 2), amino acids 318-438 comprise a PST-rich C-terminal domain with no obvious similarity to any other sequences in the databases.

### Number of amphioxus genes in the *Pax2/5/8* subfamily and phylogenetic analysis

*AmphiPax2/5/8* is probably the only amphioxus gene in the *Pax2/5/8* subfamily. First, the primers used for PCR on the embryonic cDNA should have recognized any genes in the subfamily, yet all of the resulting clones were the same (excepting minor polymorphisms). Second, PCR performed on genomic DNA pooled from 20 individuals – again with primers that should recognize all *Pax2/5/8* genes – yielded a single, 110-bp product, nearly all of which cut with *Rsa*I at base 50. After agarose gel electrophoresis of the *Rsa*I-cleaved product, only a trace of 110-bp material remained; this was excised and cloned, and several clones were sequenced. All were identical to each other and to the conserved paired box region of both the α and β DNAs of *AmphiPax2/5/8*. Thus the α and β forms are evidently derived from a single gene by alternative splicing.

The phylogenetic analysis of full-length proteins encoded by genes of the *Pax2/5/8* subfamily (Fig. 3) indicates with high confidence that the single *AmphiPax2/5/8* gene is located at the base of a divergence into separate *Pax2*, *Pax5* and *Pax8* genes in the gnathostome vertebrates. In a second phylogenetic analysis limited to comparisons of paired boxes of the same proteins (data not shown), the *Paracentrotus* sequence grouped with the vertebrate sequences, which themselves were poorly resolved; importantly, however, the relative positions of the ascidian HrPax258, *Drosophila* dPax2/5/8, amphioxus AmphiPax2/5/8 and the vertebrate sequences in the aggregate were the same as in Fig. 3.

### Embryonic coexpression of mRNA variants encoding the α and β isoforms of AmphiPax2/5/8

Fig. 4 shows reverse transcription-PCR analysis of the relative amounts of the α and β variants of *AmphiPax2/5/8* mRNA in amphioxus embryos at 7 hours (gastrula), 9 hours (gastrula/neurula transition) 12 hours (early neurula) and 14 hours (mid neurula). Neither variant was detected prior to the gastrula/neurula transition. In the early neurula, both variants began to be coexpressed, with the β variant predominating. By the mid neurula stage, the β variant still predominated over the α variant, but not as conspicuously as before.

### Developmental expression of *AmphiPax2/5/8*

In situ hybridization first detects the expression of

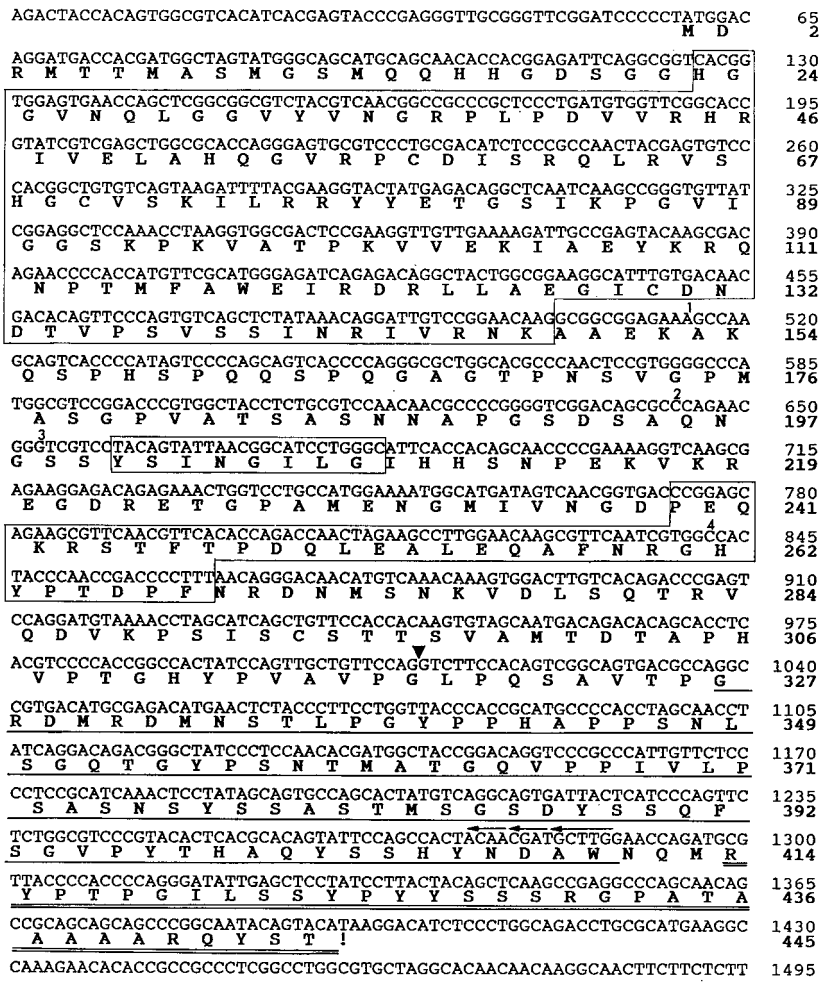


**Fig. 1.** Nucleotide and deduced amino acids of α splice variant of *AmphiPax2/5/8* (accession number AF053762). Boxed regions, from top to bottom, indicate paired domain, octapeptide and partial homeodomain. The transactivation and inhibitory domains are indicated, respectively, by single and double underlining. Superscript numbers locate the following differences in β relative to α splice variant: 1 = G; 2 = G; 3 = A; 4 = G. Arrowhead indicates start of 3′ divergence between α and β splice variants. Horizontal arrows indicate specific reverse primer used for reverse transcription-PCR.

*AmphiPax2/5/8* in the early neurula (Fig. 5A,B) in a small cluster of neural plate cells on either side of the midline at the level of the fifth muscular somite, corresponding approximately to the anteroposterior midpoint of the hindbrain (as mapped by Holland et al., 1992). By the mid-neurula stage, neural plate expression spreads anteriorly and posteriorly, except in the midline (the future floor plate of the neural tube) (Fig. 5C,D).

By the late neurula stage, the lateral margins of the neural plate have curled up and fused mid-dorsally to form the neural tube, the anterior end of which is slightly dilated and is called the cerebral vesicle (cv in Fig. 5E). Neural expression extends through the anterior half of the nerve cord, excluding the cerebral vesicle; expressing cells can be located anywhere except in the midventral floor plate cells (Fig. 5H). Elsewhere in the late neurula, expression is detectable in the nephridium (Fig. 5E,G), which is a thickening of the mesothelial wall of the first muscular somite on the left side. In addition, expression is detectable in the pharyngeal endoderm on the left side and ventrally, where the mouth and first gill slit, respectively, will form.

In embryos with an incipient mouth and early gill slits, (Fig. 5I-O), neural expression resembles that of the preceding stage except for the transitory appearance of transcripts anterodorsally in the cerebral vesicle in cells destined to differentiate into pigment cells associated with the frontal eye (Fig. 5K). In the pharyngeal endoderm, expression is detectable along the left side, where the mouth later opens, on the right side, in parts of the endostyle, which is a probable vertebrate thyroid homologue (Fig. 5M,N), and ventrally where the first and second gill slits will penetrate (Fig. 5O). Some ectodermal cells in the region of the forming mouth and gill slits likewise express *AmphiPax2/5/8*. There is also transient expression in Hatschek's left diverticulum (hd in Fig. 5J,L), which may be a non-muscular coelom and should not be confused with Hatschek's nephridium (see below). Expression in the nephridium is no longer detectable at this stage.

In the 36-hour larva (Fig. 5P,Q), the mouth and first gill slit have opened. Transcripts of *AmphiPax2/5/8* are less conspicuous in the nerve cord and no further expression is detectable in Hatschek's left diverticulum. Pharyngeal expression remains conspicuous on the right side (posteriorly in the endostyle) and ventrally near the first (open) and second (incipient) gill slit. In the 5-day larva, the only detectable transcripts are in the posterior part of the endostyle (Fig. 5R) and, in older larvae, no expression can be detected in any tissues by in situ hybridization.

## DISCUSSION

### Molecular evolution of the *Pax2/5/8* gene subfamily

*AmphiPax2/5/8* is apparently the sole representative of the *Pax2/5/8* subfamily in *Branchiostoma floridae*, because the PCR primers used on both embryonic cDNA and adult genomic DNA were designed to recognize all the genes of the subfamily, yet amplified only one gene. Our phylogenetic analyses support the conclusion that a single precursor gene in the common ancestor of amphioxus and the vertebrates gave rise in one evolutionary line to *Amphipax2/5/8* and in a second evolutionary line to *Pax2*, *Pax5* and *Pax8* genes in gnathostome vertebrates (the presence of *Pax2.1* and *Pax2.2* in zebrafish indicates additional gene duplication within the teleost lineage, as discussed by Pfeffer et al., 1998). It will be interesting to see whether agnathan vertebrates (hagfishes and lampreys) have one, two or three genes in this *Pax* subfamily. The discovery of two such genes in agnathans would lend additional support to the idea of Holland et al. (1994) that there was a first phase of gene duplication associated with the advent of the earliest vertebrates and a second phase of duplication at the origin of the gnathostomes.

### Embryonic coexpression of mRNA variants of *Pax* genes

The N-terminal two-thirds of the α and β isoforms of AmphiPax2/5/8 are colinear, while their C-terminal thirds diverge, evidently because of alternative splicing. Reverse transcription-PCR analysis, although semiquantitative, indicates a temporal regulation of alternative splicing during embryogenesis: both variants are first detected in early neurulae,

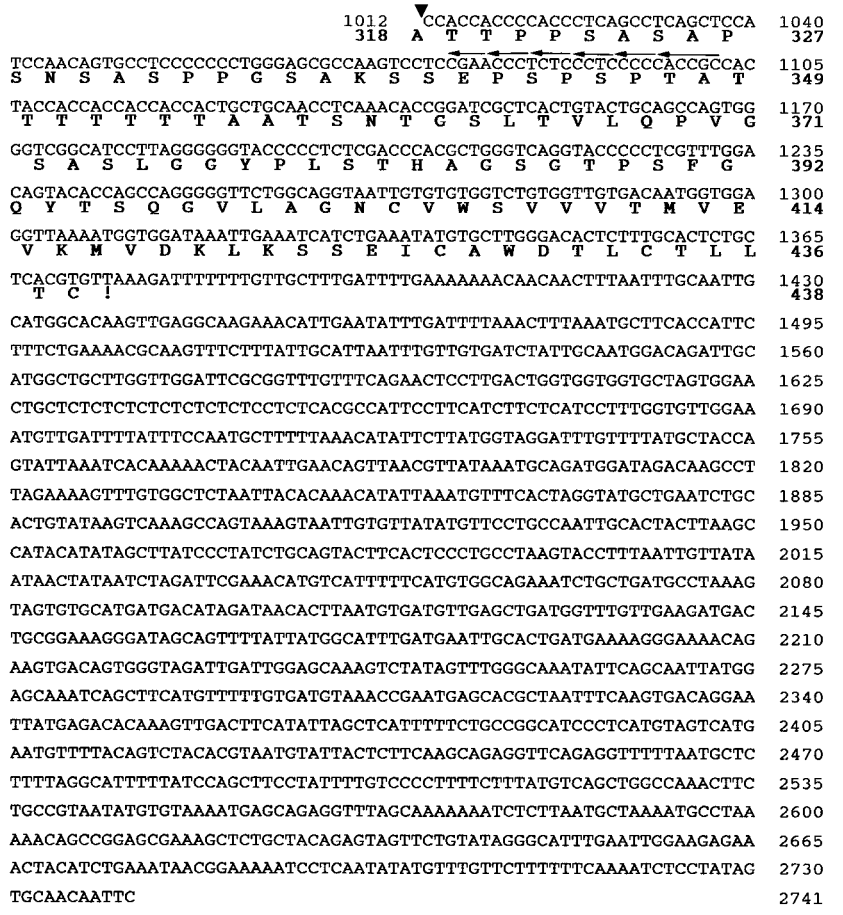



**Fig. 2.** Nucleotide and deduced amino acids of β splice variant of *AmphiPax2/5/8* (accession number AF053763). Only divergent region 3′ of alternative splice site (arrowhead) is shown. Horizontal arrows indicate specific reverse primer used for reverse transcription-PCR.

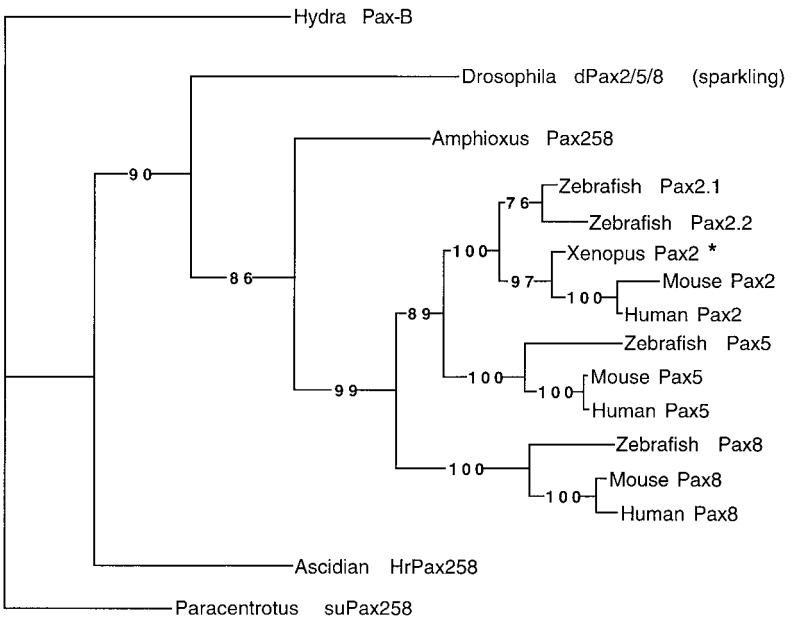



**Fig. 3.** Phylogenetic tree of proteins in Pax2/5/8 subfamily constructed from random stepwise addition of complete sequences. The numbers at the branches are bootstrap percentages (only those over 50% are shown), and hydra Pax-B was used as the outgroup. *The *Xenopus* genome also includes a *Pax5* and a *Pax8* gene (Heller and Brändli, 1999).

with the β variant predominating; in later neurulae, the β variant still predominates, but less markedly. Changes in proportions of splice variants during development have previously been reported for mouse *Pax8* and *Xenopus Pax2* (Kozmik et al., 1993; Heller and Brändli, 1997) as well as for mouse *Pax5* during cytodifferentiation of B-cells (Zwollo et al., 1997).

Heller and Brändli (1997) demonstrated that all embryonic tissues transcribing *Xenopus Pax2* expressed the full spectrum of splice variants. For human and mouse *Pax8*, however, spatial regulation of alternatively spliced products has been found by Poleev et al. (1992, 1995) and Kozmik et al. (1993). We cannot add anything definite about possible spatial regulation of alternatively spliced products of *AmphiPax2/5/8*, because our antisense riboprobe against the distinctive 3′ end of the cDNA exclusively encoding the β isoform failed to hybridize.

### *Pax2/5/8* and thyroid homologies between amphioxus and vertebrates

*Pax8* is expressed in the developing thyroid of most vertebrates (Plachov et al., 1990; Poleev et al., 1992; Mansouri et al., 1998), although *Pax-2* is expressed there instead in *Xenopus* (Heller and Brändli, 1999). These *Pax* genes are expressed in the thyroid rudiment during its differentiation from the floor of the pharynx. The thyroid of adult vertebrates also expresses *Pax8*, evidently to maintain the differentiated state by modulating cell proliferation and the expression of two thyrocyte-specific genes (thyroglobulin and thyroperoxidase) (Zannini, 1992; Fabbro et al., 1994; Rossi et al., 1995; van der Kallen et al., 1996; Macchia et al., 1998; Mansouri et al., 1998).

In amphioxus embryos, *Amphipax2/5/8* is expressed in the thickened endoderm in the right anterior region of the pharynx. At the end of the larval stage of amphioxus, this thickening, called the endostyle, migrates ventrad and then posteriad, finally taking the form of a groove along the pharyngeal floor of the juveniles and adults. Müller (1873) originally proposed that the amphioxus endostyle was the homologue of the vertebrate thyroid gland, because larval lampreys have an endostyle-like structure that becomes converted into a thyroid gland later in development. The amphioxus endostyle and the vertebrate thyroid gland have since been found to synthesize iodothyronines, thyroglobulins and thyroperoxidases (Ericson and Fredriksson, 1990). Even so, Burrow (1989) has questioned the homology between the vertebrate thyroid and the amphioxus endostyle, since the latter has no known endocrine function (it makes food-trapping extracellular secretions). Most recently, the expression of *AmphiNk2-1* (=amphioxus *TTF-1*)(Venkatesh et al., 1999) and *AmphiPax2/5/8* (present study) in the endostyle lends additional support to its proposed homology to the vertebrate thyroid gland.

### *Pax2/5/8* and renal homologies between amphioxus and vertebrates

*Pax2* and *Pax8* are transcribed in the developing vertebrate kidney (Dressler et al., 1990; Krauss et al., 1991; Püschel et al., 1992; Heller and Brändli, 1997, 1999; Pfeffer et al., 1998). In amniotes, there is a successive, anterior-to-posterior formation of a pronephros, mesonephros and metanephros, with only the last functioning as the adult kidney (Vize et al., 1997). In fishes and amphibians, the metanephros does not form and the mesonephros becomes the adult kidney; in agnathans, the pronephros is the only kidney to develop. The vertebrate pronephros typically develops bilaterally in a few anterior metameres from a

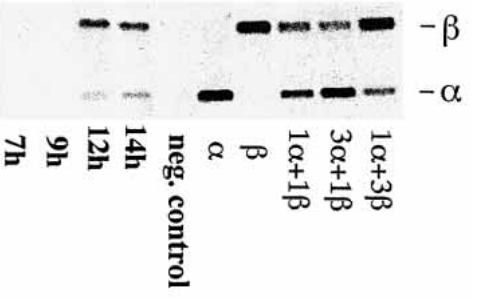
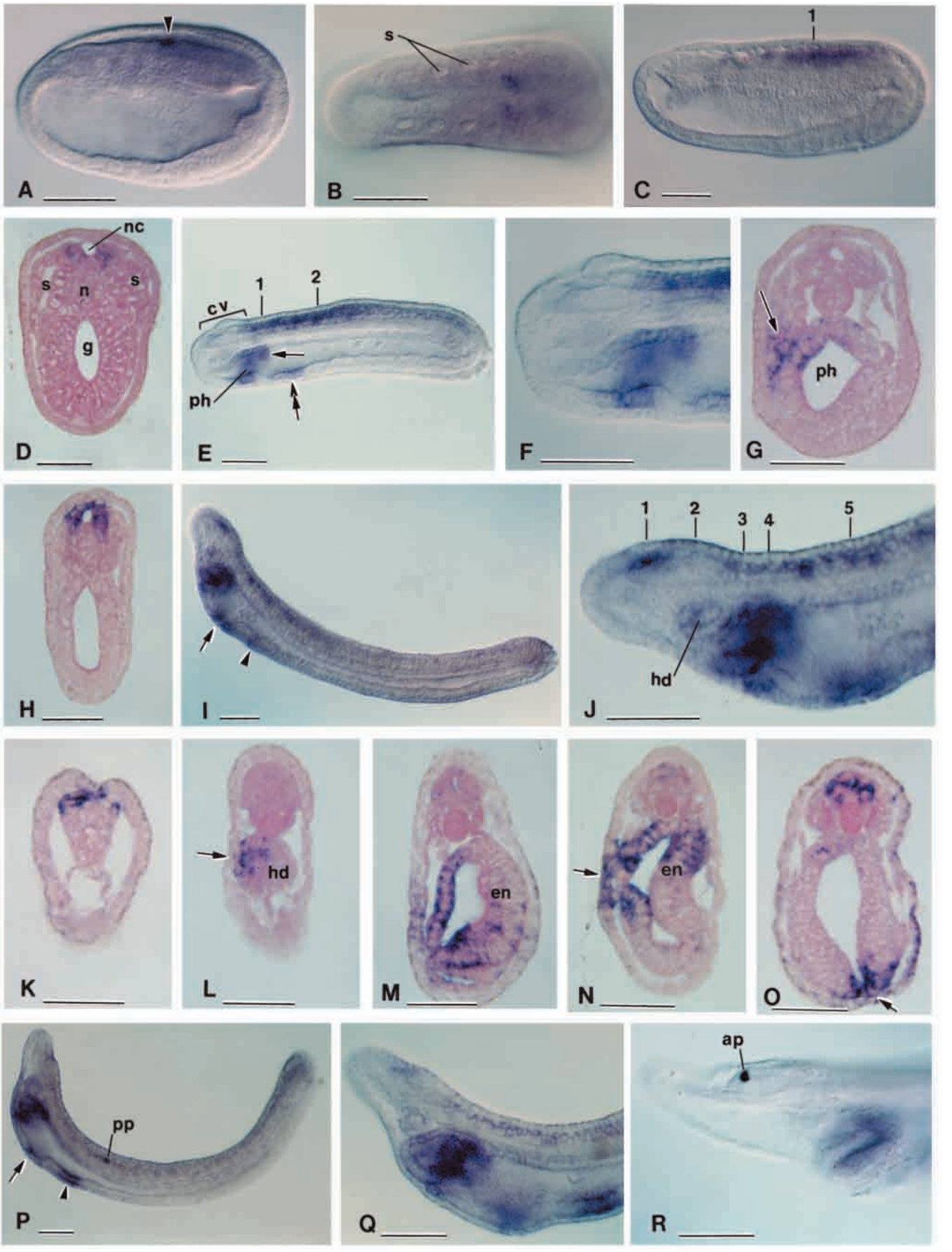



**Fig. 4.** Reverse transcription-PCR analysis showing predominance of β over α splice variant of *AmphiPax2/5/8* during embryonic development.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thickening of the somatic mesothelium of the intermediate mesoderm. On either side of the body, these mesothelial cells become rearranged into a compact bud that acquires a small lumen, the nephrocoel, and produces the pronephric tubules and their common nephric duct. The pronephric tubules and duct are subsequently completed without any contribution from the surrounding mesenchyme cells. In contrast, the mesonephros and metanephros have a dual origin: from side branches of the nephric duct as well as from nearby mesenchyme cells that form kidney tubules by a mesenchyme-to-epithelium transition. In amphioxus, the excretory system develops as a segmentally arranged series of tubules without any contribution from condensing mesenchyme. These tubules are widely thought to be homologous to the mesodermal pronephric tubules of vertebrates (Hatschek, 1884; Legros, 1910; Stach and Eisler, 1998).

The present study shows that *AmphiPax2/5/8* is expressed in the rudiment of the earliest excretory tubule (often called Hatschek's nephridium) to appear during the embryonic development, thus strengthening the homology between amphioxus excretory organs and the vertebrate pronephros, which develops under the influence of homologous *Pax* genes. This is additional evidence against the alternative idea of Goodrich (1945) that the excretory tubules of amphioxus are ectodermally derived and thus not homologous to the vertebrate kidney tubules.

**Fig. 5.** *AmphiPax2/5/8* expression during normal development. Anterior of whole mounts toward left; cross sections (counterstained pink) viewed from the posterior end of animal); whole-mount scale lines = 50 μm and section scale lines = 25 μm. (A) Early neurula side view with expression (arrowhead) in neural plate. (B) Dorsal view of preceding specimen showing muscular somites (s) and *AmphiPax2/5/8* expression in a small group of cells on either side of the neural plate. (C) Mid-neurula side view with neural plate expression. (D) Cross section through 1 in 5C, showing gut (g), somites (s), notochord (n) and neural plate beginning to curl to enclose neural canal (nc); expression in neural plate except in floor plate cells. (E) Late neurula side view with neural tube dilated anteriorly as cerebral vesicle (opposite bracket cv); neural expression in anterior half of neural tube except for cerebral vesicle; additional expression in nephridium rudiment (single arrow), on left side of pharynx and ventrally where first gill slit will form (tandem arrow). (F) Enlarged anterior end of preceding specimen. (G) Cross section through 1 in 5E showing expression in rudiment of nephridium (arrow) and in left wall of pharynx (ph) (H) Cross section through 2 in 5E showing expression in neural tube except floorplate cells. (I) Side view of embryo with incipient mouth opening and rudiments of first (arrow) and second (arrowhead) gill slits. (J) Enlarged anterior end of preceding embryo; expression in neural tube, gill slit rudiments, pharynx, Hatschek's left diverticulum (hd) and dorsoanteriorly in the cerebral vesicle. (K) Cross section through 1 in 5J showing expression in dorsoanterior cells of cerebral vesicle. (L) Cross section through 2 in 5J showing expression in Hatschek's left diverticulum which is about to fuse with ectoderm (at arrow) to form preoral ciliated pit. (M) Cross section through 3 in 5J showing expression in left wall of pharynx and in thickened right wall, which is developing into endostyle (en). (N) Cross section through 4 in 5J showing expression in part of the endostyle (en) and in regions of epidermis and left pharyngeal wall near where they are fusing (arrow) to form mouth. (O) Cross section through 5 in 5J showing expression in cells of first gill slit rudiment (arrow) and conspicuous neural tube expression except in floorplate. (P) Side view of 36-hour larva with primary pigment spot (pp); expression in pharynx, first gill slit (arrow) and rudiment of second gill slit (arrowhead); expression in neural tube becoming inconspicuous. (Q) Enlargement of anterior end of preceding specimen. (R) Side view of anterior end of 5-day larva with anterior pigment spot (ap); expression detectable only in endostyle.

### *Pax2/5/8* genes, gill slit formation and the question of otic placode homologies in chordates

In *Xenopus* embryos, *Pax2* is expressed in the ectodermal furrows of the visceral arches, which suggests that the gene might play a role in perforation of the gill slits (Heller and Brändli, 1997). For amphioxus, formation of the gill slits and mouth (which is usually considered to be a modified gill slit) may well be under similar genetic control, because the pharyngeal endoderm and the ectoderm express *AmphiPax2/5/8* in regions where the two epithelial layers will later make contact and fuse. It is possible that *AmphiPax2/5/8* may be interacting with downstream genes that control mechanochemical properties of the extracellular matrix to facilitate fusion of the adjacent endodermal and ectodermal epithelia. Edelman and Jones (1995) have previously suggested that *Pax* genes may interact with genes encoding extracellular materials and thereby influence tissue-level morphogenesis; e.g. mouse Pax8 binds to and activates the *N-CAM* gene. One kind of this morphoregulation would be the local dissolution of basal laminae to permit fusion of two adjacent epithelia that have made intimate contact. Further examples may be the regulation of the closure of the neural tube or optic fissure by mouse *Pax2* (Favor et al., 1996; Torres et al., 1996) and regulation of processes connecting the internal and external epithelia by *egl-38*, a somewhat aberrant member of the *Pax2/5/8* subfamily in *Caenorhabditis* (Chamberlin et al., 1997).

Vertebrate *Pax2* and *Pax8* are expressed in the otic placode and in the otic vesicle formed by its invagination (Püschel et al., 1992; Heller and Brändli, 1997; Herbrand et al., 1998; Pfeffer et al., 1998), and *Pax2* plays a key role in the development of the cochlea and spiral ganglion of the inner ear (Favor et al., 1996; Torres et al., 1996). In zebrafish, but evidently not in higher vertebrates, *Pax5* is expressed in addition to *Pax8* during the differentiation of the otic vesicle (Pfeffer et al., 1998). There are no previous reports that amphioxus embryos or larvae have otic placodes or otic vesicles and we found no patterns of *AmphiPax2/5/8* expression suggesting that such structures were overlooked in earlier studies.

In contrast, in the larvae of another group of invertebrate chordates (namely the ascidian tunicates), *HrPax258* is expressed in two patches of epidermis that are destined to invaginate to form the atria. This pattern of gene expression, together with the presence of cupula-like structures in ascidian atria (Bone and Ryan, 1978) prompted Wada et al. (1998) to suggest that the epidermal regions giving rise to the atria might be homologues of vertebrate otic placodes and that the atria themselves might be homologues of vertebrate otic vesicles. If these homologies are valid, it implies that otic placodes/vesicles are a fundamental chordate character that must have been lost in the evolutionary line leading to amphioxus. Alternatively, it is possible that ascidian atria represent not otic vesicles, but the outer part of gill slits, as suggested by Goldschmidt (1903); in this case, the epidermal expression of *HrPax258* would represent not otic placodes, but regions of epidermis destined to invaginate and then form the outer part of the gill slits by fusion with the endoderm. More light might be shed on the atrial homologies of tunicates by a study of *Pax2/5/8* expression

during gill slit development in appendicularians, which represent the most basal group of tunicates (Wada and Satoh, 1994) and have only one pair of simple gill slits.

Amphioxus, like ascidians, develops a peribranchial space called the atrium. Unlike ascidian atria, the one in amphioxus develops relatively late in development (in 1-month-old larvae undergoing metamorphosis) as a single, not a paired, structure. Moreover, the amphioxus atrium forms not by epidermal invagination, as in ascidians, but by the outgrowth of bilateral flanges of body wall that meet midventrally except in a region that becomes the atriopore (Stokes and Holland, 1996). In spite of these differences, some (like Minot, 1897) have claimed that the atria of ascidians and amphioxus are homologous. In 1-month-old metamorphic amphioxus larvae, we found no *AmphiPax2/5/8* expression in structures associated with atrium formation, although we did detect expression of the amphioxus actin gene *BfMA1* in the muscles (data not shown). In the opinion of Franz (1927), the amphioxus atrium is homologous with the efferent gill chambers of myxinoid hagfishes.

### *Pax2/5/8* genes and the evolution of support cells in photoreceptors

Cells expressing *AmphiPax2/5/8* near the anterior end of the amphioxus cerebral vesicle (Fig. 5K) are the presumed precursors of the frontal eye pigment cells. *AmphiPax2/5/8* expression in these neuronal support cells appears to be comparable to transcription of *Drosophila Pax2/5/8* (*sparkling*) in the developing pigment cells of the compound eyes (Fu and Noll, 1997) and to transcription of vertebrate *Pax2* in glial cells lining the choroid fissure and optic stalk/nerve of the paired eyes (Nornes et al., 1990; Krauss et al., 1991; Püschel et al., 1992; Alvarez-Bolado et al., 1997; Heller and Brändli, 1997; Macdonald et al., 1997; Otteson et al., 1998). Transcription of amphioxus *AmphiPax2/5/8* in developing pigment cells of the frontal eye is consistent with the proposal of Fu and Noll (1997) that an ancestral *Pax2* gene played a role in neuronal support or glial cells during photoreceptor development in the common precursor of the protostomes and deuterostomes. Even so, the absence of ascidian *HrPax258* expression in the sensory vesicle cells near the larval ocellus (Wada et al., 1998) does not fit with the idea of Fu and Noll (1997) and points to the need for genetic studies of photoreceptors in a wider range of animal groups.

### *Pax2/5/8* genes in the rhombospinal domain of chordates

Early neural expression of vertebrate *Pax2* appears not only in the MHB region, but also in the rhombospinal domain, where the gene is transcribed in differentiating interneurons located laterally on either side of the neural tube (Burrill et al., 1997; Pfeffer et al., 1998). In the rhombospinal region of amphioxus, the earliest detectable expression of *AmphiPax2/5/8* begins roughly in the middle of the hindbrain and spreads posteriorly and anteriorly from there. Eventually, the anterior limit of the hindbrain expression domain of *AmphiPax2/5/8* approaches a region of the neural tube where a MHB might be expected to occur if one existed; however, this spatiotemporal pattern is quite different from the early appearance of vertebrate *Pax2*, *Pax5* and *Pax8* in the vertebrate MHB region.

In the rhombospinal region of amphioxus, *AmphiPax2/5/8* can be expressed dorsoventrally anywhere except in the floor plate cells. This expression pattern may reflect a wide-spread distribution of interneurons in the dorsoventral axis of the amphioxus nerve cord; unfortunately, knowledge of the neuroanatomy of the amphioxus rhombospinal domain is still very incomplete (Bone, 1959, 1960) and needs to be reinvestigated (e.g. by the 3D electron microscopic reconstruction methods of Lacalli et al., 1994; Lacalli, 1996).

Wada et al. (1998) studied *HrPax258* transcription in larval ascidians and found that its neural expression was limited to the neck region of the central nervous system. Most of the rhombospinal region of the ascidian larval nervous system is composed solely of ependymal cell bodies and a few descending motor axons (Bone, 1981). Thus the absence of *HrPax258* expression in this posterior region of the ascidian nervous system might be related to the lack of interneurons there.

### Do invertebrate chordates have a vertebrate-like MHB?

The earliest rostrocaudal organization of the vertebrate central nervous system is considered to be fundamentally tripartite on the basis of regulatory gene expression, with an anterior region (forebrain plus anterior midbrain) separated from a posterior rhombospinal domain by an intervening MHB region (Bally-Cuif and Wassef, 1995; Joyner, 1996; Boncinelli et al., 1998). During vertebrate development, *Pax2*, *Pax5* and *Pax8* (excepting *Xenopus Pax-8*, Heller and Brändli, 1999) are transcribed in spatially and temporally overlapping patterns in the MHB region

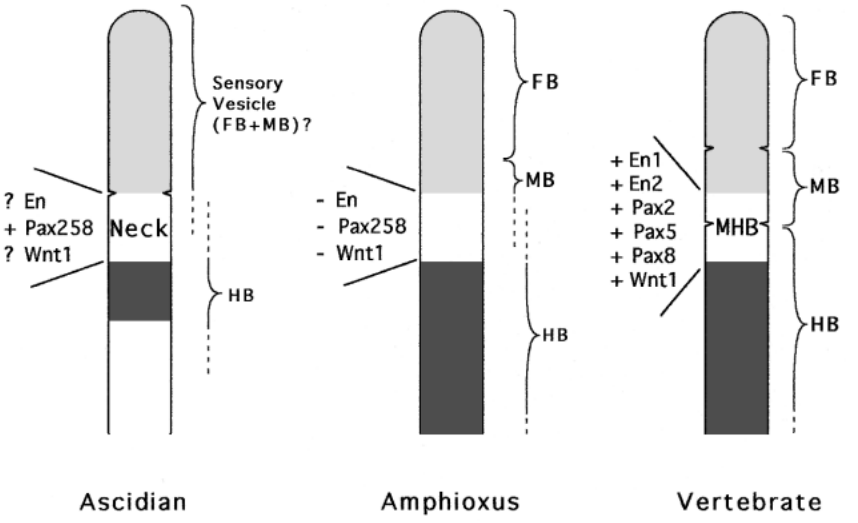


**Fig. 6.** Comparison of brain regions and some gene expression domains among chordates. Forebrain, midbrain and hindbrain, respectively, abbreviated FB, MB and HB. Expression domains of homologues of *Otx* and *Hox* genes shown, respectively, by areas of light and dark shading. The intermediate zones between *Otx* and *Hox* expression are: neck of the neural tube in ascidians, posterior midbrain and/or anterior hindbrain in amphioxus and MHB in vertebrates. For intermediate zones, developmental gene expression indicated by a plus sign if present, by a minus sign if absent or by a question mark if not yet studied.

where their functional importance has been demonstrated by the disruption of the MHB region by mutated *Pax* genes (Brand et al., 1996; Urbánek et al., 1997; Schwarz et al., 1997; Lun and Brand, 1998) and by injection of antibodies that neutralize Pax proteins (Krauss et al., 1992). In addition, chick-quail homotypic and heterotypic transplantation studies (reviewed by Joyner, 1996) have demonstrated that the vertebrate MHB region acts as an organizer, not only maintaining itself independently, but also recruiting surrounding cells to form MHB structures after its transplantation into neighboring brain regions. For establishing and maintaining the vertebrate MHB organizer region, *Pax2/5/8* subfamily genes interact with other key developmental genes including *En1*, *En2* and *Wnt1* (Joyner, 1996; Lun and Brand, 1998).

It is interesting to consider whether invertebrate chordates (tunicates and amphioxus) have a brain region comparable to the MHB of vertebrates. Wada et al. (1998) and Williams and Holland (1998) have proposed that ascidian tunicates have a MHB characterized by its position along the rostrocaudal axis, by gene expression in the MHB itself and by gene expression in neighboring regions of the neural tube; however, organizer properties are not included in the definition. In the developing central nervous system of all chordates, there is a intervening region between an anterior zone of *Otx* expression and a posterior zone of *Hox* expression (Fig. 6. In vertebrates and amphioxus, this intervening zone is a midbrain-hindbrain boundary in a general sense. In a strict sense, however, a definitive MHB should express particular genes early in its development.

In amphioxus embryos, we have found no early neural expression of amphioxus homologues of *engrailed* (Holland et al., 1997), *Wnt1* (unpublished observations) or the *Pax2/5/8* subfamily (present paper) in the region of the neural tube posterior to the *Otx* domain and anterior to the *Hox* domain (Fig. 6). Therefore, developmental gene expression does not suggest that amphioxus has a MHB. By contrast, in ascidian embryos, there is neural expression of *HrPax258* in the neck region of the neural tube, which is posterior to the *Otx* domain and anterior to the *Hox* domain. However, there is currently no information on the early neural expression of ascidian homologues of *engrailed* and *Wnt1* (Fig. 6), and the idea that ascidians have a MHB would be strengthened if additional genes critical for MHB development can be found associated with the *HrPax258* expression domain. For both ascidians and amphioxus, the question of whether a MHB is present could be further addressed by studying the developmental expression of possible *FGF8* genes in these invertebrate chordates. In the developing central nervous system of vertebrates, *FGF8* is expressed in the MHB, where it functions in the genetic cascade including *Pax* and *engrailed* genes (Lee et al., 1997). In sum, additional work on developmental gene expression in the developing nervous systems of ascidians and amphioxus will be needed to distinguish whether the MHB is a vertebrate innovation or a basal chordate character secondarily lost in amphioxus.

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