Amphioxus and ascidian Dmbx homeobox genes give clues to the vertebrate origins of midbrain development

Tokiharu Takahashi[1,2] and Peter W. H. Holland[1,*]

[1]Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK
[2]Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
*Author for correspondence (e-mail: peter.holland@zoo.ox.ac.uk)

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Summary

The ancestral chordate neural tube had a tripartite structure, comprising anterior, midbrain-hindbrain boundary (MHB) and posterior regions. The most anterior region encompasses both forebrain and midbrain in vertebrates. It is not clear when or how the distinction between these two functionally and developmentally distinct regions arose in evolution. Recently, we reported a mouse PRD-class homeobox gene, *Dmbx1*, expressed in the presumptive midbrain at early developmental stages, and the hindbrain at later stages, with exclusion from the MHB. This gene provides a route to investigate the evolution of midbrain development. We report the cloning, genomic structure, phylogeny and embryonic expression of Dmbx genes from amphioxus and from *Ciona*, representing the two most closely related lineages to the vertebrates. Our analyses show that Dmbx genes form a distinct, ancient, homeobox gene family, with highly conserved sequence and genomic organisation, albeit more divergent in *Ciona*. In amphioxus, no Dmbx expression is observed in the neural tube, supporting previous arguments that the MHB equivalent region has been secondarily modified in evolution. In *Ciona*, the *CiDmbx* gene is detected in neural cells caudal to Pax2/5/8-positive cells (MHB homologue), in the Hox-positive region, but, interestingly, not in any cells rostral to them. These results suggest that a midbrain homologue is missing in *Ciona*, and argue that midbrain development is a novelty that evolved specifically on the vertebrate lineage. We discuss the evolution of midbrain development in relation to the ancestry of the tripartite neural ground plan and the origin of the MHB organiser.

Key words: *Dmbx1*, *AmphiDmbx*, *CiDmbx*, *Ciona*, Chordate, MHB, Isthmus

Introduction

The complex brain of vertebrates is not only a product of ontogenetic processes, but also of evolutionary history. Unravelling this history can reveal which developmental processes are the most ancient, constituting the fundamental basis of vertebrate brain formation, and which processes have been superimposed later. Two groups of animals are proving particularly useful for these analyses: the cephalochordates (notably amphioxus), which constitute the most closely related invertebrates to the vertebrates; and the urochordates (including ascidians such as *Ciona*), which form the second closest branch. Both these groups of animals are chordates, sharing with vertebrates a hollow nerve cord, dorsal to a notochord.

Comparison of gene expression patterns and developmental anatomy between vertebrates, amphioxus and ascidians has yielded some surprises concerning the evolutionary history of brain pattering. For example, both ascidians and vertebrates show a basic tripartite ground plan along the anteroposterior axis of the dorsal nerve cord, comprising an anterior region expressing Otx family homeobox genes (marking the forebrain and midbrain of vertebrates), a central region expressing Pax2/5/8 genes (marking the midbrain-hindbrain boundary of vertebrates) and a Hox-expressing region (hindbrain and spinal cord of vertebrates). These zones do not, of course, correspond to the classically defined anatomical divisions of fore-, mid- and hindbrain of vertebrates, but instead reveal the probable ancestral ground plan upon which chordate brain development is based (Wada et al., 1998). Very recently, a similar tripartite pattern of gene expression has been reported for hemichordates (Lowe et al., 2003) and arthropods (Hirth et al., 2003), suggesting that this ground plan may be even older even than chordate origins.

There are several unresolved issues concerning the evolution of the tripartite neural tube, particularly concerning the central zone marked by Pax2/5/8 gene expression. In vertebrates, this zone is the site of the midbrain-hindbrain boundary organiser (MHB organiser or isthmic organiser, IsO). The MHB organiser was initially identified through transplantation experiments in chick embryos (Gardner and Barald, 1991; Martinez and Alvarado-Mallart, 1990; Martinez et al., 1991). These experiments suggest that the vertebrate MHB acts an organising centre, with inductive influences both anterior and posterior of its location. In urochordates, a homologous region was first noted in *Halocynthia* as a stripe of Pax2/5/8 expression, similar to Pax gene expression in the vertebrate MHB (Wada et al., 1998). An FGF8/17/18 gene is expressed immediately caudal to this stripe in *Ciona*, suggesting that

ascidian embryos probably do have organiser activity in this region within the visceral ganglion (Imai et al., 2002); however, this organiser activity has not been tested functionally.

One important issue of uncertainty surrounding the MHB region concerns amphioxus. Unlike vertebrates and ascidians, the cephalochordate amphioxus does not show Pax2/5/8 expression posterior to Otx gene expression and anterior to the Hox-expressing region (Kozmik et al., 1999). Furthermore, *En* and *Wnt1* genes are also not expressed in this region in amphioxus, despite their expression and function at the MHB in vertebrates (Holland et al., 1997; Holland et al., 2000). Amphioxus, therefore, lacks the MHB. Taking into account the comparative data from ascidians and hemichordates, it is most parsimonious to conclude that tripartite regionalisation is an ancient character of the vertebrate neural tube, and that secondary modification has occurred on the cephalochordate lineage (Wada et al., 1998; Williams and Holland, 1998). Such modifications might have occurred in concert with the unusual rostral extension of the notochord in amphioxus, extending beyond the tip of the cerebral vesicle.

A related issue of importance concerns the origin of the midbrain. In vertebrates, the Otx-expressing domain encompasses both forebrain and midbrain; consequently, comparative data on Otx gene expression cannot reveal when the distinction between these two structures arose. Recently, we and others reported cloning and expression of a novel PRD-class homeobox gene in the mouse, *Dmbx1* (diencephalon/mesencephalon-expressed brain homeobox gene 1) (Broccoli et al., 2002; Gogoi et al., 2002; Miyamoto et al., 2002; Ohtoshi et al., 2002; Takahashi et al., 2002; Zhang et al., 2002). Orthologues of this gene have also been reported from zebrafish (Kawahara et al., 2002), chicken (Gogoi et al., 2002) and human (Zhang et al., 2002), although not from any invertebrate. The human and mouse genes are also referred to as *Otx3*, which is erroneous as the gene is not part of the Otx gene family. In all studies, a predominant site of *Dmbx1* expression is the prospective midbrain at early embryonic stages. Indeed, *Dmbx1* gene inactivation using morpholino antisense oligonucleotide results in substantial reduction in the size of tectum and eyes in zebrafish (Kawahara et al., 2002). These results suggest that *Dmbx1* is a useful marker for the presumptive midbrain before the MHB organiser is established.

To clarify how midbrain patterning emerged during chordate evolution, we have cloned orthologues of *Dmbx1* gene from amphioxus and ascidians. We report their full sequences, phylogeny, exon-intron organisation and spatiotemporal expression patterns. There are both similarities and differences in expression pattern between ascidians and vertebrates, but the expression in amphioxus is strikingly different. These data further strengthen the case for homology between ascidian and vertebrate tripartite organisations, with secondary modification in amphioxus. Furthermore, the data suggest that midbrain development is a novel character that evolved specifically in the vertebrate lineage, superimposed onto the ancestral tripartite organisation.

Materials and methods

Animals

Adult amphioxus, *Branchiostoma floridae*, were collected in Old Tampa Bay (FL, USA) in August 2001. In vitro fertilisation, embryo culture and fixation for whole-mount in situ hybridisation were performed as previously described (Holland, 1999; Holland and Wada, 1999). Adult specimens of *Ciona intestinalis* were collected in summer 2002 from Sparke's Marina, Hayling Island, Hampshire, UK. In vitro fertilisation, embryo fixation and storage were performed as described by Boorman and Shimeld (Boorman and Shimeld, 2002).

Isolation of the amphioxus Dmbx gene

For cloning the amphioxus homologue of the *Dmbx1* gene, nested degenerate oligonucleotide primers were designed to amplify a fragment of 129 bp conserved between vertebrate Dmbx1 genes. Initial primer sequences were: F-out, 5′-CAACGTC-GGAG(CT)(AC)GNACNGC-3 with SO2T 5′-C(GT)NC(GT)-(AG)TT(CT)TT(AG)AACCA-3′; the nested reaction used F-mid, 5′-ATGCA(AG)CTNGANGCN(CT)TNGA-3′ with SO2T. A cDNA library from *B. floridae* embryos (reverse-transcribed with both random and oligo dT primers) (Langeland et al., 1998) was used as a PCR template. After cloning of the amplified band, five recombinant clones were sequenced, of which three showed high sequence similarity to vertebrate Dmbx1 genes. Screening of the *B. floridae* cDNA library with this PCR-derived clone yielded one cDNA clone including a 2.66 kb *Eco*RI fragment. This fragment was fully sequenced on both strands.

Isolation of the ascidian Dmbx gene

We identified a presumptive orthologue of *Dmbx1* through tblastn searches of the *Ciona intestinalis* genome assembly v. 1.0 (Dehal et al., 2002), accessed at the Joint Genome Institute server (http://genome.jgi-psf.org/ciona4/ciona4.home.html). It was not possible, however, to predict and assemble the complete gene sequence from the surrounding genomic sequence information alone, without cDNA information. To obtain this, we first designed four specific PCR primers (For1, For2, Rev3 and Rev4) to amplify a fragment of 319 bp from the 5′ part of the coding region of the predicted gene. Nested PCR was performed using primers For1 and Rev4, followed by For2 and Rev3, using as a template *C. intestinalis* cDNA reverse-transcribed from mRNA extracted from 5 hour embryos. After cloning of the amplified band, four recombinant clones were sequenced, all of which showed high sequence similarity to vertebrate Dmbx1 genes. To clone the 3′ half of the gene, sequence-specific primers For2 and For3 were used in conjunction with vector primers M13 Forward and T7 to amplify from a *C. intestinalis* cDNA library constructed in pBluescript (kindly provided by Dr Patrick Lemaire, Marseille, France). After PCR amplification and cloning of the amplified band, two recombinant clones were sequenced, both with high sequence similarity to vertebrate Dmbx1 genes. After combining the sequence information from these two experiments, a new sequence specific primer NewRev1 was designed near the 3′ end of the gene, and used in conjunction with For1 to amplify a band covering most of the coding sequence, from a mixture of 6 hour embryo cDNA and the cDNA library. This was cloned and two re-combinants completely sequenced to verify that the 5′ and 3′ clones are naturally contiguous in mRNA. The sequence was compatible with all sequence information obtained from the preceding experiments. Primer sequences were: For1, 5′-ATGAATTATTAT-GACGCAAT-3′; For2, 5′-TCGTGCAATGTCAGTGTTCA-3′; For3, 5′-CTCTGGCAGATTTAATACTC-3′; Rev3, 5′-CAACATTCTCT-GTTGTTTTC-3′; Rev4, 5′-CTGTTGTTTTCGATATTTTG-3′; NewRev1, 5′-AATTGAAGATTCCAAGGTTG-3′.

Sequence comparisons and molecular phylogenetic analyses

Deduced amino acid sequences were aligned using ClustalX (Thompson et al., 1997) and edited using GeneDoc (Nicholas et al., 1997) to remove regions of ambiguous alignment. Phylogenetic analyses of amino acid sequences were performed using the neighbour-joining method implemented in ClustalX, with outputs

displayed using TreeView (Page, 1996). For the analysis of PRD-class homeobox genes, we restricted the analysis to the homeodomain to maximise representation of PRD class genes. Confidence in the phylogeny was assessed by bootstrap re-sampling of the data.

Genomic organisation of Dmbx genes

A genomic clone of the amphioxus Dmbx gene was obtained from amphioxus cosmid library MPMGc117 (distributed by the Resource Centre and Primary Database, www.rzpd.de) by screening using the cDNA clone under high stringency conditions. Cosmid clone MPMGc117 G1048 was found to contain the amphioxus Dmbx gene, as confirmed by direct sequencing. Exon-intron organisation was determined by direct sequencing of the cosmid clone, using primers based on cDNA sequence. Exon-intron organisation of the ascidian Dmbx gene was determined by comparison of the cDNA sequence with the *Ciona intestinalis* genome assembly v. 1.0 (Dehal et al., 2000).

Whole-mount in situ hybridisation and sectioning

In situ hybridisation of whole-mount specimens were carried out as described by Holland (Holland, 1999) for amphioxus, and by Mazet et al. (Mazet et al., 2003) for ascidians. Probes for amphioxus and ascidian Dmbx were synthesised by in vitro transcription using a DIG RNA Labeling Mix (Roche), following the supplier's instructions. The amphioxus Dmbx template was a 1.1 kb cDNA subclone in pBluescript, containing the full open reading frame, linearised with *Not*I. The ascidian Dmbx template was a 681 bp cDNA subclone in pGEM-T Easy, covering most of the open reading frame, linearised with *Sal*I. To enable double staining with other genes, we identified cDNA clones of *Ci-Pax2/5/8-A, Ci-Fgf8/17/18* and *CiHox3* from a *Ciona* EST collection assembled at Kyoto University by Professor N. Satoh and collaborators (http://ghost.zool.kyoto-u.ac.jp/indexr1.html). Identity of the clones (Gene Collection ID: R1CiGC01B13 for *Ci-Pax2/5/8-A*, R1CiGC28f14 for *Ci-Fgf8/17/18* and R1CiGC02c13 for *CiHox3*) was verified by sequencing and comparison to published sequence (Satou et al., 2002). A *Ci-Pax2/5/8-A* riboprobe was synthesised with a fluorescein RNA Labeling Mix (Roche), and double staining performed (Mazet et al., 2003) using this probe in conjunction with DIG-labelled *Ciona* Dmbx. *Ci-Fgf8/17/18* and a *CiHox3* riboprobes were labelled with DIG and double staining performed using fluorescein-labelled *Ciona* Dmbx. Briefly, specimens were simultaneously hybridised with the DIG and fluorescein-labelled probes, before washing and sequential detection of the two labels. First, alkaline phosphatase-conjugated anti-fluorescein antibody was used, followed by staining with Fast-Red (Roche). Specimens with definite signal were treated with 0.1 M glycine-HCl (pH2.2), 0.1% Tween 20 for 10 minutes at room temperature to inactivate the first alkaline phosphatase, washed, incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody and stained blue with NBT-BCIP (Roche). After being photographed as whole mounts, amphioxus embryos were counterstained pink in 1% Ponceau S in 1% aqueous acetic acid, dehydrated in ethanol, embedded in LR White resin (TAAB) and prepared as 7.0 μm sections.

Results

Cloning of amphioxus and ascidian Dmbx genes

We isolated and sequenced a 2.66 kb cDNA derived from an amphioxus gene related to vertebrate *Dmbx1*, and eight PCR-generated 1.06 kb cDNAs of a homologous gene from an ascidian. The amino acid sequence of the longest open reading frame (ORF) was deduced for each (371 and 240 amino acids respectively), and aligned to protein sequences predicted for vertebrate *Dmbx1* genes (Fig. 1). The high degree of conservation of the homeodomain argues that these genes are members of the same homeobox gene family; we designate this the Dmbx gene family. The nucleotide sequence of *AmphiDmbx* and *CiDmbx* are accessible in the EMBL/GenBank/DDBJ databases under accession numbers AY588475 and AY588476 respectively.

Sequencing eight independent PCR clones of *CiDmbx*, and alignment to genomic sequence *C. intestinalis*, revealed considerable variation at the nucleotide level. This includes numerous synonymous substitutions that do not alter the amino acid sequence (39 sites within 720 bp of coding sequence), as well as several non-synonymous substitutions that alter the deduced protein sequence (20 sites). We conclude that these differences reflect a high degree of natural polymorphism in the *CiDmbx* gene. We also note that most of the substitutions causing amino acid changes are located at the 3′ half of the *CiDmbx* gene, where less conservation is observed between Dmbx genes of different species.

Conservation of Dmbx proteins

The homeodomain is highly conserved between amphioxus and human Dmbx genes (91.7% identity; Fig. 1), although rather divergent in *Ciona* (78.3% with human; Fig. 1). Unusually, these levels of similarity are not confined to the homeodomain, but are also seen in the N-terminal part of the protein. AmphiDmbx and human DMBX1 proteins are 88% identical from the beginning of the ORF to the end of homeodomain, while CiDmbx and human DMBX1 show 47% identity over the same region. The C-terminal half of the protein is less conserved and particularly divergent in *Ciona*. The AmphiDmbx protein does have several scattered conserved residues in this region, plus a well conserved OAR domain (Furukawa et al., 1997) near the C terminus. The OAR domain was first described in the mouse Rax protein, and is seen in several aristaless-related proteins and some other PRD-class homeodomain proteins. It functions as an intra-molecular switch to regulate the activity of the transcription factor (Brouwer et al., 2003).

Fig. 1. Comparisons of homeodomains from Dmbx family genes. Sequences of vertebrate and hydra proteins from GenBank: *Homo sapiens* (Accession Number AB037699), *Mus musculus* (AF499446), *Gallus gallus* (AF461038), *Danio rerio* (AF398526), *Takifugu rubripes* (AY071923) and *Hydra vulgaris* (AF126249). Sequences of *AmphiDmbx* (*Branchiostoma floridae*) and *CiDmbx* (*Ciona intestinalis*) cDNA sequences have been deposited in GenBank (Accession Numbers AY588475 and AY588476, respectively).

One conserved motif of interest near the N terminus (data not shown), is a six amino acid stretch [LAD(IL)IL]. This motif is similar to the GEH/en-1 consensus region (FSIDNIL) seen in several homeodomain proteins, known to function as a repressor domain (Mailhos et al., 1998). It is relevant to note that vertebrate *Dmbx1* genes are also reported to function as repressors (Kawahara et al., 2002; Zhang et al., 2002). Interestingly, an alternatively spliced form of *Dmbx1* in human, mouse, chicken and zebrafish has a five amino acid insertion (GCTFQ) within this motif. The two splice variants may interact with different partner proteins, and have different repressor activities.

Evolutionary history of Dmbx genes

To examine the phylogenetic distribution of Dmbx gene family, we conducted blast searches of GenBank. We detected no *Drosophila*, *Anopheles* or nematode genes that can be classified in the Dmbx gene family. Indeed, we detected only one gene that could be an invertebrate Dmbx gene (in addition to the amphioxus and ascidian genes reported here). This is the *Hydra vulgaris* homeobox gene *manacle* (GenBank Accession Number AF126249), which has regions of high sequence similarity to *CiDmbx* (71.7% identity over the homeodomain; Fig. 1). The *manacle* cDNA sequence was deposited on GenBank by D. M. Bridge and R. E. Steele (unpublished); expression of this gene in differentiating basal disc ectoderm is described by Bridge et al. (Bridge et al., 2000). This finding reveals that the Dmbx gene family originated before the divergence of the cnidarian and bilaterian lineages, very early in animal evolution.

The homeobox gene superfamily can be divided on the basis of homeodomain sequence into the ANTP class, the PRD class and several divergent lineages such as LIM and TALE (Galliot et al., 1999). The Dmbx homeobox gene family is clearly a member of the PRD class. For example, the human DMBX1 homeodomain has 60-65% identity with human OTX1, PTX1 and GSC (members of the PRD class), but only 38-42% identity with human HOXA1, EN1 and MSX1 (members of the ANTP class).

We conducted phylogenetic analysis using a diversity of PRD-class homeodomains. The analysis clearly groups *manacle*, *AmphiDmbx*, *CiDmbx* and vertebrate Dmbx genes together, confirming that they form a distinct gene family (Fig. 2A). They are not part of the Otx gene family. The most closely related gene families are probably Ptx, Otx and Gsc, although low support values make this conclusion tentative. This analysis is based on only the homeodomain, so should not be used to infer precise relationships, particularly within the Dmbx gene family. To address this, we undertook a second phylogenetic analysis using the full amino acid sequences of only Dmbx genes (other PRD class genes cannot be included), assigning *manacle* as the outgroup. This gave a gene phylogeny of *CiDmbx*, *AmphiDmbx* and vertebrates Dmbx genes that is entirely consistent with known relationships between the seven species (Fig. 2B). The implication is that these genes are orthologues, and that we are not sampling paralogous genes from a larger gene family.

Comparison of genomic structures of chordate Dmbx genes

We isolated an *AmphiDmbx* genomic clone and determined that the *AmphiDmbx* gene consists of five exons spanning a genomic region of 5.1 kb (Fig. 3). The homeobox is split between two exons, with an intron between homeodomain positions 46 and 47. This is a common splice site position for PRD-class homeobox genes and is seen in the Otx, Gsc and Ptx gene families (Galliot et al., 1999). Using sequence information from genome projects, in comparison to cDNA sequence, we also determined the exon-intron structures of human, mouse and pufferfish *Dmbx1* genes, and the ascidian *CiDmbx* gene. These are aligned with the *AmphiDmbx* genomic structure in Fig. 3. All vertebrate *Dmbx1* genes consist of four exons, with a conserved genomic structure. The homologue of the fourth exon is divided into two exons in amphioxus. The ascidian *CiDmbx* gene is rather different; *CiDmbx* gene consists of eight exons and is particularly divergent in gene structure over the 3′ half of the gene. This is also the region of sequence divergence.

Although the *Ciona intestinalis* genome sequence is not completely assembled, the available genomic information indicates that *CiDmbx* is localised on the same scaffold as *CiHox2* and *CiHox3* (Dehal et al., 2002). By contrast, the *Dmbx1* gene is not co-localised with any Hox clusters in mouse or human. To test whether *AmphiDmbx* is co-localised with the Hox gene cluster, we used fluorescent in situ hybridisation to amphioxus metaphase chromosomes (Castro and Holland, 2002). This revealed that, like the vertebrate situation, *AmphiDmbx* maps to a different chromosome from the Hox gene cluster (data not shown).

Expression of the *AmphiDmbx* gene

Using whole-mount in situ hybridisation and sectioning, we examined the spatiotemporal pattern of *AmphiDmbx* expression during amphioxus development. No expression was detected in blastulae, early gastrulae or mid gastrulae (data not shown). At the end of the gastrula stage, *AmphiDmbx* expression was first detected in the anterior mesendoderm (Fig. 4A). This is also clearly evident at the mid neurula stage (13 hours post fertilisation), when expression is seen to be dorsal within this tissue (Fig. 4B). At the mid neurula stage (24 hours) and late neurula stage (36 hours), the anterior mesendoderm expression extends rostrally into Hatschek's diverticula (Fig. 4D-G). Expression in the most rostral tip of the notochord is also evident at 36 hours, although this expression does not overlie the pharyngeal expression (Fig. 4E,G). Expression in anterior endoderm persists at least for a few days, and is clearly evident in swimming larvae (60 hours; Fig. 4H). At this stage, the expressing cells include part of the club-shaped gland, endostyle, pharyngeal endoderm and the preoral ciliated pit that develops from Hatschek's left diverticulum. Expression in notochord has faded at this stage. No expression is detected in the neural tube at any stage of development examined.

Expression of the *CiDmbx* gene

The developmental expression of *CiDmbx* was examined by whole-mount in situ hybridisation. No expression was detected prior to the tailbud stage (Fig. 5A,B). At the tailbud stage, expression was first detected in one bilateral pair of cells in the neural tube (Fig. 5C,D). In order to precisely locate these *CiDmbx*-expressing cells, we compared the expression of *CiDmbx* with the *Ci-Pax2/5/8-A* (Wada et al., 2003), *Ci-*

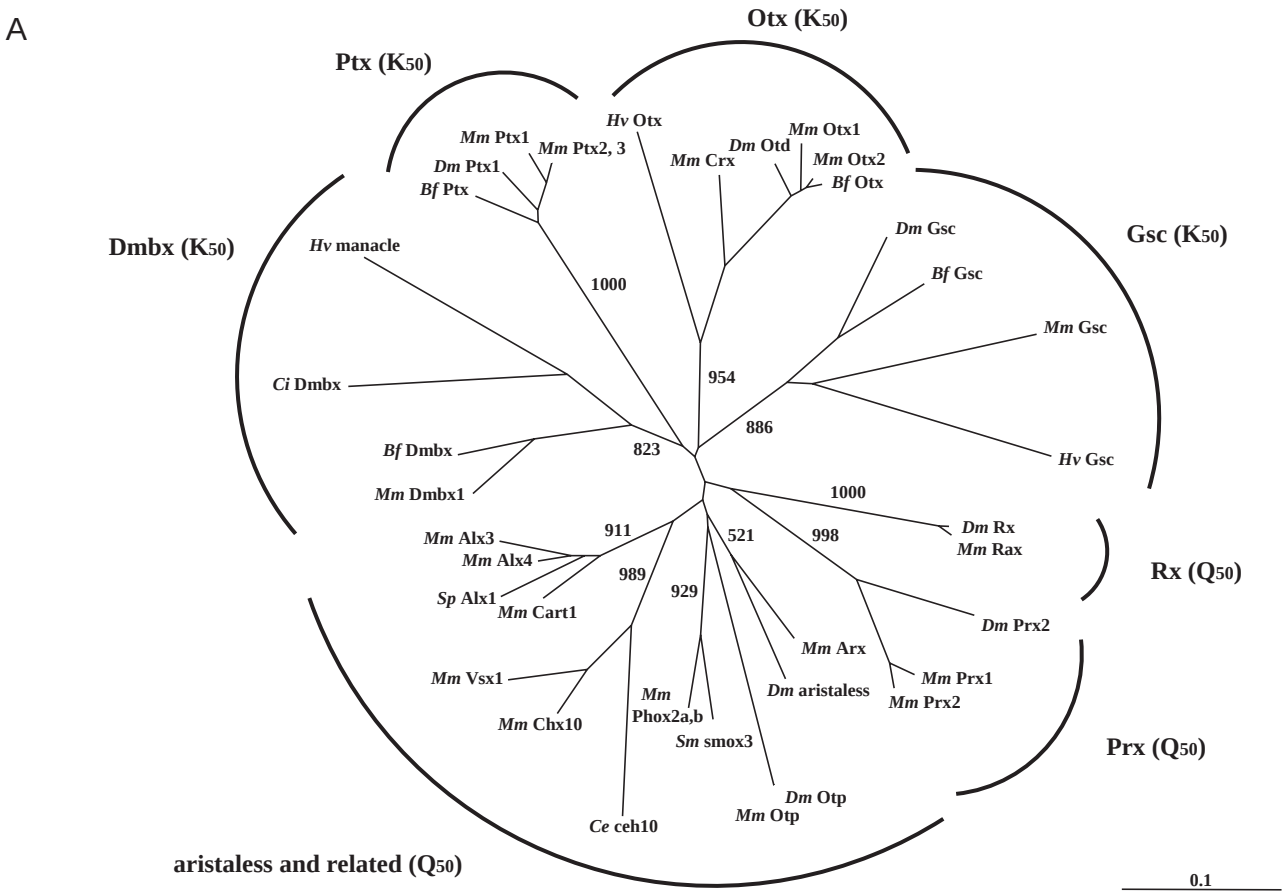

Fig. 2. (A) Unrooted phylogenetic tree of paired-like homeobox genes from the PRD class, constructed from the homeodomain only. *Bf*, *Branchiostoma floridae* (amphioxus); *Ce*, *Caenorhabditis elegans*; *Ci*, *Ciona intestinalis* (ascidian); *Dm*, *Drosophila melanogaster*; *Hv*, *Hydra vulgaris*; *Mm*, *Mus musculus*; *Sm*, *Schistosoma mansoni*. Q50 and K50 denote the amino acid of homeodomain position 50. (B) Molecular phylogenetic tree of Dmbx family proteins. This tree is constructed from the entire amino acid sequences (after removal of unalignable regions) and is rooted by *H. vulgaris* manacle protein. Both trees are constructed by the neighbour-joining method. Branch lengths are proportional to evolutionary distance corrected for multiple substitutions. Scale bar: 0.1 underlying amino acid substitutions per site. Figures on branches indicate robustness of each node, estimated from 1000 bootstrap replicates.

Fgf8/17/18 (Imai et al., 2002) and *CiHox3* (Locascio et al., 1999) genes using double in situ hybridisation. *Ci-Pax2/5/8-A* is one of two ascidian members of the vertebrate Pax2/5/8 subfamily [our phylogenetic analyses (not shown) indicate this is the same gene as reported by Imai et al. (Imai et al., 2002), except for a 174 bp insertion, presumably a differentially spliced exon]. This gene is expressed in the 'neck' region of the neural tube, immediately rostral to the cells expressing *Ci-Fgf8/17/18*, a gene descendent from the ancestor of vertebrate *Fgf8*, *Fgf17* and *Fgf18* genes (Imai et al., 2002; Wada et al., 2003). We found that *CiDmbx* (blue staining, arrowhead) is expressed in cells immediately posterior to cells expressing *Ci-Pax2/5/8-A* (red staining,

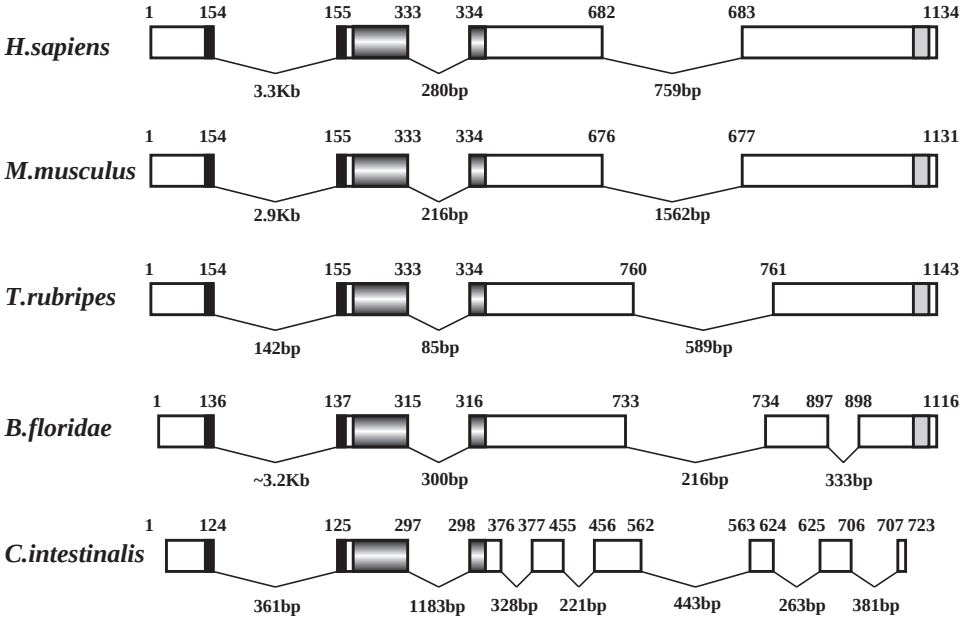

Fig. 3. Exon-intron genomic structure of Dmbx genes. Coding sequence exons are shown as rectangles; homeodomains are indicated by grey shadows, OAR domains by dotted boxes and the conserved domains similar to the GEH/eh-1 domain by black boxes. The numbers at the corner of rectangles indicate the nucleotide position at the exon-intron boundary. *AmphiDmbx* genomic sequences are available in GenBank (Accession Numbers AY588477 and AY588478). Genomic sequences of other species are from GenBank (human, NT 004852; mouse, NT 039264; *Takifugu* CAAB01001099) and the JGI website (ascidian). Each genomic structure was deduced from comparison of genomic sequence with cDNA sequence in accordance with the GU-AG splice site rule.

arrow) (Fig. 5E). The *CiDmbx* staining is coincident with *Ci-Fgf8/17/18* expression in the visceral ganglion (arrowhead, Fig. 5F). This expression also overlaps that of *CiHox3* (arrowhead, Fig. 5G). Consistent with this, *CiHox3* gene is reported to be expressed in the anteriormost region of the visceral ganglion (Locascio et al., 1999). This region is most probably homologous to the *HrHox-1* positive region at the junction of trunk and tail in another ascidian species, *H. roretzi*, which lacks a defined visceral ganglion (Nicol and Meinertzhagen, 1991; Katsuyama et al., 1995). In swimming tadpole larvae, *CiDmbx* expression is detected in the neural tube just behind the sensory vesicle; these cells are probably daughters of the cells expressing *CiDmbx* in neural tube of the tailbud stage (Fig. 5H,I).

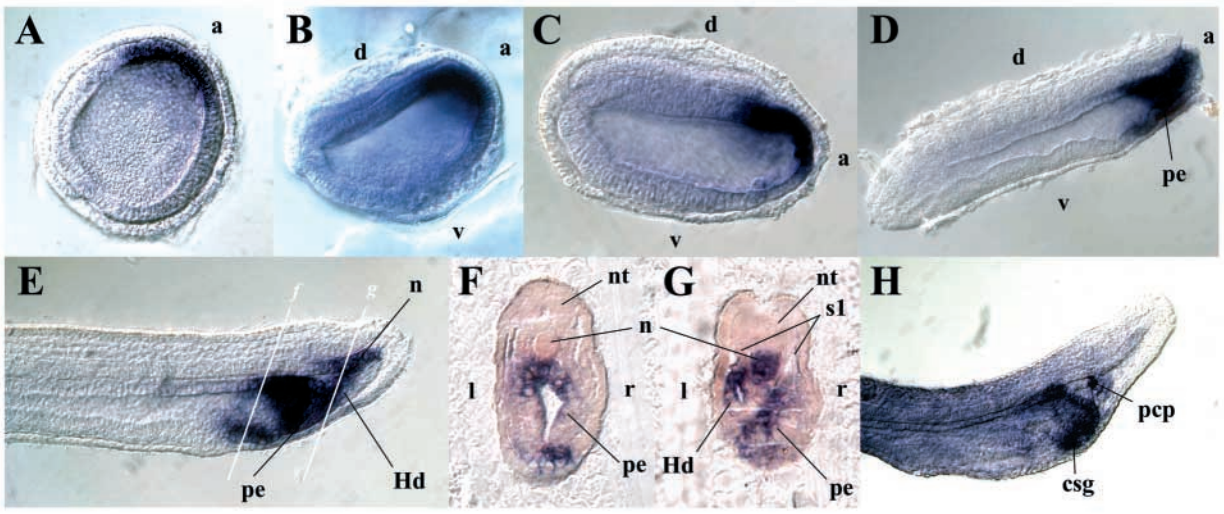

Fig. 4. Spatial and temporal expression of *AmphiDmbx* deduced by whole-mount in situ hybridisation. Anterior of whole mounts towards the right; cross-sections (counterstained pink) viewed from the posterior end of animal. (A) Lateral view of the embryo at the late gastrula stage (9 hours). *AmphiDmbx* expression is detected at the anterior mesendoderm. (B,C) Lateral views of the embryo at the neurula stage (13 hours, 20 hours). *AmphiDmbx* expression is seen in dorsal anterior mesendoderm. (D) Lateral view of the embryo at the mid neurula stage (24 hours). The anterior mesendoderm expression extends into Hatschek's diverticula. (E) Lateral view of the embryo at the late neurula stage (36 hours). Expression is detected in anterior endoderm, including Hatschek's diverticula, and also in the most rostral tip of the notochord. (F) Cross-section through *f* in E, showing expression in the anterior endoderm. (G) Cross-section through *g* in E, showing expression in anterior endoderm and notochord, but not neural tube. (H) Lateral view of 60-hour swimming larva. *AmphiDmbx* is expressed in anterior endoderm including part of the club shaped gland, endostyle and pharyngeal endoderm. Expression is also observed in a few dorsal cells of the preoral ciliated pit. No expression is detected in the neural tube at any stage of development examined. a, anterior; d, dorsal; v, ventral; r, right; l, left; csg, club shaped gland; Hd, Hatschek's diverticula; n, notochord; nt, neural tube; pcp, preoral ciliated pit; pe, pharyngeal endoderm; s1, the first somite.

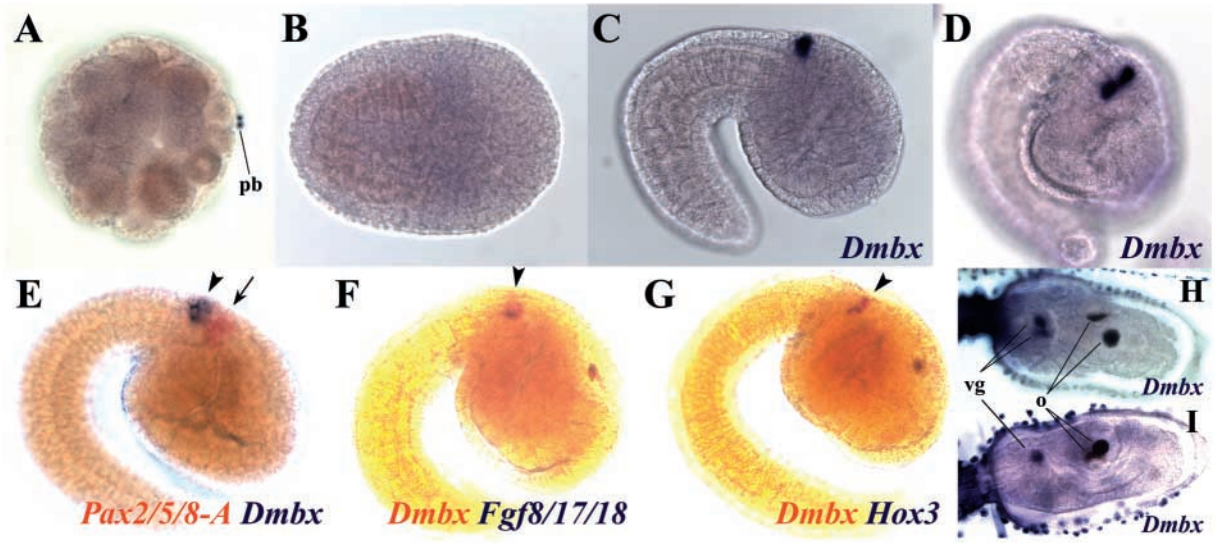

Fig. 5. Spatial and temporal expression of *CiDmbx* deduced by whole-mount in situ hybridisation. Anterior towards the right in all specimens except A. (A) Lateral view of 32-cell embryo; animal pole towards the right. (B) Dorsal view of a neurula stage embryo. No *CiDmbx* expression is observed at these stages. (C) Lateral view of mid tail-bud stage embryo. *CiDmbx* expression is detected exclusively in the neural tube. (D) Dorsal view of the same developmental stage, showing that expression is in one bilateral pair of cells. (E) Comparison of expression between *CiDmbx* (blue stain, arrowhead) and *Ci-Pax2/5/8-A* (red stain, arrow) at mid tail-bud stage (lateral view). *CiDmbx* is expressed in cells immediately posterior to cells expressing *Ci-Pax2/5/8-A*, with no gap and no overlap. (F) Comparison of *Ci-Fgf8/17/18* (blue stain) and *CiDmbx* (red stain) at mid tail-bud stage (lateral view) showing co-localised expression (arrowhead). Additional expression of *Ci-Fgf8/17/18* is detected in the trunk-lateral cells and at the tip of the tail after prolonged staining. (G) Comparison of *CiHox3* (blue stain) and *CiDmbx* (red stain) at later mid tail-bud stage embryo (lateral view), showing co-localised expression (arrowhead). Dorsal (H) and lateral (I) views of swimming tadpole larva. *CiDmbx* expression is observed in the visceral ganglion, just behind the sensory vesicle. o, otolith and ocellar pigment spots (expression negative); pb, polar bodies (expression negative); vg, visceral ganglion (expressing *CiDmbx*).

Discussion

Origin, loss and divergence of Dmbx genes

Most mammalian homeobox genes have clear orthologues in the *Drosophila melanogaster* and/or *Caenorhabditis elegans* genomes. In addition, most belong to small gene families in the human and mouse genomes, usually containing two (e.g. Emx, Otx, En, Gbx), three (e.g. Cdx, Tlx) or four (Hox cluster) paralogues. In each case, the duplicates have been traced to gene duplications that occurred at the base of vertebrate evolution (Furlong and Holland, 2002). *Dmbx1* is an unusual homeobox gene because it violates both of these general patterns: it has no clear orthologues in flies and nematodes, and no paralogues in human or mouse. Current evidence suggests there is also only a single Dmbx gene in *Takifugu*, zebrafish and chicken. The *Dmbx1* situation could be interpreted in one of two ways. One view is that *Dmbx1* is a 'new' gene that originated in vertebrate evolution. For example, it could be a cryptic member of a different homeobox gene family, and has diverged in sequence such that its vertebrate paralogues and invertebrate orthologues are not recognised. Alternatively, the Dmbx genes might constitute an ancient and distinct homeobox gene family; in this case, the Dmbx gene must have been secondarily lost from the *Drosophila* and *Caenorhabditis* genomes. After duplication in the vertebrate lineage, additional vertebrate paralogues are also though to have been lost, to leave a single copy *Dmbx1* gene in human and mouse.

The latter interpretation is the one we proposed when reporting the cloning of mouse *Dmbx1* (Takahashi et al., 2002). Our evidence at the time was circumstantial and based primarily on molecular phylogenetics of homeobox genes. The cloning of definitive Dmbx genes from amphioxus and ascidians, reported here, has confirmed this deduction. Furthermore, our analyses show that the *Hydra* homeobox gene, *manacle* (Bridge et al., 2000), also belongs to Dmbx gene family. These findings demonstrate that the Dmbx genes form a distinct, ancient, homeobox gene family. Members of this gene family have been secondarily lost in the evolutionary lineages leading to *D. melanogaster*, *C. elegans* and *Anopheles gambiae*.

Comparison of ascidian, amphioxus and vertebrate Dmbx genes reveals some unusual patterns of conservation. First, although exon-intron organisation of the gene is well conserved between human, mouse, pufferfish and amphioxus (with an extra intron in amphioxus), the ascidian *CiDmbx* gene has a very different genomic organisation. *CiDmbx* has eight exons, compared with four in human, most probably the result of four additional intron insertions. This change in gene organisation is mirrored by high levels of sequence divergence within the *Ciona* gene. We also detected a large amount of sequence polymorphism within the *Ciona CiDmbx* gene, including numerous substitutions that cause variation in the encoded protein sequence between alleles, especially in the C-terminal half of the protein. These findings provide another example of the high rate of sequence evolution reported for the ascidian genome (Ferrier and Holland, 2002; Holland and Gibson-Brown, 2003).

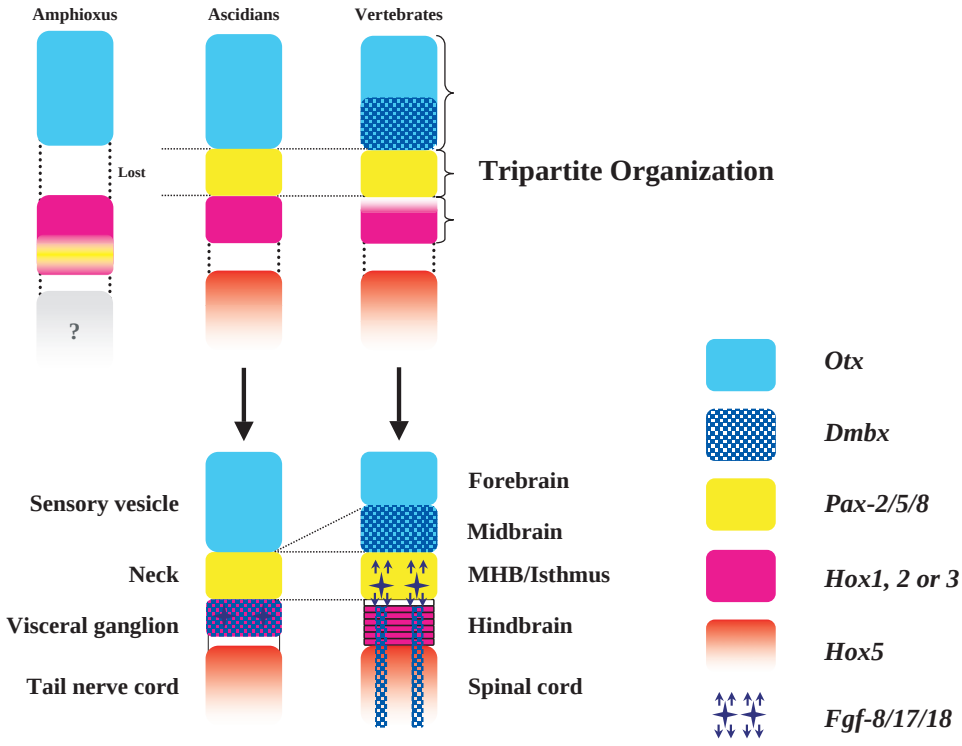

Fig. 6. Comparison of expression domains between chordate neural tubes and proposed homology of brain regions. An underlying tripartite organisation is depicted by brackets, expression of transcription factors by shading and the organiser activity of FGF8/17/18 proteins by arrows (not proven in ascidia). In all three chordate taxa, the most anterior of the three regions is marked by expression of *Otx*. This region is divided into two regions in vertebrates: an anterior region without *Dmbx* expression (forebrain) and a posterior region with *Dmbx* expression (midbrain). Amphioxus and ascidia do not possess the *Dmbx*-expressing domain (midbrain) anterior to *Pax2/5/8* expression (MHB). In vertebrates and ascidians, expression of *Pax2/5/8* family genes fills the gap between the *Otx*-expressing region and the posterior region expressing Hox genes; this is not seen in amphioxus. *Dmbx* has a later expression domain in the hindbrain of vertebrates (shown by longitudinal stripes), coincident with Hox gene expression (up to rhombomere 2); co-localisation of Hox (*CiHox3*) and Dmbx also occurs in the ascidian anterior visceral ganglion.

Evolutionary history of chordate brain patterning

The mouse *Dmbx1* gene shows a particularly interesting expression pattern in the context of the tripartite organisation of the chordate neural tube. The earliest expression site is the presumptive midbrain (at day E7.5 to E8.5), with expression extending rostrally into the diencephalon by E10. Caudally, however, this expression has a very clear spatial limit at the MHB (Broccoli et al., 2002; Gogoi et al., 2002; Miyamoto et al., 2002; Ohtoshi et al., 2002; Takahashi et al., 2002; Zhang et al., 2002). *Dmbx1* transcripts also appear in the hindbrain, where they resolve into bilateral, longitudinal stripes by E11 (possibly in specific neuronal populations), but the MHB region is strikingly devoid of *Dmbx1* expression (Takahashi et al., 2002). We hypothesise that *Dmbx1* expression is activated at a distance from the MHB, but repressed by the highest levels of FGF activity at the MHB organiser; indirect support for this comes from the observation that *Dmbx1* is also expressed immediately subjacent to the apical ectodermal ridge in limb development (Takahashi et al., 2002).

We reasoned that comparison with Dmbx gene expression in amphioxus and ascidian development would be informative in two key respects. First, the apparent absence of an MHB in amphioxus (or at least absence of a zone of *Pax2/5/8*, *En* and *Wnt1* activity between the Otx and Hox domains) (Kozmik et al., 1999; Holland et al., 1997; Holland et al., 2000), allows a test of the relation between Dmbx expression and the MHB. In short, we expect a rather different expression pattern in amphioxus. Second, early expression of *Dmbx1* is a useful midbrain marker, dividing the Otx-positive zone into anterior (forebrain) and posterior (midbrain) regions. We were curious to see whether ascidian embryos also showed this expression pattern, which may help resolve the origins of midbrain development in chordate evolution. These comparisons are facilitated by the fact that *Dmbx1* is not part of a gene family in mouse; we can be sure we are comparing the expression of single, directly orthologous genes.

In amphioxus, *AmphiDmbx* gene expression is strongest in the anterior endoderm, notochord, endostyle and part of the club-shaped gland. We did not detect any *AmphiDmbx* transcripts in the dorsal nerve cord of amphioxus at any stage of development. This finding is consistent with the suggestion, made from other gene expression data, that the anterior nerve cord of amphioxus has been secondarily modified from the ancestral tripartite organisation. Amphioxus has Otx and Hox-expressing neural domains, in regions homologues to those of vertebrates, but the intervening zone (equivalent to the MHB in vertebrates) is missing. When considered alongside the *Ciona* data (discussed below), we conclude that Dmbx gene expression in the nerve cord has been secondarily lost in the cephalochordate lineage, alongside loss of the MHB region (Fig. 6).

In ascidians, *CiDmbx* is expressed in the developing neural tube, but only in the row of neural cells posterior to Pax2/5/8-positive cells. These latter cells were used, in *Halocynthia*, to define the central zone of the tripartite neural plan (Wada et al., 1998). This row of *CiDmbx*-positive cells is coincident with the rostral limit of the Hox-expressing region. In vertebrates, this region has evolved into the hindbrain, expressing both Hox genes and *Dmbx1*. It seems logical, therefore, to conclude that the *CiDmbx* expression we describe here, and the hindbrain expression detected in mouse (see above), zebrafish (Kawahara et al., 2002) and chick (Gogoi et al., 2002), are homologous; they descend from a common ancestor with this gene expression. Interestingly, we do not detect any *CiDmbx*

expression rostral to the ascidian Pax2/5/8-positive cells. Vertebrates, by contrast, have strong midbrain expression of *Dmbx1*. This suggests that *Ciona* does not possess a neural region homologous to vertebrate midbrain (Fig. 6). It is possible that *Ciona* has lost this region or this expression domain (and, independently, amphioxus also). It is more parsimonious to conclude that absence of a distinct midbrain was the basal condition for chordates; midbrain development could be a novelty that evolved specifically on the vertebrate lineage.

Data from ascidians, *Drosophila*, hemichordates and vertebrates now concur that tripartite regionalisation of the neural tube is very ancient, and that the vertebrate fore/midbrain, MHB and hindbrain/spinal cord evolved from this tripartite organisation. In ascidians, the rostral (Otx-expressing) and caudal (Hox-expressing) zones have an intervening zone expressing a Pax2/5/8 gene (Wada et al., 1998) and an En family homeobox gene (Imai et al., 2002). In hemichordates, the central zone is marked at least by *En* (Lowe et al., 2003). In *Drosophila*, *Poxn* and *Pax2* (Pax-2/5/8 family) are each expressed at the interface of *otd* (Otx family) and *unpg* (Gbx family) genes, anterior to the Hox-expressing region (Hirth et al., 2003). However, possession of the tripartite organisation is not sufficient to indicate the existence of organiser activity; it could simply mark three distinct spatial regions. In the *Drosophila* embryo, no obvious brain phenotypes are described after mutational inactivation of fly homologues of the key genes of the vertebrate MHB, such as *pax2* and *Poxn* (Pax-2/5/8 homologues) or *branchless* (Fgf homologue) (Hirth et al., 2003). This suggests that although *Drosophila* has the tripartite organisation, it lacks MHB organiser activity; the former preceded the latter in evolution.

Turning to chordates, it is likely that MHB organiser activity had emerged by the time that the ascidian and vertebrate lineages diverged. The *Ciona Ci-Fgf8/17/18* gene is expressed immediately posterior to Pax2/5/8 (marking the middle of the three regions), in a manner reminiscent of *Fgf8* expression and function in the vertebrate MHB organiser. There are subtle differences, notably that in *Ciona Ci-Fgf8/17/18*, *CiDmbx* and *CiHox3* transcripts are co-localised posterior to Pax2/5/8, at the visceral ganglion (Imai et al., 2002). In vertebrates, *Fgf8* gene expression is caudal to the MHB isthmus, but slightly rostral to *Dmbx1* and Hox gene expression which each start in rhomobomere 2 (Irving and Mason, 2000; Takahashi et al., 2002). This difference might reflect a recently evolved inhibitory effect of FGF8 activity on Hox gene expression in vertebrates (see Irving and Mason, 2000). Until now, there is no direct evidence for the *Ciona* FGF8/17/18 protein conferring organiser activity. However, if ascidians do have MHB organiser activity, as these data suggest, then it is very interesting that *Ciona* lacks Dmbx expression anterior to the Pax2/5/8-positive cells. The implication is that the evolution of the MHB organiser preceded the evolution of a distinct midbrain.

In summary, comparative data indicate that a tripartite ground plan for brain development existed in the common ancestor of the Bilateria. At some point in the evolution of vertebrates, probably before the divergence of ascidians and vertebrates, the central region of this tripartite plan acquired MHB organiser activity, which acts to refine the developmental patterning of the adjacent regions. At the same time, these regions must have obtained competency to respond to signals from the organiser. The lineage leading to amphioxus, which diverged a little later, most probably lost the MHB region, with concomitant modification of adjacent tissues. In the evolutionary lineage leading to vertebrates, we conclude that the zone immediately rostral to the MHB organiser became further subdivided, to include a specific midbrain region marked by *Dmbx1* gene expression.

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