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Amphioxus Evx Genes: Implications for the Evolution of the Midbrain–Hindbrain Boundary and the Chordate Tailbud

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Evx genes are widely used in animal development. In vertebrates they are crucial in gastrulation, neurogenesis, appendage development and tailbud formation, whilst in protostomes they are involved in gastrulation and neurogenesis, as well as segmentation at least in *Drosophila*. We have cloned the Evx genes of amphioxus (*Branchiostoma floridae*), and analysed their expression to understand how the functions of Evx have evolved between invertebrates and vertebrates, and in particular at the origin of chordates and during their subsequent evolution. Amphioxus has two Evx genes (*AmphiEvxA* and *AmphiEvxB*) which are genomically linked. *AmphiEvxA* is prototypical to the vertebrate Evx1 and Evx2 genes with respect to its sequence and expression, whilst *AmphiEvxB* is very divergent. Mapping the expression of *AmphiEvxA* onto a phylogeny shows that a role in gastrulation, dorsal–ventral patterning and neurogenesis is probably retained throughout bilaterian animals. *AmphiEvxA* expression during tailbud development implies a role for Evx throughout the chordates in this process, whilst lack of expression at the homologous region to the vertebrate Midbrain-Hindbrain Boundary (MHB) is consistent with the elaboration of the full organiser properties of this region being a vertebrate innovation.

Key Words: **Amphioxus; Evx genes; chordates; gastrulation; neurogenesis; Midbrain–Hindbrain Boundary; tailbud development.**

INTRODUCTION

During chordate evolution several important evolutionary transitions occurred, involving the origin of new cell types, changes to body plan organization and increased genome complexity. The origin of the chordates entailed the evolution of somites/myomeres, notochord, and a post-anal tail (Gee, 1996). From within the chordates the vertebrates evolved: with extensive cephalisation, skeletonisation, neural crest and genome-wide gene duplications (with extensive maintenance and functionality). Amphioxus (Cephalochordata) is a good model system in which to investigate these evolutionary transitions, as it is the closest extant outgroup to the vertebrates (Wada and Satoh, 1994), and in terms of its genome organisation it often resembles what one would expect for the vertebrate ancestor prior to the genome-wide duplications which are hypothesized to have occurred early during vertebrate evolution (Holland *et al.*, 1994).

Evx genes are good candidates to serve as molecular markers for body plan evolution. Evx is conserved in a wide range of phyla (nematodes (Ahringer, 1996), arthropods (Macdonald *et al.*, 1986; Patel *et al.*, 1992; Brown *et al.*, 1997), vertebrates (Ruiz i Altaba and Melton, 1989; Bastian and Gruss, 1990; Joly *et al.*, 1993; Sordino *et al.*, 1996; Thaëron *et al.*, 2000), and Cnidaria (Miller and Miles, 1993)), and has a conserved role in patterning the posterior of bilaterian embryos. Although Evx genes are widely conserved in this basic function they have also been the targets of, or agents for, evolutionary change. The founding member of the group is *Drosophila melanogaster even-skipped* (*eve*), which is a Pair-Rule gene, expressed in seven

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stripes in the blastoderm embryo (Macdonald *et al.*, 1986). Comparative studies in other insects have shown that the development of this striped pattern occurs in different ways (Patel *et al.*, 1994), and may well not be ancestral for the insects (Patel *et al.*, 1992). In vertebrates there are two Evx genes (Evx1 and 2), which arose from a single ancestral gene via gene duplications in early vertebrate evolution. There are four major sites of action of these genes during vertebrate development; gastrulation (Evx1), CNS, tailbud and limb bud development (Ruiz i Altaba and Melton, 1989; Bastian and Gruss, 1991; Dush and Martin, 1992; Spyropoulos and Capecchi, 1994; Dollé *et al.*, 1994; Hérault *et al.*, 1996; Beck and Slack, 1999). A function during gastrulation is typical for the genes in all phyla so far examined (Ahringer, 1996), and so is probably an ancient role performed by Evx in a basal triploblast, vertebrate Evx2 being a derived exception (Dollé *et al.*, 1994). The CNS expression is also a phyletically widespread feature, but the details of the expression patterns show important differences (see Discussion). The tailbud, which gives rise to the postanal tail, is a synapomorphy of the chordates. This Evx function must have evolved either with the origin of chordate tailbuds, if it is an essential gene for tailbud development, or later during chordate evolution if the gene is involved with elaboration of the tailbud in higher chordates (vertebrates). The function of Evx2 in limb development is a relatively recent innovation, as the vertebrate ancestor, and probably even the most basal vertebrates, did not have paired appendages (Coates, 1994). Examination of AmphiEvx expression would thus be informative with regards to revealing possible functions of the genes that are conserved with other phyla, specific to the chordates, and were present prior to the evolution of the vertebrates.

We have found two AmphiEvx genes which are closely linked in the genome. They probably arose from a tandem duplication specific to the cephalochordate lineage. We find that *AmphiEvxA* is prototypical in sequence and expression with respect to the vertebrate Evx1 and 2 genes, whereas *AmphiEvxB* is a very derived gene. The sequence of *AmphiEvxA* is roughly equidistant between vertebrate Evx1 and 2, and it is expressed during gastrulation, posterior CNS and tailbud development.

MATERIALS AND METHODS

Gene Cloning and Sequencing

PCR was performed with degenerate homeobox forward primers eve5′a (5′-TAYMGNACNGCNTTYAC-3′, coding for YRTAFT) and eve5′b (5′-TNGARAARGARTTYTA-3′, coding for LEKEFY), and reverse primer SO2 (5′-CKNCKRTTYTGRAACCA-3′, reverse coding for WFQNRR) on DNA from a 5–24 h amphioxus embryo cDNA library (Langeland *et al.*, 1998). A first PCR reaction was performed with an annealing temperature of 44°C for 35 cycles with primers eve5′a and SO2. The reaction was then primer-purified and used as template for a nested PCR with primers eve5′b and SO2 with the same annealing conditions. The 110bp band was cloned into pBluescript SK+ vector (Stratagene) and 50 clones sequenced automatically (ABI Perkin Elmer). A fragment that was clearly from an Evx gene (clone p29) was found (as judged by BLAST searches) and an oligonucleotide based on p29 sequence (eva primer: 5′GTTGTCTCCGGTAGGTTGAGCTGCG3′) was 5′ end-labelled with T4 polynucleotide kinase and used to screen a *Branchiostoma floridae* genomic library (Garcia-Fernàndez and Holland, 1994) with standard procedures (hybridization for 48 h at 55°C in 6xSSC, 5x Denhardts, 0.05% Sodium Pyrophosphate, 0.5% SDS, 200μg/ml yeast RNA; washes 3 x 15 min at 55°C in 6xSSC, 0.05% Sodium Pyrophosphate). A positive clone λBfg1003 contained the 5′ end of the homeobox. and a 2.1 Kb *Hin*dIII subclone was used to screen single animal cosmid libraries (MPMGc117 and MPMGc118, Burgtorf *et al.*, 1998) at high stringency conditions (65°C in Church's buffer, and washed to 65°C in 1xSSC, 0.1% SDS). Clone M1917 was isolated from library MPMGc118 and sequencing of subclone pm17.9, which hybridised to the Evx homeobox probe, revealed the presence of the complete homeobox with an intron at position 46/47 of the homeodomain. This was *AmphiEvxA*.

A genomic walk was undertaken from the ends of clone M1917. The walk 5′ of *AmphiEvxA* produced no specific signals on the cosmid libraries, but the 3′ walk led to the isolation of clone F1654 (library MPMGc118), with further walking giving clone P1537 (library MPMGc117). These clones were hybridised with SO2 to screen for the presence of homeoboxes (as described in Garcia-Fernàndez and Holland, 1994), and a second signal in addition to that of *AmphiEvxA* was found. Subcloning of a 3.5Kb *Sal*I/*Not*I fragment of P1537 containing this new homeobox signal, followed by direct sequencing with the SO2 primer and then a specific primer (intronou primer 5′GGGGATGGGGCTTAGGC3′) to sequence back over the SO2 region revealed a fragment of a homeobox corresponding to the third helix region of the homeodomain and a splice acceptor site at position 46/47 of the homeodomain. BLAST searches with this small section of homeodomain revealed that it was also from an Evx gene: *AmphiEvxB*.

Hybridisation with the original *AmphiEvxA* homeobox (55°C in Church's buffer, and washed to 55°C in 1xSSC, 0.1% SDS) to shotgun subclones of clone P1537 enabled the localisation of the 5′ end of the second Evx homeobox, which was confirmed by automated sequencing (ABI Perkin Elmer).

A 958bp genomic fragment containing *AmphiEvxA* and a 3.5kb genomic fragment containing *AmphiEvxB* were used to screen a 5–24 h amphioxus cDNA library at high stringency (65°C in Church's buffer, and washed to 65°C in 1xSSC, 0.1% SDS). Ten clones were isolated from the *AmphiEvxA* screen, all of which were about 2.5kb in length, and none with *AmphiEvxB*. Clones 1.1, 3.4 and 7.3 were sequenced in their entirity by primer walking and automated sequencing. A 440bp *AmphiEvxB* genomic fragment which encompassed the third helix region and 295bp 3′ of the homeobox was generated by PCR, and used to screen the 5–24 h cDNA library at high stringency. No signals were produced. A 3–5 day larval cDNA library (a gift from Linda Holland) was screened with the same probe, and a single 1.3Kb clone was isolated. Clone pc2119 was sequenced from its 5′ end through the entire homeobox and to the stop codon 307bp downstream. The accession numbers for these sequences are AF374191 and AF374192.

Phylogenetic Analysis

The AmphiEvx sequences were aligned with a representative selection of other Evx proteins in ClustalX. The homeodomains, plus 2 upstream amino acids and 6 downstream amino acids, were

subjected to Neighbour-Joining and Maximum-Likelihood analysis. Neighbour-Joining was performed within the ClustalX package, with 1000 bootstrap replicates. Maximum-Likelihood analysis was performed with Tree-Puzzle (Dayhoff substitution model, 1 invariable and 8 gamma rates, quartet puzzling) (from http://www.zi.biologie.uni-muenchen.de/~strimmer/puzzle.html).

Embryonic in Situ Hybridisation

Whole mount embryonic *in situ* hybridisations were carried out on 10–48 h amphioxus embryos and larvae according to the protocol of Holland (1999). Antisense and sense probes of *AmphiEvxA* were made from clone 3.4, with the Roche *in vitro* transcription kit. *AmphiEvxB* antisense and sense probes were made from the 440bp clone used to screen the cDNA libraries (see above). Embryos were viewed on a Zeiss Axioscop microscope with Nomarski optics, and images captured with a Zeiss Axiocam.

RESULTS

Gene Organization

We isolated a fragment of an amphioxus Evx homeobox during PCRs with degenerate primers that recognise the first and third helix regions of Evx-like homeoboxes, on a cDNA library made from 5 to 24 h embryos (Langeland *et al.*, 1998). With this Evx fragment as a probe we isolated larger genomic fragments from phage (Garcia-Fernàndez and Holland, 1994) and cosmid libraries (Burgtorf *et al.*, 1998). A genomic walk was undertaken from the original Evx-containing cosmid, and screened for the presence of other homeoboxes by hybridisations with the third helix degenerate primer. A second partial homeobox was found approximately 35kb 3′ of the original Evx homeobox. This second fragment also resembled an Evx gene, judging from the limited sequence around this fragment, which included the third helix coding sequence and sequence 3′ of the homeobox, with an intron at position 46/47 of the homeodomain. The remainder of the second homeobox was located by cross-hybridisation with the original Evx homeobox, and its complete sequence obtained. This confirmed it as an Evx-class homeobox.

The genomic organisation of these two Evx homeoboxes is shown in Fig. 1. We have called the gene containing the original homeobox *AmphiEvxA*, and the second gene *AmphiEvxB*. The two genes are in opposite transcriptional orientations, being transcribed towards each other. Both genes have introns in their homeoboxes between homeodomain positions 46/47. This intron position is common in Evx genes. Both genes also have introns just 5′ of the homeoboxes. Again this is typical for Evx genes. The intron positions were confirmed by sequence comparison with the cDNAs (see below), although the 5′ intron of *AmphiEvxB* is not spanned by our cDNA clone, and so its presence is hypothesized by comparison to *AmphiEvxA*, and from the presence of canonical splice acceptor sites in the region expected for a typical Evx gene.

cDNA clones were isolated from a 5–24 h embryonic library for *AmphiEvxA* and a 3–5 day larval library for *AmphiEvxB*, by hybridisation with genomic fragments containing the respective homeoboxes. Despite several attempts to isolate *AmphiEvxB* from the 5–24 h library (a library renowned for its good complexity and coverage), no clones were found at this earlier stage of development. Ten independent clones were isolated for *AmphiEvxA*, all with lengths of around 2.5kb. The *AmphiEvxA* cDNA codes for a protein of 362 amino acids, and translation continues to the stop codon at cDNA postion 1185. This stop codon is almost certainly the one used, judging from comparisons to other Evx genes. The homeobox lies towards the 5′ end of the protein coding sequence, as is typical for the Evx family, and the mRNA has a long 3′UTR of 1,251 bases.

The *AmphiEvxB* cDNA clone is 1.3kb long, and is most probably incomplete at its 5′ end, as the clone terminates only 16bp 5′ to the homeobox. We did not pursue the rest of the *AmphiEvxB* sequence due to its very derived nature (sequence and expression, see below), and hence reduced relevance to evolutionary comparisons to Evx genes of other animals. The putative stop codon is at cDNA position 502bp, which leaves a long 3′UTR of approximately 0.8kb.

Sequence Comparisons

The sequence of AmphiEvxA is equally similar to vertebrate Evx1 and 2 proteins, and is intermediate between vertebrate and other invertebrate sequences. The sequence of AmphiEvxB on the other hand is very divergent. A comparison of AmphiEvxA and B homeodomain sequences with other Evx proteins is given in Fig. 2. AmphiEvxA is slightly more similar to the vertebrate proteins than the invertebrates, as expected for a gene from the sister group to the verterbates. Also it is equally similar to both of the vertebrate proteins, Evx1 and 2 (95–97% identity in the homeodomain). The zebrafish eve1 gene is an unusual, derived gene, and is neither an Evx1 or Evx2 gene (see Discussion).

Vertebrate Evx proteins are well conserved downstream of the homeodomain (Fig. 3). There are many residues common to all vertebrate Evx proteins. There are 77 “pan-Evx” residues (blue in Fig. 3), constituting 34.2–47.8% of the vertebrate Evx1/2 C-termini. Throughout these C-termini there are also diagnostic residues that distinguish the Evx1 and 2 proteins from each other (26 diagnostic residues for Evx1 and 50 for Evx 2). Of the 77 pan-Evx residues AmphiEvxA has 42 (54.5%), whilst of the Evx1 and 2 diagnostic residues AmphiEvxA has only 7/26 relative to Evx1, and 6/50 relative to Evx2. AmphiEvxB has much lower levels of similarity. The C-termini of Evx proteins act as transcriptional repressor domains, and it is thought that the prevalence of proline, alanine and glutamine residues contribute to this function (Han and Manley, 1993; Briata *et al.*, 1995). The chordate Evx C-termini consist of 21.9–39.6% proline/alanine/glutamine, and *Drosophila* Eve has 36.4%.

To further assess the apparent archetypal nature of AmphiEvxA sequence with respect to vertebrate Evx1 and 2 we

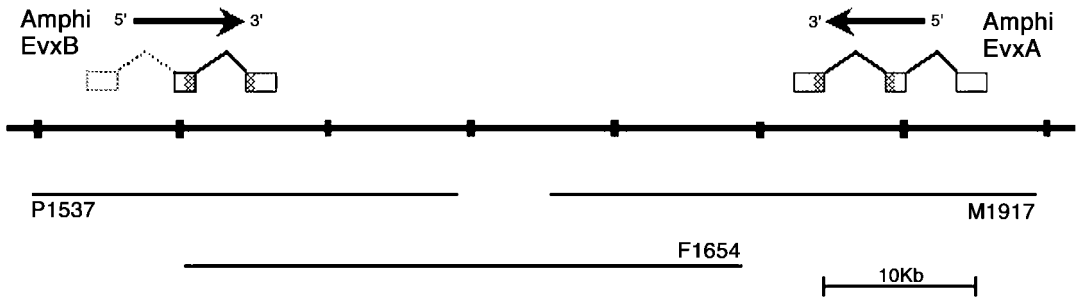


FIG. 1. Genomic organisation of *AmphiEvxA* and *AmphiEvxB*. The arrows show the transcriptional orientations. The boxes represent the exons,with the stippled regions being the homeoboxes. The 5′ exon of *AmphiEvxB* has not been sequenced, and is shown with dotted lines. The solid line underneath the genes represents the chromosome, under which are shown the cosmids of the Evx contig. Cosmids M1917 and F1654 are from library MPMGc118 and P1537 is from library MPMGc117. The intron sizes are not accurately known, and so are not precisely drawn to scale.

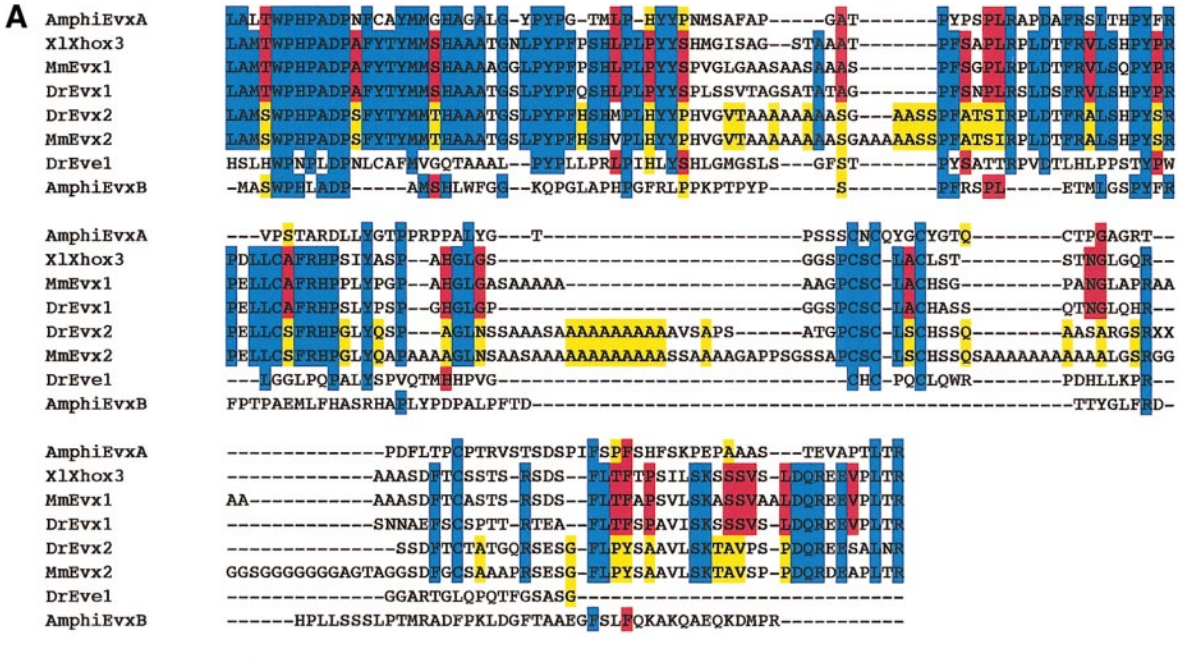
performed Neighbour-Joining (NJ) and Maximum Likelihood (ML) analyses on the sequences of Evx homeodomains with short flanks (2 amino acids 5′ and 6 amino acids 3′ of the homeodomain). Using the available Evx sequences, with some posterior Hox sequences as an outgroup, we produced the trees shown in Fig. 4. The vertebrate genes clearly resolve into the Evx1 and 2 groups (Evx1 NJ bootstrap = 86.4%, and ML Tree-Puzzle value = 77, whilst Evx2 NJ bootstrap value = 75.3% and ML Tree-Puzzle value = 57), and all of the insect Eve genes form a well-supported group (NJ bootstrap = 93.6%). The two types of vertebrate Evx are sister groups (NJ bootstrap = 89.4%), with AmphiEvxA falling immediately outside of them. This suggests that the duplication to produce vertebrate Evx1 and 2 postdated the cephalochordate-vertebrate divergence. The position of AmphiEvxA in tree A of Fig. 4 is however only weakly supported (NJ bootstrap = 48.6%). The inclusion in such trees of divergent, relatively long-branched sequences can disrupt the tree topology. Thus we repeated the analysis omitting the divergent Evx sequences (AfEve of *Acropora*, vab7 of *C.elegans*, AmphiEvxB and Eve1 of zebrafish) (Fig. 4B). Again AmphiEvxA resolved as the sister to the vertebrate Evx1 + 2 clade/group, but with much greater support (NJ bootstrap = 89.7% and ML Tree-Puzzle value = 98).

Expression

Expression of *AmphiEvxA* occurs in three distinct processes during amphioxus development: gastrulation, neurogenesis and tailbud development. Using the *AmphiEvxA* cDNA as a probe, whole-mount *in situ* hybridisations were performed on 10–48 h embryos and larvae (Fig. 5). The embryology of amphioxus has been described by Hatschek (1893) and Conklin (1932). At 10–12 h postfertilisation the embryo is at midgastrulation, forming a cup shape with the blastopore opening at the posterior. The dorsal side of the gastrula embryo can be distinguished as a slight flattening

		% identity to AmphiEvxA	% identity to AmphiEvxB
AmphiEvxA	DGDAGN VRRYRTAFTREQLARLEKEFYRENYVSRPRRCELAAQLNLPETTIKVWFQNRRMKDKRQR LALTWP	100	75
AmphiEvxB	RPLPPP ........S...T.......H.D..L..........A..................E.... M.SWPH	75	100
MmEvx1	CSASDQ M...........I.....................A..... ..M...	86	75
XlXhox3	ACAGDQ M...........I.....................A..... ..M...	86	75
DrEvx1	NYGSDQ M...........I.............S.....A..... ..M...	85	74
DrEvx2	SSSSDQ ............IG.....................A..... ..MS..	85	75
MmEvx2	GSG.DQ ............I.....................A..... ..MS..	88	76
DrEve1	LNGIDQ S..H.........T...Q.YCK.S.............A..... HS.H..	75	65
DmEve	IPADPS ..........D..G.......K.....................S..... I.VA..	82	72
SaEve	S.NDQS I.....................K.............S......S..... M.MA..	83	71
TcEve	NVNDQ. I.....................FK.....................S..... M.IA..	83	72
BmEve	SAPDP. I.....T.............MK.................Q...S..... I.VA..	81	69
CeVab7	NR.D.Q M.......S...IG...R..AK......KT.G....E.....G..... VGGLAW	69	64
AfEveC	?????? T.............S......L.......T..S...SM...S.....I.........A..R. ??????		

FIG. 2. Alignment of Evx homeodomains and six amino acid flanks, to AmphiEvxA and B. The flanks are separated from the homeodomains by a space, and dots represent identities to AmphiEvxA. The question marks are unknown residues. Mm = *Mus musculus*, Xl = *Xenopus laevis*, Dr = *Danio rerio*, Dm = *Drosophila melanogaster*, Sa = *Schistocerca americana*, Tc = *Tribolium castaneum*, Bm = *Bombyx mori*, Ce = *Caenorhabditis elegans* and Af = *Acropora formosa* (synonymous with *A.muricata*).



B

Gene	Length	% blue	# blue	# red	# yellow	% PAE
AmphiEvxA	157	26.8	42	7	6	29.9
XlXhox3	161	47.8	77	26	–	24.2
MmEvx1	174	44.3	77	26	–	37.9
DrEvx1	164	47.0	77	26	–	25.6
DrEvx2	190	40.5	77	–	50	33.2
MmEvx2	225	34.2	77	–	50	39.6
DrEve1	128	18.8	24	5	3	21.9
AmphiEvxB	135	14.8	20	4	3	29.6

FIG. 3. A) Alignment of chordate Evx C-termini to AmphiEvxA. Blue shading represents identity with all of the vertebrate proteins shown (except zebrafish Eve1). Red shading represents identity with vertebrate Evx1 proteins, and yellow shading represents identity with vertebrate Evx2 proteins. Species abbreviations are as in 2. B) Comparison amongst the chordate Evx C-termini of the percentage and number of identities to all vertebrate Evx proteins (blue), vertebrate Evx1 (red) and vertebrate Evx2 (yellow). % PAE denotes the percentage of proline, alanine and glutamine residues in the C-termini.

relative to the curve of the rest of the cup-shaped embryo. At this stage *AmphiEvxA* is expressed in a posterior, ventrally restricted domain (Fig. 5A,B). The detectable expression is located in the ectoderm, spanning a region from the posterior-most end to approximately 50% of the embryo's length at its anterior-most point, which lies on the ventral side. During gastrulation *AmphiEvxA* expression is not detected on the dorsal surface, which will go on to form the neural plate. At 15 h the embryo is a neurula, with the first few somites formed. There is an archenteron cavity and the ectodermal layer from the ventral side of the blastopore has closed over the posterior end and is progressing anteriorly, enclosing the posterior neural plate along with the ingressing lateral ectoderm. At this stage *AmphiEvxA* is expressed at the posterior of the embryo, in all three germ layers and the neural plate (data not shown), in a fashion similar to the 22 h larva described next, and shown in Fig. 5C.

The 22 h larva has 9–12 somites and the rudiment of the mouth opening and 1st gill slit. *AmphiEvxA* is still expressed in the posterior ectoderm and mesendoderm, but its most striking expression domain is in the CNS, at the level of the boundary between somites 4 and 5 in a strongly stained pair of cells. These cells are slightly offset with respect to each other, such that the left-hand cell is more anterior. In amphioxus the somites of the right side are displaced posteriorly relative to the left-hand somites, which is reflected in the staggering of the stained cell pairs in the nervous system. Similar pairs of cells stain more posteriorly, but more weakly, at approximately 1 somite intervals, with the posterior-most pairs lying within a more widespread CNS expression domain (Fig. 5C). This pattern is evident in 15 h embryos, and remains this way until approximately 22 h. Expression in the anterior cell pair (at the somite 4/5 boundary) is then no longer detectable, but remains on in the somite 5/6 cell pair and posteriorly (Fig. 5D, E). By 36 h CNS staining is only detectable as a weakly stained

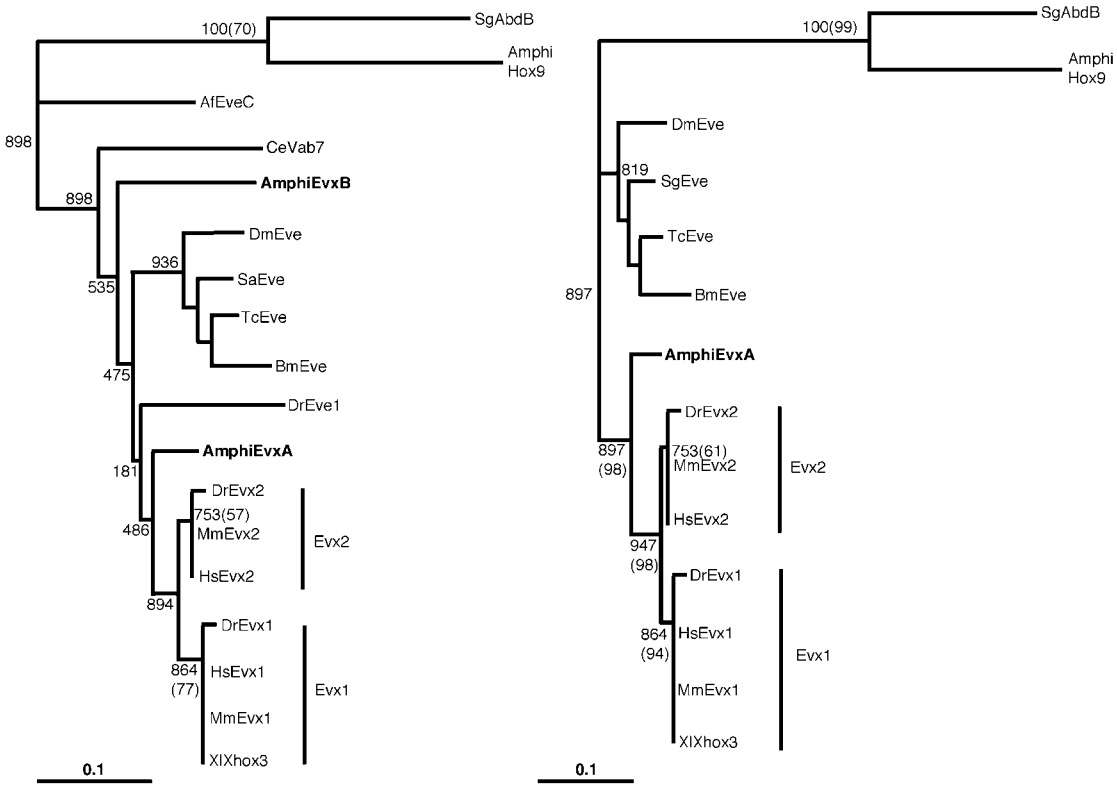


FIG. 4. Phylogenetic trees of Evx proteins, with an outgroup of two posterior Hox proteins (*Schistocerca gregaria* SgAbdB and AmphiHox9). Relevant Neighbour-Joining bootstrap values are given at the nodes, with Tree-Puzzle Maximum Likelihood values in parentheses when they are over 50. A) All Evx proteins: AmphiEvxA is the sister to the two groups of vertebrate Evx proteins. DrEve1 is neither an Evx1 or Evx2 gene, and AmphiEvxB is extremely divergent, as are CeVab7 and AfEveC. B) Tree without the divergent (long branched) Evx proteins: AmphiEvxA is clearly the sister to the two groups of vertebrate Evx proteins.

single cell-pair at the level of the somite 5/6 boundary (posterior to the pigment spot at the level of the somite 4/5 boundary) and in the posterior-most extent of the CNS (Fig. 5F).

In 36–48 h larvae the anal opening is apparent (Fig. 5F), and further elongation occurs from the postanal tailbud. *AmphiEvxA* staining is now present as a posterior subectodermal crescent, extending a short way anteriorly in the neural tube and beyond the anal opening (the gap in ventral expression in Fig. 5F).

We have been unable to detect *AmphiEvxB* expression prior to hatching. *In situ* hybridisations performed on hatched embryos and larvae stain strongly all over the ectoderm, with no discernable regionalisation (data not shown). Negative controls with sense *AmphiEvxB* probes show no staining at any time.

DISCUSSION

AmphiEvxA Is Prototypical

The position of AmphiEvxA as an outgroup to the vertebrate Evx genes allows us to distinguish the extent of conservation and the direction of evolution of features of the Evx sequences. The homeodomain of AmphiEvxA is intermediate between those of vertebrates and other invertebrates, fulfilling the expections for a gene from the invertebrate sister group to the vertebrates. The AmphiEvxA sequence is equally similar to the two vertebrate Evx families (1 and 2), and resolves as the sister to these vertebrate genes (Fig. 4). This is consistent with the duplication that produced vertebrate Evx1 and 2 having occurred after the cephalochordate and vertebrate lineages diverged, and *AmphiEvxA* resembling the ancestral Evx gene, with Evx1 and 2 diverging at similar rates after the duplication.

The transcriptional repressor function of the C-termini of Evx proteins has been hypothesised to be linked to their proline/alanine/glutamine-rich nature (Han and Manley, 1993; Briata *et al.*, 1995). The AmphiEvx proteins are also proline/alanine/glutamine-rich in their C-termini (Fig. 3), and consequently have probably retained the transcriptional repressor function. However, the chordate sequences including those of amphioxus, are not simply proline/alanine/glutamine-rich, but also show the conservation of many other residues as well, at least for the nonderived

chordate sequences (excluding AmphiEvxB and DrEve1, see below). Conservation over such long times (cephalochordates and vertebrates split at least 500MYA, and possibly as long ago as 700MYA (Nikoh *et al.*, 1997), probably indicates an evolutionary constraint on the evolution of these sequences which presumably is imparted by a functional importance. Homeodomain proteins bind to the DNA through their homeodomain, which accounts for its evolutionary conservation, but many (possibly all) homeodomain proteins also interact with other proteins, which can account for sequence conservation outside of the homeodomain, such as that seen in the C-termini of chordate Evx proteins immediately downstream of the homedomain and just prior to the stop codon (Fig. 3). The presence of sequence conservation within the chordates, that does not extend out to the protostomes may reflect certain protein-protein interactions that are specific to the chordates.

After the duplication that gave rise to Evx1 and 2, specific distinguishing residues have accumulated in the C-termini of the two different proteins. The majority of the Evx1/2 specific residues are not present in AmphiEvxA and so probably arose only in the vertebrate genes. Their conservation in such divergent lineages as mammals and amphibians (e.g., mouse Evx1 versus *Xenopus* Xhox3) and mammals versus fish (e.g., mouse Evx2 and zebrafish Evx2), probably signifies that they are of functional importance. The novel functions of vertebrate Evx genes, for example in appendage development, may be related to the "pan-vertebrate Evx" residues that are not present in AmphiEvxA, in addition to the evolution of the activation of Evx in appendage development. Furthermore the residues which distinguish Evx1 and 2 may be related to functional differences between these two proteins, for example the different expression and possible different roles in gastrulation and around the midbrain/hindbrain boundary (Dollé *et al.*, 1994; see below). Two lines of evidence are needed to confirm these hypothesized sequence significances. First, it is necessary to isolate the Evx sequences from further outgroups, such as ascidians and echinoderms. Second, the protein interactions acting through the vertebrate Evx C-termini should be examined.

The isolation of a second Evx gene in amphioxus (*AmphiEvxB*) was a surprise, due to the expectations arising from the largely ancestral nature of the amphioxus genome with respect to vertebrates, as well as the prototypical appearance of *AmphiEvxA*. We cannot completely exclude the existence of further Evx genes in vertebrates, which are orthologous to *AmphiEvxB*, although humans do not seem to possess one judging from our BLAST searches of the Human Genome. Besides, the extremely divergent sequence of AmphiEvxB leads us to suspect that this tandem duplication is specific to the cephalochordate lineage. Indeed this is not unprecedented in amphioxus (Brachyury (Holland *et al.*, 1995), HNF3 (Shimeld, 1997), myogenic bHLH (Araki *et al.*, 1996), Actins (Kusakabe *et al.*, 1999), Calmodulin-like genes (Karabinos and Bhattachorya, 2000), Emx (C.M. and J.G-F. unpublished)), and serves as a reminder that the amphioxus genome is not a static "living fossil" of the vertebrate ancestor, but is accumulating cephalochordate-specific features. Formerly reported gene duplication events in the amphioxus genome have been shown to produce genes with closely related sequences and functions. Notwithstanding, *AmphiEvxB* is by far the most striking example of sequence divergence and unrelated functions (a fast evolving gene) in amphioxus.

Comparison with AmphiEvxA confirms the unusually derived nature of zebrafish Eve1 (see Fig. 3). Zebrafish Eve1 is neither an Evx1 or Evx2 gene, and is probably a result of the hypothesized extra genome duplications that occurred in the teleost lineage within the vertebrates (Amores *et al.*, 1998). *Eve1* still however retains elements to its expression that are a subset of the canonical Evx domains, as described below (Joly *et al.*, 1993).

A Pan-Bilaterian Role for Evx: Posterior Patterning of the Gastrula?

Evx is expressed, and functions, during gastrulation in all triploblasts examined to date (Fig. 6 and Ruiz i Altaba and Melton, 1989; Patel *et al.*, 1992; Joly *et al.*, 1993; Patel *et al.*, 1994; Spyropoulos and Capecchi, 1994; Ahringer, 1996). In lower insects, nematodes and vertebrates Evx genes are expressed at the posterior terminus, predominantly in the mesoderm, but also in the ectoderm (Dush and Martin, 1992; Patel *et al.*, 1992; Joly *et al.*, 1993; Dollé *et al.*, 1994; Ahringer, 1996). *AmphiEvxA* fits this pattern, being expressed at the edge of the blastopore, which is at the posterior of the embryo.

Evx genes also show a dorsal–ventral restriction in nematodes and vertebrates. This restriction may be obscured in grasshoppers due to their gastrulation progressing from a flat blastula, but could be mechanistically related to the later segmental dorsal mesoderm expression (Patel *et al.*, 1992). The orientation of this restriction is consistent with the hypothesis of an inversion of the dorsal–ventral axis between protostomes and chordates (Arendt and Nübler-Jung, 1994), in that in nematodes *vab7* is expressed more dorsally than ventrally, whilst the converse is true in vertebrates: Evx genes are expressed more prominently on the ventral side (Dush and Martin, 1992; Joly *et al.*, 1993; Ahringer, 1996). *AmphiEvxA* fits the chordate pattern, being most widely expressed on the ventral side, and not being detected on the dorsal side (Fig. 5).

Evx is one of the best conserved of the Hox-like genes between diploblasts and bilaterians (Miller and Miles, 1993): the *Acropora* homeodomain is 80+% similar to those of triploblasts. Since diploblasts lack an organised CNS, which is the other pan-bilaterian site of Evx expression (see below), the most probable reason for this sequence constraint and similarity within the eumetazoa might be a conserved role in gastrulation. Interestingly the Cnidaria are hypothesised to be representative of the Gastraea stage of animal evolution (summarised in Nielsen, 1995). If indeed a cnidarian is comparable to a triploblast gastrula, as

proposed in the Gastraea hypothesis, then one might expect cnidarian Evx to play a prominent role in cnidarian body patterning which may even be homologous to the role of Evx in triploblast gastrulation, accounting for the strong sequence constraint.

The striking seven-stripe Pair-Rule pattern in *Drosophila* is almost certainly derived, as are its variations in other higher insects (Macdonald *et al.,* 1986; Patel *et al.,* 1992; Patel *et al.,* 1994). The segmental neural expression of *AmphiEvxA*, which arises after the somites have formed, shows that, in amphioxus Evx is responding to segmental regulation, rather than imparting segmentation as it does in *Drosophila*.

Hox Linkage of Evx

In vertebrates the Evx genes are linked to the Hox clusters. Mouse *Evx2* is so close to the Hox genes that it has come under their regulatory influence (Dollé *et al.,* 1994), with the early gastrulation expression being repressed so that *Evx2* follows the temporal colinearity of the Hox genes. Zebrafish *Evx2* is slightly further away from the Hox cluster and is weakly expressed in the tailbud. This slightly larger distance from the Hox cluster may allow zebrafish *Evx2* to escape some of the repression from the Hox cluster (Sordino *et al.,* 1996).

Whilst we have not yet established whether the AmphiEvx genes are linked to the amphioxus Hox gene cluster or not, they must be at least 85kb away from the posterior-most Hox gene so far isolated, *AmphiHox14* (Ferrier *et al.,* 2000; C.M. and J.G-F. unpublished data). The clear expression of *AmphiEvxA* during gastrulation suggests that, following the reasoning of Dollé et al. (1994), if it is indeed linked to the Hox cluster, then it is not tightly linked as it does not seem to follow the presumed temporal colinearity of the amphioxus Hox cluster (demonstrated up to Hox4; Wada *et al.,* 1999). Lack of tight linkage to the Hox gene cluster and early gastrulation expression of Evx is also apparent in *Drosophila*, beetles, grasshoppers and nematodes (Macdonald *et al.,* 1986; Patel *et al.,* 1992; Ferrier and Akam, 1996; Ahringer, 1996; Brown *et al.,* 1997). Separation of Evx from the Hox gene cluster is probably a derived condition, as Evx is linked to Hox-like genes in Cnidaria (Miller and Miles, 1993; reviewed in Ferrier and Holland, 2001). It is not clear whether there is any functional significance to the loose linkage of Evx to the Hox gene cluster, as opposed to the very tight linkage of mammalian Evx2 which clearly affects its regulation but is probably the derived condition. The position of some Evx genes next to some Hox gene clusters may simply reflect its evolutionary origin from the hypothesized tandem duplications that also generated the Hox genes (Lewis, 1978), within the context of the megaclusters of homeobox genes (Pollard and Holland, 2000).

Evx in Neurogenesis Is Ancient, whilst the Midbrain–Hindbrain Boundary (MHB) Expression Is a Vertebrate Innovation

Expression of *AmphiEvxA* is intricately regulated during neurogenesis. The distinctive pairs of stained cells at intervals of one somite length presumably reflect the involvement of the gene in the development of particular neural cells. In *Drosophila eve* is expressed in a particular subset of neurons (both motor and inter), and regulates the development of the aCC and RP2 cells (Doe *et al.,* 1988). In vertebrates Evx genes are expressed in restricted neural domains corresponding to the D1 neurons and V0 interneurons (Bastian and Gruss, 1990; Dollé *et al.,* 1994; Pierani *et al.,* 1999). In *C.elegans vab7* is expressed in a couple of neurons, and the mutant worms have a behavioural phenotype which presumably relates to defects in these cells (Ahringer, 1996). It is not clear what type of neural cell *AmphiEvxA* is being expressed in, as the descriptive work on the amphioxus CNS dealt with older larvae (Bone, 1959 and 1960; Lacalli *et al.,* 1994; Lacalli and Kelly, 1999).

In vertebrates the Evx genes are expressed around the MHB (Dollé *et al.,* 1994; Sordino *et al.,* 1996; Thaëron *et al.,* 2000), a region that acts as an organiser during brain development (reviewed in Wurst and Bally-Cuif, 2001). In mice the expression of *Evx1* relative to *Evx2* is slightly different at the MHB despite being virtually identical down the rest of the length of the neural tube (Dollé *et al.,* 1994). The evolution of such a distinct difference is suggestive of a function for these genes at this point in the neural tube. In amphioxus the homologous region of the neural tube is situated immediately behind the cerebral vesicle, level with the posterior region of somite 1, as judged from gene expression patterns and neural cell types (reviewed in Williams and Holland, 1998; S. Shimeld pers. comm.). *AmphiEvxA* is not detectably expressed in this region, the most anterior neural expression being the pair of cells level with the boundary between somites 4/5.

The lack of AmphiEvx MHB expression is analogous to the lack of *AmphiEn* MHB expression (Holland *et al.,* 1997). Engrailed is known to be involved in the development of the vertebrate MHB (reviewed in Wurst and Bally-Cuif, 2001), and consequently Williams and Holland (1998) hypothesized that the involvement of vertebrate engrailed genes in MHB patterning may be a derived role in vertebrates, and is perhaps related to the evolution of the organiser properties of this region. *AmphiEvxA* expression is consistent with this hypothesis, as Evx is another vertebrate MHB marker that is not expressed in the homologous region of the amphioxus neural tube. Further testing of this hypothesis requires investigation of more homologues of vertebrate MHB markers in amphioxus, and examination of their expression in the sister group to the cephalochordate-vertebrate clade, the urochordates, to confirm that it is not amphioxus that is the derived condition.

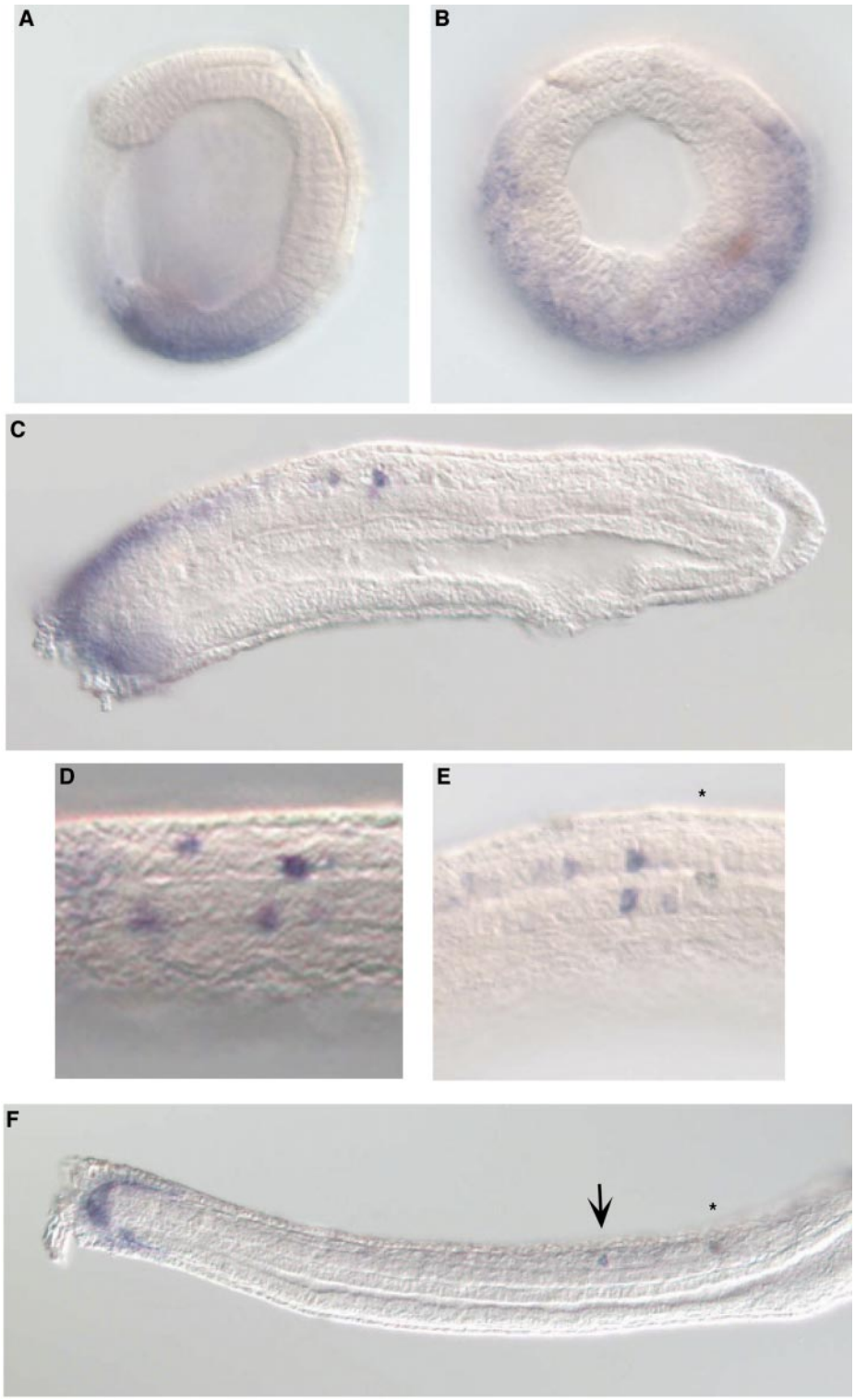


FIG. 5. Expression of *AmphiEvxA*. Anterior is to the right, except for (B), and dorsal is up in (A), (B), (C) and (F). (A) Lateral view of 7.5 h gastrula. The blastopore is on the left. Staining is on the ventral side, in the ectoderm.(B) Posterior view of 7.5 h gastrula. Staining is present ventrally and laterally. (C) Lateral view of a 22 h larva. The spots of staining midway along the larva are in the neural tube. At the posterior staining is in the endomesoderm and spreads forward in the CNS with a gradually reduced intensity. (D) Mid-dorsal view of a 22 h larva. The larva is rotated slightly off the perpendicular, such that the anterior-most stained cell appears to be on the midline, whereas in fact the

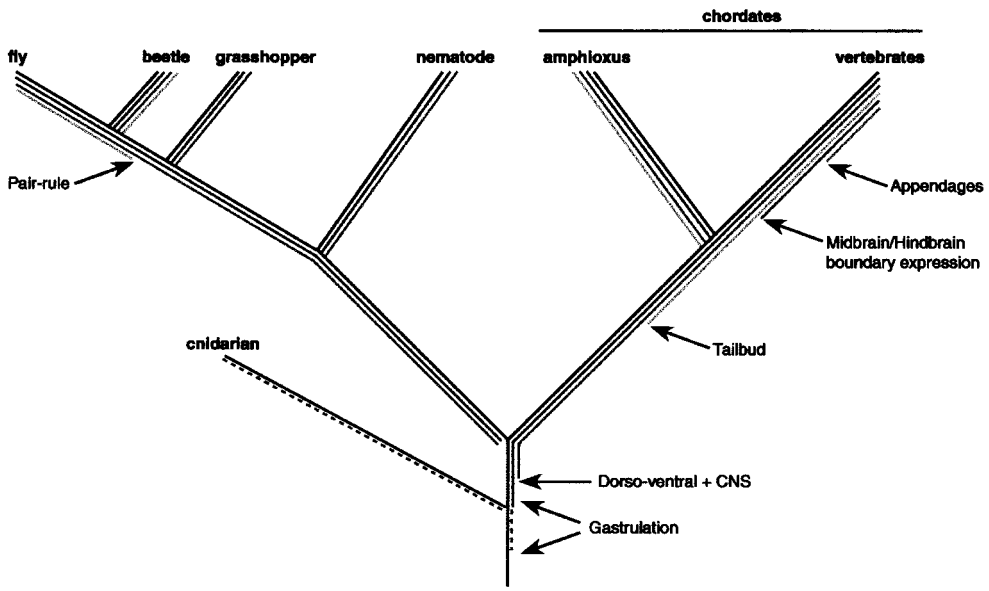


FIG. 6. Proposed scenario of the evolution of the expression of Evx genes. The phylogenetic tree is given by the black lines. The lines represent the occurrence of the named aspects of Evx gene expression. Tailbud expression is present in all chordates, whilst Midbrain-Hindbrain and appendage expression is restricted to vertebrates. Expression during gastrulation and CNS development, along with dorso-ventral restriction, are pan-triploblast features. The dotted "gastrulation" line denotes the lack of data on cnidarian Evx expression in gastrulation.

Tailbud Expression: Molecular Confirmation of a Chordate Synapomorphy

The postanal tail is a chordate synapomorphy (Gee, 1996), judging from comparative morphology. Does this synapomorphy hold up to molecular examination? The best piece of confirmatory evidence so far is *HrCdx*, the Cdx homologue of the Japanese ascidian *Halocynthia roretzi* (Katsuyama *et al.*, 1999). *HrCdx* is involved in tail formation, as is Cdx in vertebrates (Chawengsaksophak *et al.*, 1997; Isaacs *et al.*, 1998). The picture is complicated slightly however, by the ectodermal location of *HrCdx* expression and function in contrast to the mesendodermal role of Cdx in vertebrates. This may be attributable to the derived or degenerate nature of the ascidian tail endoderm. Amphioxus tail formation is probably not as derived as that of ascidians, and may provide a closer picture of how the ancestral vertebrate tailbud developed.

Vertebrate Evx genes are known to be involved in tailbud development (Beck and Slack, 1999). Here we show that *AmphiEvxA* is expressed during tailbud development, in the mesendoderm, as expected if it is functioning in a homologous way to vertebrates. *AmphiCdx* and *AmphiBra* expression during tailbud development (Holland *et al.*, 1995; Brooke *et al.*, 1998; and D.E.K.F and J.G-F unpublished data) illustrate further examples of genes whose vertebrate homologues function in this process. Thus in molecular developmental as well as morphological terms the amphioxus tailbud is homologous with that of vertebrates.

CONCLUSION

Amphioxus possesses two Evx genes, one of which, *AmphiEvxA*, is archetypal with respect to the vertebrate Evx1 and 2 genes, whilst the second, *AmphiEvxB*, is a gene with very derived sequence and expression and most probably is the result of a cephalochordate-specific duplication. AmphiEvxA sequence provides a basis on which one can distinguish the defining residues of the two vertebrate Evx

cells of each stained pair lie equidistant from the midline. The anterior cell pair are level with the boundary between somites 5 and 6, and the next cell pair are one somite further back and lie further apart than the somite 5/6 cell pair. (E) A mid-dorsal view of a slightly older larva than in (D). The somite 5/6 cell pair remain strongly stained whilst the posterior cell pair fade. The pigment spot lies at the level of the boundary between somites 4 and 5 (asterisk). (F) Lateral view of a 48 h larva. Staining is detected in the posterior endomesoderm and spreads slightly anterior in the CNS on the dorsal side of the larva, and anterior to the anal opening on the ventral side. The arrow marks a single stained cell in the CNS at the level of the boundary between somites 5 and 6. The asterisk marks the pigment spot.

families, which may help to focus future functional work to discover how Evx1 and 2 proteins carry out their different functions in combination with their differential expression. The expression of *AmphiEvxA* allows us to distinguish aspects of Evx regulation that are pan-triploblast (gastrulation, neurogenic and dorsal–ventral) from those expression domains that evolved within the chordates (tailbud) and those domains that are vertebrate-specific (MHB) (Fig. 6). *AmphiEvxA* expression is consistent with the hypothesis that the dorsal–ventral axis has been inverted between protostomes and chordates. It also distinguishes the post-anal tail as not only a morphological chordate synapomorphy, but also as a molecular one, and is consistent with the elaboration of the MHB (and possibly its organiser function) not evolving until the origin of the vertebrates by cooption of such genes as engrailed and Evx.

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