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The evolution of paired appendages in vertebrates: T-box genes in the zebrafish

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Abstract The presence of two sets of paired appendages is one of the defining features of jawed vertebrates. We are interested in identifying genetic systems that could have been responsible for the origin of the first set of such appendages, for their subsequent duplication at a different axial level, and/or for the generation of their distinct identities. It has been hypothesized that four genes of the T-box gene family (*Tbx2–Tbx5*) played important roles in the course of vertebrate limb evolution. To test this idea, we characterized the orthologs of tetrapod limb-expressed T-box genes from a teleost, *Danio rerio*. Here we report isolation of three of these genes, *tbx2*, *tbx4*, and *tbx5*. We found that their expression patterns are remarkably similar to those of their tetrapod counterparts. In particular, expression of *tbx5* and *tbx4* is restricted to pectoral and pelvic fin buds, respectively, while *tbx2* can be detected at the anterior and posterior margins of the outgrowing fin buds. This, in combination with conserved expression patterns in other tissues, suggests that the last common ancestor of teleosts and tetrapods possessed all four of these limb-expressed T-box genes (*Tbx2–Tbx5*), and that these genes had already acquired, and have subsequently maintained, their gene-specific functions. Furthermore, this evidence provides molecular support for the notion that teleost pectoral and pelvic fins and tetrapod fore- and hindlimbs, respectively, are homologous structures, as suggested by comparative morphological analyses.

Key words T-box genes · Zebrafish · Fins · Evolution · Gene duplication

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Introduction

The basic body plan of jawed vertebrates (gnathostomes) includes two sets of paired appendages. Although appendicular morphology has been dramatically modified in the course of evolution (compare the wing of a bird to a human arm), homologous relationships can be unequivocally ascertained between these structures by a comparative morphological approach. Indeed, the original formulation of the concept of "homology" by Owen stems from the comparative study of vertebrate limbs (for review see Coates 1994). Location of each set of appendages along the anterior-posterior axis of a gnathostome is relatively conserved – one at the pectoral, another at the pelvic level. Moreover, the pectoral and pelvic appendages are homologous to each other. This notion, known as serial homology, is supported by comparisons of anatomical and molecular markers (Shubin et al. 1997).

What is known about the evolutionary origin of paired appendages in vertebrates? Comparative anatomical analyses of extant chordates and palaeontological evidence are the two primary sources of information used to make inferences regarding the origin of vertebrate limbs. It is currently thought that both the closest chordate relatives of vertebrates, cephalochordates (such as amphioxus), and extant jawless fish (hagfish and lamprey), display an evolutionarily primitive limbless condition. The advent of paired appendages is first seen in the fossilized remains of osteostracans, an extinct group of jawless fish, as a single set of fins at the level of the pectoral girdle (Forey and Janvier 1993). The first fossils of jawed vertebrates are characterized by the addition of a second set of paired fins at the pelvic level (Carroll 1988). This implies that forelimbs are homologous to hindlimbs across all gnathostomes including chondrichthyans (sharks, rays, chimaeras) and osteichthyans or bony fish. The latter group is comprised of actinopterygians or ray-finned fish (of which teleosts are a subgroup) and sarcopterygians consisting of lobe-finned fish and tetrapods (Metscher and Ahlberg 1999). Recent molecular clock estimates (Kumar and Hedges 1998)

place the origin of extant agnathans at around 560 million years ago (MYA) and the split within gnathostomes between cartilaginous and bony fish at around 530 MYA. These dates imply that evolution of an anterior set of paired fins and its subsequent duplication to produce a set of posterior fins must have happened within a short span of geological time.

Is it possible to infer specific molecular events likely to have been responsible for the transformation of the body plan that produced a creature with two sets of paired appendages from the one with a single pair? The genes likely to have played a part in this transition may be the ones that now play important roles in limb development and establishment of limb identity. Certain members of the T-box gene family appear to be strong candidates for this role because of their functions in limb development and the timing of gene duplications within the family.

The T-box genes, expressed in complex spatiotemporal patterns during development, are transcription factors that bind DNA in a sequence-specific manner (Papaioannou and Silver 1998). Four of these genes (*Tbx2–Tbx5*) form a natural clade within the family, being more closely related to each other than to any other genes (Agulnik et al. 1996). The first important insight into the possible function of these genes was provided by Gibson-Brown et al. (1996), who demonstrated that while mouse *Tbx5* is expressed exclusively in the forelimbs, *Tbx4* is detected almost exclusively in the hindlimbs. It was hypothesized therefore that these two genes are involved in specifying limb identity during embryogenesis. Recent functional analyses of T-box genes in the chick (Gibson-Brown et al. 1998a; Isaac et al. 1998; Logan et al. 1998; Ohuchi et al. 1998) have reinforced the idea that *Tbx5* and *Tbx4* are likely to be responsible for determination of limb identity. These genes are expressed in lateral plate mesoderm throughout the fore- and hindlimb fields, respectively, prior to the initiation of limb bud outgrowth. This expression is retained in leg-to-wing and wing-to-leg mesenchymal tissue grafts in chicken embryos, consistent with the previously reported retention of graft identity upon such transplantation (Gibson-Brown et al. 1998a; Isaac et al. 1998). Also, in ectopic limbs induced by the application of fibroblast growth factor-soaked beads, expression of T-box genes was in direct correlation with axial level and future identity – more rostral limbs expressed *Tbx5* and developed as wing-like mosaic limbs, while more caudal limbs expressed *Tbx4* and developed as leg-like mosaic limbs (Gibson-Brown et al. 1998a; Isaac et al. 1998; Logan et al. 1998; Ohuchi et al. 1998). Furthermore, recent studies by Takeuchi et al. (1999) and Rodriguez-Esteban et al. (1999) report that ectopic expression of *Tbx4* and *Tbx5* genes in chicken limb buds results in induction of alternative identities, i.e., wing-like in the leg and leg-like in the wing. These results were interpreted as an indication of a critical role played by these two genes in establishment of limb identity. Independently, Simon et al. (1997) have shown that the newt *Tbx5* (Nv *Tbox1*) gene is expressed only during forelimb (as opposed to hindlimb) regeneration. An insight into the function of *Tbx2* was provided by Gibson-Brown et al. (1998a) who have implicated this gene in anterior-posterior patterning of chick limb buds by showing that it may be a short-range target of *sonic hedgehog*.

Some inferences regarding the functions of T-box genes during limb development can be made from analysis of mutations in the human *TBX3* and *TBX5* genes. The former cause ulnar-mammary syndrome in patients heterozygous for an apparent loss-of-function allele (Bamshad et al. 1997). A wide variety of forelimb malformations, which are characteristic of this condition, indicate a critical role played by *TBX3* in anterior-posterior and dorsal-ventral (Bamshad et al. 1995) patterning of the forelimb. Mutations in the human *TBX5* gene cause Holt-Oram syndrome (Basson et al. 1997; Li et al. 1997). Limb defects in heterozygous carriers range from subtle hand abnormalities (anterior aspects are predominantly affected) to phocomelia (severe limb shortening), revealing an important function of this gene in the process of forelimb patterning. Taken together, embryological manipulations and mutant phenotypes highlight distinct and essential roles played by *Tbx2–Tbx5* genes during limb development in tetrapods.

Since *Tbx4* and *Tbx5* genes play important roles in the determination of limb identity and thus are likely important players in vertebrate limb evolution, it would be highly instructive to establish the date of their duplication, for it was after such a duplication that they were able to evolve their gene-specific functions. It has been shown that the mouse *Tbx2* and *Tbx4* genes are tightly linked in a cluster on chromosome 11, while *Tbx3* and *Tbx5* are clustered on chromosome 5. This, considered together with their phylogenetic relationships, has been used to propose a model for their evolution through cluster formation and subsequent duplication (Agulnik et al. 1996). We have further analyzed these clusters and concluded that a large-scale chromosomal duplication event was responsible for their origin (Ruvinsky and Silver 1997). A phylogenetic analysis of several closely linked genes suggested that this duplication event (hence the divergence between *Tbx2* and *Tbx3* as well as that of *Tbx4* and *Tbx5*) was likely to have occurred prior to the divergence of actinopterygians and sarcopterygians. This time estimate strengthened a tentative connection between the T-box gene duplication and the duplication of a set of paired vertebrate limbs.

We decided to isolate *tbx2–tbx5* genes from a model teleost, *Danio rerio*, to explicitly address the following questions. Did the T-box cluster duplication had already occur prior to the divergence of actinopterygians and sarcopterygians? If so, do teleosts employ these genes in the same ways as tetrapods?

Here we report the identification and expression patterns of zebrafish *tbx2*, *tbx4*, and *tbx5* genes. Their phylogenetic analysis firmly establishes the idea that the T-box cluster duplication had already occurred in the most recent common ancestor of actinopterygians and sarc-

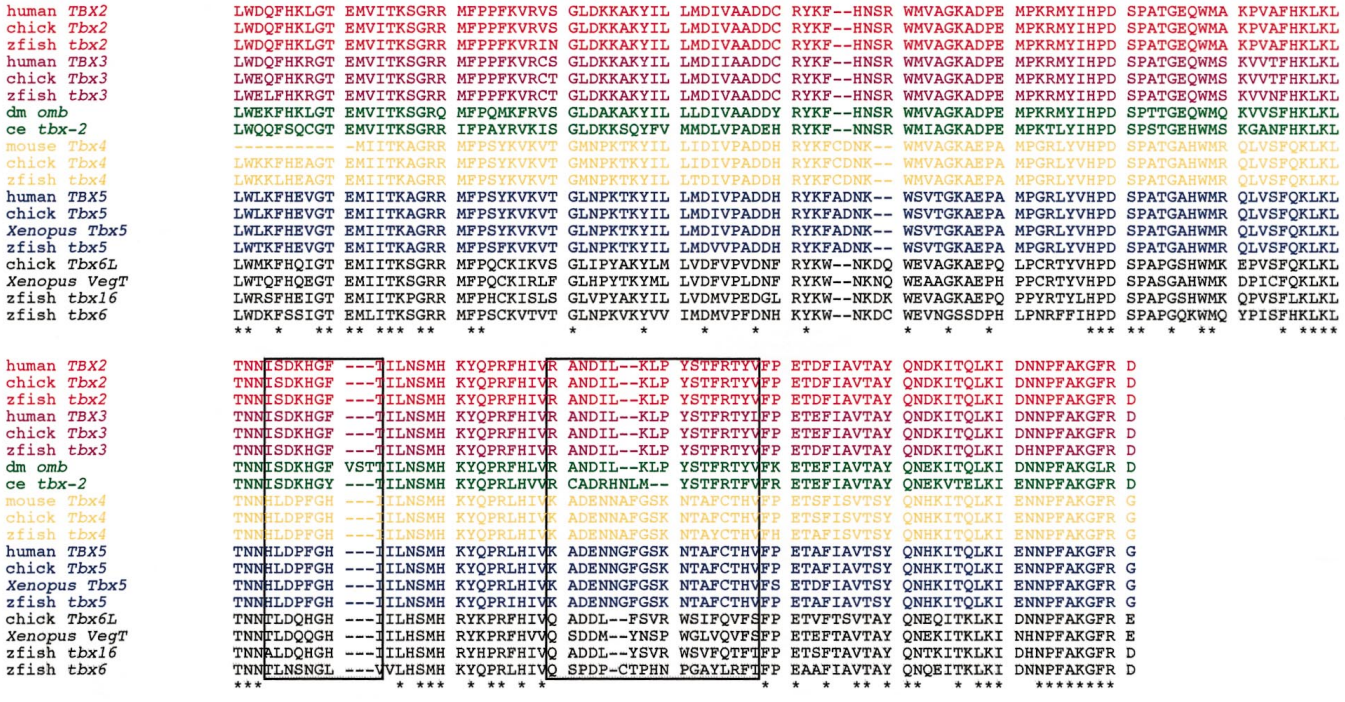


Fig. 1 An alignment of amino acid sequences of the T-box regions from the genes of the *Tbx2* subfamily and four genes of the *Tbx6* subfamily (subfamilies are defined in Papaioannou and Silver 1998). *Dashes indicate* alignment gaps and missing data (in mouse *Tbx4*); *asterisks underneath alignment indicate* amino acids that are conserved throughout the entire set; *boxed areas* were excluded from the phylogenetic analysis due to extensive sequence and length variation. *dm omb D. melanogaster optomotor blind*; *ce tbx-2 C. elegans tbx-2*

opterygians. Their expression patterns, strikingly similar to those of their tetrapod orthologs, support the idea of conserved functions and confirm a morphology-based notion of homology between teleost fins and tetrapod limbs.

Materials and methods

Isolation of cDNAs corresponding to zebrafish *tbx2*, *tbx4*, and *tbx5* genes

A cDNA clone of *tbx2* was isolated from a late gastrulation-stage embryonic λZAP II cDNA library (gift of D.J. Grunwald) by low-stringency hybridization (washes conducted at 50°C in 1×SSC) with a cocktail of mouse T-box gene probes (as in Ruvinsky et al. 1998). Polymerase chain reaction (PCR) with degenerate T-box primers was used to amplify fragments of *tbx4* and *tbx5* genes from zebrafish genomic DNA. PCR with gene-specific primers based on the sequence of these genomic fragments was used to recover the 5' ends of the *tbx4* and *tbx5* cDNAs (as detailed by Ruvinsky et al. 1998) from a 30- to 33-h postfertilization (hpf) embryonic λgt11 cDNA library (gift of K. Zinn). These 5' end fragments were used to screen this λgt11 library at high stringency (washed at 65°C in 0.1×SSC) and the recovered inserts were subcloned into pGEM-T (Promega) or pCR2.1 (Invitrogen). All clones were sequenced with an automated ABI sequencer. The sequences reported here were deposited in GenBank with the following accession numbers: *tbx2* (AF 179405), *tbx4* (AF 179406), and *tbx5* (AF 179407).

Sequence analysis

The amino acid sequences of the T-box regions were aligned by eye. Construction of the phylogenetic tree using a neighbor-joining algorithm and testing of the statistical reliability of its topology were carried out as previously described (Ruvinsky et al. 1998).

Examination of zebrafish T-box gene expression patterns

Zebrafish embryos were harvested from natural spawning and staged according to Kimmel et al. (1995). Embryos older than 24 hpf were grown in 0.003% phenylthiourea solution (Sigma) to inhibit pigment development. We used standard procedures for in situ hybridization, microscopy, and photography as reported previously (Ruvinsky et al. 1998). Full-length cDNA clones including 5' and 3' untranslated regions were used to generate digoxigenin-labeled *tbx2* and *tbx4* riboprobes of length 2.9 and 2.3 kb, respectively. A 1 kb cDNA fragment containing the 5' untranslated region, the coding region of the N-terminus of the protein and part of the T-box as well as a 2.1-kb full-length clone were used to synthesize digoxigenin-labeled *tbx5* riboprobes; these produced identical hybridization patterns.

Results

Identification of zebrafish *tbx2*, *tbx4*, and *tbx5* genes

One of the clones uncovered in a screen for T-box genes from a late gastrula cDNA library (Ruvinsky et al. 1998) contained an open reading frame which was likely a zebrafish ortholog of tetrapod *Tbx2* genes, since the initial BLAST analysis indicated that these were the highest scoring matches. Since cDNAs corresponding to *tbx4* or *tbx5* genes were not obtained in this screen, PCR amplification of zebrafish genomic DNA with degenerate T-box based primers was used to obtain *tbx4* and *tbx5* gene

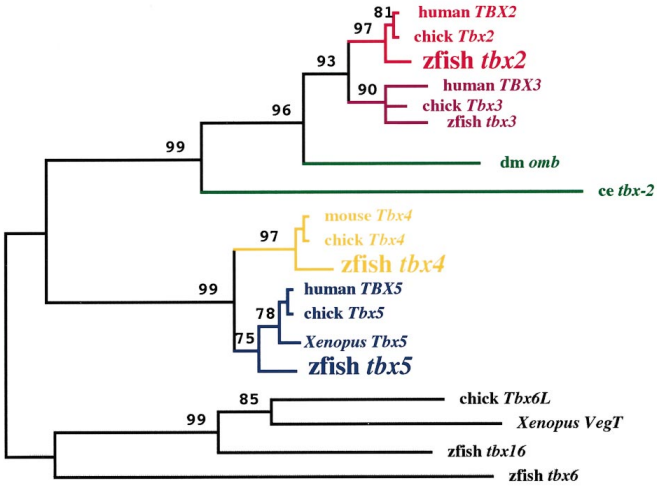


Fig. 2 A neighbor-joining tree reconstructing the phylogenetic relationships of the sequences shown in Fig. 1. The same coloring scheme is employed. *Numbers above the nodes* are confidence probability values (99 corresponding to P=0.99) and are computed as described in the text (only those above 0.75 are shown, others should be deemed unreliable and branching patterns should be considered unresolved). *Tbx2–Tbx5* genes comprise a group of most closely related paralogs (Agulnik et al. 1996). The tree is rooted using the four genes of the *Tbx6* subfamily as an outgroup based on the previously established relationships between the subfamilies of the T-box gene family (Papaioannou and Silver 1998)

fragments. Subsequent screening of a 33-hpf embryonic cDNA library resulted in the isolation of two clones: one was identified as a *tbx4*-like gene; the other was preliminarily designated *tbx5*.

Since for each gene the initiating methionine is preceded and the termination codon is followed by sequence containing multiple stop codons in all reading frames, the clones appear to represent full-length cDNAs. BLAST analyses also revealed clusters of residues with high sequence similarity between the zebrafish genes and their presumed tetrapod orthologs throughout the open reading frames (data not shown). Such extensive matches outside the T-box further suggest that the newly found zebrafish genes are orthologs of tetrapod *Tbx2*, *Tbx4*, and *Tbx5* genes.

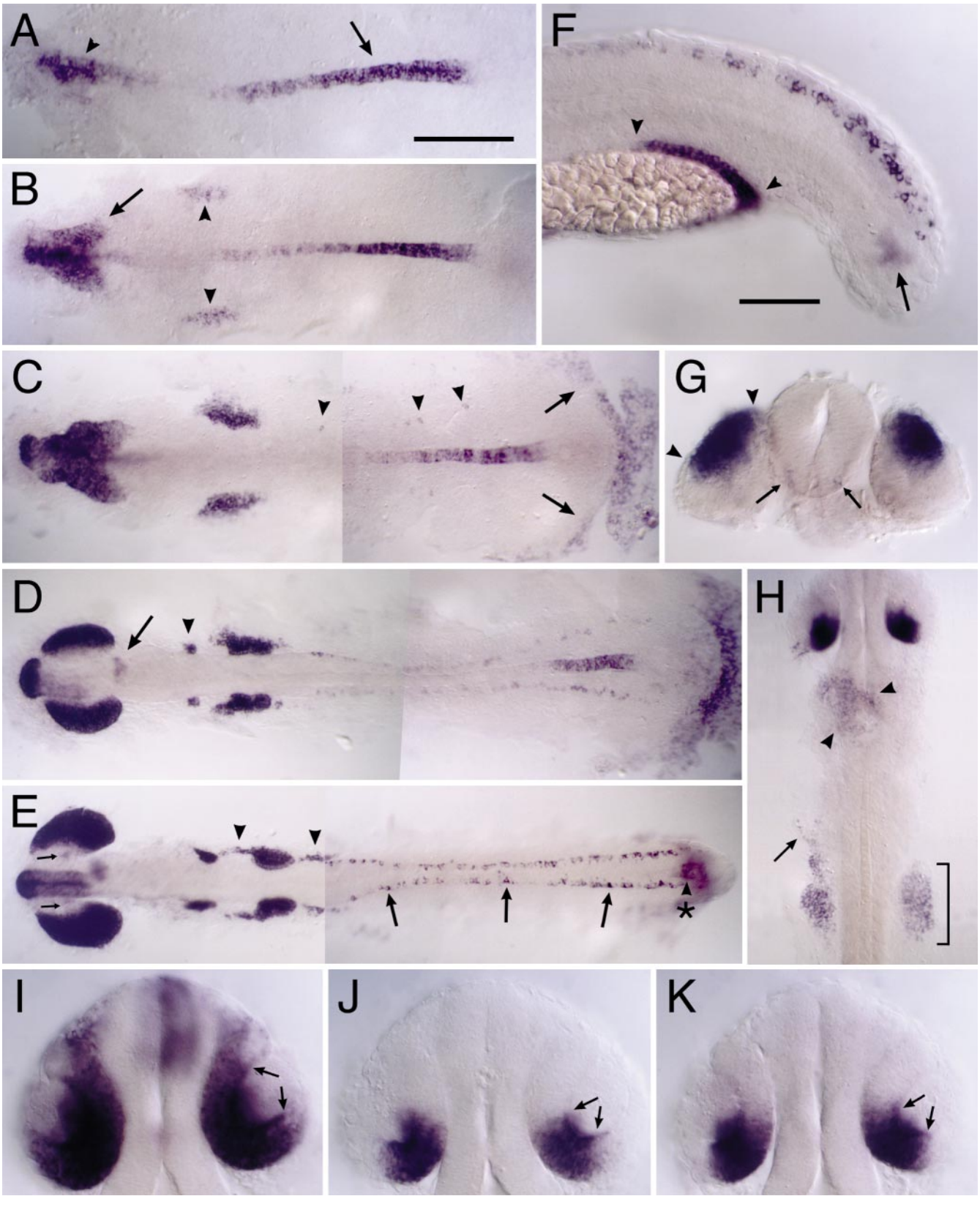
To confirm the preliminary orthology assignments we conducted a phylogenetic analysis of the natural clade consisting of currently available *Tbx2–Tbx5* genes and rooted it with several outgroup sequences. To this end we generated an alignment of their T-box regions as shown in Fig. 1. Sequences outside this domain cannot be used for phylogenetic analyses, as they cannot be reliably aligned due to a high degree of sequence and length variation between paralogs. The resulting tree (Fig. 2) strongly supports the notion that we have uncovered zebrafish orthologs of the tetrapod *Tbx2*, *Tbx4*, and *Tbx5* genes. This conclusion is borne out by the fact that each gene clusters with its presumed tetrapod counterparts (e.g., zebrafish *tbx2* with human *TBX2* and chick *Tbx2*) to the exclusion of other T-box genes (such as *Tbx3*, *Tbx4*, and *Tbx5* in the case of zebrafish *tbx2*). These clusters receive a high degree of statistical support (confidence probability values P=0.97 for *tbx2*, P=0.90 for *tbx3*, P=0.97 for *tbx4*, and P=0.75 for *tbx5*). Having established the identity of these genes, we examined their expression patterns during zebrafish embryogenesis.

Expression of *tbx2*, *tbx4*, and *tbx5* in zebrafish embryos and larvae

The distribution of transcripts from zebrafish *tbx2*, *tbx4*, and *tbx5* genes was analyzed by whole-mount in situ hybridization from the onset of gastrulation (Fig. 3). We first focus on the expression of these genes in structures other than the developing appendages. This enables us to present gene expression patterns in a proper temporal context. Furthermore, these patterns underscore the fact that the orthologous genes are expressed in very similar patterns in teleosts and tetrapods in a variety of different tissues. We then turn to a detailed examination of *tbx2*, *tbx4*, and *tbx5* expression in the pectoral and pelvic appendages.

Expression of the zebrafish *tbx2* gene was observed in many tissues of ectodermal and mesodermal origin during embryogenesis (Fig. 3A–F, I). *tbx2* transcripts were

Fig. 3 Expression patterns of *tbx2* (**A**–**F**, **I**), *tbx4* (**G**,**J**), and *tbx5* (**H**,**K**) in the zebrafish embryo. Embryos were staged according to Kimmel et al. (1995) and are shown dissected from the yolk cell and flat mounted with anterior *to the left* in **A**–**F**, and with anterior *to the top* in **H**–**K**. *Scale bars* 250 µm in **A**–**E**, **H**, and 100 µm in **F**,**G**, **I**–**K**. *tbx2* expression at bud stage (10 hpf), in the prospective ventral prosencephalon (*arrowhead*), and in the notochord (*arrow*). **B** *tbx2* expression at the 3-somite stage (11 hpf), in the otic (*arrowheads*) and optic primordia (*arrow*). **C** *tbx2* expression at the 6-somite stage (12 hpf) in lateral neurons of the prospective spinal cord (*arrowheads*) and in the ventral tissue surrounding the tail bud (*arrows*). **D** *tbx2* expression at the 10-somite stage (14 hpf) in the primordium of the trigeminal sensory ganglia (*arrowhead*) and in a small cluster of neurons in the posterior dorsal diencephalon (*arrow*). **E** *tbx2* expression at the 16-somite stage (17 hpf) in the primordium of the anterior and posterior lateral line ganglia (*arrowheads*) and ventral diencephalic neurons (*small arrows*). Note the increased number of cells expressing *tbx2* in dorsolateral positions in the spinal cord, corresponding to the Rohon-Beard sensory neurons (*arrows*). *tbx2* expression is restricted to the very caudal tip of the notochord, marking the most newly formed axial mesoderm (*arrowhead with asterisk*). **F** *tbx2* expression in the caudal portion of the embryo at 22 hpf is restricted to the newly differentiating Rohon-Beard neurons in the dorsal aspect of the tail, and the distal tip of the notochord (*arrow*). By this stage the ventral domain of *tbx2* expression has condensed around the yolk extension to form the bilateral pronephric ducts (*arrowheads*). **G** *tbx4* expression in the dorsoposterior aspect of the retinal neuroepithelium (*arrowheads*) at 24 hpf (shown in optical cross-section at the level of the posterior diencephalon) and expression in ventral diencephalic neurons (*arrows*). **H** *tbx5* expression at 22 hpf in the eyes, the heart primordium (*arrowheads*) and in the pectoral fin bud primordium (*bracket*). A slender cord of *tbx5* expressing cells is often detected extending anteriorly from the larger domain (*arrow*). **I**–**K** Comparison of the expression of *tbx2*, *tbx4*, and *tbx5* in the eye at 22 hpf. *tbx2* is broadly expressed (*arrows*) in most of the neuroepithelium of the retina (**I**). In contrast, *tbx4* (**J**) and *tbx5* (**K**) are detected in narrower, overlapping domains in the central retina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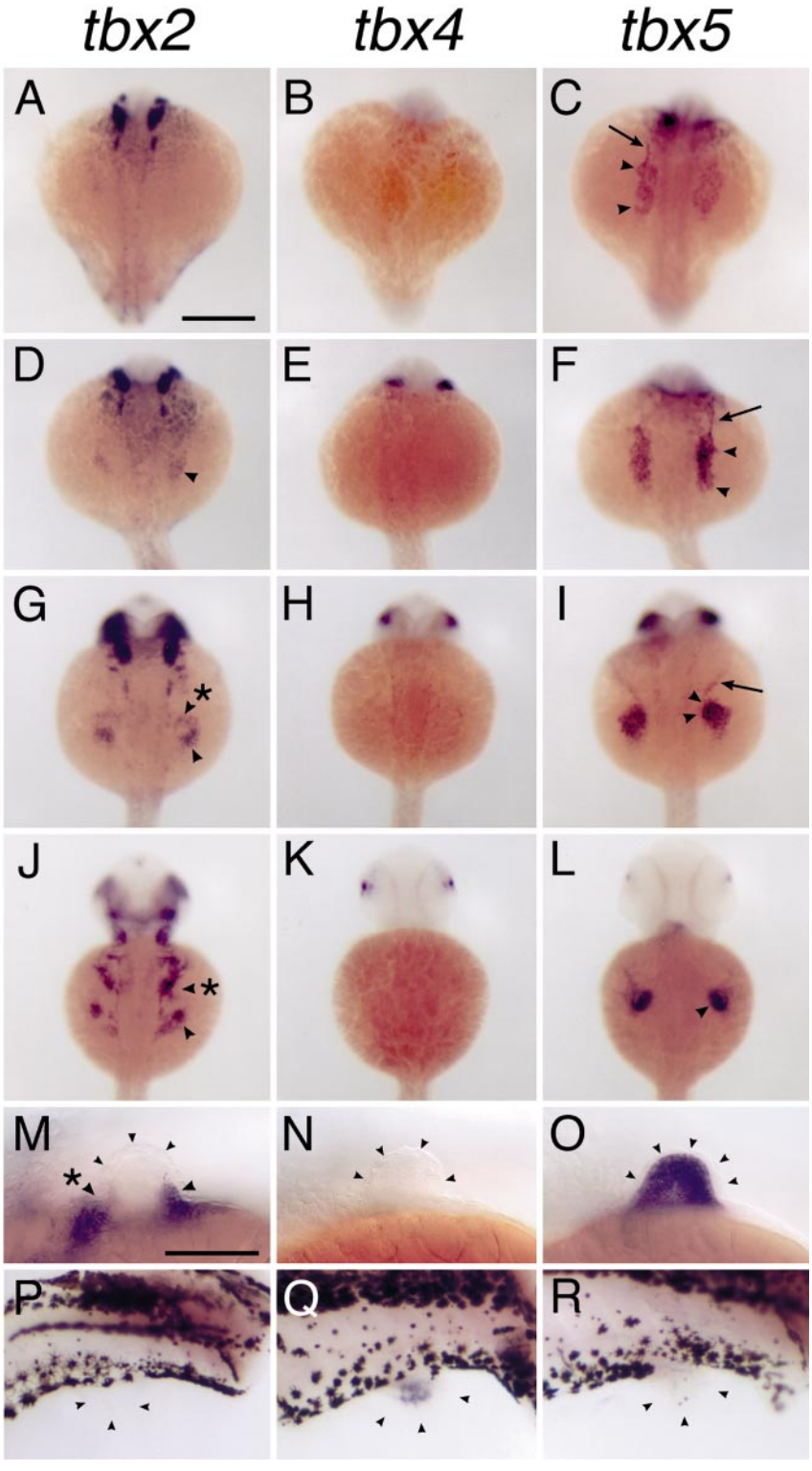

Fig. 4A–R Appendicular expression patterns of *tbx2*, *tbx4*, and *tbx5* in the zebrafish. Embryos and larvae are shown in whole mount, viewed from the dorsal aspect in **A**–**L** with anterior *to the top*. Pectoral fins in **M**–**O** and pelvic fins in **P**–**R** are shown in lateral view with anterior *to the left*. **A**,**D**,**G**,**J**,**M**,**P** Detailed expression of *tbx2*. **B**,**E**,**H**,**K**,**N**,**Q** Detailed expression of *tbx4*. **C**,**F**,**I**,**L**,**O**,**R** Detailed expression of *tbx5*. *Scale bars* 250 μm in **A**–**L, P**–**R**, and 100 μm in **M**–**O**. **A**–**C** Expression patterns at 17 hpf. *Arrowheads* (**C**) earliest expression of *tbx5* in the pectoral fin field; *arrow* anterior extension. **D**–**F** Expression patterns at 22 hpf. **D** *Arrowhead* earliest *tbx2* expression in the fin field, **F** *Arrowhead* increased levels of *tbx5* message. **G**–**I** Expression patterns at 25 hpf. The *tbx2* expression domain has separated into anterior (*arrowhead with asterisk*) and posterior (*arrowhead*) compartments (**G**) and *tbx5* expression is concentrated in the mesenchyma of the outgrowing fin bud (**I**, notations as above). **J**–**L** Expression patterns at 42 hpf; maintenance of separated *tbx2* expression domains is accompanied by expression in the flank extending anteriorly and posteriorly to the fin bud (**J**), and *tbx5* expression increases (*arrowhead*, **L**). **M**–**O** High magnification views of expression patterns in the pectoral fin bud at 42 hpf. *Small arrowheads* distal edge of the apical fold; no expression of these genes can be detected in the apical fold. **M** *tbx2* expression in the fin bud mesenchyma is extended further along the posterior (*arrowhead*) than the anterior (*arrowhead with asterisk*) margin. **N** *tbx4* is not expressed in the pectoral fin bud. **O** *tbx5* expression is found throughout the fin bud mesenchyma but at lower levels in the central, proximal portion. **P**–**R** Expression patterns in the pelvic fin at 4 wpf. *Small arrowheads* margin of the developing fin; expression of *tbx4* (**Q**) but not *tbx2* (**P**) or *tbx5* (**R**) is detected in the mesenchyma at the base of the fin

first detected at bud stage in the ventral prosencephalon and along the length of the notochord (Fig. 3A). As segmentation proceeded, *tbx2* was expressed in the optic primordia, the otic placodes (Fig. 3B), two rows of dorsally placed neurons in the spinal cord (the Rohon-Beard sensory neurons), and diffusely in cells over the yolk lying ventrally to the tail bud (Fig. 3C). Early in the segmentation period *tbx2* was expressed in a small cluster of cells in the dorsal diencephalon (likely the pineal primordium), the primordia of the trigeminal sensory ganglia and the posterior notochord (Fig. 3D). Midway through the segmentation period expression was also seen in the acoustic and lateral line sensory ganglia and was restricted to the most caudal region of the notochord

(Fig. 3E). At 22 hpf *tbx2* transcripts were present in the most posterior portion of the pronephric ducts (Fig. 3F). Examination of stages of development up to 3 days postfertilization (dpf) revealed additional *tbx2* expression in cranial nuclei, branchial arches, heart, and liver (data not shown). Recently Dheen et al. (1999) presented three zebrafish T-box genes, *tbx-a*, *tbx-b*, *tbx-c*, one of which (*tbx-c*) has an apparently identical sequence and expression pattern with *tbx2*.

In contrast, zebrafish *tbx4* and *tbx5* genes were expressed in highly restricted patterns during embryonic development. *tbx4* was expressed only in the eye, and in isolated neurons of the ventral diencephalon (Fig. 3G). *tbx5* transcripts were detected only in the eye, pectoral fin buds, and heart (Fig. 3H). *tbx5* expression in heart tissue was first detected as two bilateral stripes in the anterior lateral plate mesoderm at 16 hpf and was maintained as these primordia merged at the midline and subsequently went through heart tube and chamber formation, persisting until 3 dpf (Fig. 3H and data not shown).

tbx2, *tbx4*, and *tbx 5* expression was detected in nested domains in the developing neuroepithelium of the zebrafish retina. While *tbx2* expression was seen from 12 hpf throughout the optic primordia and lobes, *tbx4* and *tbx5* were first expressed in a restricted domain of the eye at 16 hpf. With the emergence of the lens primordium and formation of the optic cup, expression of *tbx2* in the eye became restricted to a dorsal-posterior domain (Fig. 3I). *tbx4* and *tbx 5* expression patterns were superimposable in a narrower section of the dorsal optic cup (Fig. 3J, K), such that cells expressing these genes also expressed *tbx2* and lay in the center of the *tbx2* expression domain. Expression of all three genes persisted in their respective domains until 2 dpf.

Combined, these expression data indicate a high degree of conservation between expression patterns of the zebrafish *tbx2*, *tbx4*, and *tbx5* genes and their tetrapod orthologs.

Expression of *tbx2*, *tbx4*, and *tbx5* in the developing fins of the zebrafish

Of greatest importance for testing hypotheses about a role for T-box genes in the evolution of paired appendages is the expression of these genes in the developing fins (Fig. 4). At 17 hpf (Fig. 4A–C), *tbx5* expression was detected in two bilaterally symmetrical cell sheets on the dorsal surface of the yolk cell at an axial level corresponding to somites 1–4 (Fig. 4C), consistent with expression in the larval pectoral fin field prior to the emergence of a morphologically evident fin bud (Grandel and Schulte-Merker 1998). The *tbx5* expression domain was comprised of approximately 100 cells in a rectangular patch, and a thin cord of cells that extended anteriorly from the main patch (arrow in Fig. 4C, see also Fig. 3H). By 22 hpf (Fig. 4D–F), *tbx2* was also expressed in a subset of these cells (compare Fig. 4D, F), being at highest levels in cells in the posterior aspect of this domain. At 25 hpf (Fig. 4G–I), the cells of the field have converged to form the fin bud, lying laterally to somites 1 and 2. While *tbx5* expression was strong throughout the mesenchyma of the fin bid (Fig. 4I), the *tbx2* expression domain was bipartite, with expressing cells confined to the anterior and posterior margins of the fin bud mesenchyma (Fig. 4G). By 42 hpf (Fig. 4J–O) the development of pectoral fin buds was well progressed; they possessed a differentiated apical fold, the structure thought to be equivalent to the tetrapod apical ectodermal ridge. At this time *tbx2* expression was detected in regions of mesenchyme extending along the flanks of the embryo (Fig. 4J, M), and *tbx5* expression intensified in the mesenchyme under the apical fold (Fig. 4L, O). Neither *tbx5* nor *tbx2* was expressed in the apical fold. *tbx5* expression in the mesenchyme of the fin buds was maintained until 4 dpf, whereas by 3 dpf *tbx2* could no longer be detected (data not shown). Importantly, *tbx4* was not expressed in the pectoral appendage at any of these stages (Fig. 4B, E, H, K, N).

The pelvic fin fields are formed at the level of the 9th and 10th myotomes at approximately 3 weeks postfertilization (wpf; Grandel and Schulte-Merker 1998). We were unable to detect any T-box gene expression at 3 wpf in this location (data not shown). By 4 wpf larvae had lost growth synchrony, and individuals in a clutch displayed a range of pelvic fin bud developmental stages. In 4-wpf clutches, strong expression of *tbx4* was detected in the mesenchyme of pelvic fin buds from the time of their eruption from the flanks lateral to the ventral fin fold (Fig. 4Q). This expression persisted throughout the growth of the buds and during the formation of actinotrychia (dermal fin rays). In contrast, *tbx5* and *tbx2* were not detected in any pelvic fin buds at this stage (Fig. 4P, R).

The gene expression patterns of *tbx2*, *tbx4*, and *tbx5* in the zebrafish pectoral and pelvic fins are strikingly similar to the expression patterns of their tetrapod orthologs in fore- and hindlimbs. These results indicate that the pattern of T-box gene expression in the developing appendages is conserved between teleosts and tetrapods.

Discussion

Our discovery and characterization of zebrafish *tbx2*, *tbx4*, and *tbx5* genes is important in two respects: it provides a more precise time frame for key events in the evolution of the vertebrate T-box genes, and it has implications for the evolution of T-box gene function in vertebrate limb development. Thus it allows us to refine a model seeking to connect the evolution of the T-box gene family to the evolution of the vertebrate body plan.

Establishing the timing of the T-box cluster duplication

It was shown by Agulnik et al. (1996) that mouse *Tbx2* is tightly linked to *Tbx4*, as is *Tbx3* to *Tbx5*. These map-

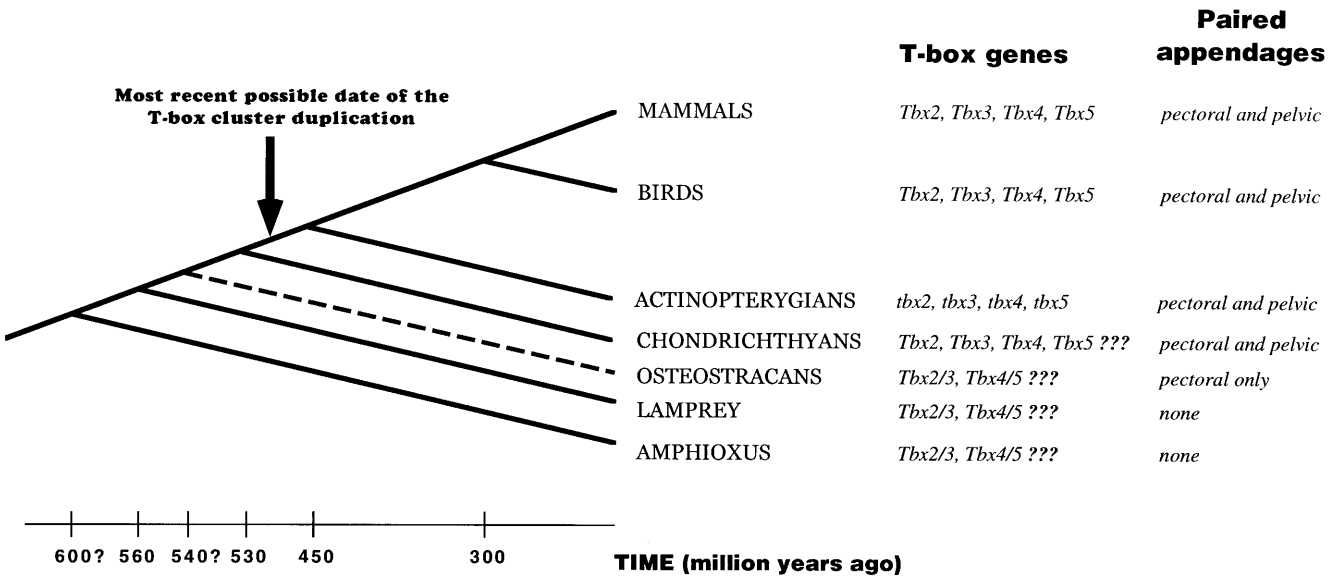

Fig. 5 A schematic representation of commonly accepted phylogenetic relationships of large groups of chordates showing correlation between the number of T-box genes (*Tbx2–Tbx5*) and the number of paired appendages. Time scale is after Kumar and Hedges (1998). *Dashed line to osteostracans* indicates extinct lineage; *question marks* indicate hypothesized dates and genes

ping data were interpreted in the light of the phylogenetic relationships of these genes (see Fig. 2) to mean that an original cluster comprised of *Tbx2/3* and *Tbx4/5* ancestor genes had undergone an en masse duplication to give rise to the two dispersed clusters seen in mammals today. This event allowed for the evolution of divergent functions between *Tbx2* and *Tbx3* as well as between *Tbx4* and *Tbx5*. The initial estimate of Agulnik et al. (1996) placed this duplication event between the divergence of protostomes from deuterostomes (over 600 MYA) and the divergence of mouse from human (around 100 MYA). Analysis of limb expression patterns of mouse *Tbx2* and *Tbx3* prompted Gibson-Brown et al. (1996) to suggest that these two genes (and also *Tbx4* and *Tbx5*) were likely to have duplicated before the split between actinopterygians and sarcopterygians. Ruvinsky and Silver (1997) independently arrived at the same conclusion by conducting a phylogenetic analysis of the genes found in large duplicated regions of the mouse genome surrounding the *Tbx2–Tbx5* containing gene clusters. However, to verify these conjectures it was essential to demonstrate the existence of true orthologs of these genes in an actinopterygian. Therefore our discovery of three of the four anticipated genes and a recent report of a zebrafish *tbx3* gene (Yonei-Tamura et al. 1999) firmly establish that the T-box cluster duplication had to have occurred prior to the split between actinopterygians and sarcopterygians around 450 MYA (Kumar and Hedges 1998), as shown in Fig. 5.

Evolution of the gene-specific functions of *Tbx2–Tbx5* genes

The presence of zebrafish *tbx2* transcript in eyes, ears, dorsal sensory neurons, pineal gland, trigeminal ganglia, pronephric ducts, heart, and pharyngeal arches (Fig. 3) indicates extensive conservation of expression with mouse and chick *Tbx2* genes (Chapman et al. 1996; Gibson-Brown et al. 1998b), suggesting that the *tbx2* ortholog in zebrafish has retained multiple gene-specific functions present in the last common ancestor of actinopterygians and sarcopterygians. In addition, the expression of *tbx5* in the zebrafish heart supports a similar claim for this gene. Expression of *tbx2* in the notochord is a unique aspect of the zebrafish gene. In the chick *Tbx4* and *Tbx5* but not *Tbx2* are expressed in the notochord (Gibson-Brown et al. 1998b) while in the mouse none of these appear to be expressed in the notochord (Chapman et al. 1996). Such discrepancy may be accounted for by the fact that the genome of the zebrafish has undergone a large-scale duplication, thus producing initially redundant copies of many genes (Amores et al. 1998; Prince et al. 1998). Indeed, Dheen et al. (1999) reported two very similar genes, *tbx-a* and *tbx-c*, which may represent the products of this duplication. While *tbx-a* is not detected in the notochord, presumably reflecting a conserved tetrapod condition, *tbx-c* (which is the same gene as our *tbx2*) is expressed in the notochord, perhaps indicating the acquisition of a novel function by a redundant gene copy.

The expression of *tbx2*, *tbx4*, and *tbx5* in nested domains in the neuroepithelium of the zebrafish retina suggests that they play a role in regionalizing this tissue. Moreover, combined with the restricted expression of *Tbx2* and *Tbx5* in the mouse and chick optic cup (Chapman et al. 1996; Gibson-Brown et al. 1998b) these data suggest that eye patterning was an ancestral function of the T-box genes studied here.

We have found that the zebrafish orthologs of tetrapod *Tbx2*, *Tbx4*, *Tbx5* genes are expressed during ap-

pendage development (Fig. 4) in spatiotemporal patterns that are remarkably similar to those reported for their mouse (Chapman et al. 1996; Gibson-Brown et al. 1996), chick (Gibson-Brown et al. 1998a; Isaac et al. 1998; Logan et al. 1998; Ohuchi et al. 1998), and newt (Simon et al. 1997) counterparts. *Tbx5* orthologs are expressed in the pectoral but not the pelvic appendages in both zebrafish and tetrapods, while the converse is true for *Tbx4* genes. Furthermore, *Tbx2* orthologs in both taxa are expressed in the anterior and posterior margins of the developing pectoral limb/fin bud, with an elevated, prolonged expression at the posterior margin. We note that *tbx2* could not be detected in the pelvic fin field or bud, as would have been predicted by the tetrapod *Tbx2* expression pattern. *tbx4* was not detectable in the pelvic fin bud until 4 wpf and required prolonged color development. Thus detection of low-level expression of *tbx2* that was transient, as is the case for the pectoral fins, may have been beyond the sensitivity of the in situ hybridization technique. Finally, the T-box genes studied here are not expressed in the apical fold/apical ectodermal ridge of the developing appendages. Thus the last common ancestor of actinopterygians and sarcopterygians must have possessed this same array of T-box genes and probably used them in the same way to specify and regulate the identity of two sets of paired appendages.

Deep homology in appendage development

Previous reports have indicated that basic mechanisms controlling limb formation are likely to be conserved among all asteichthyans. In particular, analyses of gene expression patterns during zebrafish fin development – *dlx* (Akimenko et al. 1994; Ellies et al. 1997), *engrailed* (Hatta et al. 1991), *hedgehog* (Krauss et al. 1993), *hox* (Sordino et al. 1995, 1996), and *msx* (Akimenko et al. 1995) – have revealed a remarkable degree of conservation with regards to expression patterns of orthologous genes in tetrapods. However, most of these studies (except for Sordino et al. 1995) examined only pectoral fin development, hence any inferences of homology are limited to this appendage. We demonstrated (Fig. 4) that *tbx4* and *tbx5*, the earliest markers of pelvic and pectoral limbs, have identical expression patterns between fish and tetrapods. Therefore these data provide support for a long-held morphology-based assertion of homology between pectoral fins and forelimbs as well as between pelvic fins and hindlimbs (Coates 1994; Shubin et al. 1997).

Our data (Fig. 4) indicate that *tbx2* in zebrafish is likely to be involved in fin patterning. Similarly, Yonei-Tamura et al. (1999) have demonstrated that *tbx3* is expressed in zebrafish pectoral fins. When did this limb-patterning function of *Tbx2* and *Tbx3* genes arise during evolution? *optomotor-blind* (*omb*), the *Drosophila* gene to which vertebrate *Tbx2* and *Tbx3* genes are collectively orthologous (Fig. 2) is essential for proper wing development (Grimm and Pflugfelder 1996). It is expressed in the distal compartment of the wing imaginal disc. Mutations in this gene produce phenotypes ranging from altered venation to severe reduction in distal outgrowth. *omb* is controlled by both *decapentaplegic* and *wingless* pathway signals in the wing (Grimm and Pflugfelder 1996) and in the leg (Brook and Cohen 1996). These data position *omb* as a central player in several aspects of appendage patterning in invertebrates. This in turn adds it to a growing list of genes that are implicated in developmental control of both vertebrate and invertebrate limbs (Shubin et al. 1997). Such extensive genetic similarities between structures traditionally considered analogous were interpreted by Shubin et al. (1997) to imply either convergent recruitment of conserved developmental modules or a derived condition whereby both lineages modified an appendage-patterning program inherited from a common metazoan ancestor.

The role of T-box genes during evolution of paired appendages in vertebrates

Based on the conservation of *Tbx2–Tbx5* expression during limb development, the timing of duplication events among these genes, and the palaeontological record, the following sequence of events during vertebrate evolution can be proposed to account for the origin of the two sets of paired appendages in gnathostomes (Fig. 5). We hypothesize that basal chordates similar to extant amphioxus (Chen et al. 1995) and primitively finless agnathans such as lamprey possessed a single T-box cluster comprised of *Tbx2/3* and *Tbx4/5* precursor genes. Osteostracans were the first chordates to evolve a set of paired appendages at the pectoral level and, we infer that *Tbx2/3* and *Tbx4/5* precursor genes were involved in their specification and patterning. We propose that duplication of this cluster occurred soon thereafter, such that the presence of all four *Tbx2–Tbx5* genes is a shared derived characteristic of all jawed vertebrates. This duplication generated genetic redundancy that was later exploited to reiterate a program of pectoral fin outgrowth at a different axial level. Importantly, the duplicated *Tbx5* and *Tbx4* genes were used at this stage of evolution to confer distinct identity to the pectoral and pelvic appendages, respectively. The ancestral jawed vertebrate thus possessed a set of pectoral and pelvic appendages with distinct axial morphology, a basic body plan preserved to this day. All subsequent evolution of limb morphology involved the modification of appendicular patterning programs rather than changes in limb number or location. Experimental work is currently underway to test elements of this model.

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