ORIGINAL ARTICLE

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Characterization of the zebrafish *tbx16* gene and evolution of the vertebrate T-box family

Abstract We report on a new zebrafish T-box-containing gene, *tbx16*. It encodes a message that is first detected throughout the blastoderm soon after the initiation of zygotic gene expression. Following gastrulation, expression becomes restricted to paraxial mesoderm and later primarily to the developing tail bud. To gain an evolutionary prospective on the potential function of this gene, we have analyzed its phylogenetic relationships to known T-box genes from other species. Zebrafish *tbx16* is likely orthologous to the chicken *Tbx6L* and *Xenopus Xombi/Antipodean/Brat/VegT* genes. Our analysis also shows that zebrafish *tbx6* and mouse *Tbx6* genes are paralogous to zebrafish *tbx16*. We present evidence which argues, that despite the same name and similar expression, zebrafish *tbx6* and mouse *Tbx6* genes are not orthologous to each other but instead represent relatively distant paralogs. The expression patterns of all genes are discussed in the light of their evolutionary relationships.

Key words T-box · Evolution · Zebrafish · Paraxial mesoderm · Tail bud

Introduction

A family of genes sharing a homology motif with a classical mouse *Brachyury* or *T* gene was recently described and named the T-box family (Bollag et al. 1994). These genes contain a characteristic conserved sequence that encodes a stretch of about 180 amino acids which bind DNA in a sequence-specific manner. It was shown that these transcription factor-encoding genes play critical roles during embryogenesis (Papaioannou and Silver 1997). For example, the prototypical member of the family, the *Brachyury* (*T*) gene, is found to be expressed throughout the prospective mesoderm of all vertebrates studied to date. The *Brachyury* gene product is essential for the proper development of the notochord and posterior structures in mouse and zebrafish (Chesley 1935; Halpern et al. 1993). To date, nearly 20 distinct genes have been identified that belong to the T-box gene family (Papaioannou and Silver 1997).

Recently, a number of novel T-box genes have been described in mouse (Chapman et al. 1996), chick (Knezevic et al. 1997), *Xenopus* (Lustig et al. 1996; Stennard et al. 1996; Zhang and King 1996; Horb and Thomsen 1997; to avoid confusion with the nomenclature we will refer to this gene as *Xombi*), and zebrafish (Hug et al. 1997). Shared major sites of expression for these genes are restricted to paraxial mesoderm and the tail bud of developing embryos. Our initial phylogenetic analysis indicated that some of these genes were likely to be paralogous rather than orthologous. To investigate this possibility and to study the evolution and function of vertebrate members of this family, we have isolated a number of zebrafish T-box-containing genes. Here we report the identification and initial characterization of a previously unknown gene, *tbx16*, and demonstrate its close phylogenetic relatedness to a group of recently described vertebrate T-box genes.

Materials and methods

Isolation and characterization of cDNA clones

A zebrafish λZAP II cDNA library (kindly provided by D. J. Grunwald) constructed from late gastrulation-stage embryos was screened with a cocktail of probes derived from previously cloned mouse genes *(Tbx1, Tbx3, Tbx5)*. Low-stringency hybridizations were performed at 50°C following published protocols (Agulnik et al. 1996). Bluescript plasmids containing hybridizing inserts were excised according to manufacturer's instructions (Stratagene). To obtain the 5′ end of the gene, we performed polymerase chain reaction (PCR) amplification of a directional zebrafish embryonic library (kindly provided by Bruce Appel) using a pair of nested primers designed based on the determined sequence of *tbx16*. PCR products were subcloned using a TA cloning kit (Invitrogen). Clones were sequenced using an automated ABI sequencer.

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Phylogenetic analysis

The amino acid sequence of the T-box portion of *tbx16* was aligned by eye with the homologous regions of other known T-box genes. The phylogenetic tree was produced using the neighbor-joining algorithm with Poisson-corrected amino acid distances. Confidence probability values were computed to assess the reliability of the reconstructed phylogeny. Tree construction and computation of confidence probabilities were carried out using the METREE package (Rzhetsky and Nei 1994). We use the terms paralogous and orthologous in their conventional sense to refer to homologous genes that have originated due to gene duplications and species divergences, respectively.

In situ hybridization, microscopy and photography

Whole-mount in situ hybridizations were carried out essentially as described by Thisse et al. (1993). A cDNA clone (1,600 nucleotides including the T-box and the 3′-end of the gene) was used to generate digoxigenin-labeled riboprobes. Stained embryos were mounted in 80% glycerol and photographed under a dissection or compound microscope.

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mu-Tbx6    LWKEFSAVGTEMIITKAGRRMFPACRVSVTGLDPEARYLFLLDVV
zf-tbx6    LWDKFSSIGTEMLITKSGRRMFPSCKVTVTGLNPKVKYVVIMDMV
ch-Tbx6L   LWMKFHQIGTEMIITKSGRRMFPQCKIKVSGLIPYAKYLMLVDFV
Xl-Xombi   LWSQFHQEGTEMIITKSGRRMFPQCKIRLFGLHPYAKYMLLVDFV
zf-tbx16   LWRSFHEIGTEMIITKPGRRMFPHCKISLSGLVPYAKYILLVDMV

mu-Tbx6    PVDGARYRWQGPDWEPSGKAEPRLPDRVYIHPDSPATGAHWMRQP
zf-tbx6    PFDNHKYKWNKDCWEVNGSSDPHLPNRFFIHPDSPAPGQKWMQYP
ch-Tbx6L   PVDNFRYKWNKDQWEVAGKAEPQLPCRTYVHPDSPAPGSHWMKEP
Xl-Xombi   PLDNFRYKWNKNQWEAAGKAEPHPPCRTYVHPDSPAPGAHWMKDP
zf-tbx16   PEDGLRYKWNKDKWEVAGKAEPQPPYRTYLHPDSPAPGSHWMKQP

mu-Tbx6    VSFHRVKLTNSTLDPHGHLILHSMHKYQPRIHLVRATQLCSQHWG
zf-tbx6    ISFHKLKLTNNTLNSNGLVVLHSMHKYQPRLHIVQSPDPCTPHNP
ch-Tbx6L   VSFQKLKLTNNTLDQHGHIILHSMHRYKPRFHIVQADDLFSVRWS
Xl-Xombi   ICFQKLKLTNNTLDQQGHIILHSMHRYKPRFHVVQSDDMYNSPWG
zf-tbx16   VSFLKLKLTNNALDQHGHIILHSMHRYHPRFHIVQADDLYSVRWS

mu-Tbx6    GVAS-FRFPETTFISVTAYQNPRITQLKIAANPFAKGFRE
zf-tbx6    GAYLRFTFPEAAFIAVTAYQNQEITKLKIDNNPFAKGFRD
ch-Tbx6L   IFQV-FSFPETVFTSVTAYQNEQITKLKIDNNPFAKGFRE
Xl-Xombi   LVQV-FSFPETEFTSVTAYQNEKITKLKINHNPFAKGFRE
zf-tbx16   VFQT-FTFPETSFTAVTAYQNTKITKLKIDHNPFAKGFRD
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Fig. 1 Alignment of T-box domains of several closely related genes. *Bold face* indicates that a residue has been conserved among all five sequences (*mu* mouse, *ch* chick, *Xl Xenopus laevis*, *zf* zebrafish)

Results and discussion

Identification of the zebrafish *tbx16* gene
as a member of the T-box family

We have identified two cDNA clones encoding a novel zebrafish T-box gene. In accordance with the nomenclature of Agulnik et al. (1996) and Bollag et al. (1994) we named this gene *tbx16*. Since both of the cDNA clones were truncated at the 5′ end (both started within the T-box and extended through the 3′ untranslated region; UTR), we performed PCR amplification of a cDNA library with a pair of nested primers towards the 5′ end of the cDNA. The longest obtained fragment (500 nucleotides) was subcloned and shown to span the 5′ end of the gene. This sequence contains a methionine preceded by multiple stop codons in all reading frames which suggests that we have uncovered the full-length cDNA for the coding region of *tbx16*. The resulting open reading frame encodes a protein of 470 amino acids. It is preceded by at least 80 nucleotides of 5′ UTR and followed by over 250 nucleotides of 3′ UTR including a poly-A tail. Using the complete amino acid sequence of *tbx16*, we conducted a data base search which revealed that this gene is a member of the T-box family. Extensive similarity to other family members was observed within the T-box, while virtually no matches were detected outside this domain. The overall length and position of the T-box domain within the *tbx16* gene is very similar to those of *Xenopus Xombi* and zebrafish *tbx6* genes. Alignment of T-box domains of several closely related T-box genes is shown in Fig. 1; the complete sequence is deposited into GenBank with accession number AF044977.

Expression pattern of *tbx16* during zebrafish development

As a first step towards investigating the potential role of *tbx16* during embryogenesis, we have conducted whole-mount in situ hybridizations with anti-sense RNA probes. Shortly after the mid-blastula transition (2.75 h, where h=hours of post-fertilization development at 28.5°C), expression of the *tbx16* gene is first detected uniformly throughout the blastoderm (data not shown). Beginning at sphere stage (4 h) expression of *tbx16* is progressively down-regulated at the most-animal pole regions of the blastoderm while remaining strong only in cells at the marginal region of the blastoderm. By the germ ring stage (5.7 h), the embryo has the form of an inverted bowl atop the large yolk cell. As shown in Fig. 2A, the domain of expression extends in a continuous ring around the entire marginal region of the embryo, including the future dorsal shield region. The marginal region of the zebrafish blastoderm has been shown by fate map analyses to contain the mesodermal and endodermal progenitors (Kimmel et al. 1990). In addition, the pattern of *tbx16* expression appears coincident with the initial expression of the zebrafish *no tail* (*ntl*) gene (Schulte-Merker et al. 1992), which is the zebrafish ortholog of the *Brachyury* gene. Therefore, at the onset of gastrulation, the expression pattern of the zebrafish *tbx16* gene correlates with the position of the future mesendodermal derivatives of the zebrafish embryo.

Gastrulation in the zebrafish embryo begins with the involution of cells at the dorsal shield region quickly followed by involution of cells around the entire marginal region. Expression of *tbx16* initially remains high within the first group of cells to involute at the dorsal shield region. These cells will form the pre-chordal plate mesoderm (arrowheads in Fig. 2A, D). After involution, expression of *tbx16* is strongly down-regulated but not completely absent from the pre-chordal plate mesoderm. However, *tbx16* is not detected in later involuting dorsal midline precursors which will give rise to notochord derivatives. In comparison, lateral and ventral marginal cells continue to express *tbx16* for some
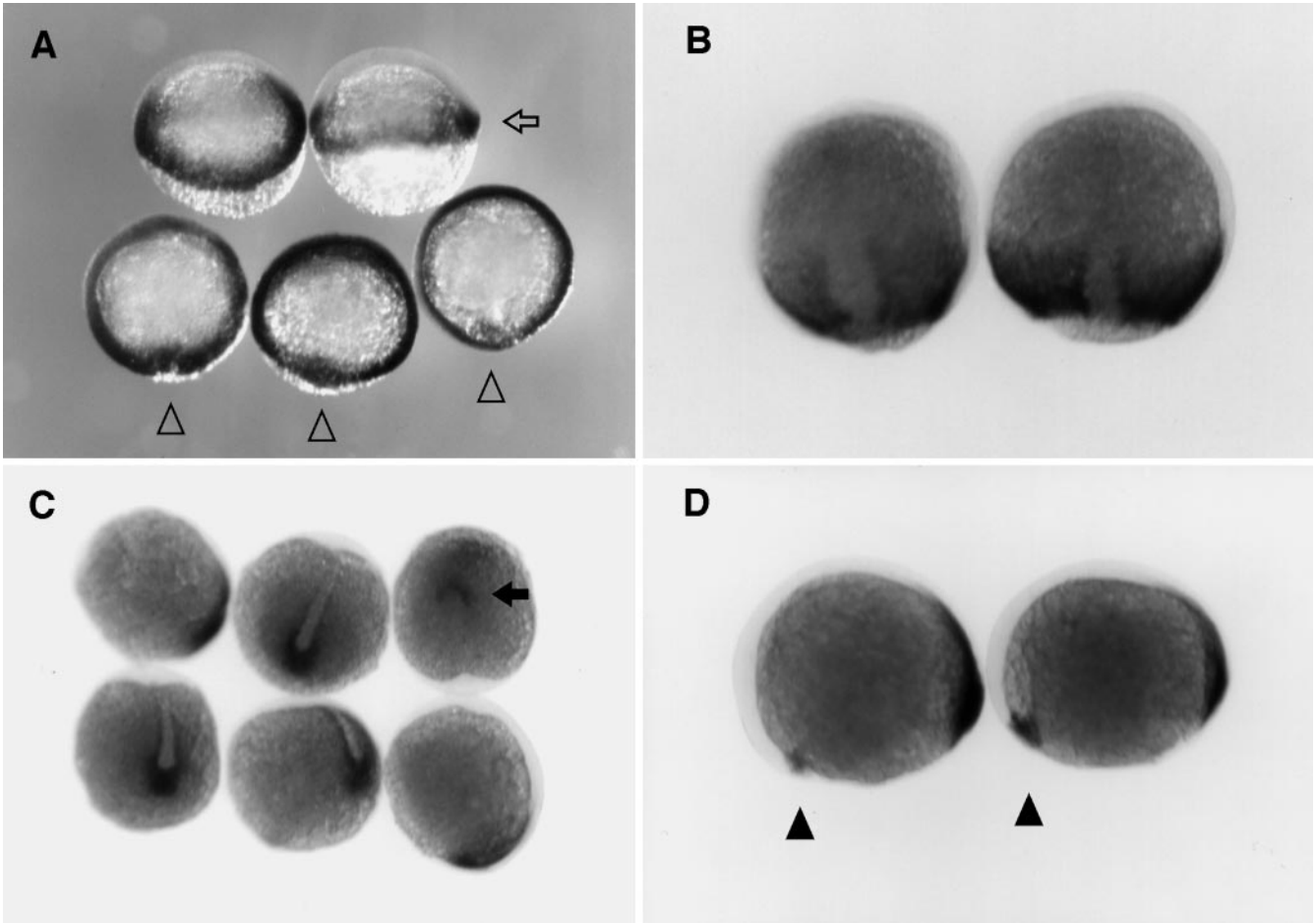
Fig. 2A–D Early expression of the zebrafish *tbx16* gene. **A** Expression of *tbx16* at germ ring stage. Expression at this time is within the entire marginal region of the blastoderm. The *bottom three embryos* are shown with animal pole facing the viewer, note that expression extends all around the marginal region including the dorsal shield region (*open arrowheads*). The embryo on the *top left* is in three-quarters view and the *top right* embryo is in profile with the animal pole towards the top of page and the dorsal shield region to the right (*arrow*). **B** Expression of *tbx16* at 85% epiboly. Expression of *tbx16* is confined to involuted lateral and ventral hypoblast cells. Note that there is no expression in the dorsal mesoderm, which gives rise to notochord derivatives. **C** Expression of *tbx16* at 1 to 3-somite stage. Embryos are in various orientations. *Arrow* points to expression in the pre-chordal plate mesoderm which will form yolk hatching gland cells. The remaining embryos highlight expression in the tail region where expression is confined to lateral and ventral mesoderm; note that expression in the axial mesoderm is absent. **D** Expression of *tbx16* at 10-somite stage. Both embryos are in profile with anterior to the left, dorsal towards the top of page. Note strong expression in the lateral mesoderm of the tail region and in the pre-chordal plate mesoderm, or "polster" region (*arrowheads*). In all panels, approximate diameter of embryos is 600 μm

time after involution (Fig. 2B). Thus, at 90% epiboly, the expression pattern of *tbx16* in the zebrafish embryo is roughly complementary to the pattern of the *ntl* gene at the same stage. Whereas the *ntl* gene is down-regulated in lateral and ventral hypoblast cells soon after involution and maintained at high levels within the dorsal

midline cells, the expression of *tbx16* remains high in the lateral and ventral hypoblast cells but is absent from the dorsal midline cells which will form the notochord. After yolk plug closure, *tbx16* is strongly re-expressed in part of the pre-chordal plate mesoderm which forms the "polster" region of the hypoblast (arrow in Fig. 2C and arrowheads in Fig. 2D). The polster region in zebrafish largely forms a specialized group of cells called yolk hatching gland cells.

As shown in Figs. 2D and 3A, at the 10-somite stage *tbx16* transcripts are detected in the anterior pre-chordal plate mesoderm and the paraxial and lateral mesoderm in the posterior tail region. By the 15-somite stage, expression is exclusively within the tail region (Fig. 3B), eventually becoming restricted to the most posterior tail tip by the 24-h stage (Fig. 3C). At that stage, expression of *tbx16* also appears within a population of dorsolaterally placed cells within the spinal cord. Staining is quite strong within the individual cells which do not appear to be segmentally arranged and most of them are located preferentially within the posterior tail region. By their position and numbers, these appear to be the Rohon-Beard cells which constitute a sub-population of interneurons in the spinal cord. Interestingly, the *Xenopus* gene related to zebrafish *tbx16*, the *Xombi* gene, is also expressed in a small group of posterior spinal cord cells (Lustig et al. 1996; Zhang and King 1996).
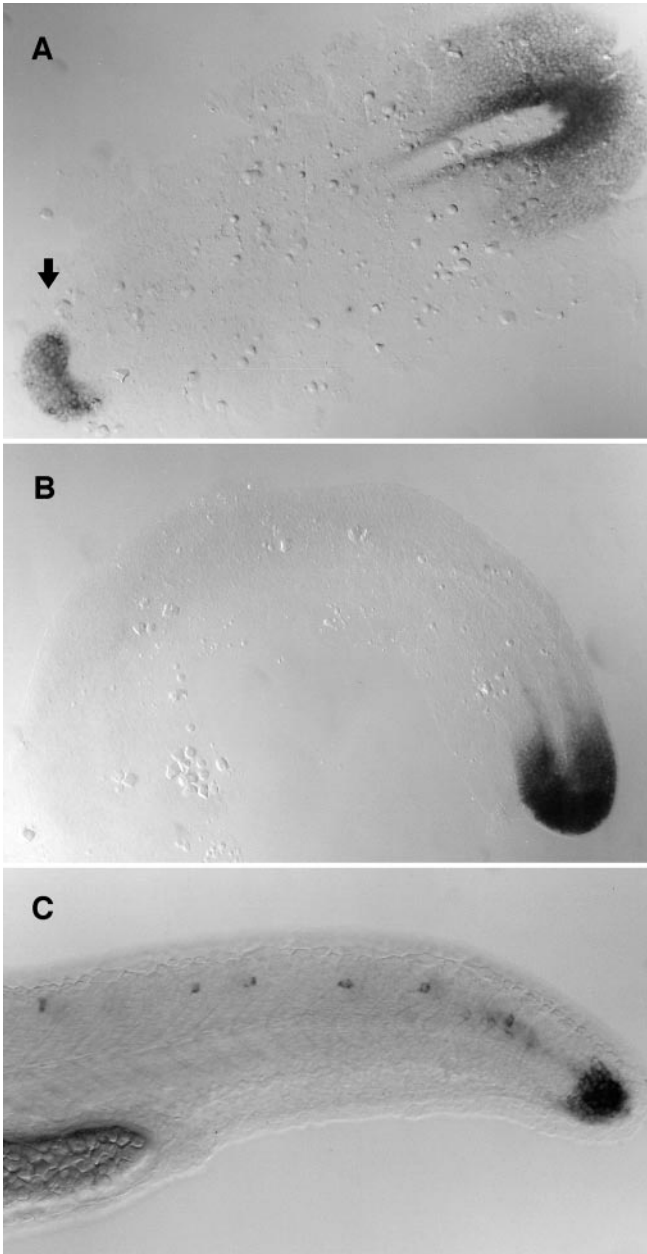
Fig. 3A–C Later expression of zebrafish *tbx16* gene. A Expression of *tbx16* at 10-somite stage. Embryo has been dissected from the yolk cell and flat mounted between coverslips with the dorsal aspect facing the viewer, anterior to the left. Note strong expression in lateral and ventral mesoderm surrounding the developing tail region. Also note strong expression in the polster region (*arrow*). B Expression of *tbx16* at 15-somite stage. Lateral view of whole-mount embryo with yolk removed, anterior to the left, dorsal towards the top of page. Expression is now confined to cells at the tip of the outgrowing tail, excluding notochord precursors. C Expression of *tbx16* at 24 h. Lateral view of whole-mount embryo, anterior to the left, dorsal towards top of page. Expression in the tail region remains strong at the very tip of tail. Strong expression is also present within dorsolaterally-located Rohon-Beard cells within the spinal cord

In summary, *tbx16* in zebrafish is expressed around the entire marginal region of the late blastula stage embryo. During gastrulation, expression is maintained in the involuting cells which will give rise to lateral and
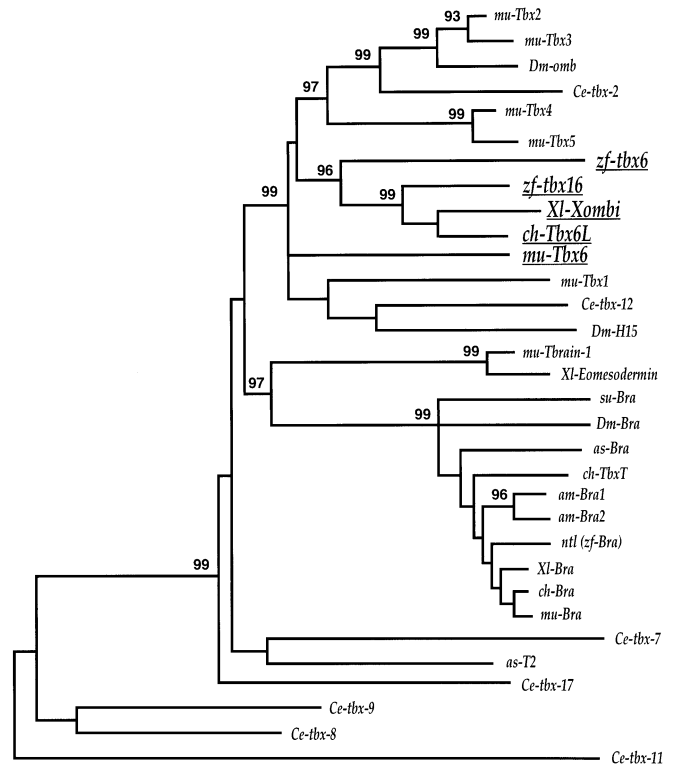

Fig. 4 Phylogenetic tree of the T-box gene family. The *values shown on the tree* indicate confidence probabilities associated with particular nodes. Thus, nodes with high values are deemed reliable (significant if $P>0.95$). It is well-established that the divergence between lineages of tetrapods (such as birds and amphibians) has occurred subsequently to the split between fish and tetrapods. Thus it is expected that avian and amphibian orthologs should be more closely related to one another, than either of them is to a fish gene. Precisely this arrangement is observed in our sequence analysis for the chicken *Tbx6L*, *Xenopus Xombi*, and zebrafish *tbx16* genes. Therefore, we infer that these genes are orthologous. Genes discussed in the text are shown in *larger font* and *underlined* (*mu* mouse, *ch* chick, *Xl Xenopus laevis*, *zf* zebrafish, *am* amphioxus, *as* ascidian, *su* sea urchin, *Dm Drosophila melanogaster*, *Ce C. elegans*)

ventral mesoderm; *tbx16* expression is excluded from axial mesoderm except for the most anterior pre-chordal plate mesoderm. During somitogenesis stages, *tbx16* is confined to the ventro-lateral mesodermal regions of the posterior body. Late expression of *tbx16* is seen at the tail tip and within a subset of posterior cells of the spinal cord, most likely Rohon-Beard cells.

Phylogenetic position of *tbx16* within the T-box family

To assess the evolutionary relatedness of *tbx16* to the other known members of this family, we generated the phylogenetic tree shown in Fig. 4. It is a result of the neighbor-joining algorithm applied to the amino acid sequences of diverse T-box genes. Only the most conserved portions of these genes, the DNA-binding domains, can be aligned with confidence, thus our analysis was limited to about 180 amino acids. Phylogenetic analyses of a large number of sequences with a small number of informative

sites are notoriously problematic, therefore we estimated statistical significance of tree reconstruction. As detailed in the figure legend, the tree implies that chicken *Tbx6L*, *Xenopus Xombi*, and zebrafish *tbx16* are orthologous genes. It should be noted that other recently reported *Xenopus* T-box genes *Antipodean*, *VegT*, and *Brat* (Stennard et al. 1996; Zhang and King 1996; Horb and Thomsen 1997) are most likely the same gene as *Xombi*. Prior to our discovery of *tbx16*, the zebrafish *tbx6* gene (Hug et al. 1997) was a candidate orthologous gene of chicken *Tbx6L* and *Xenopus Xombi*. However, this possibility is rejected now with a high degree of statistical confidence (P=0.99) making zebrafish *tbx6* paralogous to the *tbx16* gene and its anuran and avian counterparts. Interestingly, mouse *Tbx6* is not orthologous (excluded with P=0.96) to either of the two zebrafish genes. Instead, mouse *Tbx6* is no more closely related to the *tbx6/tbx16* subfamily than it is to the *Tbx2/3/4/5* or *Tbx1/tbx-12* subfamilies. Therefore, the present analysis makes it clear that the mouse *Tbx6* and zebrafish *tbx6* genes, though bearing the same name, are not orthologous genes but rather paralogs who disguise their lack of close kinship by strikingly similar expression patterns.

Finally, the third known zebrafish representative of the T-box gene family, the *ntl* gene, is only distantly related to *tbx16*. Based on the distance between the *Caenorhabditis elegans tbx-2* and its mammalian counterparts, *Tbx2* and *Tbx3*, it can be inferred with confidence which genes were contained within the genome of the most recent common ancestor of nematodes and vertebrates. Thus, it can be said of the genes discussed here that such a primitive metazoan had to have possessed at least a proto-*Brachyury*, a proto-*Tbx6* (a counterpart of which is now found in the mouse genome), and a proto-*tbx6/tbx16* gene. These data also suggest that the duplication leading to the separation of zebrafish *tbx6* and *tbx16* genes was likely to have happened early in chordate evolution prior to the divergence of fish and tetrapods. This phylogenetic analysis provides an evolutionary framework for the examination of similarities and differences among expression patterns of posteriorly expressed vertebrate T-box genes (see below).

Comparison of expression patterns of T-box family members

The T-box family of genes are thought to regulate different aspects of embryogenesis in a wide variety of organisms. The orthologous genes, chicken *Tbx6L, Xenopus Xombi*, and zebrafish *tbx16*, display overall similar expression patterns. Zygotic expression of these genes is initiated soon after mid-blastula transition in both frog and fish while the chick gene is present prior to streak formation (stage X). At the onset of gastrulation, both the frog *Xombi* and zebrafish *tbx16* genes are expressed around the entire circumference of the marginal zone (Lustig et al. 1996); similarly in the chick, expression of *Tbx6L* is found in the cells surrounding the primitive streak (Knezevic et

al. 1997). Therefore, these three related genes in chicken, frog and zebrafish embryos are expressed in cells which will later undergo involution movements during gastrulation and which were shown by fate-mapping experiments to form somitic mesoderm (Kimmel et al. 1990). Shortly after the onset of gastrulation, expression of these genes in the chicken, frog and zebrafish is absent from the dorsal-most area of the embryo where the future notochord will form. Later, the shared expression of these genes is restricted to the segmental plate mesoderm where the anterior boundary of expression regresses caudally with the wave of somitogenesis. In all three organisms, segmental plate expression is down-regulated before the level of the last formed somite, consistent with the hypothesis that loss of expression of these genes is correlated with the differentiation of mesodermal precursors and/or the processes of somitogenesis (Knezevic et al. 1997).

Despite the similarities described above, there are distinctions evident in the expression patterns of the three orthologous genes. *Xenopus Xombi* is present as a maternally deposited message in the vegetal pole of the oocytes (Lustig et al. 1996). However, zebrafish *tbx16* is not expressed maternally and is only evident by in situ and RNA blot analysis after the mid-blastula transition (data not shown). Another difference is seen in the anterior expression domain of the zebrafish *tbx16* (Fig. 2); although faint diffuse staining has been reported to be present within the head region in the frog (Lustig et al. 1996), strong staining is apparent for the zebrafish *tbx16* gene in the anterior prechordal plate mesoderm, which forms the polster region. Finally, a difference is seen between the three genes' expression during the late somitogenesis stages. Whereas both *Xenopus Xombi* and zebrafish *tbx16* transcripts are detected in the dorso-lateral spinal cord within individual cells identified as Rohon-Beard neurons (Zhang and King 1996), late expression of the *Tbx6L* gene has not been reported to occur in the dorsal region of chicken embryos.

Remarkable similarities in expression patterns are also observed between the genes described above and their paralogs: mouse *Tbx6* (Chapman et al. 1996) and zebrafish *tbx6* (Hug et al. 1997). Most notably, patterns are virtually indistinguishable in mesendodermal precursors and later in paraxial mesoderm with the anterior boundary of expression receding with the wave of somitogenesis.

The original *Brachyury* (*T*) locus of the mouse, for which this family of genes is named, has been studied extensively in frog (*Xbra*), chicken (*Ch-T*) and zebrafish (*ntl*) as reviewed by Papaioannou and Silver (1997). These *Brachyury* homologs are thought to play a role in the specification of mesoderm, particularly the axial mesoderm which gives rise to notochord derivatives. The expression patterns of the frog *Xombi*, chicken *Tbx6L* and zebrafish *tbx16* genes overlap with expression of the *Brachyury* gene in the marginal zone at the onset of gastrulation. However, as gastrulation proceeds, the expression of these three related genes becomes complementary to that of the *Brachyury* gene. Whereas *Brachyury* expression is lost in most cells shortly after involution except in those dorsal midline precursors that will give rise to notochord cells, the expres-

sion of the frog *Xombi*, chicken *Tbx6L* and zebrafish *tbx16* remains strong in the lateral and ventral mesodermal precursors and is not found within the dorsal mesoderm.

Comparison of the three zebrafish T-box genes, *ntl, tbx6* and *tbx16*, shows an early sub-division of expression patterns and presumably functions: at the onset of gastrulation the *ntl* and *tbx16* genes have overlapping expression around the circumference of the marginal region whereas the *tbx6* gene is expressed in an arc of marginal cells which excludes the most dorsally located shield region. During gastrulation, *tbx16* expression in the dorsal shield region is lost such that its expression pattern now overlaps with that of the *tbx6* gene. At the same time, *ntl* expression is lost in the ventral and lateral involuting cells such that its expression pattern is now directly complementary to that of both the *tbx6* and *tbx16* domain. During somitogenesis stages, all three genes remain strongly expressed in the developing tailbud region with the *ntl* gene also expressed in the notochord region, *tbx6* expressed in the blood precursors and *tbx16* expressed in the pre-chordal plate mesoderm as well as in certain spinal cord interneurons. Therefore, the expression patterns exhibited by different members of the T-box family in zebrafish are very dynamic, especially during early gastrulation, and could be indicative of both distinct and overlapping functions of different T-box genes. It is also possible that members of the T-box family could be dependent upon each other for proper expression (Stennard et al. 1996; Horb and Thomsen 1997), although functional studies have shown that initiation of transcription of both the mouse *Tbx6* (Chapman et al. 1996) and zebrafish *tbx6* genes (Hug et al. 1997) is independent of *Brachyury* function. A complete understanding of the specific functions of the different members of the T-box gene family will require a comparison of all members. Towards this goal, we have recently isolated several new members of the T-box family from zebrafish and are presently characterizing their expression patterns and functions in the light of their evolutionary relationships to each other.

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