

Gene expression pattern

tbx20, a new vertebrate T-box gene expressed in the cranial motor neurons and developing cardiovascular structures in zebrafish

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Abstract

The T-box genes constitute a family of transcriptional regulator genes that have been implicated in a variety of developmental processes ranging from the formation of germ layers to the regionalization of the central nervous system. In this report we describe the cloning and expression pattern of a new T-box gene from zebrafish, which we named *tbx20*. *tbx20* is an ortholog of two other T-box genes isolated from animals of different phyla – *H15* of *Drosophila melanogaster* and *tbx-12* of *Caenorhabditis elegans*, suggesting that the evolutionary origin of this gene predates the divergence between the protostomes and deuterostomes. During development, *tbx20* is expressed in embryonic structures of both mesodermal and ectodermal origins, including the heart, cranial motor neurons, and the roof of the dorsal aorta. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Zebrafish; T-box genes; Lateral plate mesoderm; Heart; Cranial motor neurons; Dorsal aorta; *TBX20*; *H15*; *tbx-12*; *cloche*

1. Results and discussion

Recent advances in molecular genetics have led to the identification and cloning of several new classes of transcriptional regulators that have important developmental functions. One class of such genes, known as the T-box genes, has been implicated in a variety of developmental processes in chordates, including the formation of mesoderm (Chapman and Papaioannou, 1998), development of the tail and the notochord (Schulte-Merker et al., 1994), patterning of the limb (Gibson-Brown et al., 1996), development of sense organs and pharyngeal arches (Chapman et al., 1996), and the regionalization of the brain (Bulfone et al., 1995). They share a highly conserved sequence motif of about 180 amino acids known as the T-box (Bollag et al., 1994), which is thought to function as a DNA-binding domain (Kispert and Herrmann, 1993). To date, about 50 different T-box genes have been isolated from several different animals including the mouse, human, chicken, *Xenopus*, zebrafish, amphioxus, ascidians, *Drosophila*, *Caenorhabditis elegans*, and hydra, suggesting a very ancient origin of this gene family (Papaioannou and Silver, 1998; Technau and Bode, 1999).

In an effort to further our understanding of the evolution and functional diversification of T-box genes in chordates,

we have recently isolated several new T-box genes from the zebrafish, *Danio rerio* (e.g. Ruvinsky et al., 1998, 2000). In one of the clones we identified a novel T-box sequence highly similar to those of *H15*, a *Drosophila* T-box gene (Brook and Cohen, 1996), as well as *TBX20*, a human T-box gene recently reported in GenBank database (accession number: AJ237589). Detailed comparison of the amino acid sequence of this clone with that of *Drosophila H15* revealed that sequence similarity extended several amino acids outside of the T-box domain (Fig. 1A), which strongly suggested that this gene is likely to be the zebrafish ortholog of the *Drosophila H15* gene. This hypothesis was further confirmed by phylogenetic analysis of known T-box sequences, which indicated that *tbx20* is closely related to *Drosophila H15* as well as *tbx-12* of *C. elegans* (Agulnik et al., 1997), forming a new subgroup of T-box genes within the *Tbx1* subfamily (Fig. 1B; Papaioannou and Silver, 1998).

Expression of *tbx20* was detected from the bud stage to 72 hours post fertilization (hpf) in a variety of embryonic structures, including the primordia of the heart and dorsal aorta, neurons within the brain and pituitary gland, and the mesenchyme cells of the pharynx and the yolk tube extension (Fig. 2). The first strong expression of *tbx20* was detected at the bud stage (10 hpf) as a pair of curved bilateral stripes lying within the anterior lateral plate mesoderm (Fig. 2A). This region is known to contain precursor cells

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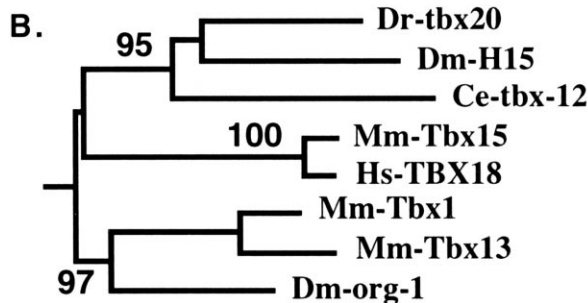
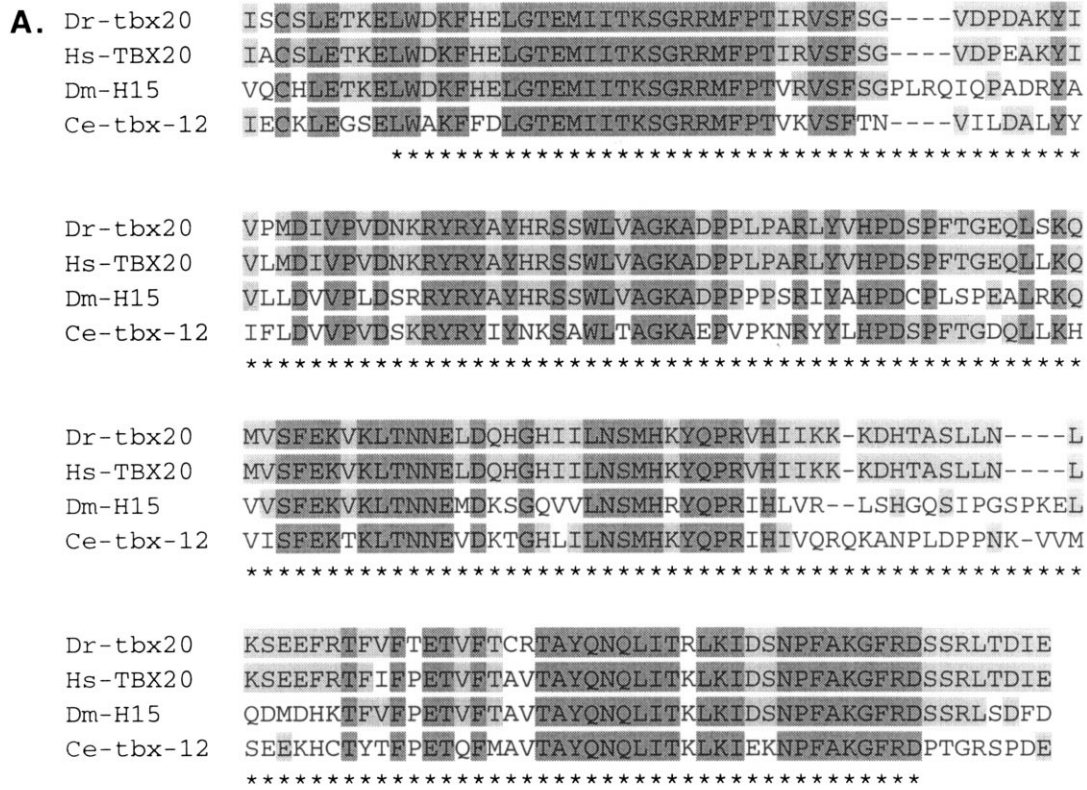


Fig. 1. Sequence analysis of the zebrafish *tbx20* gene. (A) Alignment and amino acid sequence comparisons of the T-box motifs and flanking sequences from *tbx20* and its orthologs. Gaps are introduced to facilitate the alignment. Amino acid residues that are identical in all genes and between *tbx20* and other genes are shaded in dark and light gray colors, respectively. Gaps are represented by ‘-’ and amino acid sequences corresponding to T-box motifs are marked with ‘*’. (B) Neighbor-joining tree showing evolutionary relationships among selected members of the T-box gene family. Numbers on nodes are the bootstrap values showing statistical supports for each node. Only part of the tree including members of *Tbx1* subfamily genes (defined in Papaioannou and Silver, 1998) is shown here. Mm: *Mus musculus* (mouse). Dr: *Danio rerio* (zebrafish). Dm: *Drosophila melanogaster* (fruitfly). Ce: *Caenorhabditis elegans* (nematode worm). Hs: *Homo sapiens* (human).

for the myocardium, the muscular outer layer of the heart (Stainier and Fishman, 1992). Consistent with this, subsequent *tbx20* expression within this region closely followed known patterns of heart morphogenesis in zebrafish (e.g. Stainier et al., 1993; Lee et al., 1996; Chen et al., 1997), including convergence toward the midline (Fig. 2A–C), fusion at the midline (Fig. 2D), formation of the cone, and jogging (Fig. 2I,J) and looping (Fig. 2L) of the tube. At 36 h of development, expression was detected along the entire length of the embryonic heart, including the atrium, ventricle, and cardiac outflow tracts (Fig. 2L). Comparison with the expression domains of the heart-specific homeobox gene

nkx2.5 (Chen and Fishman, 1996; Lee et al., 1996) indicated that *tbx20* was initially expressed in much broader domains within the anterior lateral plate mesoderm (Fig. 3A,G), but with the progression of development, expression became gradually restricted to the heart-forming area through the loss of expression in non-cardiogenic cells such that by 24 h of development virtually no difference in expression domains was noted between *tbx20* and *nkx2.5* (Fig. 3B,H).

By the 10-somite stage, in addition to the expression within the anterior lateral plate mesoderm, new expression domains of *tbx20* were established within the hindbrain in the form of bilateral clusters of cells lying at the level of the

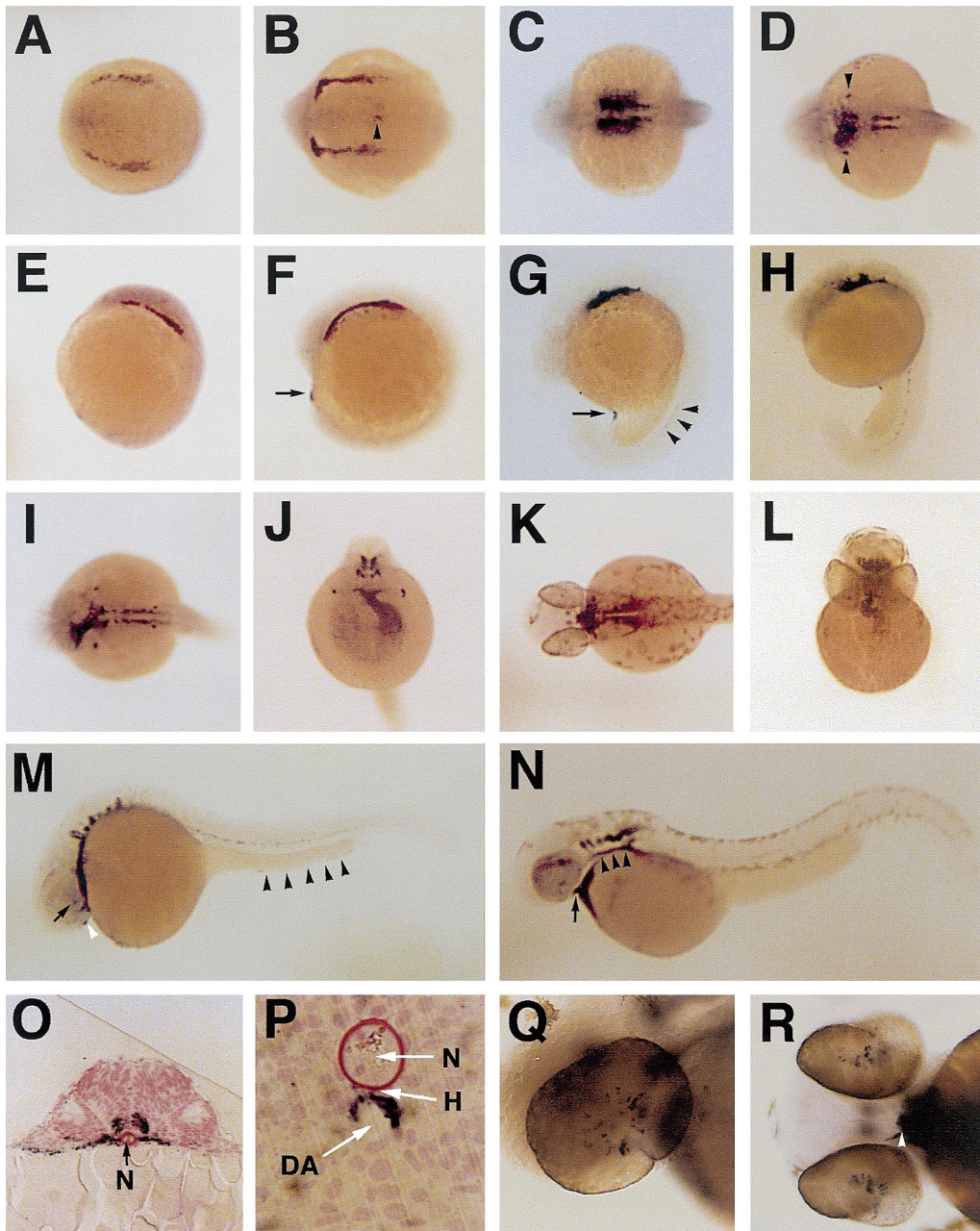


Fig. 2. Expression of *tbx20* during zebrafish development. (A,E) Bud stage. Animal pole (A) and lateral (E) views. Expression is noted within the anterior lateral plate mesoderm, including the prospective bilateral heart primordia. (B,F) Ten-somite stage. Animal pole (B) and lateral (F) views. Additional expression domains appear within the hindbrain (B: arrowhead) and at the advancing margin of the tail bud (F: arrow). (C,G) Fifteen-somite stage. Animal pole (C) and lateral (G) views. More hindbrain neurons begin to express *tbx20*. Expression is also present in a subset of angioblasts that will later form the roof of the dorsal aorta (G: arrowheads). (D,H) Twenty-somite stage. Animal pole (D) and lateral (H) views. The fusion between the bilateral heart primordia is under way. Expression is also seen in a pair of cell clusters wedged between the primordia of mandibular and hyoid arches (D: arrowheads). (I,J,M) Twenty-four hours post fertilization. Dorsal (I), frontal (J), and lateral (M) views. Expression occurs along the entire length of the heart tube, in the hindbrain branchiomotor neurons, and in the dorsal half of the dorsal aorta primordium. Expression within the anlage of the pituitary gland (M: white arrowhead) and the putative midbrain motor nuclei (M: arrow) also starts to appear at this stage. Ventral mesenchyme cells of the yolk tube extension expressing *tbx20* are seen to spread anteriorly along the length of the yolk tube (M: arrowheads). (K,L,N) Thirty-six hours post fertilization. Dorsal (K), frontal (L), and lateral (N) views. Expression is clearly seen in the motor nuclei of mid- and hindbrain, within the pituitary gland (N, arrow), and the mesenchyme cells of unknown identity lying dorsal to the pharynx (N: arrowheads). (O,P) Cross-sectional views of a 24-h old embryo. Sections are made at the level of the hindbrain (O) and posterior trunk (P). Expression is seen in the motor neurons within the hindbrain and para-notochordal mesenchyme cells overlying the pharynx (O), as well as within the sub-hypochordal mesenchyme cells occupying the roof of the dorsal aorta (P). N, notochord; H, hypochord; DA, the lumen of the dorsal aorta. (Q,R) High magnification views of the expression of *tbx20* within the eyes of a 36-h embryo (Q: lateral view, R: ventral view). Note the expression within the pituitary gland (R: white arrowhead).

fourth rhombomere (r4) (arrowhead in Fig. 2B). Between this stage and 24 hpf, additional clusters of cells expressing *tbx20* appeared in rapid succession within the hindbrain in the sequence of r2, r3, r5, r6 and r7, and caudal hindbrain (Fig. 2B,C,D,I). The overall patterns of *tbx20* expression within the hindbrain were identical to that of *islet1* at comparable stages (Fig. 3C,I), indicating that the hindbrain cells expressing *tbx20* are the precursors of hindbrain motor

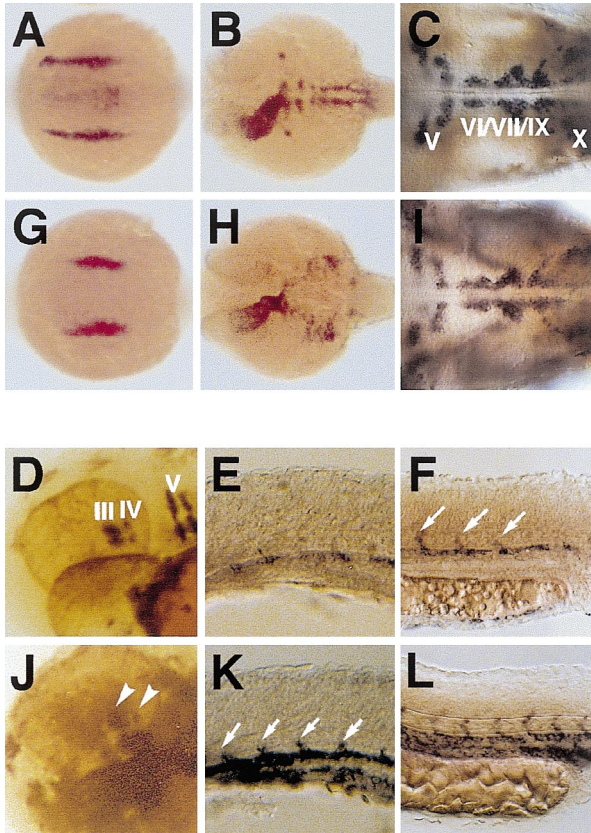


Fig. 3. Comparison of the expression domains of *tbx20* with other genes involved in the development of central nervous system and cardiovascular structures. (A,G) Dorsal views of the 10-somite stage embryos stained with *tbx20* (A) and *nkx2.5* (G). The initial expression domain of *tbx20* within the anterior lateral mesoderm is much broader than that of *nkx2.5*. (B,H) Dorsal views of the 24-h old embryos probed for *tbx20* (B) and *nkx2.5* (H) expression. Expression domains of both genes within the heart tube are almost identical. (C,I) Dorsal views of the hindbrain of 36-hpf embryos showing *tbx20* (C) and *islet1* (I) expression. Both genes are expressed in the branchiomotor neurons (V, trigeminal; VII, facial; IX, glossopharyngeal; X, vagus) and the neurons within the abducens (VI) nuclei. At this stage the precursors of VI, VII, and IX neurons are not completely segregated from each other (Chandrasekhar et al., 1999). (D,J) Dorso-lateral view of the head region of 36-hpf embryos showing the expression of *tbx20* (D) and *islet1* (J). Both genes show the expression in the presumptive oculomotor (III) and trochlear (IV) neurons. (E,K) Lateral views of the anterior trunk region of the 20-somite stage embryos showing expression of *tbx20* (E) and *flk1* (K), the latter of which marks angioblasts. *tbx20* expression is concentrated in clusters of cells lying at or near the segmental borders where the angiogenic sprouts (K: arrows) are forming. (F,L) Lateral views of the posterior trunk region of 24-hpf embryos showing the expression of *tbx20* (F) and *flk1* (L). *tbx20* expression is now occasionally seen within the cells forming intersegmental arteries (F: arrows), as well as the roof of the dorsal aorta. Anterior is to the left in all panels.

neurons of the abducens (VI) and branchiomotor (V, VII, IX, X) nuclei (see Chandrasekhar et al., 1997). By 24 hpf, additional small groups of cells located within the ventral midbrain, within the optic cup near the choroid fissure, and within the anlage of the pituitary gland began to show expression of *tbx20* (Fig. 2M), which became more prominent at 36 hpf (Fig. 2N,Q,R). The midbrain expression domains again co-localize with the expression of *islet1* at the same stage (Fig. 3D,J), which strongly suggests that these midbrain cells are also the precursors of motor neurons, most likely those of the oculomotor (III) and trochlear (IV) nuclei, which are known to be present in the tegmental part of the zebrafish midbrain (Wullmann et al., 1996; but also see Chandrasekhar et al., 1999). Expression within these presumptive neuronal and neuroendocrine cells remained strong up to 48 hpf and was still recognizable in some motor nuclei at least until day 3 of embryogenesis.

Between the 15-somite stage and 24 hpf, *tbx20* expression was also seen in a loose string of cells lying ventral or immediately lateral to the hypochord (Fig. 2G,H,M,P), a row of specialized midline cells with flat morphology that are located directly underneath the notochord (Kimmel et al., 1995). Distribution of the cells expressing *tbx20* in this sub-hypochordal domain, which extends from the level of the first pair of somites to just a few segments anterior to the last pair of somites (Fig. 2H,M), initially showed a weak periodicity, with most cells found in aggregates near the segmental borders (Figs. 2H and 3E). However, by 24 h of development, this periodicity had largely disappeared, and cells expressing *tbx20* were found rather uniformly along the body axis (Fig. 2M). In cross sections of 24-h old embryos, these *tbx20*-expressing cells were found among cells lining a hollow space that corresponds to the lumen of future dorsal aorta (Fig. 2P), indicating that these cells are likely to be the angioblasts, or the precursor cells of the endothelium (Liao et al., 1997). Consistent with this view, no *tbx20*-expressing cells were seen in the trunk of embryos homozygous for the mutation *cloche* (Fig. 4), which prevents the formation of blood and blood vessel precursor cells in the embryo (Stainier et al., 1995). Some

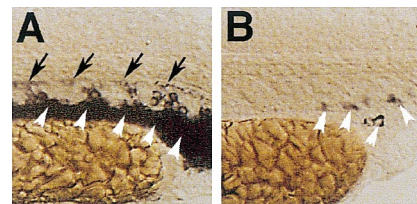


Fig. 4. Expression of *tbx20* in the posterior trunk of wild-type (A) and *cloche* mutant (B) embryos. Embryos are stained with *tbx20* (black arrows) and *gata1* (white arrowheads) probes, the latter of which marks prospective red blood cells. Expression of *tbx20* in sub-hypochordal mesenchyme cells (black arrows in A) is missing in *cloche* mutants, which fail to form progenitor cells for both blood and blood vessels (Stainier et al., 1995). Note the severe reduction of *gata1* expression in *cloche* mutants. Lateral views with the anterior to the left and dorsal to the top in both panels.

of the *tbx20*-expressing cells were occasionally seen along the borders between adjacent somites at 24 hpf (arrows in Fig. 3F), which is coincident with the dorsalward migration of angioblasts known to occur at this time to form intersegmental blood vessels (Fig. 3L; also see Fouquet et al., 1997; Sumoy et al., 1997). Expression in these prospective vascular cells was transient, and no longer detected after 30 h of development.

Finally, with the onset of somitogenesis, expression of *tbx20* was also seen in the cells lying along the anterior margin of the advancing tailbud (Fig. 2F, arrow). With the eversion of the tail, these cells became progressively localized to the ventral posterior portion of the yolk tube, slightly anterior to the future anus (Fig. 2G, arrow). These cells were later seen to spread anteriorly along the yolk tube on its ventral side (Fig. 2M, arrowheads), eventually forming a thin stripe of mesenchyme cells lying along the entire length of the yolk tube extension. At present, we do not know the identity of these cells, although their location suggests that they might give rise to the mesenchyme cells of the future pre-anal finfold. *tbx20* expression was also noted from the 16-somite stage to at least 48 hpf in a bilateral cluster of cells found between the primordia of mandibular and hyoid arches (Fig. 2D,I,J,K), which appears to correspond to recently described arch-associated noradrenergic neurosecretory cells that are likely to be homologous to the carotid body glomus cells of mammals involved in the control of blood oxygen levels (see Guo et al., 1999). Other area of *tbx20* expression includes a layer of mesenchyme cells overlying the roof of the pharynx (Fig. 2N, arrowheads; Fig. 2O), which also expresses zebrafish type II collagen (*col2a1*; Yan et al., 1995) at this stage.

2. Materials and methods

2.1. Isolation and characterization of *tbx20*

A zebrafish λ gt11 (Stratagene) cDNA library (gift from Dr Kai Zinn, Division of Biology, California Institute of Technology) constructed from 30- to 33-h-old embryos was screened under low stringency conditions (50°C) following standard procedures (Sambrook et al., 1989), using a mixture of ³²P-labeled oligonucleotide probes prepared from various mouse T-box sequences. Positive plaques were individually isolated, and the included cDNAs were subcloned into the pBluescript (Stratagene). Identity of each clone was determined by a partial sequencing of the T-box region and a subsequent BLAST search against GenBank database. One clone was found to contain a T-box sequence highly similar to that of *Drosophila H15* (Brook and Cohen, 1996), as well as that of human *TBX20* (GenBank Accession No: AJ237589), and was therefore named *tbx20*. Since the initial clone lacked a valid stop codon and a poly(A)⁺ tail, additional cloning was performed by a 3' RACE against cDNAs from 24 hpf

embryos, using nested primer sets designed to amplify the missing 3' end. The resulting product contained a stop codon in the correct reading frame followed by a 610-base pair 3' UTR including a poly(A)⁺ tail, indicating that the missing 3' end of *tbx20* was successfully recovered by this procedure. The full-length sequence of *tbx20* cDNA was then determined and deposited in the GenBank data base under the accession number AF253325.

2.2. Phylogenetic analysis

Evolutionary relationships between *tbx20* and other T-box genes were estimated by phylogenetic analysis using neighbor-joining algorithm applied to the Poisson-corrected amino acid sequence distances (Kumar et al., 1993), excluding the gaps. Amino acid sequences of T-box domains from various T-box genes were first aligned using Clustal W's alignment algorithm (Thompson et al., 1994), and distances and trees were then calculated using MEGA phylogenetic analysis software package (Kumar et al., 1993).

2.3. Whole-mount *in situ* hybridization

Embryos used in whole-mount *in situ* hybridization were collected from natural spawnings and kept in embryo medium at 28.5°C until fixation. Staging was done using the criteria described in Kimmel et al. (1995). Whole-mount *in situ* hybridization was carried out essentially as described in Thisse et al. (1995). Digoxigenin-labeled riboprobes were prepared by *in vitro* transcription of full-length or near full-length cDNAs of *tbx20*, *islet1*, *flk1*, *nkx2.5*, *gata1*, and purified through a CHROMA spin column (Clonetech). Stained embryos were cleared in 80% glycerol and either photographed as a whole-mount under a dissection microscope, or examined under a compound microscope as a flat mount after removal of the yolk. In some cases, embryos were processed further for histological examinations on semi-thin sections. Selected embryos were dehydrated through graded ethanol series after staining, and then embedded in epon/araldite resins (Polysciences Inc.). Serial cross sections (10 μ m) were made using a diamond knife (DuPont) on an LKB ultramicrotome. Sections were then briefly counterstained with Eosin Y (1%) and coverslipped under Permount (Fisher) for microscopic examination and photography.

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