

Gene expression pattern

Cloning of zebrafish T-box genes *tbx15* and *tbx18* and their expression during embryonic development

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Abstract

Members of the T-box (*tbx*) gene family encode developmentally regulated transcription factors, several of which are implicated in human hereditary diseases. We have cloned the paralogous genes *tbx15* and *tbx18* in zebrafish and have characterised their expression in detail. *tbx15* is expressed in paraxial head mesenchyme and its derivatives, the extraocular and jaw musculature and the posterior neurocranium. Further areas of *tbx15* expression are in the anterior somitic mesoderm, in periocular mesenchyme and in the pectoral fin mesenchyme throughout larval development. Areas of strong *tbx18* expression are found in the developing somitic and presomitic mesoderm, in the heart and in pectoral fin mesenchyme, as well as the ventral neuroectoderm and the developing palate. Both genes exhibit particular differences in expression compared to their murine orthologs. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Results and discussion

T-box (*tbx*) genes encode a large family of transcription factors, whose importance in development is emphasised by the identification of several mutations in *tbx* genes linked to human hereditary syndromes (reviewed in Smith, 1999; Papaioannou, 2001). To identify *tbx* genes that are expressed during limb and head development, we cloned two novel zebrafish T-box genes which we call *tbx15* and *tbx18* (Fig. 1A). Both genes are members of the *Tbx1*-subfamily, of which so far only *tbx20*, also called *hrT*, had been reported in zebrafish (Ahn et al., 2000; Griffin et al., 2000). Phylogenetic analyses confirmed their orthology to murine and human *Tbx15* and *Tbx18*, respectively (Fig. 1B) (Agulnik et al., 1998; Yi et al., 1999; Kraus et al., 2001). Both genes, together with human *Tbx22* (Laugier-Anfossi and Villard, 2000), constitute a group with a single homologue in amphioxus, *tbx15/18/22* (Ruvinsky et al., 2000). Human *Tbx15* shows linkage with acromegaloid facial appearance, a dominant human syndrome that affects many of the tissues that express *Tbx15* in the mouse (Agulnik et al., 1998).

Expression of *tbx15* is first detected at 13 h post fertilisation (hpf) bilaterally in paraxial head mesenchyme (not shown). Expression increases in the paraxial head mesenchyme by 17 hpf, flanking rhombomere 3 and extending posteriorly to the trunk mesoderm. Expression is excluded from the notochord and otic vesicles (Fig. 2A–C). *tbx15* is expressed segmentally in somites in a medial position lateral to the notochord within the first 6–8 somites at 17 hpf (Fig. 2D, E). Expression then expands to the entire somite at 27 hpf, but is absent from tail somites at all developmental stages (Fig. 2F and not shown).

By 24 hpf the first pharyngeal arch mesenchyme, and a layer of extraocular mesenchyme surrounding the eye, begin to express *tbx15* (Fig. 2G, H). During the second day of development expression in the paraxial head mesenchyme extends dorsally, anterior and posterior to the ear and resembles the cartilaginous condensations of the posterior neurocranium (Fig. 2I, J). Fate mapping and extirpation studies in other vertebrates suggest that the posterior neurocranium is of mesodermal origin (Langille and Hall, 1989; Le Lievre, 1978), thus *tbx15* may play a role in the development of the neurocranial commissures and auditory capsules. Strong expression is detected in extraocular and dorsal jaw muscles as early as 36 hpf, particularly in the medial rectus and adductor mandibulae, and is observed in all pharyngeal muscles of the mandibular and hyoid arches at 60 hpf

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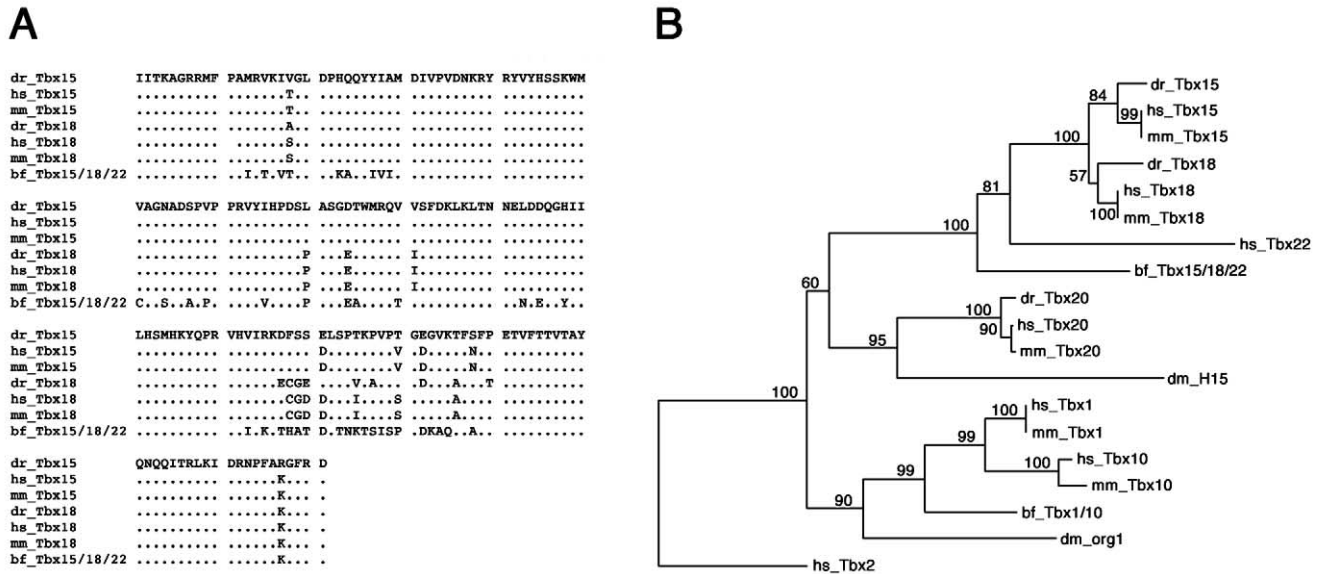


Fig. 1. Sequence alignment and phylogeny of *Tbx15/18* genes. (A) Alignment of predicted amino acid sequence of Tbx15 and Tbx18 orthologs from zebrafish (dr; GenBank accessions AF448504 and AF448503), human (hs; Tbx15 assembled from human contigs AL390796, AL446025 and AL357045; SwissProt 095935), mouse (mm; AAC32316; AAG48598) and Amphioxus (bf; AAG34891). Because the isolated sequences of zebrafish *tbx15* lack the 5'-end of the gene, the 171 common amino acids within the T-box were aligned for phylogenetic analyses. The alignment was performed using PILEUP; periods depict identical amino acids. (B) Phylogenetic tree of the Tbx1-subfamily, aligned using amino acid sequences as shown in (A), and rooted with human Tbx2. Note that zebrafish *tbx15* and *tbx18* are orthologs of the mammalian genes. Reliability of internal nodes was tested using 1000 bootstrap replications; values are presented above particular branches. dr: *Danio rerio*; hs: *Homo sapiens*; mm: *Mus musculus*; dm: *Drosophila melanogaster*; bf: *Branchiostoma floridae*.

(Fig. 2K). Within the developing fins, *tbx15* is expressed in the pectoral fin bud mesenchyme starting at 27 hpf, prior to distal outgrowth of the fin bud proper. Throughout pectoral fin outgrowth expression remains strong in the entire fin bud mesoderm (Fig. 2L–N). Areas of strong *tbx15* expression are found in larval fins at 30 days post fertilisation (dpf) (8.5 mm body length) in the distal tips of lepidotrichs (Fig. 2O). Pelvic and unpaired fins were not assayed for expression.

Expression of *tbx18* is first detected from 11 hpf onwards, shortly after the onset of somitogenesis, in the somitic and presomitic mesoderm (not shown). At 19 hpf expression in the somitic mesoderm is strong in the anterior half and medial aspect of posterior somites. More mature anterior somites express *tbx18* in dorsal and ventral regions of the somites, where they are restricted to the somitic mesoderm proximal to the notochord (Fig. 3A–C). *tbx18* expression gradually ceases within the somites in an anteroposterior direction, such that expression at 29 hpf is restricted to the posterior 13–15 somites and is absent from somites by 40 hpf. Interestingly, from 17 hpf onwards, expression of *tbx15* gradually appears in place of *tbx18* in the somites (compare Figs. 3C and 2E, F). At 24 hpf, *tbx18* is expressed in two bilateral domains of single-cell width, extending from within the mesencephalon posteriorly into rhombomere 2. The timing and neuroectodermal location of this expression identify this tissue as the developing medial longitudinal fascicles (Fig. 3D, E). At 30 hpf *tbx18* transcript is detected in the paraxial head mesenchyme, flanking the otic vesicles medially and

dorsally (Fig. 3F, G). This expression is strongly maintained at least up to 3 dpf (Fig. 3L).

tbx18 expression in the heart is detected from 36 hpf onwards in the sinus venosus, atrium and ventricle. In addition, strong expression is found in the septum transversum from 48 hpf onwards (Fig. 3H–J). *tbx18* is strongly expressed in the facial area anterior to and surrounding the mouth and in an area lining the olfactory epithelium (Fig. 3K, K'). From 40 hpf onwards the developing palate strongly expresses *tbx18* (not shown and Fig. 3K, L).

In developing pectoral fins, *tbx18* expression is not detected prior to 27 hpf. During outgrowth of the apical fold, expression is restricted to the median part of the fin bud mesenchyme, and then expands to the entire mesenchyme. Expression of *tbx18* is later restricted to the margin of the pectoral fin buds and is expressed in the interradial zones of the pectoral fins of 30 day old larvae (Fig. 3M–Q).

Expression of zebrafish *tbx15* in the cranial paraxial mesoderm, the head musculature and the somites are novel features not reported in the mouse. However, expression surrounding the eyes, in the branchial region and in forelimbs are reminiscent of the mouse expression pattern (Agulnik et al., 1998). Likewise, we find both similarities and differences in expression between the orthologs of *tbx18*. Somitic expression of *tbx18* is not restricted to the sclerotome in zebrafish, and cranial paraxial expression is not observed during somitogenesis stages in zebrafish. Moreover, expression of *tbx18* was not observed in the central nervous system and in the palate of the mouse (Kraus et al., 2001).

2. Material and methods

2.1. Fish stocks

Breeding zebrafish of the London wild type strain were reared and staged at 28.5°C according to Westerfield (1995).

2.2. Cloning of *T-box* genes

Partially degenerate primers were synthesised to a portion of the T-box with regions of amino acid identity between mouse and human Tbx proteins (HEVGTEM: 5'-ccaa-gacctgagcat/cgaa/ggtiggiaci-gaa/gatg-3'; PFAKGFR: 5'-

gcagttatcgatcg/ta/gaaiccc/ttigca/gaaigg-3') that contained *XhoI* and *Clal* restriction sites, respectively, for subcloning of the 544 bp amplification product into pBluescript SK. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed with these primers on first strand cDNA, prepared from 48 hpf wild type embryos using a kit (Pharmacia Biotech). PCR conditions: 36 cycles of 1 min, 94°C; 2 min, 54°C; 1.5 min, 72°C; followed by an extension step of 7 min, 72°C. Sequencing and phylogenetic analyses identified two clones as homologues of *tbx15* and *tbx18* in humans and mouse. To isolate the 3'-sequence of *tbx15* primer 5'-gttcctactggagaaggagtgaagac-3' was used for 3'-RACE on 24 hpf cDNA following the manufacturer's

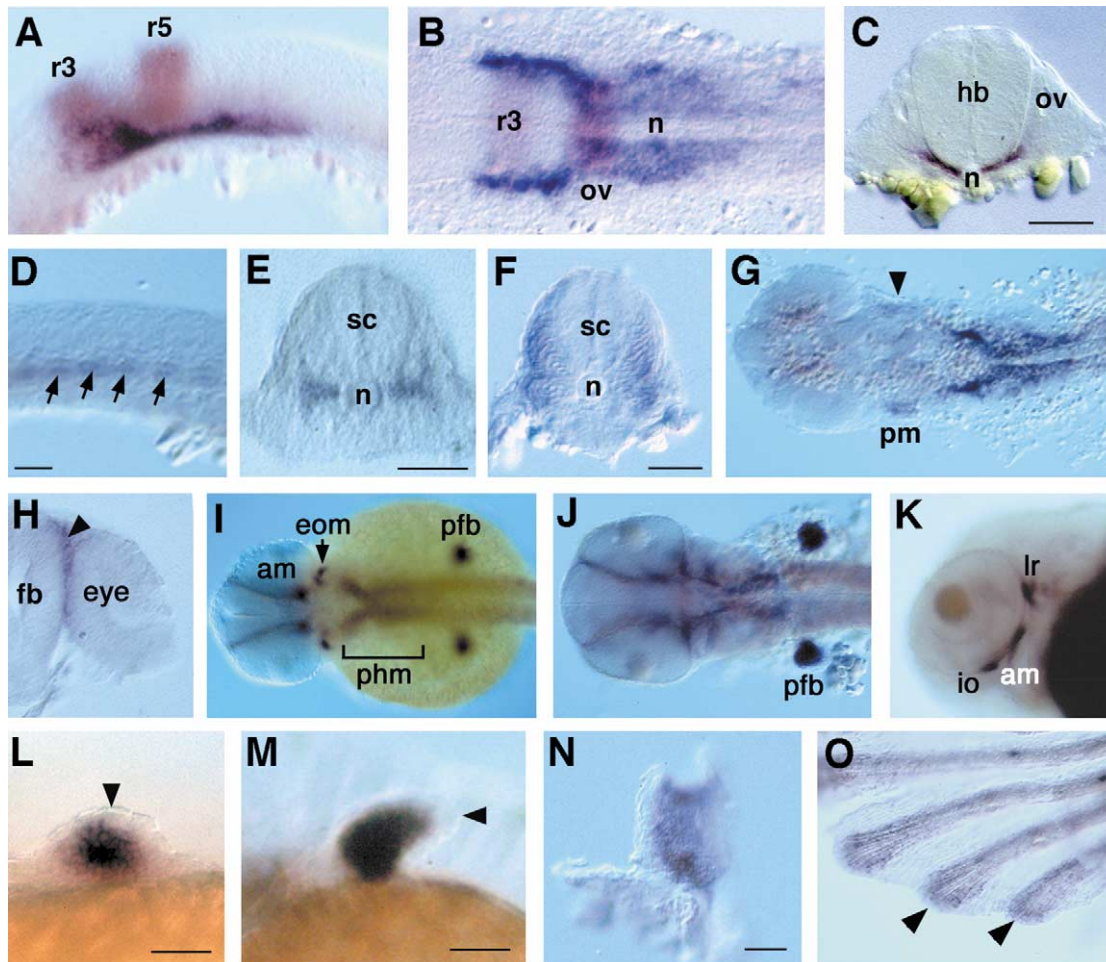


Fig. 2. Expression of *tbx15* during wild type zebrafish development. (A,B) Double in situ hybridisation showing *tbx15* expression (purple) at 17 hpf in the paraxial head mesenchyme, extending rostrally underneath and lateral to rhombomere 3 (r3), counterstained with *krox-20* in r3 and r5 (red); (B) dorsal view. (C) At 18 hpf expression is restricted to the paraxial mesenchyme, but excluded from r5 and otic vesicles. (D,E) Somitic expression at 17 hpf in 10–11 rostral somites in the region of the horizontal myoseptum (arrows) expands to the entire somite at 27 hpf (F); (E,F) transverse sections at the level of the fifth somite. (G) Expression is detected in the first pharyngeal arch (at 24 hpf) and (H) in mesenchymal cells separating forebrain and eye. (I,J) *tbx15* expression in extraocular and pharyngeal arch muscles, paraxial head mesoderm and pectoral fin bud mesenchyme at (I) 30 hpf and (J) 48 hpf. (K) Strong expression in eye and pharyngeal muscles; ventro-lateral view at 60 hpf. (L–O) *tbx15* transcript in developing pectoral fins; expression is exclusively mesodermal, and at 36 h (L) is strong in the medial part of the fin bud and absent from the epidermis (arrowhead); mesodermal expression remains strong throughout the fin bud at (M) 48 hpf and (N) 3 dpf; (O) At 30 dpf *tbx15* is strongly expressed in the margins and distal tips of lepidotrichs (arrowheads). (A,D,K–N) lateral views, (B,G,I,J) dorsal views, (C,E,F,H) transverse sections, (O) ventral view. am, adductor mandibulae; eom, extraocular muscles; fb, forebrain; hb, hindbrain; io, inferior oblique; lr, lateral rectus; n, notochord; ov, otic vesicle; pfb, pectoral fin bud; phm, paraxial head mesenchyme; pm, pharyngeal arch mesenchyme; r, rhombomere; sc, spinal cord; Scale bars, 100 μ m.

protocol (Smart RACE Kit, Clontech). The *tbx18* fragment overlapped with a zebrafish EST (GenBank accession number AI666969) encoding a partial *tbx18* clone. The 3'-end of *tbx18* was amplified by 3'-RACE with primer 5'-TTCGCTCTCCGCAGACTCC-3' from 75 hpf cDNA.

2.3. In situ hybridisation

Whole mount in situ hybridisation was performed as described previously (Begemann and Ingham, 2000).

2.4. Phylogenetic analysis

Amino acid sequences of the alignable T-box domains from members of the Tbx1-subfamily were aligned using

the ClustalX program (Thompson et al., 1997). A tree was constructed based on Poisson-corrected distances using the neighbour joining algorithm implemented in TREECON (Van de Peer and De Wachter, 1993).

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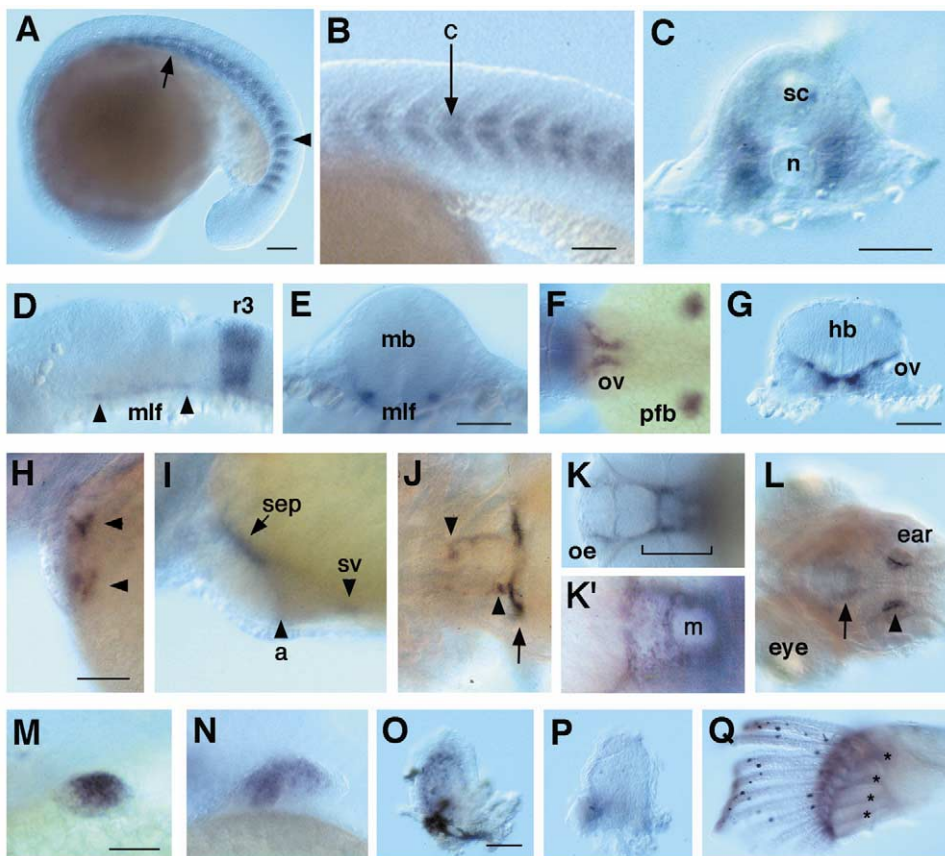


Fig. 3. Expression of *tbx18* during wild type zebrafish development. (A–C) Somitic expression of *tbx18*. (A) At 19 hpf expression is strong in the anterior halves of posterior somites (arrowhead); more mature somites express *tbx18* above and below the horizontal myoseptum (arrow). (B) Higher magnification, 24 hpf; expression is restricted to the median part of each somite; a transverse section (C) at the level indicated by the arrow reveals the restriction of expression to the somites. (D,E) The ventral neuroectoderm expresses *tbx18* at 24 hpf in the medial longitudinal fascicles; r3 is indicated by *krox-20* expression. (F,G) *tbx18* transcript is detected at 30 hpf in the paraxial head mesenchyme, flanking the otic vesicles medially and dorsally. Note expression in the pectoral fin mesenchyme. (H–J) *tbx18* transcript in the developing heart at 36 hpf (H), 48 hpf (I) and 4 dpf (J); strong expression is observed in the septum transversum (arrow); arrowheads point to sites of *tbx18* expression in atrium and sinus venosus. (K,K') Ventral views of the head at 40 hpf; *tbx18* is expressed anterior to and surrounding the mouth (bracket indicates region depicted magnified in a different embryo in K') and in an area flanking the olfactory epithelium; (L) Ventral view of the head of a 3-day-old embryo (lower jaw removed), showing *tbx18* expression in the palate (arrow) and in paraxial mesoderm between notochord and ear. (M–Q) *tbx18* expression in developing pectoral fins; expression is initiated shortly before 30 hpf (M) in the median part of the fin bud mesenchyme and expands to the entire mesenchyme by 48 hpf (N); (O) at 3 dpf expression is stronger in the anterior mesenchyme and the mesenchymal margin; at 4 dpf (P) expression is restricted to cells bordering the non-expressing apical fold; (Q) pectoral fin at 30 dpf; expression of *tbx18* in the interradial zone (asterisks); dark spots on the pectoral fin rays are melanocytes. (Distal is to the left, anterior to the top. (C,E,G) transverse sections, (A,B,D,H,I,M–P) lateral views, (J–L,Q) ventral views. (F) dorsal view. a, atrium; hb, hindbrain; m, mouth; mb, midbrain; mlf, medial longitudinal fascicle; n, notochord; oe, olfactory epithelium; ov, otic vesicle; pfb, pectoral fin bud; r, rhombomere; sep, septum transversum; sc, spinal cord; sv, sinus venosus; Scale bars, 100 μm.

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