Gene Expression Patterns 10 (2010) 24–30

Contents lists available at ScienceDirect

Gene Expression Patterns

journal homepage: www.elsevier.com/locate/gep

Identification and characterisation of the developmental expression pattern of *tbx5b*, a novel *tbx5* gene in zebrafish

Ricard Albalat [b], Mireia Baquero [a], Carolina Minguillón [a,*]

[a] CSIC-Instituto de Biología Molecular de Barcelona, Parc Científic de Barcelona, C/Baldiri i Reixac, 10, 08028 Barcelona, Spain
[b] Departament de Genètica, Facultat de Biologia, and Institut de Recerca de la Biodiversitat (IrBio), Universitat de Barcelona, Avda. Diagonal, 645, 08028 Barcelona, Spain

ARTICLE INFO

Article history:
Received 9 October 2009
Received in revised form 9 November 2009
Accepted 10 November 2009
Available online 17 November 2009

Keywords:
Teleost
Zebrafish
T-box
tbx5
Gene duplication
Heart development
Eye development
Pectoral fin development

ABSTRACT

Tbx5 is a T-box transcription factor that has been characterised in most vertebrate lineages and is widely expressed during the development of various embryonic structures, including the heart, the eyes and the anterior set of paired appendages (tetrapod forelimbs and fish pectoral fins). Mutations in *TBX5* cause Holt–Oram syndrome, an autosomal dominant human "heart-hand" condition characterised by upper limb and heart malformations. In zebrafish, embryos with compromised *tbx5* function show a complete absence of pectoral fins, whereas heart and eye development are not so highly disturbed. Here, we identify a new *tbx5* gene in zebrafish that we have called *tbx5b*. This duplicate gene is present in all teleost genomes whose sequence is available, suggesting it resulted from the teleost-specific genome duplication event that took place during fish evolution. We show that *tbx5b* has lost the characteristic forelimb/pectoral fin expression of Tbx5 genes but has retained the eye and heart expression, partially overlapping with that of its paralogue, now referred to as *tbx5a*. Functional redundancy of *tbx5a* and *tbx5b* in the eye and heart would therefore explain the mild phenotypes observed during development of these organs in fish embryos with compromised *tbx5a* function.

1. Results and discussion

T-box genes encode a family of transcription factors characterised by the presence of a dimerisation/DNA binding domain, the T-box, that has been used to further subdivide this family into subfamilies based on their sequence similarity. From these, the Tbx2/3/4/5 subfamily has received much attention due to the essential roles its members play during embryonic development. In vertebrates, this subfamily is composed of four genes – *Tbx2*, *Tbx3*, *Tbx4* and *Tbx5* – grouped in two clusters, *i.e.* the *Tbx2–Tbx4* and the *Tbx3–Tbx5* gene pairs. The initial cluster arose by the tandem duplication of a single *Tbx2/3/4/5* gene long before the divergence of protostomes and deuterostomes, generating a *Tbx2/3–Tbx4/5* cluster as still found in the most basal chordate amphioxus (Horton et al., 2008). After the divergence of the different chordate subphyla, the *Tbx2/3–Tbx4/5* pair was further duplicated due to the whole-genome duplications that took place during vertebrate evolution, giving rise to the two clusters *Tbx2–Tbx4* and *Tbx3–Tbx5* currently found in many vertebrate species (Agulnik et al., 1996). The paralogues *Tbx4* and *Tbx5* have been extensively studied due to their limb-type specific expression pattern. Briefly, *Tbx5* is expressed in the anterior set of vertebrate appendages (*i.e.* tetrapod forelimbs and fish pectoral fins), whereas *Tbx4* is expressed in the posterior set (*i.e.* tetrapod hindlimbs and fish pelvic fins) [reviewed in Logan (2003)]. However, gene deletion/replacement experiments have shown these genes do not play a role in determining limb-type identity but rather they have equivalent (and thus interchangeable) roles during the initiation of limb outgrowth. Essentially, mouse and fish mutant/morphant for *Tbx5* lack the anterior set of paired appendages whereas hindlimbs fail to develop in knockout *Tbx4* mice. Moreover, limb-delivered *Tbx4* can rescue the no-forelimb phenotype of the *Tbx5* conditional knockout and the rescued embryos develop phenotypically normal forelimbs (Agarwal et al., 2003; Ahn et al., 2002; Garrity et al., 2002; Minguillon et al., 2005; Naiche and Papaioannou, 2003; Rallis et al., 2003).

1.1. tbx5 gene duplication in the teleost lineage

The pivotal role *Tbx5* plays during forelimb/pectoral fin development was first identified in zebrafish embryos using a morpholino knockdown approach. Morphant *tbx5* embryos failed to initiate fin bud formation, leading to the complete loss of pectoral fins, due to the lack of directed migration of individual lateral plate mesoderm (LPM) cells into the prospective fin bud region (Ahn et al., 2002). Other tissues with prominent *tbx5* expression such as the developing heart or eyes (Begemann and Ingham, 2000; Ruvinsky et al., 2000; Tamura et al., 1999) showed however only

* Corresponding author. Tel.: +34 93 4020234; fax: +34 93 4034979.
E-mail address: carolina.minguillon@ibmb.csic.es (C. Minguillón).

1567-133X/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.gep.2009.11.003

subtle or no phenotypic abnormalities in *tbx5* morphant embryos, compared to the severe defects in fin formation. Similarly, *tbx5* mutant embryos, *heartstrings*, lack pectoral fins but their heart appears to develop normally through heart tube stages (Garrity et al., 2002). These observations pointed to the existence of extra *tbx5* copies in the zebrafish genome that may rescue the lack of function or morpholino-induced defects due to physiological redundancy in tbx5 activity. To test this possibility, we surveyed five teleost genomes – *Danio rerio, Gasterosteus aculeatus, Oryzias latipes, Takifugu rubripes* and *Tetraodon nigroviridis* – for *tbx5* genes. We have identified two putative *tbx5* copies in each fish genome, which we have called *tbx5a* and *tbx5b*. The *tbx5a* genes correspond to the previously reported forms linked to *tbx3* genes in the *tbx3–tbx5* cluster, while *tbx5b* stands for the genes identified here. We have found no *tbx3* duplicates in the vicinity of these newly identified *tbx5b* genes.

The *tbx5* nature of the tbx5b proteins was inferred initially by best-hit BLASTP searches against human proteins database (E-values from $9e^{-150}$ to $9e^{-118}$ with human TBX5) and confirmed by phylogenetic reconstructions. To establish the orthologous and paralogous relationships of the fish *tbx5* genes, we performed phylogenetic analyses adding the new tbx5b sequences to a vertebrate Tbx4–Tbx5 phylogeny. The amphioxus single *AmphiTbx4/5* gene sequence was used as outgroup. Tree topology split the Tbx4 and the Tbx5 sequences in two clusters, with the new fish sequences clustering at the base of the Tbx5 group (Fig. 1). The exclusion of AmphiTbx4/5 and use of Tbx4 as outgroup did not modify tree topology: the existence of a tetrapod Tbx5 – fish tbx5a group with fish tbx5b members clustering at a basal position was still strongly supported (100%) (data not shown). The basal position of tbx5b forms and the presence of a sole *Tbx5* gene in tetrapods could be explained as a *Tbx5* duplication predating the divergence of fish and tetrapods followed by the loss of the Tbx5b members in the tetrapod lineage. However, an alternative evolutionary scenario compatible with the tree topology was possible: taking into consideration the genome duplication that specifically affected the teleost lineage (the R3 hypothesis) (Amores et al., 1998; Meyer and Schartl, 1999), *tbx5b* genes may represent fish-specific forms derived from such a duplication event. After duplication, one of the *tbx5* copies – the one that yielded the current "b" forms – diverged rapidly due to increased evolutionary rates. Amino acid distances between fish tbx5 sequences are in average 4.5-fold higher for tbx5b than for tbx5a. This higher rate agrees with the different branch lengths of tbx5a and tbx5b clusters in the phylogenetic tree (Fig. 1) and supports increased evolutionary rates for fish *tbx5b* genes after duplication. Asymmetric evolutionary rates among paralogs may affect tree topologies, and those members of a protein family evolving at faster rates, the *tbx5b* forms in this case, would branch artifactually at a more basal position inside the family (Fares et al., 2006; Hahn, 2007).

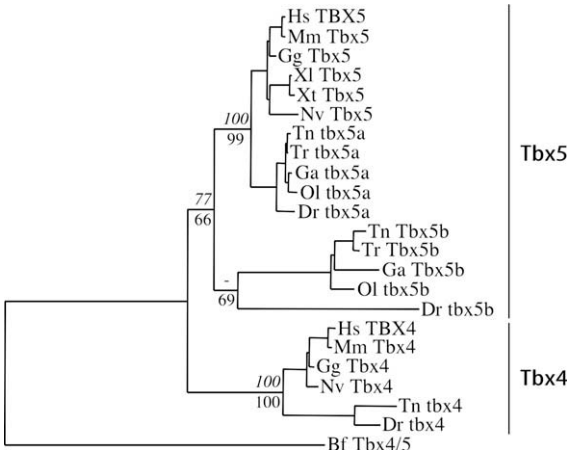

Fig. 1. Phylogenetic tree of the Tbx4/5 subfamily of T-box proteins generated by maximum-likelihood (ML) method. The same tree topology was supported by neighbour-joining (NJ) method. *B. floridae* Tbx4/5 was used as outgroup. Figures at the nodes are percentage of bootstrap values supporting each node ($n = 1000$ for ML, and $n = 1000$ for NJ, in italics). Tree topology split the Tbx4 and the Tbx5 sequences in two clusters, with the fish tbx5b sequences within the Tbx5 group. We hypothesize that fish *tbx5b* genes derived from a genome duplication specific of fish lineage (the R3), and the basal position of fish tbx5b within the Tbx5 cluster would be caused by accelerated evolutionary rates of the duplicates (see text for details). Bf, *Branchiostoma floridae*; Dr, *Danio rerio*; Ga, *Gasterosteus aculeatus*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Nv, *Notophthalmus viridescens*; Ol, *Oryzias latipes*; Tn, *Tetraodon nigroviridis*; Tr, *Takifugu rubripes*; Xl, *Xenopus laevis*; Xt, *Xenopus tropicalis*.

1.2. Synteny analysis of tbx5 genes in teleosts

The R3 origin of the *tbx5a* and *tbx5b* genes can be tested because it predicts that (1) both fish *tbx5* genes should be located in genomic regions with conserved synteny within the region surrounding tetrapod *Tbx5* genes (e.g., the region surrounding the human *TBX5* gene in chromosome 12), and (2) that a certain degree of syntenic conservation should be observed among genomic neighbourhoods of the fish chromosomes containing *tbx5* genes. To test these predictions, we performed dotplot and circleplot analyses with the genomes of the four teleost species implemented in the Synteny Database (Cañestro et al., 2009; Catchen et al., 2009). Dotplot analysis comparing 20 Mb surrounding human *TBX5* in chromosome 12 (Hsa12) with the genomes of the four teleost species clearly identified conserved synteny between Hsa12 and the two *tbx5*-containing chromosomes of *O. latipes, G. aculeatus* and *T. nigroviridis* species – *O. latipes* chromosomes 12 and 9, *G. aculeatus* Group XIV and Group XIII, and *T. nigroviridis* chromosomes 4 and 12 – but with only one zebrafish chromosome, number 5, that contained both *tbx5* copies (Fig. 2A–D). The conserved synteny in the two *tbx5*-containing chromosomes with human chromosome 12 is in agreement with the first prediction. Additionally, circleplots between the *tbx5*-genomic neighbourhoods and each fish chromosome showed paralogous syntenic conservation among neighbourhoods of the fish chromosomes containing *tbx5* genes (Fig. 2E–G). This is in agreement with the second prediction. These data therefore provided strong evidence of a fish-specific *tbx5* duplication due to a total or partial genome duplication episode, and suggested a later rearrangement of the *tbx5b* gene in *D. rerio*. Although we cannot formally discard other complex patterns of gene duplications and losses, we favour the most parsimonious hypothesis of an R3 origin for the two *tbx5* genes in teleosts, followed by an accelerated evolutionary rate of the *tbx5b* copies leading to an artifactual basal position in phylogenetic tree reconstructions.

1.3. tbx5a and tbx5b expression patterns during early zebrafish development

Zebrafish *tbx5b* prediction was verified by comparison with two EST sequences found in public databases and the PCR amplification of the *tbx5b* cDNA. To elucidate the expression pattern of *tbx5b* during teleost development, we performed whole-mount *in situ* hybridisation analyses of zebrafish embryos using a fragment of the amplified cDNA as a probe and compared it to that of *tbx5a*. The earliest signal of *tbx5b* was observed at 14 hpf (hours post-fertilisation) in the developing eye (Fig. 3A, asterisk), where *tbx5a* was also co-expressed (Fig. 3A′, asterisk). However, *tbx5b* expression was not observed in the lateral plate mesoderm (LPM), where *tbx5a* is conspicuously expressed (Fig. 3A′), even at long exposure conditions (Fig. 3A). *tbx5b* was only detected in bilateral stripes in the LPM from 17 hpf onwards (Fig. 3B). Nevertheless, the expression patterns of both genes in the LPM were distinct: *tbx5a* was expressed in a longer anterior–posterior domain than *tbx5b* (compare