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ORIGINAL ARTICLE

Identification and developmental expression of two *Tbx1/10*-related genes in the agnathan *Lethenteron japonicum*

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Abstract We have identified two *Tbx1/10*-related genes, *LjTbx1/10A* and *LjTbx1/10B*, from the Japanese river lamprey *Lethenteron japonicum*. We used in situ hybridization to characterize their expression pattern during embryonic development. *LjTbx1/10A* and *LjTbx1/10B* shared common expression in the pharyngeal arches and otic vesicle, although their levels and timing of expression differed markedly. *LjTbx1/10A* was highly expressed in the mesodermal core of pharyngeal arches and the adjacent endoderm throughout pharyngeal arch development, whereas *LjTbx1/10B* expression was only transiently upregulated in forming pharyngeal pouches. *LjTbx1/10A* transcripts first appeared at stage 25 in otic vesicles, whereas *LjTbx1/10B* transcripts could already be detected in the developing otic placode at stage 20. These results suggest that lamprey *LjTbx1/10A* and *LjTbx1/10B* may play distinct roles in the patterning and development of the pharyngeal apparatus. It appears that lamprey *Tbx1/10* genes have undergone subfunctionalization independent from gnathostomes, with regard to both regulation and function.

Keywords T-box · *Tbx1* · *Tbx10* · Lamprey

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Introduction

The Tbx gene family of transcription factors is found in many metazoan species and shares a conserved deoxyribonucleic acid (DNA)-binding domain called the T-box. These genes are involved in a number of developmental processes in diverse organisms, including *Caenorhabditis elegans*, *Drosophila*, zebrafish, *Xenopus*, chick, and mice (Papaioannou 2001). Vertebrate T-box genes are divided into five major subfamilies (eight groups) namely, *Brachyury*, *Tbx1* (*Tbx1/10*, *Tbx15/18/22*, *Tbx20*), *Tbx2/3/4/5* (*Tbx2/3* and *Tbx4/5*), *Tbx6*, and *Tbr/Eomes/TBX21* (Minguillon and Logan 2003). Because T-box sequences are highly conserved among organisms, reliable phylogenetic relationships can generally be established. Therefore, comparative studies may provide clues as to how novel functions have evolved.

The invertebrate chordate species ascidian *Ciona intestinalis* and amphioxus have only one *Tbx1/10* gene, whereas gnathostome vertebrates possess separate genes for *Tbx1* and *Tbx10*. In *Ciona*, *Ci-Tbx1/10* transcripts can be detected in dorsal endodermal cells until hatching (Takatori et al. 2004). Amphioxus *Tbx1/10* (*AmphiTbx1/10*) is expressed in a bilateral segmented pattern in the ventral half of somites and branchial arches of neurulae (Mahadevan et al. 2004). Somitic expression persists in 1-day-old larvae but is absent in 2-day-old larvae. Pharyngeal expression of *AmphiTbx1/10* is restricted to the first three branchial

arches and disappears in 4-day-old larvae (Mahadevan et al. 2004).

Vertebrate *Tbx1* was first isolated in mouse where its expression was detected in pharyngeal arches and otic vesicles (Bollag et al. 1994). More detailed expression patterns were later described in lung epithelium, sclerotomes, tongue mesenchyme and developing tooth buds (Chapman et al. 1996). *Tbx1* expression in pharyngeal arches, otic vesicles and sclerotome has now been reported in many vertebrates, including newt (Simon et al. 1997), *Xenopus laevis* (Ataliotis et al. 2005), *Xenopus tropicalis* (Showell et al. 2006), chick (Garg et al. 2001), and human (Chieffo et al. 1997). Similarly, *Tbx1* is expressed in the pharyngeal arches and otic vesicles of zebrafish (Kochilas et al. 2003).

Tbx1 has been linked to DiGeorge Syndrome (DGS; OMIM 18840) and Velocardiofacial Syndrome (VCFS; OMIM 192430) in humans (Chieffo et al. 1997). Abnormalities in individuals with DGS/VCFS include craniofacial defects, aortic arch malformations, thymus and parathyroid hypoplasia, conotruncal cardiac defects, and hearing loss (Ryan et al. 1997). *Tbx1* −/− mice phenocopy many of the characteristics of DGS/VCFS; thus, *Tbx1* has been proposed as a candidate gene for these conditions (Jerome and Papaioannou 2001).

In contrast to *Tbx1*, very little is known about *Tbx10*. In adult humans, *Tbx10* is expressed in the brain, ovary, uterus, pituitary, and fetal kidney, as measured by reverse transcriptase polymerase chain reaction (RT-PCR; Law et al. 1998). In mice, *Tbx10* is expressed during development, as well as in adult ovary, uterus, and kidney, also as measured by RT-PCR (Law et al. 1998). More detailed analyses later showed that *Tbx10* is expressed in cells flanking the dorsal edge of rhombomere 4 of the developing hindbrain (Bush et al. 2003). Expression then spreads ventrolaterally to rhombomere 6, where it is weakly expressed. *Tbx10* is also weakly expressed in tail somites and in condensing mesenchyme of the inner ear (Bush et al. 2003). Recently, it has been shown that *Tbx10* is the gene affected in *Dancer* (*Dc*) mutant mice, a model in which a *Tbx10* transcript lacking exon 1 but with a functional T-box domain is ectopically expressed resulting in cleft lip and palate (Bush et al. 2004).

Agnathan lampreys are an important model system for making developmental and embryological comparisons not only because of their phylogenic position but also because lampreys retain several ancestral characteristics including jawless oral apparatus derived from the pharyngeal arches. This allow ones to determine the events that gave rise to the evolutionary novelties. Only one *Lampetra fluviatilis* (European river lamprey) *Tbx1/10* gene, *LfTbx1*, has been reported (Sauka-Spengler et al. 2002). In this study, we identified two *Tbx1/10*-related genes, *LjTbx1/10A* and *LjTbx1/10B*, in *Lethenteron japonicum* (Japanese river lamprey) and examined their expression patterns in developing lamprey embryos. Our results indicate that the two lamprey *LjTbx1/10* genes may play distinct roles in patterning of the pharyngeal apparatus.

Materials and methods

Embryo collection

Adult male and female lampreys (*L. japonicum*) were purchased from Ebetsu fishery cooperative, Hokkaido, Japan, during the breeding season (early June). Spawning was induced and embryos were reared to the desired developmental stages at 16°C in 10% Steinberg's solution (Steinberg 1957). Lamprey embryos were staged according to Tahara's staging of *L. reissneri*, a species closely related to *L. japonicum* (Tahara 1988). For in situ hybridization, embryos were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) then dehydrated in a graded methanol series and stored in 100% methanol at −20°C.

Isolation of cDNA fragments

The lamprey *Tbx1/10A* and *Tbx1/10B* fragments were amplified using degenerate primers in forward (sequence encoding TKAGRRM; nested AEPAMPG) and reverse (PFAKGFR) orientations and several complementary DNAs (cDNAs) of the novel lamprey T-box genes were identified. Two cDNAs closely related to the mammalian *Tbx1* and *Tbx10* genes were further characterized. 3′ and 5′ rapid amplification of cDNA ends (RACE) using the First Choice RLM-RACE kit (Ambion) were performed to obtain longer fragments of the lamprey *Tbx1/10*-related genes. *LjTbx1/10A* and *LjTbx1/10B* partial coding sequences have been submitted to GenBank under accession numbers EF422068 and EF422069, respectively.

Phylogenetic analysis

Amino acid sequences of the T-box domains of LjTbx1/10A and LjTbx1/10B were aligned with those of other Tbx1/10 subfamily genes. Unalignable regions were excluded from analysis. The molecular phylogenetic tree was inferred with the neighbor-joining method (Saitou and Nei 1987) on the alignment editor XCED in which MAFFT programs are implemented (Katoh et al. 2002). 155 amino acid sites were used for the inference assuming JTT + Γ model.

In situ hybridization

Whole-mount in situ hybridization was performed using protocols modified from Murakami et al. (2001). Embryos

were bleached by a series of 50, 100, 50% ethanol in PBS with 0.1% Tween 20 (PBT) and washed several times in PBT. Embryos were then cleared in 50% glycerol in PBT. Selected embryos were processed for frozen sections by dehydration in 30% sucrose in PBS followed by embedding in 7.5% gelatin in 15% sucrose for cryosections (15 μm).

Results and discussion

Identification of two *Tbx1/10*-related sequences in the lamprey *L. japonicum*

To identify *Tbx1/10*-related genes in stage 22 to 28 lamprey *L. japonicum* embryos, we used RT-PCR with degenerate primers for the T-box. As expected, several 336-bp fragments of T-box genes were amplified. Two of the products with sequence similar to gnathostome *Tbx1* and *Tbx10* and were further characterized. A combination of 3′ and 5′ RACE was used to extend their sequences and yielded 495- and 505-bp fragments, respectively. Sequence analysis of the T-box of these fragments and comparison with members of the *Tbx1/10* subfamily from a variety of other species showed that these two clones are orthologs of the *Tbx1/10* gene family. Thus, we termed these genes *LjTbx1/10A* and *LjTbx1/10B*. We estimated the number of synonymous substitutions (*Ks*) between *L. fluviatilis Tbx1* (Sauka-Spengler et al. 2002) and *LjTbx1/10A* to be 0.281 (*Ka*= 0.003) and between *LfTbx1* and *LjTbx1/10B* to be 1.284 (*Ka*=0.134). In terms of the standard level of synonymous substitutions between lamprey species previously described (Kuraku and Kuratani 2006), this strongly suggests that *LjTbx1/10A* is an ortholog of *LfTbx1*.

To determine the phylogeny of *LjTbx1/10A* and *LjTbx1/10B*, a phylogenetic tree was constructed based on the amino acid sequences of the conserved T-box regions using the neighbor-joining method (Fig. 1). In neighbor-joining analysis, the LjTbx1/10B sequence was part of the group containing all the gnathostome Tbx10 sequences (Fig. 1). However, this grouping was not supported by the maximum-likelihood analysis. Results of the molecular phylogenetic tree inference, which is based on the maximum likelihood, did not converge in a specific tree topology, indicating that *Tbx1/10* may have undergone independent duplication in the lamprey lineage. Therefore, it remains unclear whether lamprey *Tbx1/10A* and *Tbx1/10B* represent true orthologs of gnathostome *Tbx1* and *Tbx10* or duplication of *Tbx1/10* in the lamprey lineage.

Expression of *LjTbx1/10A*

LjTbx1/10A transcripts first appeared at stage 23 in the region of the pharyngeal arches (Fig. 2a,b). At stage 24, signals intensified in the mandibular arch, hyoid arch, and branchial arches 1–3 (Fig. 2c,d). During subsequent stages, *LjTbx1/10A* transcripts appeared in successively developing pharyngeal arches (Fig. 2e–m,r). At stage 26.5, the mandibular arch undergoes a transformation that leads to labial muscle formation. At this stage, *LjTbx1/10A* transcripts were observed in the upper and lower lips, as well as

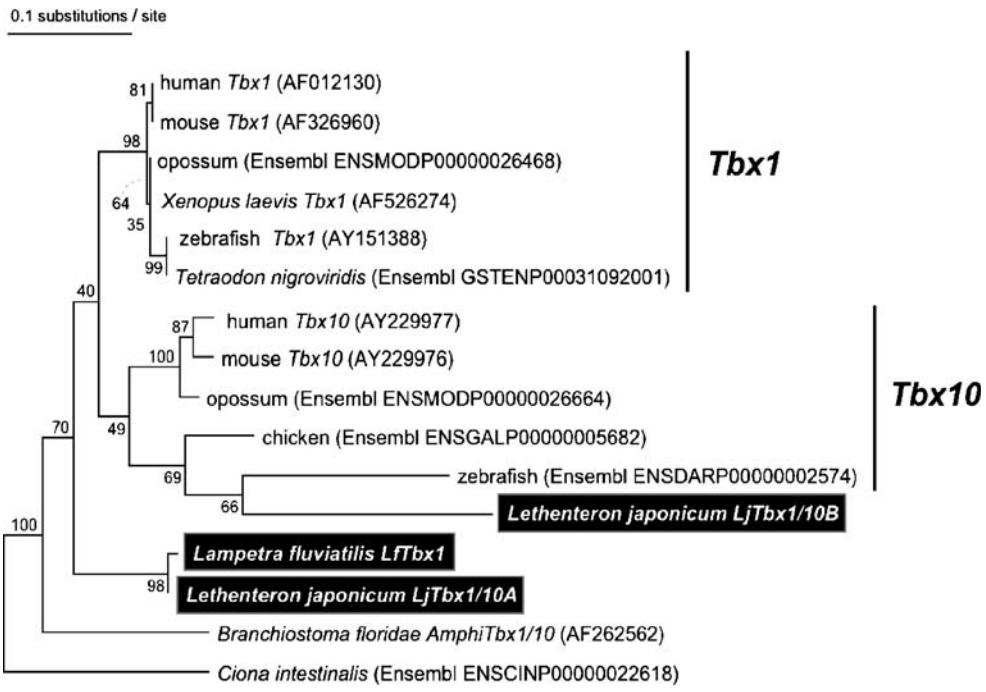

Fig. 1 Phylogenetic comparison of *Tbx1/10*-related genes. Phylogenetic relationship between the Tbx1/10 subfamily members as shown by a consensus tree derived using the neighbor-joining method. *Numbers above the nodes* indicate bootstrap values

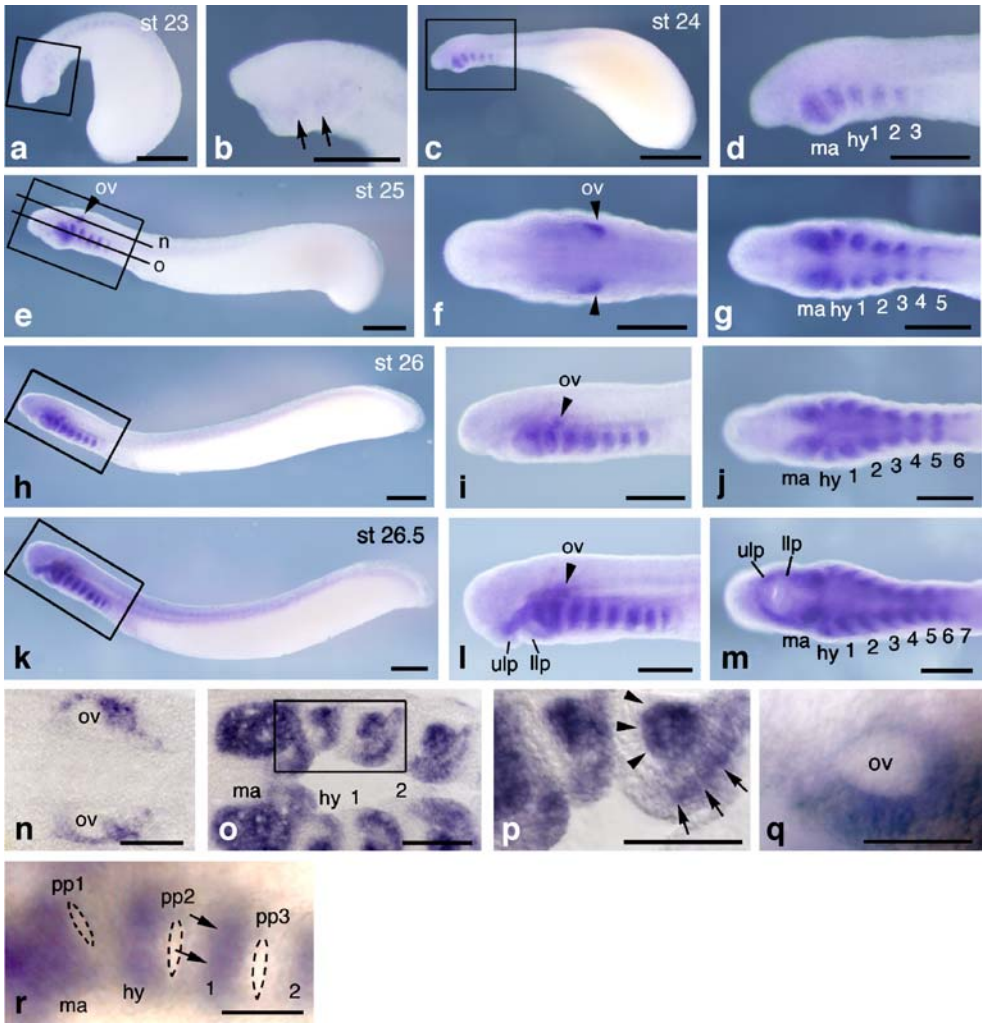

Fig. 2 Expression of *LjTbx1/10A* during embryonic development. **a**, **c**, **e**, **h**, **k** Lateral view of stage 23, 24, 25, 26, and 26.5 embryos, respectively. **b**, **d**, **f**, **g**, **i**, **j**, **l**, **m** Higher magnification of the head region of the photographs shown in **a**, **c**, **e**, **h**, and **k**, respectively. **b**, **d**, **i**, **l** Lateral view. **f** Dorsal view. **g**, **j**, **m** Ventral view. Hybridization signals were seen in the pharyngeal arches and in the lateral wall of the otic vesicles (*arrowheads*). **e** *Thin lines* indicate the plane of sections for **n** and **o**. In the head of stage 26.5 embryos, transcripts were detected in the lower and upper lips (**l**, **m**). **n**, **o** Horizontal sections of a stage 25 embryo. **p** Higher magnification of the photographs shown in **o**. **q**, **r** Lateral view of the otic vesicle (**q**) and the pharyngeal arches (**r**) of a stage 26 embryo. Anterior is to the *left*, and dorsal is at the *top*. The pharyngeal pouches are *outlined*. Transcripts were detected in the mesodermal core (**p**, *arrowheads*) and the posterior endodermal wall of the arches (**p** and **r**, *arrows*) Transcripts were also present in the posterior epithelium of the otic vesicle (**n**, **q**). *hy*, Hyoid arch, *llp* lower lip, *ulp* upper lip, *ma* mandibular *arch*, *ov* otic vesicle, *pp1–3*, the first to third pharyngeal pouches; *numbers 1–7* indicate the first to seventh branchial arches. *Scale bars*=500 μm in **a**–**m**, 100 μm in **n**–**r**

in the hyoid and branchial arches (Fig. 2l,m). In horizontal sections of the pharyngeal arches of stage 25 embryos (Fig. 2o), *LjTbx1/10A* transcripts were detected both in the mesodermal core (Fig. 2p, arrowheads) and in the posterior endoderm (Fig. 2p, arrows). *LjTbx1/10A* was also expressed in the developing otic vesicle (Fig. 2f,i,l,q). Expression appeared in the lateral–posterior regions of the otic epithelium at stage 25 (Fig. 2e,f,n) and persisted at least through stage 26.5 (Fig. 2l, arrowheads).

Although the pattern of *LjTbx1/10A* expression was almost identical to that of *LfTbx1* in *L. fluviatilis* (Sauka-Spengler et al. 2002), *LjTbx1/10A* transcripts were first detected at a slightly later stage.

Expression of *LjTbx1/10B*

LjTbx1/10B transcripts were first detected at stage 20 in a few cells of the otic placode (Fig. 3a, arrowhead). During subsequent stages, expression in the posterior epithelium of the otic placode persisted and intensified (Fig. 3b–g,q). At stage 24, the ectoderm of the otic placode invaginates, forming the otic vesicle, which then separates from the surface ectoderm (Streit 2001). In the otic vesicle of stage 25 embryos, *LjTbx1/10B* transcripts were restricted to the lateral–posterior region of the epithelium (Fig. 3g,n,q), but by stage 26.5, the number of *LjTbx1/10B*-expressing cells in this region was greatly reduced (Fig. 3m). *LjTbx1/10B*

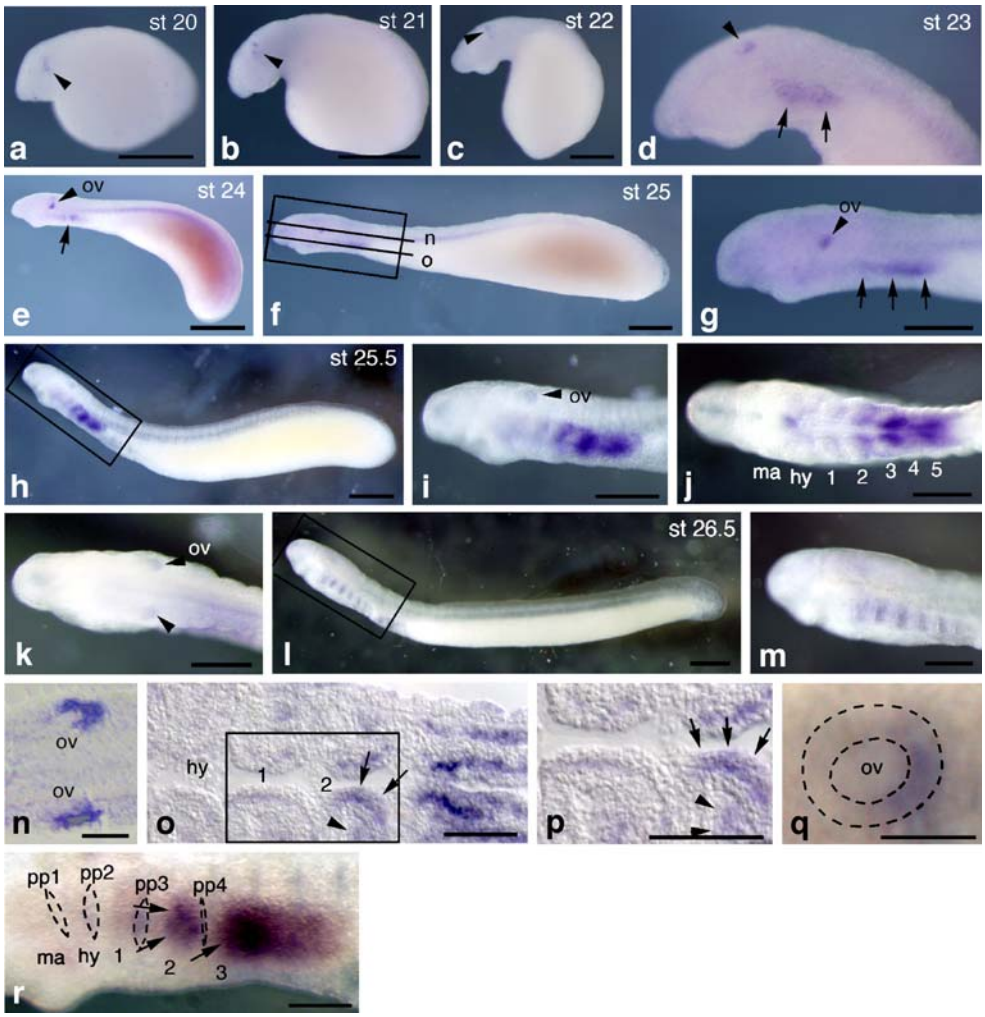

Fig. 3 Expression of *LjTbx1/10B* during embryonic development. **a**, **b**, **c**, **e**, **f**, **h**, **l** Lateral view of stage 20, 21, 22, 24, 25, 25.5, and 26.5 embryos, respectively. **f** *Thin lines* indicate the plane of sections for **n** and **o**. **d** Lateral view of the head region of a stage 23 embryo. **g**, **i**, **j**, **k**, **m** Higher magnification of the head region shown in **f**, **h**, and **l**, respectively. **g**, **m**, **o** Lateral view. **j** Ventral view. **k** Dorsal view. Hybridization signals were seen in the otic vesicles (*arrowheads*) and in the developing pharynx (*arrows*). **n**, **o** Horizontal sections of a stage 25 embryo. **p** Higher magnification of the photograph shown in **o**. **q**, **r** Lateral view of the otic vesicle (**q**) and the pharyngeal arches (**r**) of a stage 24 embryo. Anterior is to the *left*, and dorsal is at the *top*. The otic vesicle (**q**) and the pharyngeal arches (**r**) are *outlined*. Transcripts were detected in the mesodermal core (**o** and **p**, *arrowheads*) and the posterior endodermal wall of the arches (**o**, **p**, and **r**, *arrows*). Transcripts were also detected in the posterior epithelium of the otic vesicle (**n**, **q**). *hy Hyoid* arch, *ma* mandibular arch, *ov* otic vesicle, *pp1–4* the first to fourth pharyngeal pouches; *numbers 1–5* indicate the first to fifth branchial arches. *Scale bars*=500 μm in **a**–**m**, 100 μm in **n**–**r**

expression was also observed in the region forming pharyngeal pouches. Transcripts of *LjTbx1/10B* first appeared in the posterior regions of the pharynx in stage 23 embryos (Fig. 3d) and the signal remained in posterior pharyngeal arches until stage 25 (Fig. 3f,g). At stage 25.5, *LjTbx1/10B* was strongly expressed in pharyngeal arches during pouch outgrowth but seemed to be downregulated once the pouches formed (Fig. 3h–j). By stage 26.5, *LjTbx1/10B* expression in the pharyngeal arches was reduced (Fig. 3l,m) and was undetectable thereafter (data not shown). As shown in Fig. 3o, we analyzed pharyngeal expression of *LjTbx1/10B* in horizontal sections of stage 25 embryos. Intense *LjTbx1/10B* staining was detected in the posterior endoderm of the forming pharyngeal arches (Fig. 3o,p,r). *LjTbx1/10* was also expressed in the mesodermal core of developing arches (Fig. 3o,p).

Comparison of *LjTbx1/10A* and *LjTbx1/10B* expression: insight into the evolution of *Tbx1/10* and its developmental function

LjTbx1/10A and *LjTbx1/10B* share similar expression domains in the developing otic vesicles and pharyngeal arches, but the levels and timing of expression differ. *LjTbx1/10B* transcripts were already visible in developing otic placodes of stage 20 embryos, whereas *LjTbx1/10A*

transcripts were not detectable in the otic vesicle until stage 25 (compare Figs. 2e,f, and 3a). During development of pharyngeal arches, *LjTbx1/10A* was expressed strongly in the pharyngeal endoderm and mesodermal core (Fig. 2), whereas *LjTbx1/10B* transcripts were abundant in the endoderm and mesoderm of the forming pharyngeal pouches but were downregulated once the pouches formed (Fig. 3).

Based on analyses of the gene expression pattern in cephalochordate Amphioxus *Tbx1/10*, it has been suggested that branchial arches and somites are the ancestral expression domains of *Tbx1/10* in chordates (Mahadevan et al. 2004). The two lamprey *Tbx1/10* genes, *LjTbx1/10A* and *LjTbx1/10B*, are expressed in a spatially and temporally distinct manner and seem to have retained their ancestral expression domains in pharyngeal arches. In mouse and zebrafish embryos, *Tbx1* expression in the endoderm of forming pharyngeal pouches is involved in development and patterning of the pharyngeal apparatus and its derivatives (Piotrowski et al. 2003; Arnold et al. 2006). *Tbx1* also plays a role in the proliferation of endodermal cells at sites of pharyngeal pouch outgrowth during mouse embryogenesis (Xu et al. 2005). During lamprey pharyngeal development, *LjTbx1/10B* but not *LjTbx1/10A* is transiently expressed in the endoderm of the forming pharyngeal pouches. Thus, it seems likely that lamprey *LjTbx1/10B* plays a role in proliferation of pharyngeal endodermal cells, similar to that of mouse *Tbx1*. Alternatively, it is possible that *LjTbx1/10B* plays a role in shaping the pouches (Arnold et al. 2006). Intense expression of *LjTbx1/10A* in the mesodermal core, on the other hand, seems to be responsible for the patterning and development of mesodermal cell derivatives that comprise velar muscles. Unlike the lamprey *Tbx1/10* genes, mammalian *Tbx10* is not expressed in the developing facial region, suggesting that *Tbx10* lost its ancestral pharyngeal expression at some point within the vertebrate lineage (Bush et al. 2004). In contrast, *Tbx1/10* seems to have lost its ancestral somatic expression either before the divergence of gnathostomes and lampreys or in the lamprey lineage because *Tbx1* and to a lesser degree *Tbx10* are expressed in developing somites in mice (Chapman et al. 1996; Bush et al. 2003).

Similar to lampreys, spatially restricted expression of *Tbx1* in a posterior domain of the otic epithelium has also been described in zebrafish and mice (Piotrowski et al. 2003; Raft et al. 2004; Hammond and Whitfield 2006). Recent studies in zebrafish and mouse embryos have shown that *Tbx1* plays a crucial role in the developing ear (Jerome and Papaioannou 2001; Piotrowski et al. 2003). In zebrafish, the *van gogh* mutation disrupts *Tbx1*, resulting in reduced otic vesicle size and a lack of semicircular canals and sensory cristae of the ear (Piotrowski et al. 2003). Sensory maculae are, however, present in these mutants (Piotrowski et al. 2003). In mice, mutations in *Tbx1* lead to smaller and thickened otic vesicles, resulting in ears with poorly developed pinnae and misformed semicircular canals (Jerome and Papaioannou 2001). More recently, it has been shown that *Tbx1* regulates the growth of a group of otic epithelial cells in the developing ear and then determines their fate (Xu et al. 2007). Therefore, *Tbx1/10* seems to have gained a novel expression domain and probably a novel function (Hammond and Whitfield 2006) in otic vesicle patterning, before the divergence of lampreys and gnathostomes.

Although neither *LjTbx1/10A* nor *LjTbx1/10B* transcripts were identified in the hindbrain, mammalian *Tbx10* has previously been shown to be expressed in rhombomeres 4 and 6 of the hindbrain (Bush et al. 2003), suggesting that *Tbx10* may have independently gained novel expression domains at some point within the gnathostome lineage. Alternatively, this expression domain may have been lost secondarily in the cyclostome lineage.

The present phylogenetic analyses did not reveal whether the two lamprey *Tbx1/10* genes are true orthologs of gnathostome *Tbx1* or *Tbx10* or the result of an independent duplication event in the lamprey lineage. Extensive genomic analyses will be required to answer this question.

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