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ARTICLE

Molecular Structure and Developmental Expression of Three Muscle-Type Troponin T Genes in Zebrafish

Chung-Der Hsiao, Wei-Yuan Tsai, Long-Shyan Horng, and Huai-Jen Tsai*

Troponin T (Tnnt), a troponin component, interacts with tropomyosin and is crucial to the regulation of striated muscle contraction. To gain insight into the molecular evolution and developmental regulation of Tnnt gene (*Tnnt*) in lower vertebrates, zebrafish *Tnnt1* (slow *Tnnt*), *Tnnt2* (cardiac *Tnnt*), and *Tnnt3b* (fast *Tnnt* isoform b) were characterized. The polypeptides of zebrafish *Tnnt1*, *Tnnt2*, and *Tnnt3b* were conserved in the central tropomyosin- and C-terminal troponin I-binding domains. However, the N-terminal hypervariable regions were highly extended and rich in glutamic acid in polypeptides of *Tnnt1* and *Tnnt2*, but not *Tnnt3b*. The *Tnnt2* and *Tnnt3b* contain introns, whereas *Tnnt1* is intron-free. During development, large to small, alternatively spliced variants were detected in *Tnnt2*, but not in *Tnnt1* or *Tnnt3*. Whole-mount in situ hybridization showed zebrafish *Tnnt1* and *Tnnt2* are activated during early somitogenesis (10 hr postfertilization, hpf) and cardiogenesis (14 hpf), respectively, but *Tnnt3b* is not activated until middle somitogenesis (18 hpf). *Tnnt2* and *Tnnt3b* expression was cardiac- and fast-muscle specific, but *Tnnt1* was expressed in both slow and fast muscles. We propose that three, distinct, muscle-type *Tnnt* evolved after the divergence of fish and deuterostome invertebrates. In zebrafish, the developmental regulation of *Tnnt* during somitogenesis and cardiogenesis is more restricted and simpler than in tetrapods. These new findings may provide insight into the developmental regulation and molecular evolution of vertebrate *Tnnt*. *Developmental Dynamics 227: 266–279, 2003.* © 2003 Wiley-Liss, Inc.

Key words: zebrafish; troponin T; expression pattern; alternative splicing; gene structure; molecular evolution

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INTRODUCTION

In vertebrates, striated muscle is composed of three distinct fiber types: cardiac, slow-twitch, and fast-twitch (Oota and Saitou, 1999). Transgenic techniques have been used to study how *trans*-acting transcription factors and *cis*-acting elements control cardiac and skeletal muscle-fiber specificity in mammals (Nakayama et al., 1996; Calvo et al., 1999; Wang et al., 2000; Koban et al., 2001; Yan et al., 2001). However, the regulatory elements that restrict the transcription of genes encoding contractile proteins specific to fish cardiac or slow- or fast-twitch skeletal muscles have been the subject of only a few studies (Kusakabe et al., 1999; Ju et al., 1999; Xu et al., 1999; Tan and Du, 2002). Fish are excellent models for studying the developmental and fiber-specific regulation of muscle diversity. Compared with mammals, the fish genome is more compact (Venkatesh et al., 2000), so it is easier to dissect the enhancers and silencers that control tissue-specific expression (Aparicio et al., 1995; Muller et al., 2002). In addition, a fish embryo is transparent (Briggs, 2002), so the processes of cardiac and skeletal muscle commitment, differentiation, and maturation can be observed directly in vivo. Finally, the organization of slow-twitch and fast-twitch muscles in fish is more homogenous than in birds and mammals. In fish, slow- and fast-twitch muscles

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are spatially separate: slow muscles form a superficial layer, and fast muscles a deep layer (Waterman, 1969; Koumans and Akster, 1995). These traits make it easy to assay developmental regulation of muscle-fiber specificity in fish.

Troponin T (Tnnt) is the tropomyosin-binding subunit of the troponin complex and is crucial to the regulation of striated muscle contraction (reviewed by Perry, 1998; Filatov et al., 1999). The occurrence of the Tnnt gene (*Tnnt*) is associated with the evolution of muscle tissues. For example, a single *Tnnt* has been identified in the smooth muscle of *Caenorhabditis elegans* (Myers et al., 1996), in the striated muscle of *Drosophila melanogaster* (Fyrberg et al., 1990, Benoist et al., 1998), and in the adductor muscle of scallops (Inoue et al., 1996, 1998). In deuterostome invertebrates, two distinct *Tnnt* were identified in the smooth and striated muscles of ascidians (Endo et al., 1996, 1997). In tetrapods, three fiber-specific *Tnnt* have evolved to regulate muscle contraction in cardiac (Cooper and Ordahl, 1984), slow-twitch (Gahlmann et al., 1987), and fast-twitch (Wilkinson et al., 1984; Bucher et al., 1999) muscles. In addition, each fiber-specific *Tnnt* can yield a variety of isoforms by alternative splicing (reviewed by Schiaffino and Reggiani, 1996; Perry, 1998). Transitional expression of alternatively spliced *Tnnt* during different developmental stages and in different muscle types can modulate muscle calcium sensitivity and muscle performance in tetrapods (Jin et al., 1996; Schiaffino and Reggiani, 1996; Perry, 1998; Marden et al., 1999). In fish, several stage- (Yamano et al., 1991; Johnston et al., 1997) or tissue- (Thys et al., 1998, 2001) specific *Tnnt* have been identified at the protein level. Unfortunately, little is known about the genomic structure and developmental regulation of *Tnnt* at the gene level (Waddleton et al., 1999; Xu et al., 2000). To better understand the mechanism controlling *Tnnt* muscle fiber diversification in lower vertebrates, we isolated three distinct fiber types of zebrafish *Tnnt* and compared their patterns of spatiotemporal expression to those of their tetrapod counterparts.

RESULTS

Identification and Characterization of Zebrafish *Tnnt*

A BLAST search of the zebrafish expression sequence tag (EST) database identified three zebrafish EST clones: AI883430, AW455177, and AI353765, that exhibited high sequence homology with the trout *Tnnt1* (Waddleton et al., 1999), zebrafish *Tnnt* (Xu et al., 2000), and human *Tnnt2* (Mesnard et al., 1993), respectively. We also identified two potential *Xenopus* EST clones, BG514147 and BJ055552, that exhibited high sequence homology with chicken *Tnnt2* (Cooper and Ordahl, 1984) and mouse *Tnnt3* (Wang and Jin, 1997), respectively. EST clone-specific primers were designed to amplify potential *Tnnt* cDNAs from zebrafish and *Xenopus* by rapid amplification of cDNA ends (RACE). Full-length cDNA of zebrafish and *Xenopus Tnnt* were assembled with sequences derived from 3′- and 5′-RACE. Three zebrafish and two *Xenopus Tnnt2* clones were identified. Based on sequence analysis, the three zebrafish and two *Xenopus Tnnt2* clones were alternatively spliced variants derived from a single gene. In zebrafish, the longest, isolated *Tnnt2* clone was identical to that reported by Sehnert et al. (2002). Two other alternatively spliced variants lacked 18 and 10 amino acid residues, respectively, in the N-terminal hypervariable region (Fig. 1A). In contrast, only one clone each of *Tnnt1* and *Tnnt3* was isolated from zebrafish. The *Tnnt3* clone reported here and the *Tnnt* clone reported by Xu et al. (2000) exhibited many amino acid residue substitutions throughout the open reading frame and had distinct nucleotide sequences in both the 5′- and 3′-untranslated regions. Thus, these two *Tnnt3* clones seemed to be encoded by a distinct gene. Because they are co-orthologous to tetrapod *Tnnt3* (Fig. 2), we named them *Tnnt3a* (=*Tnnt*, Xu et al., 2000) and *Tnnt3b* (Table 1 summarizes their molecular features).

The number of deduced amino acid residues in *Tnnt1* (290) and *Tnnt2* (282) was much greater than in *Tnnt3a* (230) and *Tnnt3b* (232). Moreover, there were many more negatively charged residues (primarily glutamic acid) in *Tnnt1* and *Tnnt2* than in *Tnnt3b*. Thus, *Tnnt1* and *Tnnt2* have acidic pI and are highly negatively charged at pH 7, whereas *Tnnt3b* has a basic pI and is positively charged at pH 7. To better understand the relationship among different *Tnnt*, the zebrafish and *Xenopus Tnnt* sequences were aligned based on deduced amino acid sequences (Fig.1A). The three types of zebrafish *Tnnt* and two types of *Xenopus Tnnt* were highly conserved in the central tropomyosin-binding domain and C-terminal troponin I-binding domain segments, but they were highly divergent in the N-terminal hypervariable region. The deduced amino acid sequences of zebrafish *Tnnt2* and *Tnnt3b* exhibited high sequence identity with tetrapod *Tnnt2* (57.4–64.5%) and *Tnnt3* (67.7–69.0%), respectively, whereas *Tnnt1* exhibited moderate identity with tetrapod *Tnnt1* (52.5–54.2%). Unexpectedly, the amino acid identity of zebrafish and trout *Tnnt1* (48.9%) was lower than that between zebrafish and tetrapod *Tnnt1*. Thus, *Tnnt1* were more divergent than *Tnnt2* and *Tnnt3* in teleost branch. Of interest, in *Tnnt1*, the number of amino acid residues in the N-terminal hypervariable region decreased from 85 in zebrafish to 53 in humans, but in *Tnnt3* they increased from 25 in zebrafish to 46 in humans (Fig. 1B).

Phylogenetic Analysis of *Tnnt*

To examine the evolutionary relationship between teleost and tetrapod *Tnnt*, we conducted a phylogenetic tree based on unspliced primary sequences of *Tnnt*. Ascidian *Tnnt* from embryonic striated muscle and the adult body wall (Endo et al., 1996, 1997) were included for comparison. The occurrence of three distinct types of *Tnnt* evolved after the divergence of deuterostome invertebrate and teleost lineages (Fig. 2). Zebrafish *Tnnt2* and *Tnnt3* clustered

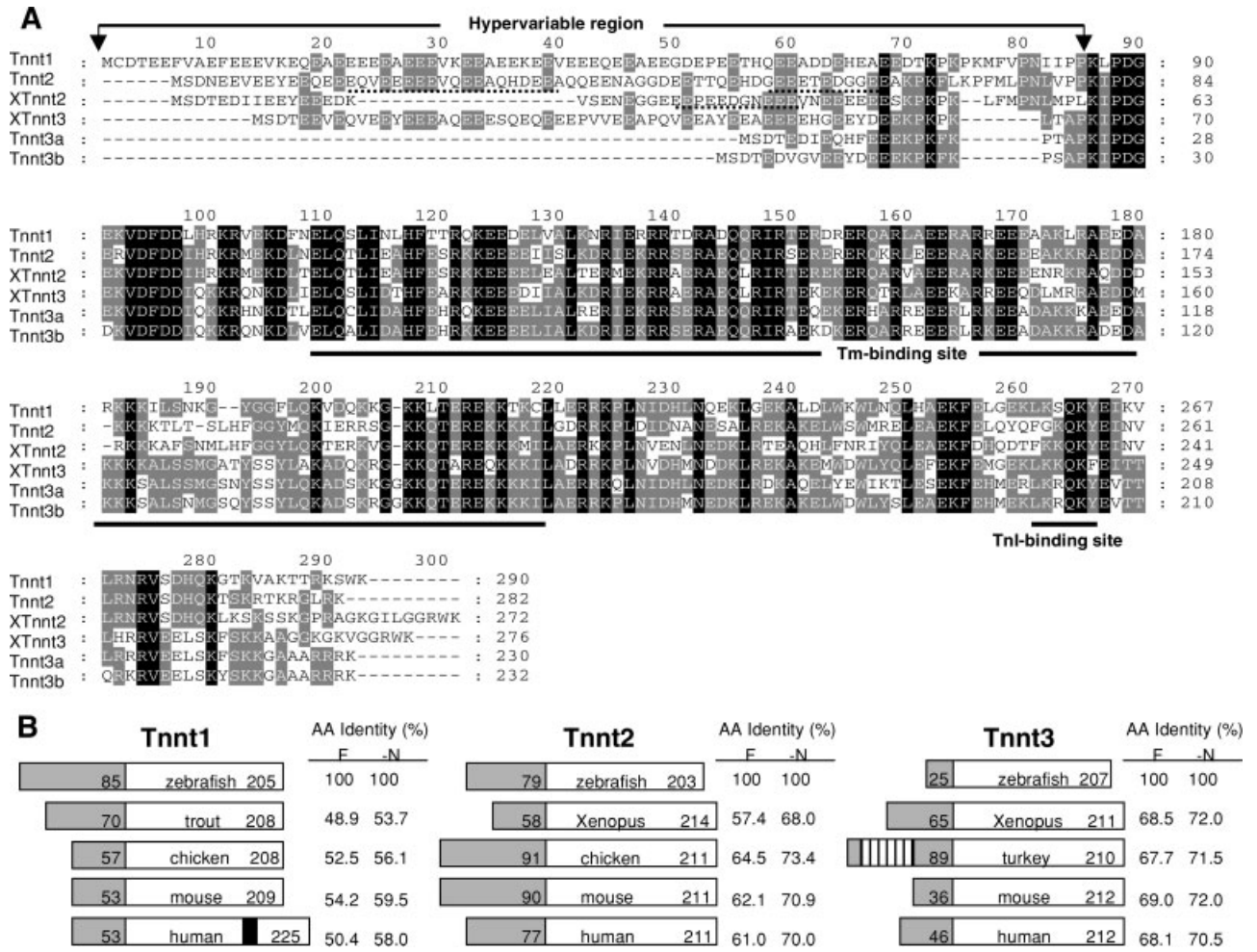

Fig. 1. Sequence alignment and homology analysis of *Tnnt* isolated in this study. **A:** The aligned, deduced amino acid sequences of zebrafish *Tnnt1*, *Tnnt2*, *Tnnt3a*, *Tnnt3b*, and *Xenopus XTnnt2*, *XTnnt3*. Gaps introduced during the alignment are shown by hyphens. Amino acid residues conserved among the *Tnnt* family members are highlighted in black; residues conserved in at least 50% are highlighted in gray. Putative tropomyosin (Tm)- and troponin I (TnI)-binding domains (Pearlstone et al., 1977) are indicated with solid underlines. Alternatively spliced exons detected in zebrafish and *Xenopus Tnnt2* are indicated with dotted underlines. **B:** Schematic representation of the evolution among vertebrates of *Tnnt* N-terminal hypervariable regions. The gray boxes indicate the N-terminal hypervariable regions (residue numbers are on the right side). The empty boxes indicate the full-length *Tnnt* excluding the N-terminal hypervariable region. The black box indicates the 3′ alternatively spliced exon of human *Tnnt1* (Gahlmann et al., 1987). The hatched box indicates the metal-binding cluster of turkey *Tnnt3* (Jin and Samanez, 2001). The right panels list the amino acid identity between zebrafish and other species for each type of muscle *Tnnt*. F, full-length amino acid sequences; -N, partial sequences excluding N-terminal hypervariable region.

with their tetrapod counterparts into two distinct monophyletic groups. Orthologous relationship between zebrafish and tetrapods in the *Tnnt2* and *Tnnt3* branches were strongly supported. In fish, the amino acid substitution rate in the *Tnnt1* branch was faster than in the *Tnnt2* and *Tnnt3* branches. There was not an unambiguous orthologous relationship between zebrafish and tetrapod *Tnnt1* (Fig. 2). To determine whether this phenomenon was caused by sequence variation in the N-terminal hypervariable region, we constructed a phylogenetic tree based on partial *Tnnt* sequences that omitted the N-terminal hypervariable region. However, there was no significant difference in the tree topology (data not shown). That is, fish *Tnnt1* was more closely related to the *Tnnt2* clade than to the tetrapod *Tnnt1* clade.

Zebrafish *Tnnt* Gene Structure

During the initial cloning of partial sequences (N-terminal hypervariable region) of zebrafish *Tnnt1*, the polymerase chain reaction (PCR) products amplified from cDNA and genomic DNA by primers *Tnnt1*-F and *Tnnt1*-R were identical in size. Furthermore, several primers that span the entire *Tnnt1* cDNA were used for genomic PCR and reverse transcriptase-PCR (RT-PCR). This strategy consistently produced PCR products with the same size and sequences (Fig. 3A). In addition, using PCR-based genomic walking, we obtained a genomic clone that covered the entire *Tnnt1* (including 0.3-kb-upstream and 0.7-kb-downstream regions; Fig. 3A). These results confirm that zebrafish *Tnnt1* lacks introns. By using bioinformatics, we searched for *Tnnt1* homologs in fugu draft genome sequences. We identified one genomic clone (FT:

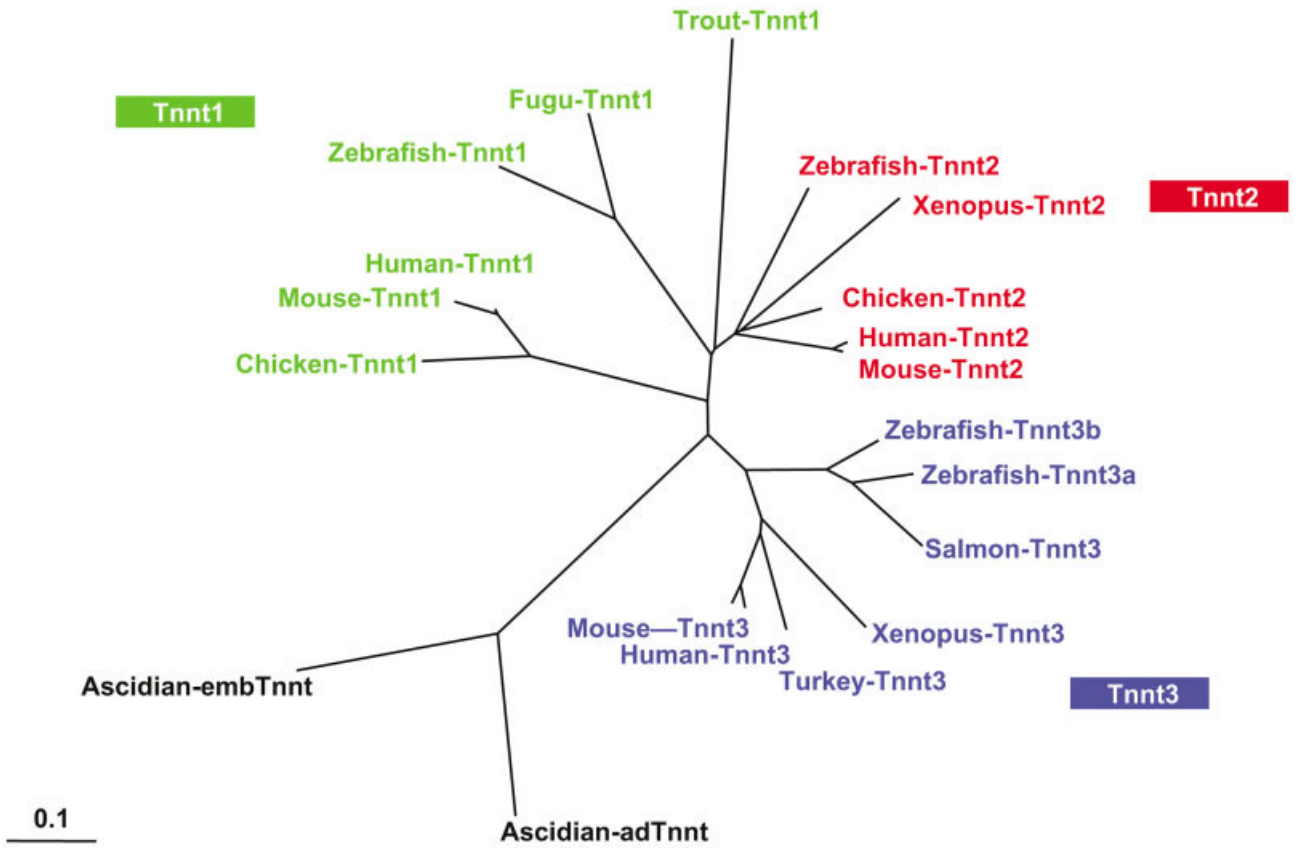

Fig. 2. An unrooted radial gene tree for *Tnnt* among vertebrates. The gene tree was constructed with the neighbor-joining method (Pearson et al., 1999), using 1,000 bootstrap values. The marker length of 0.1 corresponds to 10% sequence difference. The *Tnnt1*, *Tnnt2*, and *Tnnt3* clades are marked in green, red, and blue, respectively. See the Experimental Procedure section for details on the sources of *Tnnt*. (Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.)

TABLE 1. Summary of the Troponin T cDNAs Used in This Study

Gene names	Gene abbreviations	cDNA length (bp)	Coding region (aa)	Mr (kDa)	pI	GenBank accession no.	References
Zebrafish							
Slow troponin T	*Tnnt1*	1461	290	34.7	5.0	AF425255	This study
Cardiac troponin T	*Tnnt2*	1047	282	34.0	5.1	AF434187	Sehnert et al. (2002); this study
Fast troponin T isoform a	*Tnnt3a*	1098	230	27.8	9.9	AF180889	Xu et al. (2000)
Fast troponin T isoform b	*Tnnt3b*	1099	232	26.5	9.9	AF425741	This study
Xenopus							
Cardiac troponin T	*XTnnt2*	1047	272	32.6	5.5	AF467919	This study
Fast troponin T	*XTnnt3*	1521	276	32.9	5.1	AY114144	This study

T005154) that corresponds to fugu *Tnnt1*. Gene prediction analysis showed it was also intronless. In contrast, numerous introns interrupt *Tnnt1* orthologs in chickens (12 introns; Hirao et al., 2001), mice (13 introns; Huang et al., 1999; Huang and Jin, 1999), and humans (13 introns; Barton et al., 1999; Fig. 3B). This unexpected finding prompted us to examine the gene structure of zebrafish *Tnnt2* and *Tnnt3*. An incomplete (24 kb) genomic clone of zebrafish *Tnnt2*, corresponding to exons 1 to 13, was reported by Sehnert et al. (2002). By using gene mining and PCR-based genomic walking, we obtained complete genomic clones of zebrafish *Tnnt2* (34 kb), *Tnnt3a* (12 kb), and *Tnnt3b* (15 kb; Fig. 3C). Like chicken (9 kb and 18 exons; Cooper and Ordahl, 1985), rat (19 kb and 16 exons; Jin et al., 1992), and human *Tnnt2* (17 kb and 17 exons; Farza et al., 1998), zebrafish *Tnnt2* has a complex intron–exon organization (17 exons), but at 34 kb, it is two- to nearly fourfold larger than its tetrapod counterparts. In contrast, the gene structures of zebrafish *Tnnt3a*

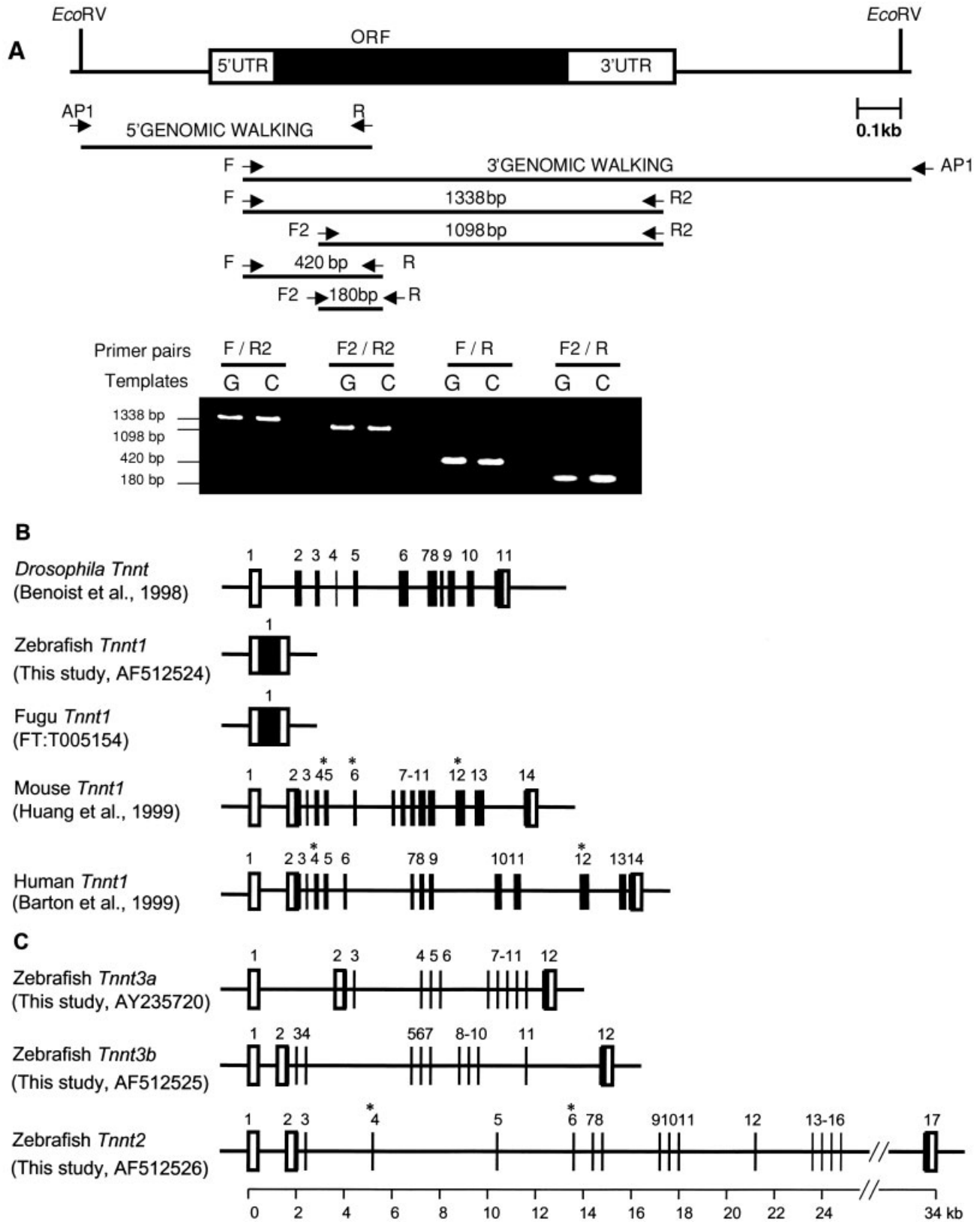

Fig. 3. Comparison of *Tnnt* gene structure in different vertebrates. **A:** A schematic, scale diagram of *Tnnt1* gene structure. Black box, coding region. Empty boxes, untranslated regions. Solid lines indicate 5′- and 3′-flanking regions. The primers used to clone the flanking regions of genomic DNA, and for RT-PCR on cDNA, are indicated below. PCR fragments amplified from genomic DNA (G) and cDNA (C) using different combinations of *Tnnt1*-specific primer pairs were the same size. **B:** The complete gene structures of zebrafish and fugu *Tnnt1* are compared with that of *Drosophila Tnnt* (Benoist et al., 1998), mouse *Tnnt1* (Huang et al., 1999), and human *Tnnt1* (Barton et al., 1999). **C:** The complete gene structures of zebrafish *Tnnt3a*, *Tnnt3b*, and *Tnnt2*. Exons are shown as boxes: black boxes are open reading frames, empty boxes are 5′- or 3′-untranslated regions. Asterisks denote the alternatively spliced exons.

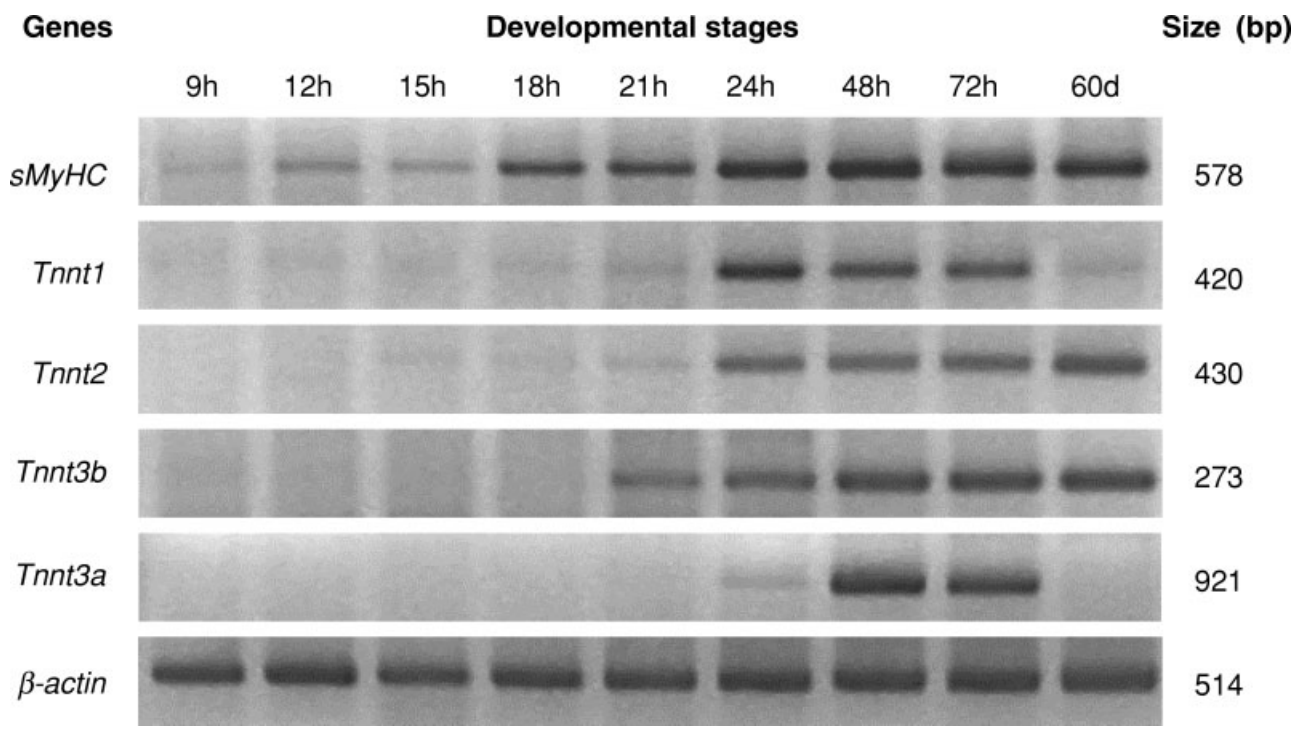

Fig. 4. *Tnnt* temporal expression patterns in developing zebrafish embryos as detected by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from different developmental stages, and primers specific for *sMyHC*, *Tnnt1*, *Tnnt2*, *Tnnt3a*, and *Tnnt3b* were used for RT-PCR. β-*actin* was used as an internal control. Numbers in the right panel indicate the molecular mass of RT-PCR products.

(12 kb and 11 exons) and *Tnnt3b* (15 kb and 11 exons) are smaller and simpler than their tetrapod counterparts in quail (33 kb and 25 exons; Bucher et al., 1999) and rat (16 kb and 19 exons; Breitbart and Nadal-Ginard, 1986). This comparison highlights the unique, relatively compact genomic organization of *Tnnt1* within the zebrafish *Tnnt* family.

Temporal Expression of *Tnnt* in Zebrafish

We compared the spatiotemporal expression of *Tnnt* in zebrafish, a lower vertebrate, with that in tetrapods. Total RNA was extracted from zebrafish embryos at eight different stages, ranging from 9 hpf (90% epiboly) to 72 hpf (hatched fry), and from adult fish. RT-PCR was performed to compare the relative abundance of *Tnnt* transcripts. *Tnnt1* was the first of the three types of *Tnnt* to be detected (Fig. 4). *Tnnt1* was activated approximately 12 hpf and was expressed vigorously 24 hpf. However, *Tnnt1* expression was very low in adults. *Tnnt2* transcripts first appeared 15 hpf and remained constant through adulthood. *Tnnt3a* (24 hpf) and *Tnnt3b* (21 hpf) transcripts appeared much later than *Tnnt1* (12 hpf) and *Tnnt2* (15 hpf). Each exhibited a distinct expression profile during development: *Tnnt3a* was expressed transiently in embryos, whereas *Tnnt3b* expression was constant from the embryonic stage through adulthood. Compared with *sMyHC*, which is expressed specifically in slow muscle, and *β-actin*, which is expressed constantly in the entire body, only low levels of *Tnnt1* and *Tnnt3a* were expressed in adults.

To detect alternatively spliced *Tnnt* variants in zebrafish, RT-PCR was carried out by using primers designed to amplify various fragments of the N-terminal hypervariable region or the entire *Tnnt* open reading frame. There was no variation in the size of the RT-PCR products produced by the primers for *Tnnt1*, *Tnnt3a*, and *Tnnt3b* (data not shown). In contrast, one *Tnnt2* primer pair (*Tnnt2*-F and *Tnnt2*-R3) produced RT-PCR products of three sizes (Fig. 5A,B). The expected, 318-bp fragment (exon 1 to exon 8) was detected in embryos and adults (Fig. 5B). Two additional bands (282-bp and 264-bp), which first appeared 72 hpf, were expressed predominately in adults. The two bands were cloned and sequenced, yielding 282-bp and 264-bp amplified products that corresponded to a splicing variant lacking exon 6 (designated *Tnnt2*-6E) and exon 4 (designated *Tnnt2*-4E), respectively (Fig. 5C).

Spatial Expression of *Tnnt* in Zebrafish

To examine the spatial expression of *Tnnt*, whole-mount in situ hybridization (WISH) was performed on zebrafish embryos collected 9 to 72 hpf. WISH detected *Tnnt* transcripts earlier than RT-PCR. We found the onset of *Tnnt2* expression in the bilateral precardiac mesoderm (Fig. 6A,F) occurred 2.5 hr earlier than reported in a previous study of the developmental expression of *Tnnt2* in zebrafish (Sehnert et al., 2002). *Tnnt2*-expressing cells rapidly migrated toward the notochord 17 hpf (Fig.

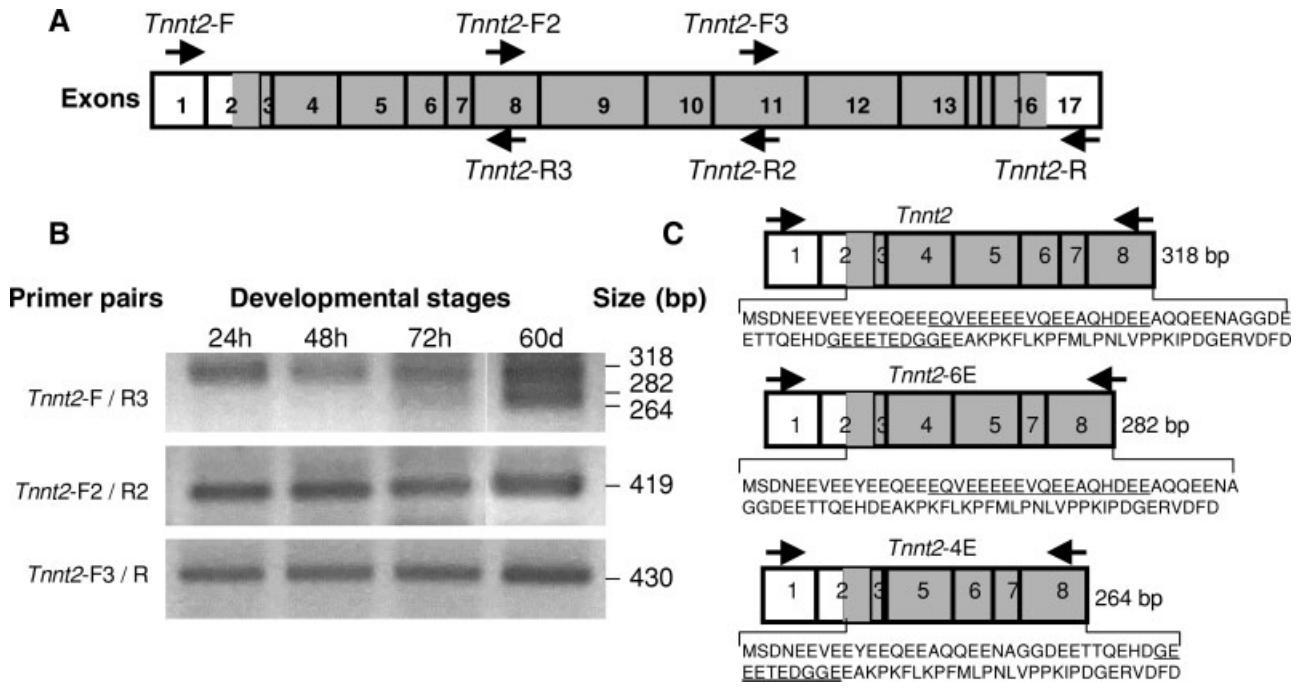

Fig. 5. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *Tnnt2* alternatively spliced variants during zebrafish development. **A:** Schematic representation of the positions of the primers used to perform RT-PCR. Empty boxes indicate untranslated regions; solid boxes show open reading frames. **B:** Age-dependent expression of alternatively spliced, *Tnnt2* variants. Alternatively spliced variants were detected by RT-PCR using the primer pair combinations indicated. Two, alternatively spliced variants were detected by primer pair *Tnnt2*-F and *Tnnt2*-R3 in adult fish. **C:** Schematic illustration of the alternative splicing involved in the generation of *Tnnt2* (unspliced form), *Tnnt2*-6E (excluding exon 6), and *Tnnt2*-4E (excluding exon 4). The alternatively spliced exons are underlined.

6B,G) and fused to become a single heart cone 18 hpf. From 24 hpf onward, *Tnnt2* transcripts were up-regulated and expressed robustly in the looping heart tube (Fig. 6C,D,H,I). By 48 hpf, *Tnnt2* transcripts were specifically and uniformly detected in both the atrium and ventricle (Fig. 6E,J). No *Tnnt2* transcripts were detected in skeletal muscles. Thus, unlike its tetrapod counterparts, expression of zebrafish *Tnnt2* was highly cardiac-specific. To better understand the ontogeny of *Tnnt2,* the expression of several cardiac-specific contractile proteins during early cardiogenesis was determined on transcription level. We found *cmlc2* (Fig. 6L), *cmlc1* (Fig. 6M), *vmhc* (Fig. 6N), and *cTnC* (Fig. 6O) are synchronously activated 17–18 hpf, whereas *Tnnt2* (Fig. 6K) is activated 14–15 hpf.

Compared with *sMyHC,* a slow-muscle-specific marker activated 9 hpf (Fig. 7A), *Tnnt1* was activated in adaxial cells (slow muscle precursors) 10 hpf, during early somitogenesis (Fig. 7F). By 15 hpf, *sMyHC* (Fig. 7B) and *Tnnt1* (Fig. 7G) were vigorously expressed in all developing somites and adaxial cells. *Tnnt3a* and *Tnnt3b* were not detected until 16.5 and 18 hpf, respectively. By 18 hpf, all developing somites stained positive for *sMyHC* (Fig. 7C) and *Tnnt1* (Fig. 7H), but only 10 somites stained positive for *Tnnt3a* (Fig. 7M) and only one was positive for *Tnnt3b* (Fig. 7R). Although *Tnnt3a* and *Tnnt3b* were activated much later than *sMyHC* and *Tnnt1,* they were sharply up-regulated from 18 hpf onward. By 24 hpf, only 3 and 5 newly forming somites were unstained for *Tnnt3a* (Fig. 7N) and *Tnnt3b* (Fig. 7S), respectively. To verify the muscle fiber-specificity of *Tnnt1* and *Tnnt3* in zebrafish, trunk-level tissue sections were examined. Compared with the specific staining of *sMyHC* in slow muscles (Fig. 7E), *Tnnt1* transcripts (Fig. 7J) were detected in superficial slow and inner fast muscles, whereas *Tnnt3a* (Fig. 7O) and *Tnnt3b* (Fig. 7T) transcripts were fast-muscle–specific. From 36 hpf onward, there was a wave of *Tnnt1* down-regulation (Fig. 8E) that moved in a rostral–caudal direction, beginning in the oldest somites. *sMyHC* (Fig. 8A), *Tnnt3a* (Fig. 8I), and *Tnnt3b* (Fig. 8M) were not down-regulated. Transverse section (data not shown) and high-magnifi-

Fig. 6. Whole-mount in situ hybridization of *Tnnt2* and other cardiac-specific genes during zebrafish cardiogenesis. Embryos were hybridized with *Tnnt2* **(A–K)**, *cmlc2* **(L)**, *cmlc1* **(M)**, *vmhc* **(N)**, and *cTnC* **(O)** riboprobes, respectively. A–E: Lateral views, anterior of embryo to the left. F–I,K–O: Dorsal views, anterior of embryo to the top. J: Frontal view of embryo. Embryonic stages are indicated in each panel. h, hour; a, atrium; ht, heart tube; mp, myocardium precursor; v, ventricle. Scale bar = 100 μm in all pictures.

Fig. 7. Whole-mount in situ hybridization of *Tnnt1*, *Tnnt3a*, *Tnnt3b*, and *sMyHC* during zebrafish early somitogenesis. Embryos were hybridized with *sMyHC* **(A–E)**, *Tnnt1* **(F–J)**, *Tnnt3a* **(K–O)**, and *Tnnt3b* **(P–T)** riboprobes, respectively. A,B,F,G,K,L,P,Q: Dorsal views. C,D,H,I,M,N,R,S: Lateral views. E,J,Q,T: Transverse sections at trunk level. The anterior is to the left in all whole-mount stained embryos. Dotted lines indicate the section level. Embryonic stages are indicated in each panel. h, hour. Scale bar = 200 μm in D,I,N,S, 140 μm in C,H,M,R, 100 μm in A,B,F,G,K,L,P,Q, 25 μm in E,J,O,T.

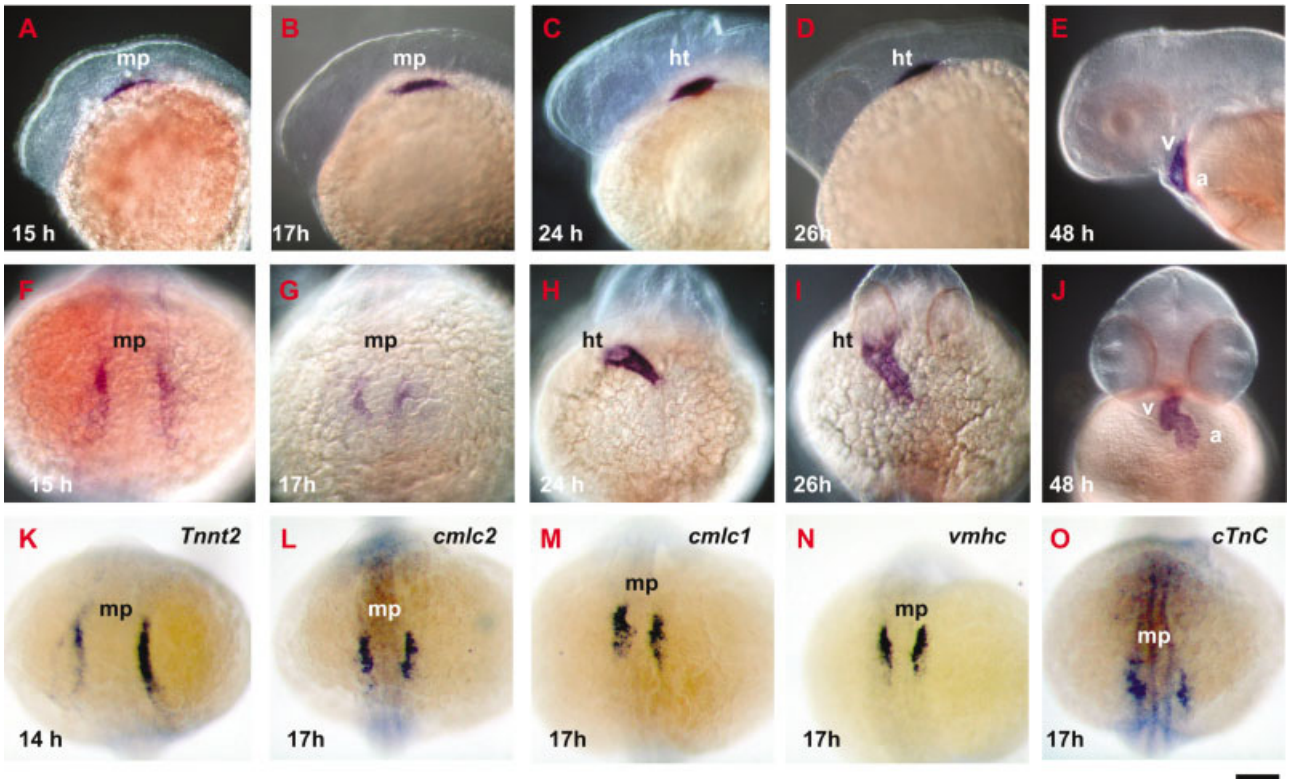
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Fig. 6.

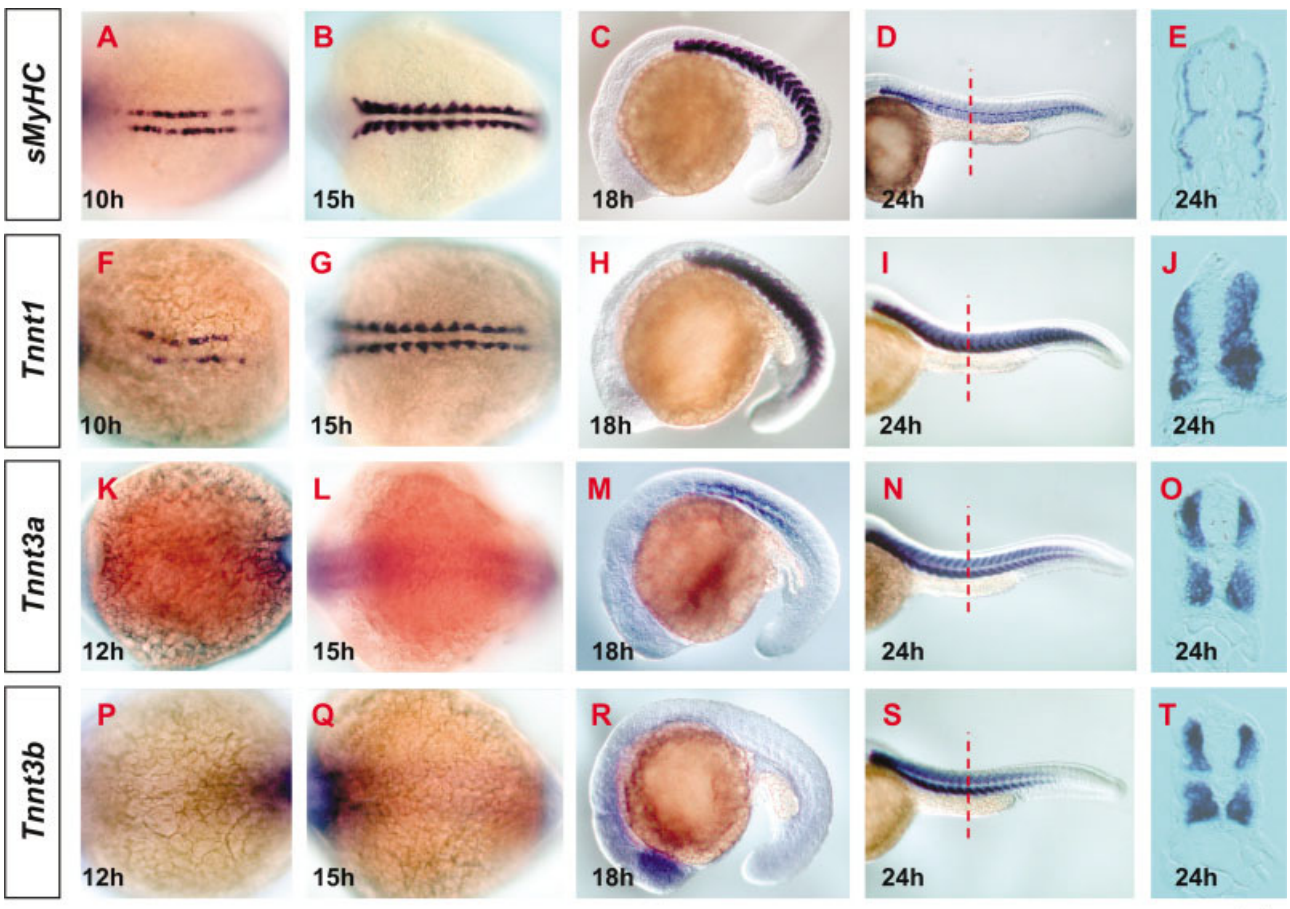
sMyHC
A
10h
B
15h
C
18h
D
24h
E
24h
Tnnt1
F
10h
G
15h
H
18h
I
24h
J
24h
Tnnt3a
K
12h
L
15h
M
18h
N
24h
O
24h
Tnnt3b
P
12h
Q
15h
R
18h
S
24h
T
24h

Fig. 7.

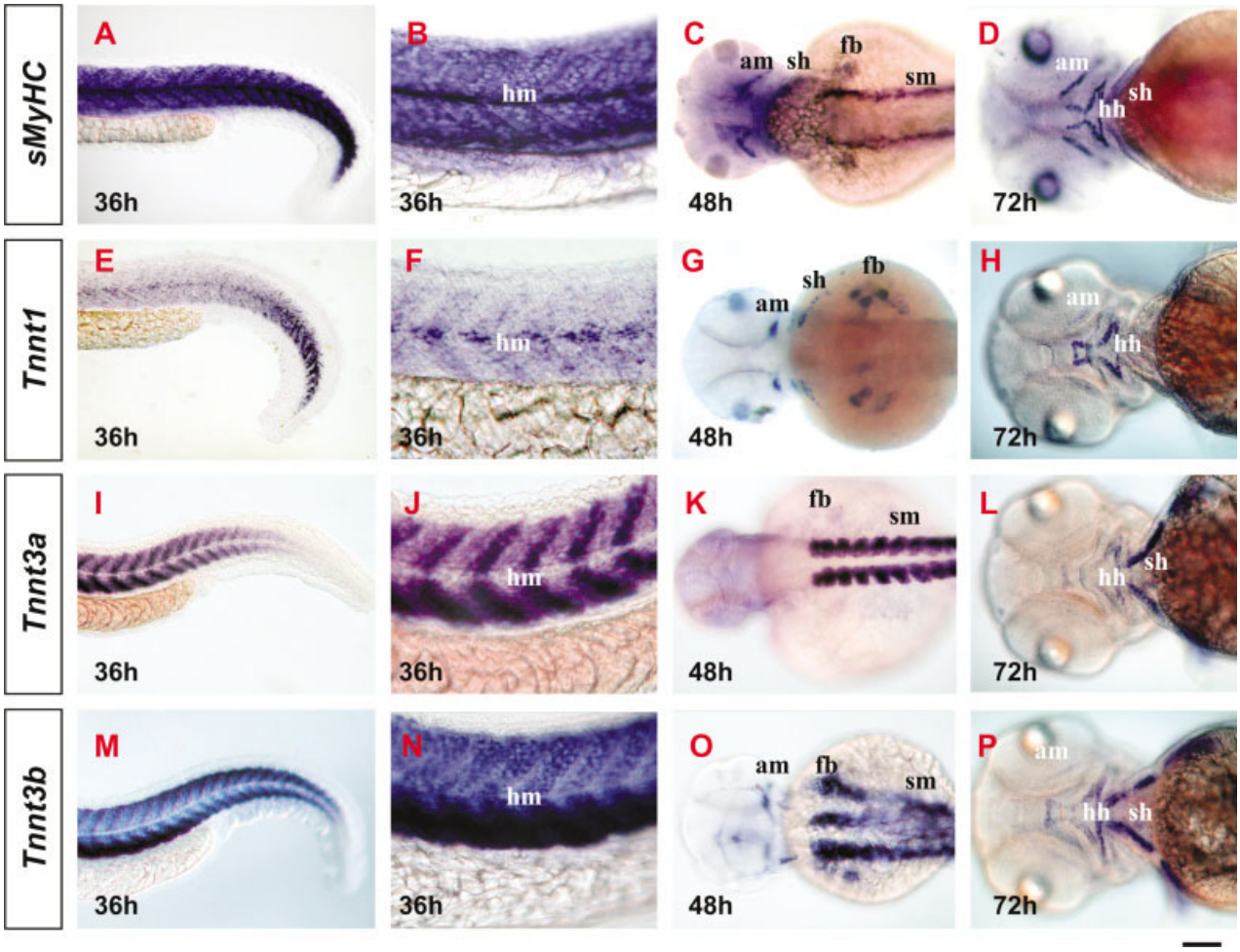

Fig. 8. Whole-mount in situ hybridization of *Tnnt1*, *Tnnt3a*, *Tnnt3b*, and *sMyHC* genes during zebrafish late somitogenesis. Embryos were hybridized with *sMyHC* **(A–D)**, *Tnnt1* **(E–H)**, *Tnnt3a* **(I–L)**, and *Tnnt3b* **(M–P)** riboprobes, respectively. C,G,K,O: Dorsal views. A,B,E,F,I,J,M,N: Lateral views. D,H,L,P: Ventral views. The anterior is to the left in all pictures. Embryonic stages are indicated in each panel. am, adductor mandibulae; fb, fin bud; hh, hyohyoideus; hm, horizontal myospetum; sh, sternohyoideus; sm, somites. Muscle nomenclature is based on Schilling and Kimmel (1997). Scale bar = 50 μm in B,F,J,N, 100 μm in A,C–E,G–I,K–M,O,P.

cation view (Fig. 8F) 36 hpf revealed that *Tnnt1* was down-regulated in fast muscles, but it was still strongly expressed in pioneer slow muscles. By 48 hpf, *Tnnt1* staining were undetectable in trunk muscles, but they had been up-regulated in fin buds and jaw and head muscles (Fig. 8G). At the same time, *Tnnt3b* (Fig. 8O) but not *Tnnt3a* (Fig. 8K) was weakly activated in fin buds and jaw and head muscles. The staining intensity of *sMyHC* (Fig. 8D), *Tnnt1* (Fig. 8H), *Tnnt3a* (Fig. 8L), and *Tnnt3b* (Fig. 8P) in craniofacial muscles 72 hpf varied in different muscle fibers. For example, the sternohyoideus was heavily stained by *Tnnt3a* and *Tnnt3b* but weakly stained by *sMyHC* and *Tnnt1*. Jaw muscles were heavily stained by *sMyHC*, *Tnnt1*, and *Tnnt3b* but weakly stained by *Tnnt3a*.

DISCUSSION

Molecular Characterization of *Tnnt* in Zebrafish

In tetrapods, muscle-specific and development-regulated *Tnnt* have been characterized thoroughly at the protein and gene levels (Jin and Lin, 1988; Sabry and Dhoot, 1991; Wang and Jin, 1997; Ogut et al., 1999; Yonemura et al., 2000). However, few tetrapod orthologs have been characterized at the gene level in teleosts (Waddleton et al., 1999; Xu et al., 2000; Sehnert et al., 2002). In this study, we isolated three zebrafish *Tnnt* and two *Xenopus Tnnt* cDNAs with an EST-based approach. Like their tetrapod counterparts, the deduced amino acids of zebrafish and *Xenopus Tnnt* are highly conserved in the tropomyosin- and troponin I-binding domains, but they diverse greatly in the N-terminal hypervariable region. Among vertebrates, fish *Tnnt1* has the longest N-terminal extension, whereas *Tnnt3* has the shortest N-terminal extension. Extension of the N-terminal hypervariable region in vertebrates, and the C-terminal region in invertebrates, plays a role in modulating the conformation of Tnnt isoforms (Benoist et al., 1998; Jin and Samanez, 2001). As negatively charged residues in the N-terminal hypervari-

TABLE 2. Comparison of the Muscle Specificity of Three Troponin T Gene Transcripts in Early Developmental Stage Among Vertebrates

	Tnnt1	*Tnnt2*	*Tnnt3*
Slow muscle	Zebrafish[a] Chicken[b] Mouse[c,d]	Mouse[c]	Chicken[b,f]
Cardiac muscle	Mouse[c,d]	Zebrafish[a,e] Chicken[b] Mouse[c]	
Fast muscle	Zebrafish[a] Chicken[b] Mouse[c,d]	Chicken[b] Mouse[c]	Zebrafish[a,g] Chicken[b] Mouse[c]

[a]This study.
[b]Yonemura et al., 2002.
[c]Wang et al., 2001.
[d]Krishan et al., 2000.
[e]Sehnert et al., 2002.
[f]Nakada et al., 2000.
[g]Xu et al., 2000.

able region of *Tnnt* increase, sensitivity of the myofibrillar contractile apparatus to Ca^{2+} (Ogut and Saitou, 1999) and tolerance of acidosis also increase (Ogut and Jin, 1998). Studies that generate pCa/tension curves are needed to determine whether variation in the N-terminal hypervariable region of zebrafish *Tnnt1* and *Tnnt3* is involved in regulating contraction in red and white muscles.

During somitogenesis and cardiogenesis in tetrapods, changes in *Tnnt* isoforms expression resulting from alternative splicing (i.e., from large to small, and acidic to basic) are common (Jin and Lin, 1988, Wang and Jin, 1997; Yonemura et al., 2002). In birds and mammals, alternative splicing is most frequent in *Tnnt3* (Wang and Jin, 1997; Bucher et al., 1999) and *Tnnt2* (Jin et al., 1996), and less frequent in *Tnnt1* (Huang et al., 1999; Yonemura et al., 2000). As development proceeded, large to small alternatively spliced variants of *Tnnt2* isoforms were detected in both zebrafish and *Xenopus*. Thus, our data provide strong evidence that alternative splicing is an evolutionarily conserved mechanism for generating Tnnt2 isoforms in both teleosts and tetrapods. However, in zebrafish, unlike tetrapods, no alternatively spliced variants were detected in *Tnnt1*, *Tnnt3a*, and *Tnnt3b*. Because zebrafish *Tnnt1* lacks introns, the absence of alternatively spliced variants is easily explained. However, it is difficult to explain the lack of alternatively spliced variants of *Tnnt3a* and *Tnnt3b*. In chickens, RNA-binding proteins and intronic elements related to muscle-specific splicing in skeletal and cardiac muscles have been identified in *Tnnt* (Ryan and Cooper, 1996; Charlet et al., 2002). Further studies are needed on the RNA-binding proteins and intronic elements of *Tnnt3* in zebrafish.

Evolutionary Significance of Zebrafish *Tnnt1*

Homology, phylogenetic, and expression analyses indicate the three fiber types of zebrafish *Tnnt* evolved before teleost and tetrapod lineages diverged. However, the three distinct *Tnnt* probably evolved after deuterostome invertebrates and fish diverged. Before our study, there was no information on the gene structure of *Tnnt* in lower vertebrates, so the mechanism by which the three fiber types of *Tnnt* evolved was unknown. The position of one constitutively spliced intron of *Drosophila Tnnt* coincides precisely with rat *Tnnt3*, leading Fyrberg et al. (1990) to hypothesize that *Tnnt* predates the divergence of invertebrates and vertebrates. However, we found that both zebrafish and fugu *Tnnt1* lack introns. We propose two working hypotheses to address this issue. First, *Tnnt1* introns were lost in teleost lineage but gradually increased and expanded gene size in tetrapod lineage. In this case, fish *Tnnt1* may represent the vertebrate *Tnnt1* prototype. Similar evolutionary trends have also been proposed for bony fish *rhodopsin* (Fitzgibbon et al., 1995) and fugu *SART1* (Bolland and Hewitt, 2001). Second, due to genome duplication in fish (Postlethwait et al., 1998; Taylor et al., 2001), zebrafish may have evolved two or more copies of *Tnnt1*, as occurred with *Tnnt3*. Searching for more *Tnnt1* genes, and studying their genomic structure in zebrafish and other lower vertebrates will provide more insight into the molecular evolution of *Tnnt1*.

Early Activation of *Tnnt1* and *Tnnt2* During Somitogenesis and Cardiogenesis

Slow muscles develop much earlier than fast muscles (van Swearingen and Lance-Jones, 1995, Stockdale, 1997) in the trunk, limb, and craniofacial regions of vertebrates (Wachtler and Christ, 1992, Shuler and Dalrymple, 2001). The differential development of slow and fast muscles results in the asynchronous activation of the slow and fast isoforms of muscle contractile proteins during somitogenesis. In tetrapods, the slow isoforms of muscle-specific proteins, such as the myosin heavy chain (Dhoot, 1986), troponin I (Kyprianou et al., 1997), and troponin T (Krishan et al., 2000, Wang et al., 2001), predominate during early development. The fast isoforms of these proteins predominate later in development. Zebrafish *Tnnt* exhibits a similar pattern. The onset of zebrafish *Tnnt1* expression is 6 to 8 hr earlier than that of *Tnnt3a* and *Tnnt3b* and also is much earlier than that of several fast isoforms of muscle contractile proteins (Xu et al., 2000). The precocious expression of *Tnnt1* may help assemble the tropomyosin–troponin complex during early somitogenesis in both slow and fast muscles.

In zebrafish, the ontogenic expression of muscle contractile proteins during cardiogenesis has not been

studied as much as expression of skeletal muscle proteins. Most of the cardiac-specific contractile proteins we examined were synchronously activated 17–18 hpf, whereas *Tnnt2* was activated about 14 hpf. The precocious expression of *Tnnt2* indicates it is crucial to assemble the tropomyosin–troponin complex during early cardiogenesis. This hypothesis is supported by zebrafish *Tnnt2* mutants (Sehnert et al., 2002) and knock-down expression of *Tnnt2* by morpholino oligos, which completely stop the heart beat (Sehnert et al., 2002; Hsiao et al., unpublished observations).

Tnnt Expression in Fish Is More Restricted Than in Tetrapods

In fetal to adult tetrapods, transitional expression of *Tnnt* transcripts in many different fiber-types is common (summarized in Table 2). In chickens, *Tnnt1* transcripts have been detected in slow, fast, and mixed muscles (Yonemura et al., 2002). In mice, *Tnnt1* transcripts are expressed in all striated muscles during early fetal stages, but are restricted to slow muscle fibers during postnatal development (Krishan et al., 2000; Wang et al., 2001). Mouse *Tnnt2* transcripts occur in developing skeletal muscles and the heart during the embryonic and fetal stages, but expression in skeletal muscles is selectively repressed after the late fetal stages (Wang et al., 2001). In zebrafish, only *Tnnt1* is expressed in both slow and fast muscles. In contrast, *Tnnt2* and *Tnnt3* exhibit high cardiac- and fast-muscle specificity during cardiogenesis and somitogenesis, respectively. This simplified muscle contractile protein expression pattern also was reported in *Xenopus* slow and fast troponin I (Warkman and Atkinson, 2002). Based on this evidence, we propose that the *cis*-acting elements controlling tissue- or stage-specific expression of *Tnnt* in fish are simpler than their tetrapod counterparts. Transgenic zebrafish lines controlled by *Tnnt* promoters should yield insights into the developmental regulation of muscle fiber specificity of *Tnnt*.

EXPERIMENTAL PROCEDURES

Extraction of Nucleic Acids

To obtain genomic DNA, an adult AB strain zebrafish (*Danio rerio*) was anesthetized by immersion in a tank of water containing 300 μg/ml ethyl-*m*-aminobenzoate tricane methane sulfonate (MS-222; Sigma). Its anal fin was removed, and genomic DNA was isolated from the fresh fin by using the procedures described by Hsiao et al. (2001). To obtain total RNA, zebrafish embryos aged 24 to 72 hpf and *Xenopus* (*Xenopus laevis*) embryos aged 1 to 7 dpf were collected and immediately stored in liquid nitrogen. The whole, frozen embryos were homogenized with TRIzol reagent (Gibco BRL), and their total RNA was extracted according to the manufacturer's instructions.

Molecular Cloning of *Tnnt* cDNAs

The EST database maintained at NCBI (http://ncbi.nlm.nih.org) was searched for sequence annotations indicative of possible homology to zebrafish or *Xenopus Tnnt*. To clone full-length cDNA clones of *Tnnt*, 5′- and 3′-RACE were performed with EST clone-specific primers. First-strand cDNA was synthesized from 3 μg of total RNA using a MATCHMAKER Library Construction & Screening Kit (Clonetech). This method involved synthesis of full-length cDNA using CDS III oligo(dT) primer (5′-ATTCTAGAGGCCGAGGCGGCCGACATG-d$(T)_{30}$VN-3′), coupled with (dC) tailing by PowerScript reverse transcriptase, followed by template switching and extension with SMART III oligonucleotide (5′-AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG-3′). The RNA/cDNA hybrid was then preamplified by long-distance PCR with the primer set 5′-PCR (5′-TTCCACCCAAGCAGTGGTATCAACGCAGAGTGG-3′) / 3′-PCR (5′-GTATCGATGCCCACCCTCTAGAGGCCGAGGCGGCCGACA-3′). For 3′-RACE, each preamplified PCR product was performed with primer sets *Tnnt1*-F (5′-AGAAGTAGCACCATGTGCGACAC-3′), *Tnnt3b*-F (5′-CTGTAGGTCTTGAGAGGGCCG-3′), *Tnnt2*-F (5′-TTCCTCTGCATCTCTGCTGCGTGTC-3′), *XTnnt2*-F(5′-CCCAAGATTGTGGTGAAAACATGTCTGATAC-3′), *XTnnt3*-F (5′-GAAAAAGTCTCACAACTGCTGCGAC-3′) and 3′-PCR, respectively. For 5′-RACE, each preamplified PCR product was performed with primer sets of *Tnnt1*-R (5′-TTCAATTCGGTTCTTCAACGCTAC-3′), *Tnnt3b*-R (5′-AATGTTCAGAGGCTTGCGTC-3′), *Tnnt2*-R (5′-AGGTAAAATCTATATTGTTCAGTGAAATCTAACCG-3′), *XTnnt2*-R (5′-ACTCTGTTTAAATGTATGTTTTCAACTCTAAC-3′), *XTnnt3*-R (5′-ATGAATTACAGGGCACAACGAACGC-3′) and 5′-PCR, respectively. Thirty-five cycles of PCR amplification were performed by EXTaq DNA polymerase (Takara). Each cycle consisted of denaturation for 40 sec at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C. The last extension step was extended for 10 min at 72°C. The PCR products were cloned into pGEM-T Easy vector (Promega) and both strands were sequenced on an ABI 373A sequencer.

Bioinformatic Analysis of *Tnnt* Sequences

Nucleotide sequences were translated by using the sequence utilities available through the BCM Search Launcher interface (http://searchlauncher.bcm.tmc.edu). Multiple sequence alignment of the deduced amino acid sequences of unspliced *Tnnt* were performed by using ClustalW (Thompson et al., 1994), and phylogenetic trees were constructed by the neighbor-joining method (Pearson et al., 1999) through the DDBJ interface (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). Confidence in the branch nodes was assessed with 1,000 bootstrap replicates. To identify potential *Tnnt1* genomic sequences in other fish species, we used zebrafish *Tnnt1* to BLAST search fugu draft genome sequences at the HGMP interface (http://fugu.hgmp.mrc.ac.uk). The intron–exon organization of potential fugu *Tnnt1* was predicted by GENSCAN (Burge and Karlin, 1997). The accession numbers of sequences used in Figures 1 and 2 are *Tnnt1* from humans (*Homo sapiens*, P13805), mouse (*Mus musculus*,

NP_035748), chicken (*Gallus gallus*, JC4970), brown trout (*Salmo trutta*, AAB58912), fugu (*Fugu rubripes*, FT: T005154), and zebrafish (AF425255); *Tnnt2* from humans (P45379), mouse (BAB19881), chicken (TPCHTC), *Xenopus* (AF467919), and zebrafish (AF434187); and *Tnnt3* from humans (NM_006757), mouse (NM_011620), turkey (*Meleagris gallopavo*, AF210256), *Xenopus* (AY114144), zebrafish (*Tnnt3a*, AAF78472 and *Tnnt3b*, AF425741), Atlantic salmon (*S. salar*, AAC24595); embryonic striated muscle *Tnnt* (D85077) and adult body wall *Tnnt* (D50867) from an ascidian (*Halocynthia rorezi*).

Genomic Structure Determination

A PCR-based strategy was used to elucidate the genomic organization of zebrafish *Tnnt1* by using a Universal Genomic Walker Kit (Clonetech). In brief, 3 μg of genomic DNA was digested with *Dra*I, *Eco*RV, *Pvu*I, *Sca*I, or *Stu*I and then ligated to Genome Walker Adapters. For 3′-genomic walking, the adapter-ligated genomic DNA fragments were amplified by using the primer set *Tnnt1*-F/AP1 (5′-GTAATACGACTCACTATAGGGC-3′). For 5′-genomic walking, the adapter-ligated genomic DNA fragments were first amplified by using the AP1 and *Tnnt1*-R primer set, and then amplified with the AP2 (5′-ACTATAGGGCACGCGTGGT-3′) and *Tnnt1*-R2 (5′-TGATGTGTCTCTTCAGGTTCGTC-3′) nested primer set (Fig. 3A). For *Tnnt2*, *Tnnt3a*, and *Tnnt3b*, the primer pairs *Tnnt2*-F/*Tnnt2*-R4 (5′-ATGTCCAGAGGTTTGCGTCGATCAC-3′), *Tnnt3a*-F (5′-GGACATTGAGCAGCATTTCG-3′)/*Tnnt3a*-R (5′-CATGATGTCACACTATTGTTTAGCAAACCT-3′), and *Tnnt3b*-F/*Tnnt3b*-R were used to amplify genomic DNA by long-distance PCR amplification with LATaq DNA polymerase (Takara). Each of the thirty-five cycles of PCR amplification was comprised of denaturation for 30 sec at 94°C, 10 min of annealing and elongation at 68°C. The PCR products were cloned into pGEM-T Easy vector (Promega), and both strands were sequenced on an ABI 373A sequencer.

RT-PCR Analysis

To detect the temporal expression of zebrafish *Tnnt*, RT-PCR was performed, as described in the cDNA cloning section, on the total RNA isolated from 9, 12, 15, 18, 24, 48, 72 hpf, and adult zebrafish (60 dpf). The primer pairs *Tnnt1*-F/*Tnnt1*-R, *Tnnt3a*-F/*Tnnt3a*-R, *Tnnt3b*-F/*Tnnt3b*-R, *Tnnt2*-F3 (5′-GTCTGCACTTCGGCGGTTACA-3′)/*Tnnt2*-R, and *sMyHC*-F (5′-CAGGCTCCCAGAATGAGATTGAAG-3′)/*sMyHC*-R (5′-AGCTTCATTCCTGCTGCGAG-3′) were used to amplify cDNA fragments of 420, 921, 273, 430, and 578 bp, respectively. To exclude false-positive bands, which could result from genomic DNA contamination, the total RNA was digested with RNase-free DNase I before RT-PCR. To confirm that the absence of a *Tnnt* cDNA product was not due to a deficiency in cDNA preparation, the 514-bp fragment of the zebrafish *β-actin* (Kelly and Reversade, 1997) PCR product served as an RNA quality control in each tissue sample.

Detection of Alternative RT-PCR Products of *Tnnt*

To examine the potential alternative splicing products of zebrafish *Tnnt2*, total RNA isolated from 24, 48, 72 hpf, and adult zebrafish (60 dpf) was subjected to RT-PCR as described in the cDNA cloning section. The primer pairs *Tnnt2*-F/*Tnnt2*-R3 (5′-GGTGGATGTCATCAAAATCGACTC-3′), *Tnnt2*-F2 (5′-AAGATTCCAGATGGAGAAAGAGTCG-3′)/*Tnnt2*-R2 (5′-ATGTCCAGAGGTTTGCGTCGATCAC-3′), and *Tnnt2*-F3/*Tnnt2*-R were used to amplify cDNA fragments with predicted sizes of 318, 419 and 430 bp, respectively.

WISH

Zebrafish embryos were obtained by natural mating and were reared at 28.5°C in water containing 0.2 mM phenylthiocarbamide (Sigma) to suppress melanin formation. Embryos younger than 24 hpf were staged on the basis of somite number, whereas older embryos were staged on the basis of hpf (Kimmel et al., 1995). Embryos were dechorionated by pronase (10 μg/ml) digestion and fixed overnight at 4°C in PBS containing 4% paraformaldehyde. Digoxigenin (DIG) -labeled RNA probes for *Tnnt1*, *Tnnt2*, *Tnnt3a* (Xu et al., 2000), *Tnnt3b*, *sMyHC* (AF425742), *cmlc2* (Yelon et al., 1999), *cmlc1* (Reiter et al., 2001), *vmhc* (Yelon et al., 1999), and *cTnC* (AF434188), were used to perform WISH according to procedures described by Thisse et al. (1993). Color reactions were carried out in alkaline phosphatase buffer using nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche) as substrates. Stained embryos were placed in 100% glycerol, and evaluated with a differential interference contrast microscope (DMR, Leica) with a color digital camera (COOLPIX 996, Nikon) attached. For histologic examination, some stained embryos were embedded in paraffin and sectioned at 10-μm intervals.

NOTE ADDED IN PROOF

During the revision of this manuscript, one publication has appeared that is consistent with our data. Antin et al. (2002) reported the precocious expression of Tnnt2 mRNA in early chick embryos. They found most gene transcripts of cardiac muscle contratile protein are detected after HH stage 11, while Tnnt2 mRNA is detected as early as HH stage 5.

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REFERENCES

Antin PB, Zhang W, Bales MA, Garriock RJ, Yatskievych TA. 2002. Precocious ex-

pression of cardiac troponin T in early chick embryos is independent of bone morphogenetic protein signaling. Dev Dyn 225:135–141.

Aparicio S, Morrison A, Gould A, Gilthorpe J, Chaudhuri C, Rigby P, Krumlauf R, Brenner S. 1995. Detecting conserved regulatory elements with the model genome of the Japanese puffer fish, *Fugu rubripes*. Proc Natl Acad Sci U S A 92:1684–1688.

Barton PJ, Cullen ME, Townsend PJ, Brand NJ, Mullen AJ, Norman DA, Bhavsar PK, Yacoub MH. 1999. Close physical linkage of human troponin genes: organization, sequence, and expression of the locus encoding cardiac troponin I and slow skeletal troponin T. Genomics 57:102–109.

Benoist P, Mas JA, Marco R, Cervera M. 1998. Differential muscle-type expression of the *Drosophila* troponin T gene. A 3-base pair microexon is involved in visceral and adult hypodermic muscle specification. J Biol Chem 273:7538–7546.

Bolland DJ, Hewitt JE. 2001. Intron loss in the SART1 genes of *Fugu rubripes* and *Tetraodon nigroviridis*. Gene 271:43–49.

Breitbart RE, Nadal-Ginard B. 1986. Complete nucleotide sequence of the fast skeletal troponin T gene. Alternatively spliced exons exhibit unusual interspecies divergence. J Mol Biol 188:313–324.

Briggs JP. 2002. The zebrafish: a new model organism for integrative physiology. Am J Physiol Regul Integr Comp Physiol 282:R3–R9.

Bucher EA, Dhoot GK, Emerson MM, Ober M, Emerson CP. 1999. Structure and evolution of the alternatively spliced fast troponin T isoform gene. J Biol Chem 274:17661–17670.

Burge C, Karlin S. 1997. Prediction of complete gene structures in human genomic DNA. J Mol Biol 268:78–94.

Calvo S, Venepally P, Cheng J, Buonanno A. 1999. Fiber-type-specific transcription of the troponin I slow gene is regulated by multiple elements. Mol Cell Biol 19:515–525.

Charlet BN, Logan P, Singh G, Cooper TA. 2002. Dynamic antagonism between ETR-3 and PTB regulates cell type-specific alternative splicing. Mol Cell 9:649–658.

Cooper TA, Ordahl CP. 1984. A single cardiac troponin T gene regulated by different programs in cardiac and skeletal muscle development. Science 226: 979–982.

Cooper TA, Ordahl CP. 1985. A single cardiac troponin T gene generates embryonic and adult isoforms via developmentally regulated alternate splicing. J Biol Chem 260:11140–11148.

Dhoot GK. 1986. Selective synthesis and degradation of slow skeletal myosin heavy chains in developing muscle fibers. Muscle Nerve 9:155–164.

Endo T, Matsumoto K, Hama T, Ohtsuka Y, Katsura G, Obinata T. 1996. Distinct troponin T genes are expressed in embryonic/larval tail striated muscle and adult body wall smooth muscle of ascidian. J Biol Chem 271:27855–27862.

Endo T, Matsumoto K, Hama T, Ohtsuka Y, Katsura G, Obinata T. 1997. Temporal and spatial expression of distinct troponin T genes in embryonic/larval tail striated muscles and adult body wall smooth muscle of ascidian. Cell Struct Funct 22:197–203.

Farza H, Townsend PJ, Carrier L, Barton PJ, Mesnard L, Bahrend E, Forissier JF, Fiszman M, Yacoub MH, Schwartz K. 1998. Genomic organisation, alternative splicing and polymorphisms of the human cardiac troponin T gene. J Mol Cell Cardiol 30:1247–1253.

Filatov VL, Katrukha AG, Bulargina TV, Gusev NB. 1999. Troponin: structure, properties, and mechanism of functioning. Biochemistry 64:969–985.

Fitzgibbon JA, Hope SJ, Slobodyanyuk SJ, Bellingham J, Bowmaker JK, Hunt DM. 1995. The rhodopsin-encoding gene of bony fish lacks introns. Gene 164:273–277.

Fyrberg E, Fyrberg CC, Beall C, Saville DL. 1990. *Drosophila melanogaster*. Troponin-T mutations engender three distinct syndromes of myofibrillar abnormalities. J Mol Biol 216:657–675.

Gahlmann R, Troutt AB, Wade RP, Gunning P, Kedes L. 1987. Alternative splicing generates variants in important functional domains of human slow skeletal troponin T. J Biol Chem 262:16122–16126.

Hirao C, Hosoda K, Yonemura I, Miyazaki JI. 2001. Muscle subdivision: 1. Regulation of muscle genes. Dev Growth Differ 43:S71.

Hsiao CD, Hsieh FJ, Tsai HJ. 2001. Enhanced expression and stable transmission of transgenes flanked by inverted terminal repeats from adeno-associated virus in zebrafish. Dev Dyn 220:323–336.

Huang QQ, Jin JP. 1999. Preserved close linkage between the genes encoding troponin and troponin T, reflecting an evolution of adapter proteins coupling the Ca(2+) signaling of contractility. J Mol Evol 49:780–788.

Huang QQ, Chen A, Jin JP. 1999. Genomic sequence and structural organization of mouse slow skeletal muscle troponin T gene. Gene 229:1–10.

Inoue A, Ojima T, Nishita K. 1996. Cloning and sequencing of a cDNA for Akazara scallop troponin T. J Biochem 120: 834–837.

Inoue A, Ojima T, Nishita K. 1998. Cloning and sequencing of a cDNA for troponin T of Ezo-giant scallop striated muscle. Fish Sci 64:459–463.

Jin JP, Lin JJC. 1988. Rapid purification of mammalian cardiac troponin T and its isoform switching: in rat hearts during development. J Biol Chem 263:7305–7315.

Jin JP, Samanez RA. 2001. Evolution of a metal-binding cluster in the NH(2)-terminal variable region of avian fast skeletal muscle troponin T: functional divergence on the basis of tolerance to structural drifting. J Mol Evol 52:103–116.

Jin JP, Huang QQ, Yeh HI, Lin JJ. 1992. Complete nucleotide sequence and structural organization of rat cardiac troponin T gene. A single gene generates embryonic and adult isoforms via developmentally regulated alternative splicing. J Mol Biol 227:1269–1276.

Jin JP, Wang J, Zhang J. 1996. Expression of cDNAs encoding mouse cardiac troponin T isoforms: characterization of a large sample of independent clones. Gene 168:217–221.

Johnston I, Cole N, Viera V, Davidson I. 1997. Temperature and developmental plasticity of muscle phenotype in herring larvae. J Exp Biol 200:849–868.

Ju B, Xu Y, He J, Liao J, Yan T, Hew CL, Lam TJ, Gong Z. 1999. Faithful expression of green fluorescent protein (GFP) in transgenic zebrafish embryos under control of zebrafish gene promoters. Dev Genet 25:158–167.

Kelly GM, Reversade B. 1997. Characterization of a cDNA encoding a novel band 4.1-like protein in zebrafish. Biochem Cell Biol 75:623–632.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995. Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310.

Koban MU, Brugh SA, Riordon DR, Dellow KA, Yang HT, Tweedie D, Boheler KRA. 2001. Distant upstream region of the rat multipartite Na(+)-Ca(2+) exchanger NCX1 gene promoter is sufficient to confer cardiac-specific expression. Mech Dev 109:267–279.

Koumans JTM, Akster HA. 1995. Myogenic cells in development and growth of fish. Comp Biochem Physiol 110A:3–20.

Krishan K, Morgan MJ, Zhao W, Dhoot GK. 2000. Slow troponin T mRNA in striated muscles is expressed in both cell type and developmental stage specific manner. J Muscle Res Cell Motil 21:527–536.

Kusakabe R, Kusakabe T, Suzuki N. 1999. In vivo analysis of two striated muscle actin promoters reveals combinations of multiple regulatory modules required for skeletal and cardiac muscle-specific gene expression. Int J Dev Biol 43:541–554.

Kyprianou P, Madgwick A, Morgan M, Krishan K, Dhoot GK. 1997. Expression pattern of troponin I and distinct alternatively spliced developmental isoforms of troponin T in vitro and in neonatally denervated rat skeletal muscles. Basic Appl Myol 7:287–293.

Marden JH, Fitzhugh GH, Wolf MR, Arnold KD, Bowan B. 1999. Alternative splicing, muscle calcium sensitivity, and the modulation of dragonfly flight performance. Proc Natl Acad Sci U S A 96: 15304–15309.

Mesnard L, Samson F, Espinasse I, Durand J, Neveux JY, Mercadier JJ. 1993. Molecular cloning and developmental expression of human cardiac troponin T. FEBS Lett 328:139–144.

Muller F, Blader P, Strahle U. 2002. Search for enhancers: teleost models in comparative genomic and transgenic analysis of cis regulatory elements. Bioessays 24:564–572.

Myers CD, Goh PY, Allen TS, Bucher EA, Bogaert T. 1996. Developmental genetic analysis of troponin T mutations in striated and nonstriated muscle cells of *Caenorhabditis elegans*. J Cell Biol 132: 1061–1077.

Nakada K, Miyazaki JI, Saba R, Hirabayashi T. 1997. Natural occurrence of fast- and fast/slow-muscle chimeric fibers in the expression of troponin T isoforms. Exp Cell Res 235:93–99.

Nakayama M, Stauffer J, Cheng J, Banerjee-Basu S, Wawrousek E, Buonanno A. 1996. Common core sequences are found in skeletal muscle slow- and fast-fiber-type-specific regulatory elements. Mol Cell Biol 16:2408–2417.

Ogut O, Jin JP. 1998. Developmentally regulated, alternative RNA splicing-generated pectoral muscle-specific troponin T isoforms and role of the NH_2-terminal hypervariable region in the tolerance to acidosis. J Biol Chem 273: 27858–27866.

Ogut O, Granzier H, Jin JP. 1999. Acidic and basic troponin T isoforms in mature fast-twitch skeletal muscle and effect on contractility. Am J Physiol 276:C1162–C1170.

Oota S, Saitou N. 1999. Phylogenetic relationship of muscle tissues deduced from superimposition of gene trees. Mol Biol Evol 16:856–867.

Pearlstone JR, Johnson P, Carpenter MR, Smillie LB. 1977. Primary structure of rabbit skeletal muscle troponin-T. Sequence determination of the NH_2-terminal fragment CB3 and the complete sequence of troponin-T. J Biol Chem 252:983–989.

Pearson WR, Robins G, Zhang T. 1999. Generalized neighbor-joining: more reliable phylogenetic tree reconstruction. Mol Biol Evol 16:806–816.

Perry SV. 1998. Troponin T: genetics, properties and function. J Muscle Res Cell Motil 19:575–602.

Postlethwait JH, Yan YL, Gates MA, Horne S, Amores A, Brownlie A, Donovan A, Egan ES, Force A, Gong Z, Goutel C, Fritz A, Kelsh R, Knapik E, Liao E, Paw B, Ransom D, Singer A, Thomson M, Abduljabbar TS, Yelick P, Beier D, Joly JS, Larhammar D, Rosa F. 1998. Vertebrate genome evolution and the zebrafish gene map. Nat Genet 18:345–349.

Reiter JF, Verkade H, Stainier DY. 2001. Bmp2b and Oep promote early myocardial differentiation through their regulation of gata5. Dev Biol 234:330–338.

Ryan KJ, Cooper TA. 1996. Muscle-specific splicing enhancers regulate inclusion of the cardiac troponin T alternative exon in embryonic skeletal muscle. Mol Cell Biol 16:4014–4023.

Sabry MA, Dhoot GK. 1991. Identification of and pattern of transitions of cardiac, adult slow and slow skeletal muscle-like embryonic isoforms of troponin T in developing rat and human skeletal muscles. J Muscle Res Cell Motil 12:262–270.

Schiaffino S, Reggiani C. 1996. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. Phys Rev 76:371–423.

Schilling TF, Kimmel CB. 1997. Muscoskeletal patterning in the pharyngeal segments of the zebrafish embryo. Development 124:2945–2960.

Sehnert AJ, Huq A, Weinstein BM, Walker C, Fishman M, Stainier DY. 2002. Cardiac troponin T is essential in sarcomere assembly and cardiac contractility. Nat Genet 31:106–110.

Shuler CF, Dalrymple KR. 2001. Molecular regulation of tongue and craniofacial muscle differentiation. Crit Rev Oral Biol Med 12:3–17.

Stockdale FE. 1997. Mechanisms of formation of muscle fiber types. Cell Struct Funct 22:37–43.

Tan X, Du J. 2002. Differential expression of two MyoD genes in fast and slow muscles of gilthead seabream (*Sparus aurata*). Dev Genes Evol 212:207–217.

Taylor JS, Van de Peer Y, Braasch I, Meyer A. 2001. Comparative genomics provides evidence for an ancient genome duplication event in fish. Philos Trans R Soc Lond B Biol Sci 356:1661–1679.

Thisse C, Thisse B, Schilling TF, Postlethwait JH. 1993. Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. Development 119:1203–1215.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Thys TM, Blank JM, Schachat FH. 1998. Rostral-caudal variation in troponin T and parvalbumin correlates with differences in relaxation rates of cod axial muscle. J Exp Biol 201:2993–3001.

Thys TM, Blank JM, Coughlin DJ, Schachat F. 2001. Longitudinal variation in muscle protein expression and contraction kinetics of largemouth bass axial muscle. J Exp Biol 204:4249–4257.

van Swearingen J, Lance-Jones C. 1995. Slow and fast muscle fibers are preferentially derived from myoblasts migrating into the chick limb bud at different developmental times. Dev Biol 170:321–337.

Venkatesh B, Gilligan P, Brenner S. 2000. Fugu: a compact vertebrate reference genome. FEBS Lett 476:3–7.

Wachtler F, Christ B. 1992. The basic embryology of skeletal muscle formation in vertebrates: an avian model. Semin Dev Biol 3:217–227.

Waddleton DM, Jackman DM, Bieger T, Heeley DH. 1999. Charactersation of Troponin-T from salmonid fish. J Muscle Res Cell Motil 20:315–324.

Wang J, Jin JP. 1997. Primary structure and developmental acidic to basic transition of 13 alternatively spliced mouse fast skeletal muscle troponin T isoforms. Gene 193:105–114.

Wang Q, Sigmund CD, Lin JJ. 2000. Identification of cis elements in the cardiac troponin T gene conferring specific expression in cardiac muscle of transgenic mice. Circ Res 86:478–484.

Wang Q, Reiter RS, Huang QQ, Jin JP, Lin JJ. 2001. Comparative studies on the expression patterns of three troponin T genes during mouse development. Anat Rec 263:72–84.

Warkman AS, Atkinson BG. 2002. The slow isoform of Xenopus troponin I is expressed in developing skeletal muscle but not in the heart. Mech Dev 115:143–146.

Waterman RE. 1969. Development of the lateral musculature in the teleost, *Brachydanio rerio*: a fine structural study. Am J Anat 125:457–493.

Wilkinson JM, Moir AJG, Waterfield MD. 1984. The expression of multiple forms of troponin T in chicken fast muscle may result from differential splicing of a single gene. Eur J Biochem 143:47–56.

Xu Y, He J, Tian HL, Chan CH, Liao J, Yan T, Lam TJ, Gong Z. 1999. Fast skeletal muscle-specific expression of a zebrafish myosin light chain 2 gene and characterization of its promoter by direct injection into skeletal muscle. DNA Cell Biol 18:85–95.

Xu Y, He J, Wang X, Lim TM, Gong Z. 2000. Asynchronous activation of 10 muscle-specific protein (MSP) genes during zebrafish somitogenesis. Dev Dyn 219:201–215.

Yamano K, Miwa S, Obinata T, Inui Y. 1991. Thyroid hormone regulates developmental changes in muscle during flounder metamorphosis. Gen Comp Endocrinol 81:464–472.

Yan Z, Serrano AL, Schiaffino S, Bassel-Duby R, Williams RS. 2001. Regulatory elements governing transcription in specialized myofiber subtypes. J Biol Chem 276:17361–17366.

Yelon D, Horne SA, Stainier DY. 1999. Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. Dev Biol 214:23–37.

Yonemura I, Hirabayashi T, Miyazaki J. 2000. Heterogeneity of chicken slow skeletal muscle troponin T mRNA. J Exp Zool 286:149–156.

Yonemura I, Mitani Y, Nakada K, Akutsu S, Miyazaki J. 2002. Developmental changes of cardiac and slow skeletal muscle troponin T expression in chicken cardiac and skeletal muscles. Zool Sci 19:215–223.