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計畫主持人：蔡懷楨
計畫參與人員：王玉雪、蕭崇德、蔡維原

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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
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, 1997). In tetrapods, three were identified in the smooth and the adductor muscle of scallops (Inoue et al., 1990, Benoist et al., 1998), and in Drosophila melanogaster (Fyrberg et al., 1993, 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 (Gahrnmann 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 spatio-temporal 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: A1883430, 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 clones-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 pl and are highly negatively charged at pH 7, whereas Tnnt3b has a basic pl 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
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:
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. 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>
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<th>Gene names</th>
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<th>Coding region (aa)</th>
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TABLE 1. Summary of the Troponin T cDNAs Used in This Study
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.
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.
were detected by RT-PCR using the primer pair combinations indicated. Two, alternatively spliced variants were detected by primer pair solid boxes show open reading frames.

Age-dependent expression of alternatively spliced, 

**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.

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–K: Dorsal views, anterior of embryo to the top. J: Frontal view of embryo. Embryonic stages are indicated in each panel. h, hour; a, atrium; h, 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,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,Q,T.
Fig. 6.

Fig. 7.
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, Tnnt3a (Fig. 8K) but not Tnnt3b (Fig. 8P) 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 sternothyroides 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 diverge 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-
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 Fyberg 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), troponin T (Krishan et al., 2000, Wang et al., 2001), and troponin C (Krishan et al., 2001), predominate during early development. The fast isoforms of these proteins predominate later in development. Zebrafish Tnnt1 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 fetal stages but are restricted to slow muscle fibers during postnatal development (Krishan et al., 2002; Wang et al., 2001). In zebrafish, only Tnnt1 is expressed in all slow and fast muscles during early embryonic and fetal stages, but expression in skeletal muscles is selectively repressed after the late fetal stages (Wang et al., 2001). To obtain total RNA, zebrafish embryos 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 MATCH-MAKER Library Construction & Screening Kit (Clonetech). This method involved synthesis of full-length cDNA using CDS III oligo(dT) primer (5’-ATTTCTAGAGCCGCAAGGGGCGC-3’), coupled with (dC) tailing by PowerScript reverse transcriptase, followed by template switching and extension with SMART III oligonucleotide (5’-AACCGATGTGATCAACGCCAGAGGCGCATTATGGCCGGG-3’). The RNA/cDNA hybrid was then preamplified by long-distance PCR with the primer set 5’-PCR (5’-TTCCACC-CGCGATCCCGATTATCAAACGCGAGTGG-3’) / 3’-PCR (5’-GTATCGATGCCTCACATGAAACGCGGGCGGCAAG-3’). For 3’-RACE, each preamplified PCR product was performed with primer sets Tnnt1-F (5’-AGAAGATGACATCTGGGAGACACCCGGCAGG-3’), Tnnt3b-F (5’-CTTGAGTGCTCTGCGGCGGCGG-3’), Tnnt2-F (5’-TTCCTGTGACATCTGCGGCGCAGTG-3’), Tnnt2-F (5’-CCCAAGATGGTGGTAACACATGCTGATAC-3’), Tnnt3-F (5’-GAAAGGTCTCACAACCTGAGTCGC-GAC-3’), and 3’-PCR, respectively. For 5’-RACE, each preamplified PCR product was performed with primer sets Tnnt1-R (5’-CTCAATCGGTCTCAACGGTCATAGC-3’), Tnnt3b-R (5’-AATGTCCAGGGCTCGTAC-3’), Tnnt2-R (5’-AGTGAATATCTATATTGTGCAATGAAATACTTACGCGG-3’), XTnnt2-R (5’-ACTCTGTGTTAAATGTATTGTTTTCAACTTACAC-3’), XTnnt3-R (5’-ATGAAATTACGCCAGGCCACACTGAAACGCGGGCAG-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 unsliced Tnnt were performed by 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 HGMIP 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,
In brief, 3′ genomic walker kit (Clonetech). 3′ genomic walking, the adapter ligated genomic DNA fragments were amplified using the primer set Tnnt1-F/A1 (5′-GTAATACGACTCACTATAGGGC-3′) and Tnnt1-R set (5′-CATGATGTCACACTATT-CACTATAGGGC-3′) nested primer set (Fig. 3A). For Tnnt2, Tnnt3a, and Tnnt3b, the primer pairs Tnnt2-F/Tnnt2-R (5′-ATGTCCAGAGGTTTGCGTCGATCAC-3′) and Tnnt3a-F (5′-GGACATAGCACAGGATITTGC-3′)/Tnnt3a-R (5′-CATGATGTCACACTATTGTTAGCAAACCT-3′), and Tnnt3b-F/Tnnt3b-R were used to amplify genomic DNA by long-distance PCR amplification with LA Taq 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, 16, 24, 48, 72 hpf, and adult zebrafish (60 dpf). The primer pairs Tnnt1-F/Tnnt1-R, Tnnt3a-F/Tnnt3a-R, Tnnt3b-F/Tnnt3b-R, Tnnt2F (5′-GTCATCAAAATCGACTC-3′)/Tnnt2-R, and sMyHC-F (5′-CAGGCTCCAGAAATGAGATTGAG-3′)/sMyHC-R (5′-AGCTTCATCTCCTGCGAG-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 Tnnt2F/Tnnt2R (5′-GGTGGATGTCATCAAAATCGACT-3′) and Tnnt2-F (5′-AAGATTCAGATGGAGAAAGAGTGCG-3′)/Tnnt2-R (5′-ATGTCCAGAGGTTTGCGTCGATCAC-3′), and Tnnt2-F/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 Thissen 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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