Distinct Troponin T Genes Are Expressed in Embryonic/Larval Tail Striated Muscle and Adult Body Wall Smooth Muscle of Ascidian*

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During development of the ascidian Halocynthia roretzi, the tadpole larva hatched from the tailbud embryo metamorphoses to the sessile adult with a body wall muscle. Although the adult body wall muscle is morphologically nonsarcomeric smooth muscle, it contains troponin complex consisting of three subunits (T, I, and C) as do vertebrate striated muscles. Different from vertebrate troponins, however, the smooth muscle troponin promotes actomyosin Mg²⁺-ATPase activity in the presence of high concentration of Ca²⁺, and this promoting property is attributable to troponin T. To address whether the embryonic/larval tail striated muscle and the adult smooth muscle utilize identical or different regulatory machinery, we cloned troponin T cDNAs from each cDNA library. The embryonic and the adult troponin Ts were encoded by distinct genes and shared only <60% identity with each other. Northern blotting and whole mount in situ hybridization revealed that these isoforms were specifically expressed in the embryonic/larval tail striated muscle and the adult smooth muscle, respectively. These results may imply that these isoforms regulate actin-myosin interaction in different manners. The adult troponin T under forced expression in mouse fibroblasts was unexpectedly located in the nuclei. However, a truncated protein with a deletion including a cluster of basic amino acids colocalized with tropomyosin on actin filaments. Thus, complex formation with troponin I and C immediately after the synthesis is likely to be essential for the protein to properly localize on the thin filaments.

Troponin (Tn) complex, which consists of three subunits T, I, and C, is the Ca²⁺-dependent negative regulator of vertebrate striated muscle contraction (1, 2). This protein complex is associated with tropomyosin (Tm) on the thin filaments with a periodicity of 38 nm. Tn does not exist in vertebrate smooth muscle, and its contraction is primarily regulated by Ca²⁺/calmodulin-dependent phosphorylation of myosin light chain (3, 4). Thin filament-linked regulatory systems including caldesmon and calponin associated with Tm may further modulate smooth muscle contraction status (5, 6).

Tn/Tm regulatory system also prevails in a variety of invertebrate striated and obliquely striated muscles (7). Tns in these invertebrate muscles also inhibit actin-myosin interaction dependently on Ca²⁺. The presence of Tn has not been demonstrated in smooth or nonstriated muscles except for the adult body wall muscle of the ascidian Halocynthia roretzi (8, 9), a protochordate, and the oviduct myoepithelial sheath of Caenorhabditis elegans (10). The ascidian body wall muscle is composed of multinucleated but nonsarcomeric smooth muscle cells (11). Different from Tns from the other sources, the ascidian Tn activates in the presence of high concentration of Ca²⁺-actomyosin Mg²⁺-ATPase and superprecipitation (9, 12), both of which reflect the degree of actin-myosin interaction. Later, a similar activating property was also reported for Tns isolated from striated adductor muscle of Akazara scallop (13). The activating property of ascidian Tn is ascribable to TnT, because the isolated TnT promotes actomyosin Mg²⁺-ATPase activity and superprecipitation regardless of Ca²⁺ concentration (9). Reconstitution with the TnT and the isolated TnI and C restores the intact Tn activity. The ascidian TnT shares with vertebrate counterparts some other properties including Tm-binding ability (9), basic pi, consisting of multiple isoelectric forms on two-dimensional isoelectric focusing SDS-polyacrylamide gel electrophoresis (PAGE), and cross-reactivity to the monoclonal antibody NT302 (14) raised against chicken skeletal muscle TnT (15).

During development of the ascidian, the tailbud embryo hatches out to generate the swimming tadpole larva, which metamorphoses to the sessile adult. The tails of the embryo and the larva contain mononucleated striated muscle cells or their precursor cells. A myosin heavy chain gene and an α-actin gene are specifically expressed in the striated muscle cells and the precursor cells but not in the adult smooth muscle (16, 17). In contrast, a Tm gene is expressed in the adult body wall muscle as well as striated muscles of adult heart and larval tail in another ascidian Ciona intestinalis (18). As to Tn subunits, there has been no report regarding their expression at the embryonic or larval stage. Thus, it remains to be determined whether the adult TnT accelerates actin-myosin interaction, how its multiple isoelectric forms are generated, and how the embryonic or larval striated muscle contraction is regulated. To these ultimate ends, we cloned the TnT cDNAs by screening adult body wall muscle and tailbud embryo cDNA expression libraries with NT302. The embryonic and adult proteins encoded by these cDNAs were only <60% identical to each other. In addition, they were
specifically expressed in the embryonic/larval tail striated muscle and the adult smooth muscle, respectively, suggesting their functional difference. Forced expression of the adult TnT in cultured mammalian nonmuscle cells further suggests that complex formation with the other troponin subunits is required for the protein to associate with thin filaments or microfilaments in vivo.

**Experimental Procedures**

Construction and Screening of cDNA Libraries—Total RNA was prepared from the adult body wall smooth muscle of *H. roretzi* by the guanidium thiocyanate method (19). Poly(A)^+ RNA was isolated by column chromatography with oligo(dT) cellulose (type 3, Collaborative Research, Inc.). One microgram of total RNA was reverse transcribed and cDNA was synthesized, and cDNA libraries were constructed in Agt10 and Agt11 (Stratagene) as described (21). The unamplified total libraries in Agt10 and Agt11 contained 2.4 × 10^6 and 5.3 × 10^5 recombinants, respectively. The amplified Agt11 cDNA library and the tailbud embryo Agt11 cDNA library (22), which was presented by Drs. K. W. Makabe and N. Satoh, were screened with the monoclonal antibody NT302 raised against chicken pectoralis skeletal muscle TnT (15).

Library (22), which was presented by Drs. K. W. Makabe and N. Satoh, was screened with the antibody, and three positive clones (aTnT1, 2, and 3) were isolated from −2 × 10^6 plaques. Their nucleotide sequences indicated that they were overlapping cDNAs of the same mRNA species. The longest clone, aTnT2, was 923 bp (when the poly(A) tail was excluded), which represented nucleotides 208-1130 in Fig. 1A, and contained an open reading frame encoding a 248-amino acid protein. The sequence around the initiation codon was GtaAaaATG, where uppercase but not lowercase letters met the consensus sequence for the translation initiation of vertebrate mRNAs (32). Canonical poly(A) addition signal was not present in this sequence. Considering the site of poly(A) tail, which started at 1131 in Fig. 1A, one of ATAAAA or ATGAA or ATTAT may serve as the signal.

To investigate whether similar but distinct TnT mRNAs exist in the body wall muscle, we further screened the Agt10 cDNA library with aTnT2 and isolated ~30 clones from ~4 × 10^6 plaques. One of the longest clones, aTnT19, was 1,164 bp and completely overlapped with aTnT2 between nucleotides 208 and 1130 (Fig. 1A). aTnT19 contained additional 207 bp of nucleotides upstream of the 5′-end of aTnT2 and additional 34 bp of nucleotides downstream of the 3′-end. Because there was an in-frame termination codon (T200AA) only 27 bp upstream of A230TG, this ATG should serve as the initiation codon. Consequently, aTnT19 shared the open reading frame with aTnT2. In aTnT19, AATATA is likely to represent a poly(A) addition signal (33), which was 11 bp apart from the poly(A) addition site. Because there were two putative poly(A) addition signals in this clone, at least two mRNA species seem to be generated from a single gene by the alternative 3′ end processing of a single pre-mRNA (34). These two mRNAs, however, code for the identical proteins. A protein encoded by aTnT2 and aTnT19 consisted of 248 amino acids with a calculated molecular mass of 29,770 Da and a calculated pI of 9.56. These values are close to those estimated by SDS-PAGE (9) and two-dimensional isolectric focusing SDS-PAGE (14).

We next screened the tailbud embryo Agt11 cDNA expression library with NT302 to determine which type of regulatory system governs in the tail striated muscle of *H. roretzi* tailbud embryo and tadpole larva. Two positive clones (eTnT11 and 14) were obtained from ~2 × 10^5 plaques. Both clones were 1,012 bp long, and their nucleotide sequences were identical to each other (Fig. 1B). Because no in-frame termination codon was present upstream of the A230TG in these clones, they may contain a partial coding sequence lacking the 5′-terminal sequence including the initiation codon. If this is true for these clones, they code for an N-terminal-truncated protein consisting of 242 amino acids with a calculated molecular mass of 28,615 Da and a calculated pI of 10.22. There were three ATAAA sequences in the most translated region (Fig. 1B). aTnT19 A5′most sequence is likely to serve as a poly(A) addition signal in these clones. Because doublet mRNA bands were detected in the larva by Northern blotting (see Fig. 4), either one of the other
**FIG. 1. Nucleotide sequences and predicted amino acid sequences of aTnT19, aTnT2, and eTnT11/14.**

A, the nucleotide sequence of aTnT19 (1,164 bp when the poly(A) tail is excluded) and aTnT2 (923 bp when the poly(A) tail is excluded) cDNAs and predicted amino acid sequence. aTnT19-specific nucleotide sequences at 5’ and 3’ ends that aTnT2 lacks are shown in white on black. Asterisks represent the first nucleotides of in-frame termination codons. Double underlines indicate the poly(A) addition signal for aTnT19. Single underlines denote putative poly(A) addition signals for aTnT2. Dotted underlines indicate basic amino acid clusters. The C-terminal sequence started from the Ala141 marked by + is deleted in the truncated TnTDAva.

B, the nucleotide sequence of eTnT11/eTnT14 cDNAs (1,012 bp) and predicted amino acid sequence. Asterisks represent the first nucleotide of in-frame termination codon. Single underlines denote possible poly(A) addition signals. Dotted underlines indicate a basic amino acid cluster.
two sequences may also function as a poly(A) addition signal (see below). The identity of sequence between aTnT2/aTnT19 and eTnT11/eTnT14 was only ~60%, and identical sequences were extremely dispersed. This implies that they are derived from different genes.

Comparison of the Amino Acid Sequence among Ascidian and Vertebrate TnTs—The amino acid sequences of the proteins encoded by aTnT2/aTnT19 (hereafter the protein is referred to as adTnT) and by eTnT11 (the protein is designated as embTnT) were compared with each other and with those of chicken skeletal (35) and cardiac (36) and rabbit skeletal (37) and cardiac (38) muscle TnTs (Table I). adTnT and embTnT were only 58.9% identical to each other, but they were still more similar to each other than to vertebrate TnTs. In addition, it is worth noting that the identity between skeletal and cardiac muscle TnTs is only 54% and 56% in chicken and rabbit, respectively.

To examine the evolutionary relationships of adTnT and embTnT to known TnTs of other species, their amino acid sequences were aligned and analyzed by UPGMA (39). Among various species of mammalians and chicken, fast skeletal muscle TnTs formed one clade and cardiac muscle TnTs clustered within another clade (Fig. 2). Human slow skeletal muscle TnT was more closely related to the cardiac muscle proteins than to the fast skeletal muscle ones. adTnT and embTnT formed another clade than those to which the skeletal and cardiac muscle TnTs belong. C. elegans (10) and Drosophila (40, 41) TnTs diverged at an early stage of evolution from these deuterostome proteins. Thus, the evolutionary relationships of TnT proteins reflect phylogenetic relationships of these animal species. Neither of the H. roretzi TnTs had a long C-terminal extension, which C. elegans and Drosophila TnTs but not vertebrate TnTs possess (Fig. 3). The fact may also indicate that the ascidian TnTs are more closely related to vertebrate TnTs than to the protostome proteins.

Analyses of cyanogen bromide-digested fragments of rabbit skeletal muscle TnT have shown that Tm-binding ability resides in Glu71-Ser150 (42, 43). Analyses of chymotryptic fragments and measurements of the relative reactivity of lysine residues with acetic anhydride have revealed that TnI-binding domain is Lys23-Tyr257 (44–47). When the amino acid sequences of adTnT and embTnT were aligned with those of the rabbit skeletal and cardiac muscle TnTs to search similarity according to Lipman and Pearson (48), central regions were more conserved than the N- and C-terminal regions (Fig. 3). The above Tm- and TnI-binding sites were located in these conserved areas. Adult H. roretzi TnT binds to Tm as determined by electron microscopic observations of the binding to Tm paracrystals (9). In addition, the fact that the ability of adult TnT to accelerate actin-myosin interaction is inhibited by TnI (9) implies that the TnT interacts with TnI. The identity between adTnT and embTnT in the corresponding areas to Tm binding and TnI binding was 60.0% and 80.0%, respectively, which was higher than the identity in the whole length of the molecules. This may indicate that embTnT also has abilities to interact with Tm and TnI, implying that it functions as TnT.

In rabbit skeletal muscle TnT, Ser1 is acetylated and phosphorylatable by casein kinase (37, 49). Ser150 or Ser156 and Ser152 or Ser157 are also phosphorylatable by phosphorylase kinase (49). Existence of these multiple phosphorylation sites in addition to isoforms produced by alternative splicing may account for multiple isoelectric variants of TnT (14, 15, 50). If Met1 in adTnT is removed by a methionine-specific aminopeptidase, the first residue is Ser as in rabbit skeletal and cardiac muscle TnTs, and this Ser might be also acetylated and phosphorylated. In this context, it should be noted that this Ser, as well as Ser1 in rabbit skeletal and cardiac muscle TnTs, fits in the consensus phosphorylation site by casein kinase II (S/TXX(D/E)) (51) (Figs. 1 and 3). Residues corresponding to S150E/156 and S152E/157 in rabbit skeletal muscle TnT were, however, replaced by other amino acids in adTnT and embTnT.

Existence of embTnT in the Embryonic/Larval Tail Striated Muscles—We next carried out Northern blotting probed with aTnT2 and eTnT11 cDNAs to examine whether multiple mRNA species exist in the adult H. roretzi body wall muscle and the larva, as well as whether similar mRNA species are present in avian and mammalian muscle cells and tissues. aTnT2 hybridized to the adult body wall muscle mRNA as apparently a single broad band of ~1.1 kilobases but neither to the larval mRNA nor to the vertebrate muscle mRNAs examined (Fig. 4B). eTnT11 hybridized to the larval mRNA as double bands of ~1.6 and ~1.4 kilobases but not to the adult body wall muscle or vertebrate muscle mRNAs (Fig. 4A). As described above, these doublet bands might correspond to mRNAs generated by alternative 3’ end processing because the difference in the size of the mRNA bands is close to the length between the proximal and distal putative poly(A) addition signals. The rat skeletal muscle cDNA TnT15 (27) hybridized to mRNAs from mouse skeletal muscle, mouse C2C12, and rat L6E9-B myotubes but not to mRNAs from the adult H. roretzi body wall muscle or mouse cardiac muscle (data not shown). The hybridization was also detected as apparently single broad bands of ~1.1 kilobases.

Although eTnT11 was specifically expressed in the larva, it was necessary to examine whether eTnT11 was expressed in the tail striated muscle or other tissues such as heart muscle and visceral smooth muscle because poly(A)’ RNA derived from whole body of the tailbud embryos was used to construct the cDNA library (22). To address this issue, we applied whole mount in situ hybridization probed with the antisense eTnT11 transcript. The hybridization was already detected in the immature tail muscle cells of the early and middle stages of the embryos (Fig. 5, A and B). Even after the development of various organs in the late stage of the embryos and the hatched larvae, the hybridization was restricted to the striated muscle cells and excluded from heart or guts (Fig. 5, C and D). Antisense aTnT2 transcript did not hybridize to mRNAs in any tissues of the embryos or the larvae (data not shown). These results indicate that embTnT is exclusively present in the tail striated muscle at stages of the tailbud embryos and the tadpole larvae, whereas adTnT is restricted to the adult body wall muscle.

Localization of Transfected H. roretzi TnT in Mammalian Fibroblasts—Electron microscopic observations of the binding ability of the purified adult body wall muscle TnT to Tm paracrystals have shown that the TnT binds to both Tms from H. roretzi body wall muscle and rabbit skeletal muscle (9).
Although adTnT is so far the sole TnT cloned from the body wall muscle, we cannot rule out a possibility that the adTnT is distinct from the purified body wall muscle TnT in the properties including Tm-binding ability. To investigate whether adTnT is able to bind to Tm in vivo, we transfected the recombinant plasmid, pSRTnT, harboring aTnT2 cDNA under the control of strong SRa promoter (29) to the mouse fibroblast cell line C3H10T1/2. Immunofluorescence microscopy by the staining with NT302 showed that the exogenous adTnT unexpectedly accumulated in the nuclei instead of being associated with Tm-containing microfilament bundles (Fig. 6, A and B). In addition to the dense nuclear localization, diffused cytoplasmic distribution of the TnT was also discerned in some cells (Fig. 6, C and D). This ectopic nuclear location suggests either that adTnT has a sequence corresponding to the nuclear localization signal (NLS) or that adTnT is imported to the nucleus in association with some nuclear protein. The diffused cytoplasmic distribution may imply either that adTnT is not able to bind to fibroblast Tm or that some modification that occurred in the cells on adTnT molecule prevents the TnT from binding to the Tm.

We scrutinized the amino acid sequence of adTnT and noticed two clusters of basic amino acids: the N-terminal (R107K--K116KRK) and the C-terminal (K150R---K157KKK) (Fig. 1A). Because these basic amino acid clusters are similar to the NLS of the SV40 large T antigen (PKKKRKV) or the bipartite NLS of nucleoplasmin (KRPAAIKKAGQKKK) (52, 53), they may have served as NLSs in the transfected cells. To confirm this postulation, we transfected the cells with the plasmid pSRTnTD2Ava, which contains aTnT2 cDNA deleted downstream of the AvaI site. This deletion-mutated cDNA specifies a truncated adTnT lacking the C-terminal cluster (Fig. 1A). The truncated protein was located along the microfilament bundles detected by rhodamine-phalloidin staining.
Double immunostaining with NT302 and anti-Tm showed that the exogenous TnT and endogenous Tm were colocalized on the microfilament bundles (Fig. 7, A and B). Observations at a higher magnification revealed that they coexisted with a periodic distribution (Fig. 7, C and D). This microfilament-associated location coincided with the nuclear distribution in some cells (Figs. 6E and 7A), whereas almost exclusive cytoplasmic distribution was also detected in other cells (Fig. 6G). These results and our unpublished observations suggest that the sequence including the C-terminal basic amino acid cluster is responsible at least in part for the nuclear localization of adTnT. Furthermore, they imply that adTnT is indeed able to interact with Tm in vivo when some modification that takes place in the C-terminal portion is hindered.

DISCUSSION

In the present study, we cloned two distinct TnT molecules from adult and tailbud embryo of the ascidian H. roretzi. This is the first case of cloning deuterostome invertebrate TnTs. Probable Tm- and TnI-binding ability of these TnTs was suggested by the existence of the conserved sequences in these proteins. N. Sawada and T. Endo, unpublished observations.

(Fig. 6, E–H). Double immunostaining with NT302 and anti-Tm showed that the exogenous TnT and endogenous Tm were colocalized on the microfilament bundles (Fig. 7, A and B). Observations at a higher magnification revealed that they coexisted with a periodic distribution (Fig. 7, C and D). This microfilament-associated location coincided with the nuclear distribution in some cells (Figs. 6E and 7A), whereas almost exclusive cytoplasmic distribution was also detected in other cells (Fig. 6G).
TnTs corresponding to Tm- and Tnl-binding domains in rabbit skeletal muscle TnT. Colocalization of the truncated adTnT and endogenous nonmuscle Tm in the transfected cells corroborated its Tn-binding ability. Because H. roretzi body wall muscle TnTs has the accelerating effect on the actomyosin Mg$^{2+}$-ATPase activity (9), different from vertebrate striated muscle TnTs, there may be a domain in adTnT responsible for the effect. Although Tn from the Akazara scallop striated muscle, like H. roretzi Tn, activates actomyosin Mg$^{2+}$-ATPase activity under high Ca$^{2+}$ concentration, its TnT-like 40-kDa protein itself does not have an activating effect (13). Thus, even if the sequence of the Akazara TnT-like protein is determined, comparison of the sequence of the TnT-like protein with that of adTnT does not seem to be informative to identify the domain responsible for the activation. Instead, experiments with mutated recombinant adTnT proteins are required to determine the activation domain and more definite Tm-binding domain as well as Tnl-binding domain. All known isoforms of TnT, including the protostome TnTs, share the feature of a highly acidic N terminus, whereas adult H. roretzi TnT had only a short stretch of Glu at the N terminus (Fig. 3). Consequently, this might be a region worth being examined for the promoting effect.

Although aTnT2 and aTnT19 were derived from distinct mRNA species, they coded for the identical protein (adTnT). In addition, eTnT11-encoded protein (embTnT) was not detected in the adult body wall muscle. Thus, so far only a single TnT protein has been identified in the adult smooth muscle. Isolelectric focusing SDS-PAGE analyses, however, have detected multiple isoelectric forms of body wall muscle TnT (14). At least some of these forms are likely to be generated by post-translational modifications such as phosphorylation. In fact, there are several possible phosphorylation sites in adTnT by known protein serine/threonine kinases: (S/T)TX(D/E) for casein kinase II (51) and (S/T)P for Cdc2 (54, 55). S$^{O}$EE, T$^{O}$RE, S$^{O}$REE, and S$^{O}$EE meet these consensus sequences. Particularly, the first site is likely to be phosphorylated by casein kinase II because this sequence is similar to S$^{O}$DEE, which is phosphorylatable by casein kinase (presumably casein kinase II, considering the sequence), in rabbit skeletal muscle TnT. If variability of the activation domain would be ascribable at least in part to the functional difference between embTnT and adTnT. Nevertheless both types of TnTs are likely to interact with the same species of Tm, because adTnT seems to interact even with the mouse fibroblast Tm. These postulations may explain the different temporal expression pattern of Tm and TnT.

Despite the thin filament-associated localization of H. roretzi TnT in the body wall muscle (9), the exogenously expressed entire length of adTnT was mainly located in the nuclei of the mouse fibroblasts. Lower levels of the protein also diffusely distributed in the cytoplasm of some of the cells. These ectopic distributions do not seem to be due to heterology of host cells but owing to the primary or higher structure as well as a post-translational modification of the TnT, which is present as an orphan molecule in the heterologous host cells. Deletion of the sequence including the C-terminal basic amino acid cluster suggested that this region functioned as the NLS. The deletion also suggested that some modification in this sequence was responsible for the diffused cytoplasmic distribution. It is remarkable that the deleted sequence contains several phosphorylation consensus sites as discussed above. Comparison of the putative NLS with those of other TnTs indicates that this basic amino acid cluster is conserved in some other TnTs including embTnT and chicken cardiac muscle TnT (36). Thus, expression of these TnTs in non-sarcomeric cells may also result in nuclear localization of the proteins. If Tn and Tc are synthesized concurrently with the intact TnT in vivo, however, prompt complex formation may occur. Association of the Tn complex with thin filament-linked Tm in vivo suggests that the complex formation results in concealment of the NLS and the modification sites. Hence we have to be careful when we analyze the localization and effects of certain proteins by introducing the proteins or their cDNAs into cells. This is particularly applicable to subunit proteins composing a complexed molecule.

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Ascidian Smooth and Striated Muscle Troponin Ts
