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Primary Structure of Troponin I Isoforms from the Ascidian *Halocynthia roretzi*[1]

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The solitary ascidian *Halocynthia roretzi* possesses three types of muscle: the larval tail striated muscle, the adult heart striated muscle, and the adult body wall smooth muscle. The troponin complex is observed in all types of muscle, and the isoform sequences and expression patterns of two of the three troponin components, troponins C and T, have been reported. In this study, we have determined cDNA sequences of the three TnI isoforms from *H. roretzi*. One of the three isoforms (adult TnI), expressed in adult body wall smooth muscle and heart muscle, was composed of 173 amino acids, being similar to vertebrate fast and slow skeletal TnIs in length. The other two isoforms (larval TnIα and TnIβ) were isolated from a cDNA library of larvae. Both larval TnIs were composed of 142 amino acids, with truncation amounting to *ca.* 30 amino acid residues at the C-termini. These larval TnIs are the smallest known TnIs. The position of the last intron of these TnIs was also determined. When compared with vertebrate TnI genes, the last intron of the ascidian adult TnI gene is located at 6 nucleotides downstream, and the introns of the two larval TnIs are positioned at 9 nucleotides upstream. These results suggest that *H. roretzi* TnI is encoded by at least three genes.

Key words: cDNA sequence, *Halocynthia roretzi*, isoforms, troponin I.

Troponin (Tn) is a main regulatory protein of striated muscle contraction and is constructed from three components, TnT, I, and C. TnC is the Ca^{2+} sensor of the Tn complex and TnT is the tropomyosin-binding subunit. In relaxed muscle, TnI binds to actin, and inhibits the interaction between actin and myosin. TnI also binds to TnC and T. The Ca^{2+} binding to TnC affects the TnC-TnI interaction; the inhibition of the actin-myosin interaction by TnI is removed, and contraction starts (*1, 2*). Tn has been observed only in striated muscle, and no Tn has been isolated from smooth muscle except in three instances: the adult body wall muscle of the ascidian *Halocynthia roretzi* (*3*), the adductor muscle of the scallop *Chlamys nipponensis akazara* (*4*), and the oviduct myoepithelial sheath of the nematode *Caenorhabditis elegans* (*5*).

The ascidian belongs to Urochordata, which is one of two subphyla of protochordates. The other subphylum is Cephalochordata, known as amphioxus, which is regarded as the closest invertebrate group to vertebrates (*6*). During development, the ascidian changes its shape drastically from the tadpole-like larva to the sessile adult. Three types of muscle cells are observed in the ascidian: monocellular striated muscle cells of larval tail muscle (*7*), unicellular striated muscle cells of adult heart muscle (*8*), and multinucleate smooth muscle cells of adult body wall muscle (*9, 10*).

Recently, the cDNA sequences and tissue-specific expression patterns of TnC and TnT isoforms from the ascidian *Halocynthia roretzi* were determined (*11, 12*). The ascidian TnC gene is a single copy gene, and two isoforms are produced through alternative splicing. One of the two isoforms is larval TnC, which is expressed in the larval striated muscle, and the other is adult TnC, which is present in heart muscle and body wall smooth muscle. The amino acid sequence identity between these two TnC isoforms is 91%, and there seems to be no functional differences between them (*11*). On the other hand, the larval striated muscle TnT and the adult body wall smooth muscle TnT are encoded by distinct genes, and the identity between them is less than 60% (*12*).

In mammalian and avian muscles, three distinct isoforms of TnI: fast skeletal TnI (fTnI), slow skeletal TnI (sTnI), and cardiac TnI (cTnI) have been identified. These three isoforms are encoded by different genes, and are specifically expressed in fast skeletal, slow skeletal, and cardiac muscle, respectively (*13, 14*). Among invertebrates, the TnI sequences from the crayfish *Astacus leptodactylus* (*15*), the fruitfly *Drosophila melanogaster* (*16, 17*), and the nematode *Caenorhabditis elegans* (*18*) have been determined. In *Drosophila*, three TnI isoforms are produced from the same gene through differential RNA processing (*16, 17*).

In this study, we isolated and sequenced three distinct cDNAs of TnI isoforms from the ascidian *Halocynthia roretzi*, and showed that the three isoforms are encoded by different genes.

[1] The nucleotide sequences have been submitted to the DDBJ under the accession numbers AB001685 (*H. roretzi* body wall muscle TnI cDNA), AB001686 (*H. roretzi* larval TnIα cDNA), and AB001687 (*H. roretzi* larval TnIβ cDNA).

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MATERIALS AND METHODS

The ascidian *Halocynthia roretzi* was obtained from the Marine Biological Station of Asamushi, Tohoku University, and at a seafood market in Sendai.

Washed adult body wall muscle myofibrils (*19*) were extracted with 0.4 M LiCl at pH 4.5, and fractionated with 40–60% saturation of ammonium sulfate. Protein was dissolved in 6 M guanidine-HCl containing 0.5 M Tris-HCl buffer, pH 8.5 and 10 mM EDTA, then reduced with 10 mM dithiothreitol and carboxymethylated with 20 mM iodoacetic acid. Tn components were separated by reverse-phase HPLC using a column (6×150 mm) of Asahipak ODP-320 (Asahi Kasei Kogyo). The chromatogram was developed with a linear gradient concentration of acetonitrile in 0.1% trifluoroacetic acid.

Reduced and carboxymethylated TnI was digested with lysyl endopeptidase (Wako Pure Chemicals) or CNBr. Peptides were separated by reverse-phase HPLC using a column (4.6×150 mm) of ODS 80TM (Tosoh). The amino acid sequence of peptides was determined on an automated protein sequencer (Applied Biosystems Model 477A coupled with a 120A PTH-amino acid analyzer). The N-terminal acetyl group was removed by treatment with trifluoroacetic acid at 60°C for 45 min (*20*).

Total RNA of adult body wall muscle and heart muscle was prepared according to the acid guanidium thiocyanate-phenol-chloroform method (*21*), and mRNA was purified with an Oligotex dT-30 Super column (Japan Roche). The single-stranded cDNA was synthesized with a First-Strand cDNA Synthesis Kit (Pharmacia), using oligo-dT_{17} as a primer.

The cDNA of body wall muscle TnI was amplified by polymerase chain reaction (PCR) (*22*) using Ex *Taq* DNA polymerase (Takara). The redundant oligomer used for PCR was 5′-AAYGAYCARGARATHGARGA-3′, where R represents A and G, Y represents C and T, and H represents A, C, and T. This oligomer was designed based on the partial amino acid sequence of body wall muscle TnI, NDQEIED (residues 82–88). The oligo-dT adaptor, 5′-GGGATCCGAATTC$(T)_{17}$-3′, was used as another primer.

The 5′ end of cDNA was determined as follows. The *Eco*RI-ended double-stranded cDNA was synthesized from mRNA using a TimeSaver cDNA Synthesis Kit (Pharmacia). The *Eco*RI cassette (Takara) was ligated to both ends of the cDNA. The 5′ upstream region was amplified with PCR using cassette-specific primer C1, 5′-GTACATATTGTCGTTAGAACGCG-3′, and non-redundant reverse primer, 5′-ATAATGAGCCGTTACAGTTC-3′ (complementary to the nucleotide positions 523–542 in Fig. 1A).

The cDNA library of larvae was constructed in λgt10 using mRNA prepared from mid-tailbud stage embryos. The larval TnI cDNAs were also amplified by PCR using a cDNA library as a template. The 3′-half of the cDNAs was amplified with the λgt10-specific reverse primer and the same redundant oligomer used for body wall muscle TnI amplification. The primers used for amplification of the 5′-halves were the λgt10-specific forward primer and non-redundant reverse primer, 5′-CAAGGCGGATCTCGACATCT-3′ (complementary to the nucleotide positions 455–474 in Fig. 1B).

The genomic DNA was prepared from a single specimen of *H. roretzi* by the conventional phenol-chloroform method. To amplify the genomic DNAs encoding the C-terminus region of the isoform, the following primers were

(A); adult TnI

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                                                    -23  GCGAACAGAATCAACAACGCAAG  -1

ATGACGCATCAGCGCAAGCAAAATCTCAAATCTCTGATGCTTAACAAGGCCCGCGAAGATTTGAAACGCGAGGCGGAAGTTAAAGCTGAA  90
 M  T  H  Q  R  N  Q  N  L  K  S  L  M  L  N  K  A  R  E  D  L  K  R  E  A  E  V  K  A  E   30

GAGAAAAAGAAAATTCTCAACAGCAGAATCGAACCGCTCTCTAACCTTGGTGGCATGTCAGAGCAAGACCTGAAGGATCTTTGCAGAGAA 180
 E  K  K  K  I  L  N  S  R  I  E  P  L  S  N  L  G  G  M  S  E  Q  D  L  K  D  L  C  R  E   60

CTGCACGCGAAAATTGAAAAAGTCGACGAACAAAGATACGACATCGAGGTGAAAGTCAACAAGAATGACCAAGAGATAGAGGATCTTAAC 270
 L  H  A  K  I  E  K  V  D  E  Q  R  Y  D  I  E  V  K  V  N  K  N  D  Q  E  I  E  D  L  N   90

CAGAGGATATTCGATCTTCGCGGCAAGTTCAAACGACCTCCACTGCGCAGAGTCCGCATGTCAGCCGACCAAATGCTCCGCGCCCTCCTC 360
 Q  R  I  F  D  L  R  G  K  F  K  R  P  P  L  R  R  V  R  M  S  A  D  Q  M  L  R  A  L  L  120

GGATCCAAGCACAAAGTGTCTATGGATCTCCGATCAAGCCTAAAATCCGTCAAGAAGGAAGAAACCAAGAAAGATGAGGCTGAAGTAAAA 450
 G  S  K  H  K  V  S  M  D  L  R  S  S  L  K  S  V  K  K  E  E  T  K  K  D  E  A  E  V  K  150

GACTGGAGAGAGAGCGTGGAAGCTAAAACTGGTGGTATGGGAGAAATGAAGGCTGTGTTCGAGGGTCAGTGAGAACTGTAACGGCTCATT 540
 D  W  R  E  S  V  E  A  K  T  G  G  M  G  E  M  K  A  V  F  E  G  Q  *                      173

ATCACAACAACAATATTTCACATAGTTAATTACAAGATATTCTGTAAACCTACTAATTCTGTTATGGCAAAAGTTTCATATTTTCATATT 600

TCAACCTGACCGCTTGAGAACGCAATTTCTTTGGAACAACATATACCATGTTAACGATAGTATATCTACTTGTATAGAATACAACACGGT 720

AATCTCAn                                                                                    727
```

Fig. 1A

used: 5′-G(CT)ATGTC(AT)GC(CT)GACCAAATG-3′ (corresponding to the nucleotide positions 326-345 in Fig. 1, A and B), the adult/larval TnIs common forward primer, and 5′-ATAATGAGCCGTTACAGTTC-3′ (complementary to the positions 423-542 in Fig. 1A), the adult TnI specific reverse primer, or 5′-AGCATAGTGTATTCTCCAGG-3′ (complementary to the positions 478-497 in Fig. 1B), the larval TnIα specific forward primer, or 5′-TCCTGTAATCTTCATGAG-3′ (complementary to the positions 537-554 in Fig. 1B), and the larval TnIβ specific forward primer. These primers were also used for RT-PCR, to test the tissue-specific expression of TnI isoforms in adult and larvae.

All the amplified products were subcloned into the pCR II plasmid vector (TA-cloning kit; Invitrogen) or pUC18 for sequencing. The sequences of the products were determined by the dideoxy chain termination method with a Dye Primer Cycle Sequencing Kit (Applied Biosystems) and an automated DNA sequencer (Applied Biosystems 373A).

RESULTS AND DISCUSSION

The N-terminus of ascidian body wall muscle TnI was blocked, so the N-terminal acetyl group was removed by treatment with TFA. The amino acid sequence of the deacetylated TnI was Thr-His-Gln-Arg-Asn-Gln. The

(B); larval TnIs

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-81  TGAGCATTCAGTAACTACTACTTCCTTTACTAGCACATACGCTTACAAATACTAAATAAAAAAATGACAGAATCTACACGCAAA   -1
                  -60  .A.T.C................G..........---.......G.................   -1

 M  T  H  Q  R  K  L  Q  L  K  S  L  L  L  N  R  A  R  E  D  L  K  R  E  A  E  Q  K  A  E    30
ATGACCCACCAGCGCAAACTGCAGCTCAAGTCTCTTTTGCTCAACAGAGCCCGGGAGGATTTGAAAAGAGAAGCTGAACAAAAAGCAGAA   90
............................................G....................G.......AG...............   90
 .  .  .  .  .  .  .  .  .  .  .  .  .  .  S  .  .  .  .  .  .  .  .  .  R  .  .  .  .  .    30

 E  K  K  K  I  L  N  N  R  I  E  S  L  G  D  L  S  S  M  S  Q  Q  E  L  M  E  L  C  R  E    60
GAGAAAAAGAAGATTTTGAACAACAGAATCGAATCTCTCGGGGACTTATCTAGCATGTCGCAACAAGAACTGATGGAATTATGCCGAGAA  180
....................G.....................A......G....................T......G.....A......  180
 .  .  .  .  .  .  S  .  .  .  .  .  .  .  .  .  A  .  .  .  .  .  .  .  .  .  .  .  .  .    60

 L  H  A  K  T  D  K  V  D  D  E  R  F  D  I  E  L  K  V  K  K  N  D  Q  E  I  E  E  L  N    90
CTCCACGCAAAAACAGACAAAGTCGACGATGAAAGATTTGACATCGAATTAAAAGTGAAAAAGAACGACCAAGAGATCGAAGAACTAAAT  270
..........................................................................................  270
 .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .    90

 Q  K  I  F  E  L  R  G  K  F  K  R  P  P  L  R  R  V  R  M  S  A  D  Q  M  L  R  A  L  L   120
CAGAAAATCTTTGAACTCCGAGGTAAATTCAAACGCCCACCTCTGAGACGTGTCCGTATGTCTGCTGACCAAATGTTGCGCGCCCTCCTG  360
........A...............................................C.....................A....T......  360
 .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .   120

 G  S  K  H  K  V  T  M  D  L  R  S  N  L  K  T  V  K  E  T  K  K  *                        144
GGATCAAAACACAAAGTTACAATGGATCTTAGATCCAACCTCAAGACAGTCAAAGAGACAAAGAAATAGACGACCAACAGAGGATGCAAA  450
.....G........................C..............A..T........G............................T...  450
 .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  A  .  .  *                        144

                        ↓
TCGAAGATGTCGAGATCCGCCTTGAAACCTGGAGAATACACTATGCTATAATGGAATTTTCAATTATTTAAGAATGATACTTGCAATAAA  540
...G.....................GGGAAAAAT.C.A.TTAAG.TG.TGTT.TTTTGAGCT.TACC..GT.CCG.ATA..ACAA..CTC.  540

ACAGTACTACAG                                                                                552
TG.AG.T.....GATTTGAATACTGTATTTGATAAAGAATAAAAATATTGGAn                                       592
```

Fig. 1. **The cDNA and deduced amino acid sequences of *H. roretzi* TnI isoforms.** (A) Adult body wall muscle TnI cDNA sequence and the derived amino acid sequence. The broken-underlined peptides were determined directly by an automated protein sequencer. (B) Upper line: the cDNA and deduced amino acid sequences of larval striated muscle TnIα; lower line: the cDNA and deduced amino acid sequences of larval striated muscle TnIβ. Identical nucleotides and amino acids to those in TnIα are indicated by dots (.), and deletions are shown by bars (-). In the downstream region from the nucleotide position 475, indicated by an arrow (↓), no significant similarity is observed between TnIα and TnIβ. The stop codon is indicated by an asterisk (*). Possible polyadenylation signals are underlined.

amino acid sequence at other regions was determined using peptides obtained by lysyl endopeptidase or CNBr digestion, as shown in Fig. 1A. Based on these partial amino acid sequences, the cDNA of the adult body wall muscle TnI was amplified by PCR. The complete cDNA sequence of 750 nucleotides was constructed from two overlapping fragments (Fig. 1A). No difference was observed in the sequences at the overlapping region. The open reading frame was composed of 522 nucleotides and encodes a protein of 173 amino acid residues, including the initial Met, and the sequence was identical to that determined by peptide sequencing. The mature TnI was generated by removal of the initiating Met, followed by acetylation. In the cDNA sequence, a typical polyadenylation signal (AATAAA) was present at nucleotide position 707.

In the larval cDNA library, two cDNAs encoding the larval TnIs, TnIα, and TnIβ, were detected. The cDNA of the larval TnIα was composed of 636 nucleotides and that of the TnIβ was composed of 652 nucleotides, as shown in Fig. 1B. There was no sequence discrepancy in the overlapping regions. The open reading frames of both isoforms are 432 nucleotides and encode a protein of 142 amino acid residues, including the initial Met (Fig. 1B). These TnIs are the shortest of all known TnIs. Between the open reading frames of larval TnIα and TnIβ, there are only 19 nucleotide substitutions (95.6% identity), producing 5 amino acid differences (96.5% identity). However, in the downstream region from the nucleotide position 475 (indicated by arrow in Fig. 1B), there is no significant similarity between two isoforms. Though both isoforms possess a typical polyade-

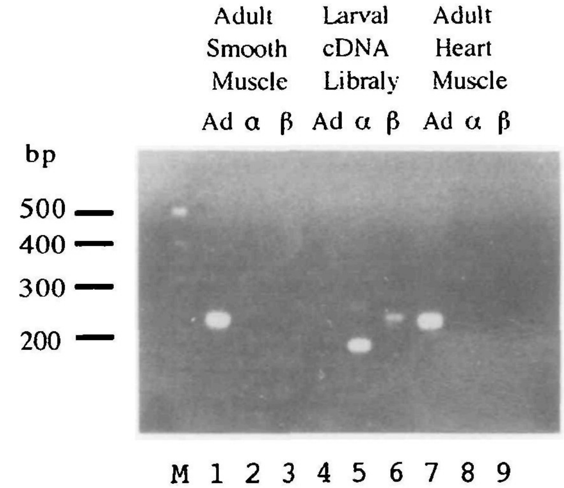

Fig. 2. **Tissue-specific expression of TnI isoforms.** Lane M, molecular markers. Lanes 1-3, the cDNA prepared from adult body wall muscle was used as the PCR template. Lanes 4-6, the tailbud embryo cDNA library in λgt10 was used. Lanes 7-9, the cDNA prepared from adult heart muscle was used. Lanes 1, 4, and 7 (indicated by Ad), the adult TnI specific reverse primer was used for PCR. The amplification of a 217-bp product was expected. Lanes 2, 5, and 8 (indicated by α), the larval TnIα specific forward primer was used, and a 172-bp product was expected. Lanes 3, 6, and 9 (indicated by β), the larval TnIβ specific forward primer was used, and a 229-bp product was expected. It was confirmed by sequencing that the larger product (245 bp) in lane 5 contains an intron. As the forward primer, the adult/larval TnIs common primer was used.

```
                10        20        30        40        50        60        70        80
                                                      *    **  *         *       **  *
Ad TnI                                            MTHQRKQNLKSLMLNKAREDLKREAEVKAEEKKKILNSR
La TnI                                            MTHQRKLQLKSLLLSRAREDLKREREQKAEEKKKILSNR
Qu fTnI                                  MSDEEKKRRAATARRQHLKSAMLQLAVTEIEKEAAAKEVEKQNYLAEH
Hu sTnI                                  MPEVERKPKITASRKLLLKSLMLAKAKECWEQEHEEREAEKVRYLAER
Mo cTnI  MADESSDAAGEPQPAPAPVRRRSSANYRAYATEPHAKKKSKISASRKLQLKTLMLQIAKQEMEREAEERRGEKGRVLRTR

                90       100       110       120       130       140       150       160
            *          *   **  ***    **  * * * *      *   *      ******** ***** *** * **
Ad TnI   IEPLSNLGGMSEQDLKDLCRELHAKIEKVDEQRYDIEVKVNKNDQEIEDLNQRIFDLRGKFKRPPLRRVRMSADQMLRAL
La TnI   IESLGDLASMSQQELMELCRELHAKTDKVDDERFDIELKVKKNDQEIEELNQKIFELRGKFKRPPLRRVRMSADQMLRAL
Qu fTnI  CPPL-SLPG-SMQELQELCKKLHAKIDSVDEERYDTEVKLQKTNKELEDLSQKLFDLRGKFKRPPLRRVRMSADAMLRAL
Hu sTnI  IPTL-QTRGLSLSALQDLCRELHAKVEVVDEERYDIEAKCLHNTREIKDLKLKVMDLRGKFKRPPLRRVRVSADAMLRAL
Mo cTnI  CQPL-ELDGLGFEELQDLCRQLHARVDKVDEERYDVEAKVTKNITEIADLTQKIYDLRGKFKRPTLRRVRISADAMMQAL

                170       180       190       200       210       220
         **       ***  ** **     *
Ad TnI   LGSKHKVSMDLRSSLKSVKKEETKKDEA--EVKDWRESVEAKTGGMGEMKAVFEGQ
La TnI   LGSKHKVTMDLRSNLKTVK--EAKK
Qu fTnI  LGSKHKVNMDLRANLKQVKKEDTEKEKDLRDVGDWRKNIEEKSGMEGRKKMFEAGES
Hu sTnI  LGSKHKVSMDLRANLKSVKKEDTEKERPV-EVGDWRKNVEAMSGMEGRKKMFDAANAPTSQ
Mo cTnI  LGTRAKESLDLRAHLKQVKKEDIEKENR--EVGDWRKNIDALSGMEGRKKKFEG
                               ↑  ↑ ↑
                               La VeAd
```

Fig. 3. **Alignment of the amino acid sequences of *H. roretzi* TnIs and those of vertebrates.** The alignment of amino acid sequences was mainly done with GeneWorks release 2.5 (IntelliGenetics). Ad TnI and La TnI, *H. roretzi* adult-type and larval-type TnI; Qu fTnI, quail fast skeletal TnI (*21*); Hu sTnI, human slow skeletal TnI (*22*); Mo cTnI, mouse cardiac TnI (*23*). The actin/TnC-binding domain is underlined. The residues conserved in all chains are indicated by asterisks (*) and gaps are shown by bars (-). The position of the last intron is indicated by an arrow (↑); La, the intron of *H. roretzi* larval-type TnI gene; Ad, the intron of *H. roretzi* adult-type TnI; Ve, the intron of three vertebrate TnI isoforms.

nylation signal at nucleotide positions 535 and 578, it is suggested that insertion or deletion occurred following gene duplication.

In order to determine the tissue-specific expression of these TnI isoforms, RT-PCR was performed using isoform-specific primers. As shown in Fig. 2, the larval TnIα and TnIβ cDNA were amplified only when using a tailbud embryo cDNA library as the template. In contrast, the adult body wall muscle TnI was detected in the adult body wall muscle cDNA and not in the embryo cDNA library. The expression of ascidian TnI isoforms seems to be regulated specifically in larval and adult tissues, as in the cases of TnC (*11*) and TnT isoforms (*12*). In addition, we also conducted RT-PCR on heart muscle mRNA. In the heart muscle, only the adult-type TnI isoform seems to be expressed. Indeed, the open reading frame sequences of TnI cDNA from the body wall muscle and heart muscle were identical (data not shown).

The amino acid sequences of the ascidian adult and larval TnIs were aligned with those of the vertebrate TnI isoforms, quail fTnI (*23*), human sTnI (*24*), and mouse cTnI (*25*), as shown in Fig. 3, and the identity among these sequences is listed in Table I. The ascidian adult and larval TnIs show 77-79% identity to each other, but show lower identity to vertebrate TnIs (52-59% identity). Within the actin/TnC-binding domain, all known mammal and avian TnIs possess the sequence motif, RPXLR (Fig. 3, underlined). In this domain, invertebrate (*15-18*) and fish (*Clupea harengus*) (*26*) TnIs contain the KPXLK motif, and cTnI of the frog *Xenopus laevis* (*27*), contains the KPXLR motif. Hodgson *et al.* (*26*) have proposed that the KPXLK motif is an ancestor motif, and during the evolution of vertebrates, it evolved to the RPXLR motif in the tetrapod lineage after the tetrapod-teleost divergence. However, all the ascidian TnI isoforms possess the RPXLR motif, so the Lys to Arg substitutions in the actin/TnC-

TABLE I. **The identity (%) among sequences of *Halocynthia roretzi* and vertebrate TnIs.**

	Ad TnI	La TnI	Qu	Hu	Mo
Ad TnI		77.5	57.3	59.1	53.0
La TnIα			56.4	56.7	52.3
Qu fTnI				56.1	56.7
Hu sTnI					61.5
Mo cTnI					

(A); adult TnI

```
                                                    GCATGTCAGCCGACCAAATGCTCCGCGCCCTCCTC  360
GGATCCAAGCACAAAGTGTCTATGGATCTCCGATCAAGCCTAAAATCCGTCAAGAAGGAAGAAACCAAGAAAGATGAG
gtaatgtactccgtgtgtgaaatgggtgtgtattattgtttttttaatccgatgctagcatatacatattccaatacaatgtttttttttaa  (90)
gtgagtagcagaaaatgcgtcataactcgatgtctaggcctgatttgattggaaatatctaacagagattgcgtcactgttagccgtttt (180)
taacaactagaaatcggataattattatatagattctatcattataacaggacagggccgaaaatagtcccactaataatcattatttac (270)
atgtgtactgctttcatatagttaatatgtgtttattctcagctatttgtcattcatgtaatgcacgagaactacagggtgaatactgta (360)
cagaatccacctatcgagtattcctcgccaataaaatatgtctacgcatcattttactgataacaactgatccagcaaaataacaagatt (450)
ttaacatgggtttatgtttattcttcaagcaaagatcttggtattatcagttatcttttctaactttcag                    (520)
                                                                    GCTGAAGTAAAA  450
GACTGGAGAGAGAGCGTGGAAGCTAAAACTGGTGGTATGGGAGAAATGAAGGCTGTGTTCGAGGGTCAGTGAGAACTGTAACGGCTCATT  540
AT                                                                                  542
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(B); larval TnIs

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                                                    GTATGTCTGCTGACCAAATGTTGCGCGCCCTCCTG  360
                                                    .C.................A.....T......  360

GGATCAAAACACAAAGTTACAATGGATCTTAGATCCAACCTCAAGACAGTCAAAGAG
.....G.....................C.............A..T.........

gtaacatccgcactaaccttgtgttatgtattatttgtgtttgcaatgtgtttaaactttctattttcacag                  (73)
.................g...a...at.....g.........---.......c..............t....                  (70)

                                                                    ACAAAGAAATAGACGACCAACAGAGGATGCAAA  450
                                                                    G.............................T...  450

TCGAAGATGTCGAGATCCGCCTTGAAACCTGGAGAATACACTATGCT                                           497
...G.....................GGGAAAAAT.C.A.TTAAG.TG.TGTTTTTTTGAGCTATACCTTGTACCGTATAACACAAAACTCA  540

TGAAGATTACAGGA                                                                            554
```

Fig. 4. **The partial genomic sequences of *H. roretzi* TnI isoforms.** (A) The partial sequence of adult body wall muscle TnI gene. (B) Upper line: the partial genomic sequence of larval striated muscle TnIα; lower line: larval striated muscle TnIβ. Identical nucleotides to those in TnIα are indicated by dots (.), and deletions are shown by bars (-). The exons are indicated by capital letters, and given the same numbers as in the cDNAs (see Fig. 1). The sequences of introns are shown by small letters, and numbers of nucleotides are parenthesized. The primer sites used for PCR amplification are underlined.

TABLE II. **The tissue-specific expression pattern of the three troponin components of *Halocynthia roretzi*.**

	Larval tail muscle (striated muscle)	Adult body wall muscle (smooth muscle)	Adult heart muscle (striated muscle)
TnC*	larval type TnC	adult type TnC	adult type TnC
TnI	larval type TnIα larval type TnIβ	adult type TnI	adult type TnI
TnT	larval type TnT	adult type TnT	(not determined)

*Two isoforms of TnC are produced from a single gene through alternative splicing.

binding domain might not have occurred in one direction.

The ascidian adult TnI is similar to vertebrate fTnI and sTnI in length. On the other hand, the larval TnIs are shorter than other TnIs, having been truncated by *ca.* 30 amino acid residues at the C-termini. A C-terminal truncated mutant of chicken fTnI (*28*), TnI_{1-156} (lacking the C-terminal 26 amino acids), shows slightly impaired Ca^{2+} regulation of the actomyosin ATPase activity (*ca.* 80%), but retains full inhibitory capacity and the ability to form a Tn complex. The ascidian larval TnIs may possess similar functional features.

In the genes of quail fTnI (*23*), human sTnI (*29*), and mouse cTnI (*30*), the 7th intron is located at the same position (Fig. 3, indicated by arrow). As regards the amino acid alignment, ascidian larval TnIs C-terminal truncation occurs at the same point, as if the truncation were caused by the absence of the following exon. To investigate the intron localization of the ascidian TnIs genes, each C-terminus-encoding region was amplified by PCR. The ascidian adult TnI, larval TnIα, and TnIβ genes possess an intron of 520, 73, and 70 bp length at the each C-terminus encoding region, respectively, as shown in Fig. 4. Each ascidian intron has phase 0 (between two triplet codons), as in vertebrates, thought the positions are slightly different. When compared with vertebrate TnI genes, the intron of the ascidian adult TnI gene is located at 6 nucleotides downstream, and the introns of the two larval TnIs are positioned at 9 nucleotides upstream (Fig. 3, indicated by arrow). The last exons of the larval TnI genes encode 3 amino acids. In addition, no intron is observed near nucleotide position 475 of the larval TnI genes. Thus, there should be no intron involvement in the insertion or deletion at nucleotide position 475, if it exists.

The expression patterns of the three ascidian Tn components are summarized in Table II. Between larval striated muscle and adult body wall smooth muscle, different isoforms of all three Tn components are expressed. In heart muscle, though it is a striated muscle, the same isoforms of TnC and TnI are present as in body wall smooth muscle. As for TnT of heart muscle, there has been no report yet. The nature of the functional difference between larval and adult Tn complexes remains to be established.

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REFERENCES

1. Ohtsuki, I., Maruyama, K., and Ebashi, S. (1986) Regulatory and cytoskeletal proteins of vertebrate skeletal muscle. *Adv. Protein Chem.* **38**, 1-67
2. Farah, C.S. and Reinach, F.C. (1995) The troponin complex and regulation of muscle contraction. *FASEB J.* **9**, 755-767
3. Endo, T. and Obinata, T. (1981) Troponin and its components from ascidian smooth muscle. *J. Biochem.* **89**, 1599-1608
4. Ojima, T. and Nishita, K. (1986) Isolation of troponins from striated and smooth adductor muscle of *Akazara* scallop. *J. Biochem.* **100**, 821-824
5. Myers, C.D., Goh, P.-Y., Allen, T.S., Bucher, E.A., and 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
6. Wada, H. and Satoh, N. (1994) Details of the evolutionary history from invertebrates to vertebrates, as deduced from the sequences of 18S rDNA. *Proc. Natl. Acad. Sci. USA* **91**, 1801-1804
7. Satoh, N. (1994) *Developmental Biology of Ascidian*, Cambridge University Press, New York
8. Kalk, M. (1970) The organization of a tunicate heart. *Tissue Cell* **2**, 99-118
9. Shinohara, Y. and Konishi, K. (1982) Ultrastructure of the body-wall muscle of the ascidian *Halocynthia roretzi*: Smooth muscle cell with multiple nuclei. *J. Exp. Zool.* **221**, 137-142
10. Terakado, K. and Obinata, T. (1987) Structure of multinucleated smooth muscle cells of the ascidian *Halocynthia roretzi*. *Cell Tissue Res.* **247**, 85-94
11. Yuasa, H.J., Sato, S., Yamamoto, H., and Takagi, T. (1997) The structure of the ascidian, *Halocynthia roretzi*, troponin C gene. *J. Biochem.* **121**, 671-676
12. Endo, T., Matsumoto, K., Hama, T., Ohtsuka, Y., Katsura, G., and 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
13. Koppe, R.I., Hallauer, P.L., Karpati, G., and Hasting, K.E.M. (1989) cDNA clone and expression analysis of rodent fast and slow skeletal muscle troponin I mRNAs. *J. Biol. Chem.* **264**, 14327-14333
14. Murphy, A.M., Jones, L., Sims, H.F., and Strauss, A.W. (1991) Molecular cloning of rat cardiac troponin I and analysis of troponin I isoform expression in developing rat heart. *Biochemistry* **30**, 707-712
15. Kobayashi, T., Takagi, T., Konishi, T., and Cox, J.A. (1989) Amino acid sequence of crayfish troponin I. *J. Biol. Chem.* **264**, 1551-1557
16. Barbas, J.A., Galceran, J., Krah-Jentgens, I., de la Pompa, J.L., Canal, I., Pong, O., and Ferrús, A. (1991) Troponin I is encoded in the haplolethal region of the *Shaker* gene complex of *Drosophila*. *Genes Dev.* **5**, 132-140
17. Beall, C.J. and Fyrberg, E. (1991) Muscle abnormalities in *Drosophila melanogaster heldup* mutants are caused by missing or aberrant troponin-I isoforms. *J. Cell Biol.* **114**, 941-951
18. Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J., Coulson, A., Craxton, M., Dear, S., Du, Z., Durbin, R., Favello, A., Fulton, L., Gardner, A., Green, P., Hawkins, T., Hillier, L., Jier, M., Johnston, L., Jones, M., Kershaw, J., Kirsten, J., Laister, N., Latreille, P., Lightning, J., Lloyd, C., McMurray, A., Mortimore, B., O'Callaghan, M., Parsons, J., Percy, C., Rifken, L., Roopra, A., Saunders, D., Shownkeen, R., Smaldon, N., Smith, A., Sonnhammer, E., Staden, R., Sulston, J., Thierry-Mieg, J., Thomas, K., Vaudin, M., Vaughan, K., Waterston, R., Watson, A., Weinstock, L., Wilkinson-Sproat, J., and Wohldman, P. (1994) 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*. *Nature* **368**, 32-38
19. Takagi, T. and Konishi, K. (1983) Amino acid sequence of troponin C obtained from ascidian (*Halocynthia roretzi*) body wall muscle. *J. Biochem.* **94**, 1753-1760
20. Wellner, D., Panneerselvam, C., and Horecker, B.L. (1990) Sequencing of peptides and proteins with blocked N-terminal amino acids: N-acetylserine or N-acetylthreonine. *Proc. Natl. Acad. Sci. USA* **87**, 1947-1949
21. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159

22. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491
23. Baldwin, A.S., Jr., Kittler, E.L.W., and Emerson, C.P., Jr. (1985) Structure, evolution, and regulation of a fast skeletal muscle troponin I gene. *Proc. Natl. Acad. Sci. USA* **82**, 8080-8084
24. Wade, R., Eddy, R., Shows, T.B., and Kedes, L. (1990) cDNA sequence, tissue-specific expression, and chromosomal mapping of the human slow-twitch skeletal muscle isoform of troponin I. *Genomics* **7**, 346-357
25. Guo, X., Wattanapermpool, J., Palmiter, K.A., Murphy, A.M., and Solaro, R.J. (1994) Mutagenesis of cardiac troponin I. Role of the unique NH_2-terminal peptide in myofilament activation. *J. Biol. Chem.* **269**, 15210-15216
26. Hodgson, P.A., Leaver, M.J., George, S.G., MacLean, D.W., and Hastings, K.E.M. (1996) Molecular cloning of troponin I expressed in fast white muscle of a teleost fish, the atlantic herring (*Culpea harengus* L.). *Biochim. Biophys. Acta* **1306**, 142-146
27. Drysdale, T.A., Tonissen, K.F., Patterson, K.D., Crawford, M.J., and Krieg, P.A. (1994) Cardiac troponin I is a heart-specific marker in the *Xenopus embryo*: expression during abnormal heart morphogenesis. *Dev. Biol.* **165**, 432-441
28. Farah, C.S., Miyamoto, C.A., Ramos, C.H.I., Da Silva, A.C.R., Quaggio, R.B., Fujimori, K., Smillie, L.B., and Reinach, F.C. (1994) Structural and regulatory functions of the NH_2- and COOH-terminal regions of skeletal muscle troponin I. *J. Biol. Chem.* **269**, 5230-5240
29. Corin, S.J., Juhasz, O., Zhu, L., Conley, P., Kedes, L., and Wade, R. (1994) Structure and expression of the human slow twitch skeletal muscle troponin I gene. *J. Biol. Chem.* **269**, 10651-10659
30. Ausoni, S., Campione, M., Picard, A., Moretti, P., Vitadello, M., De Nardi, C., and Schiaffino, S. (1994) Structure and regulation of the mouse cardiac troponin I gene. *J. Biol. Chem.* **269**, 339-346