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The Structural Organization of Ascidian *Halocynthia roretzi* Troponin I Genes[1]

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The organization of troponin I (TnI) genes from the ascidian *Halocynthia roretzi* have been determined. *Halocynthia* possesses roughly two types of TnI isoforms. One type is a single-copied adult *TnI* (*adTnI*) gene, which contains eight exons and seven introns. *adTnI* expresses two isoforms, the shorter body wall muscle *TnI* and the longer cardiac *TnI*, through alternative splicing. The mRNAs of these *TnI* isoforms may undergo trans-splicing of the 5′-leader sequences, like the *TnI* mRNA of another ascidian species, *Ciona intestinalis*. The other type comprises multi-copied larval *TnI* (*laTnI*) genes. *Halocynthia* has at least three *laTnIs* (α, β, and γ), which are composed of five exons and four introns, and two of them (α and γ) are clustered in tandem. All *laTnIs* have B- and M-regions within their 5′-upstream regions, which have been discovered to be the regulatory elements of *Halocynthia* larval actin genes. The expression of *Halocynthia laTnIs* and larval actins may be regulated in the same manner. It is known that *Ciona* does not possess a larva-specific TnI isoform. The phylogenetic tree of ascidian TnIs suggests that *laTnIs* might have only been generated within the Pleurogona lineage after Enterogona/Pleurogona divergence, and this scenario well agrees with the absence of *laTnIs* in *Ciona*.

Key words: ascidian (*Halocynthia roretzi*), genomic structures, molecular evolution, *trans*-splicing, troponin I.

Troponin I (TnI) is one of the three components of the troponin complex, which is a main regulator of striated muscle contraction. In mammals and birds, three TnI isoforms are known; fast-skeletal TnI (fTnI), slow-skeletal TnI (sTnI), and cardiac TnI (cTnI). They are encoded by distinct genes, and are specifically expressed in fast skeletal, slow skeletal, and cardiac muscles, respectively. As for amphibians, only the sequence of *Xenopus* cTnI has been determined (*1*). However, recent EST analysis showed that *Xenopus* also has fTnI and sTnI (*2*), suggesting that amphibians also possess three TnI isoforms, which may be expressed in the same manner as in mammals and birds. The TnI organization in fish has not been clearly revealed, but at least three distinct TnI isoforms are expressed in skeletal muscle (*3*). On the other hand, smooth muscle contraction of vertebrates is regulated by calmodulin-dependent myosin light chain kinase, and no troponin expression is observed.

Ascidians, belonging to the Urochordata, have three types of muscular tissues: larval tail muscle (striated muscle), adult cardiac muscle (striated muscle), and adult body wall muscle (smooth muscle). The contraction of all of them seems to be regulated by the troponin complex, and specially the last is a rare case of troponin expression in smooth muscle. The ascidian *Halocynthia reretzi* has roughly two types of TnI isoforms (*4*). One is the larval TnIs (laTnIs), which are expressed in larval tail muscle, and there are at least two laTnI isoforms (laTnIα and laTnIβ). The other is the adult TnI (adTnI) expressed in adult tissues, which we previously reported as the body wall muscle TnI (bwTnI), and the cardiac TnI (cTnI) seemed to be identical (although this is corrected in this study). On the other hand, the TnI organization of another ascidian species, *Ciona intestinalis*, has also been reported (*5*). *Ciona* has at least two TnI isoforms, the shorter bwTnI and the longer cTnI, which are expressed from an identical gene through alternative splicing. In addition, *Ciona* seems to possess only a single *TnI* gene, and none corresponding to *Halocynthia laTnI* genes were observed (Dr K.E.M. Hastings of McGill University, personal correspondence).

In this study, we determined the structures of *Halocynthia TnI* genes, and revealed their accurate expression patterns. We also isolated *TnI* cDNAs from another two ascidian species, *Polyandrocarpa misakiensis* and *Chelyosoma siboja*, and discussed the evolution of ascidian TnIs.

MATERIALS AND METHODS

Reconfirmation of the 5′-Untranslated Region of the Body

[1] The determined nucleotide sequences have been submitted to DDBJ under accession numbers AB001685 (*Halocynthia roretzi* body-wall muscle *TnI* cDNA; corrected), AB077758 (*H. roretzi* cardiac *TnI* cDNA), AB077763 (*H. roretzi* adult *TnI* gene), AB077761 (*H. roretzi* larval *TnIα* and larval *TnIγ* genes), AB077762 (*H. roretzi* larval *TnIβ* gene), AB077759 (*Chelyosoma siboja TnI* cDNA), and AB077760 (*Polyandrocarpa misakiensis TnI* partial cDNA).

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Wall Muscle and the Cardiac TnI cDNAs—Single-stranded cDNAs of the body wall and cardiac muscles were prepared as previously described (*4*). The 5′-untranslated region of *bwTnI* cDNA was amplified by 5′-RACE (*6*). A poly-C tail was added to the 3′-end of cDNAs with terminal deoxynucleotidyl transferase (Takara), and PCR was performed with primer sets, the oligo-dG adaptor, 5′-GGGATCCGAATTCAAGCTTG$_{17}$-3′, and a reverse primer R1, 5′-ATAATGAGCCGTTACAGTTC-3′ (complementary to nucleotide positions 784 to 803 in Fig. 1). The *cTnI* cDNA was amplified with the following primer set, the forward primer F1, 5′-GTGTTAATTAACATCTACTG-3′ (corresponding to nucleotide positions –42 to –23 in Fig. 1), and the reverse primer R1.

Isolation of Genomic Clones of Ascidian TnIs—A genomic library was constructed as previously described (7), and was screened using the open reading frames of *bwTnI* and *laTnIα* cDNAs as probes, which were labeled with DIG-DNA Labeling Mixture (Roche Diagnostics). Hybridization and washing were carried out according to the manufacturer's protocols, and positive clones were detected with a DIG Luminescent Detection Kit (Roche Diagnostics).

Southern Hybridization—Genomic DNA was digested with restriction enzymes, separated on 0.7% agarose gels, and then transferred to nylon membranes. A 180 bp fragment of *Halocynthia bwTnI* cDNA (corresponding to exon V, see Fig. 3a) was labeled with DIG-DNA Labeling Mixture (Roche Diagnostics). Hybridization and washing were performed basically according to the manufacturer's instructions (Roche Diagnostics), and the *adTnI* gene was detected using a DIG Luminescent Detection Kit (Roche Diagnostics).

Cloning of TnI cDNAs from Two Other Ascidian Species—A lambda gt11 cDNA library of colonial ascidian *Polyandrocarpa misakiensis* was constructed as previously described (*8*), and was screened with DIG-labeled *Halocynthia bwTnI* cDNA. A solitary ascidian *Chelyosoma siboja* was collected at the Asamushi Marine Biological Station, Tohoku University, Aomori. Total RNA was prepared from the muscular tissue of branchial or atrial siphons by the acid guanidinium thiocyanate method (*9*), and mRNA was purified with an Oligotex dT-30 Super (Roche Diagnostics). The single-stranded cDNA was synthesized using a First-Strand cDNA Synthesis Kit (Pharmacia). The 3′-half of *Chelyosoma TnI* cDNA was amplified by PCR using Ex *Taq* DNA polymerase (Takara). The redundant oligomer used for the amplification was 5′-GGNAARTTYAARMGNCCNCC-3′, it was designed based on the amino acid sequence GKFKRPP, which is the well conserved actin/troponin C binding site of chordate TnIs (*10*). Oligo-dT adaptor 5′-GGGATCCGAATTCT$_{17}$-3′ was used as another primer. The 5′-untranslated region of *Chelyosoma TnI* cDNA was amplified by 5′-RACE as in the case of *Halocynthia*, except that a non-redundant reverse primer, 5′-GAAGCCACATGATCATTC-3′, was used.

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

RESULTS AND DISCUSSION

N-Terminal Region of the Body Wall Muscle TnI—Using the 5′-RACE method, we can amplify the *Halocynthia bwTnI* cDNA containing the complete 5′-untranslated re-

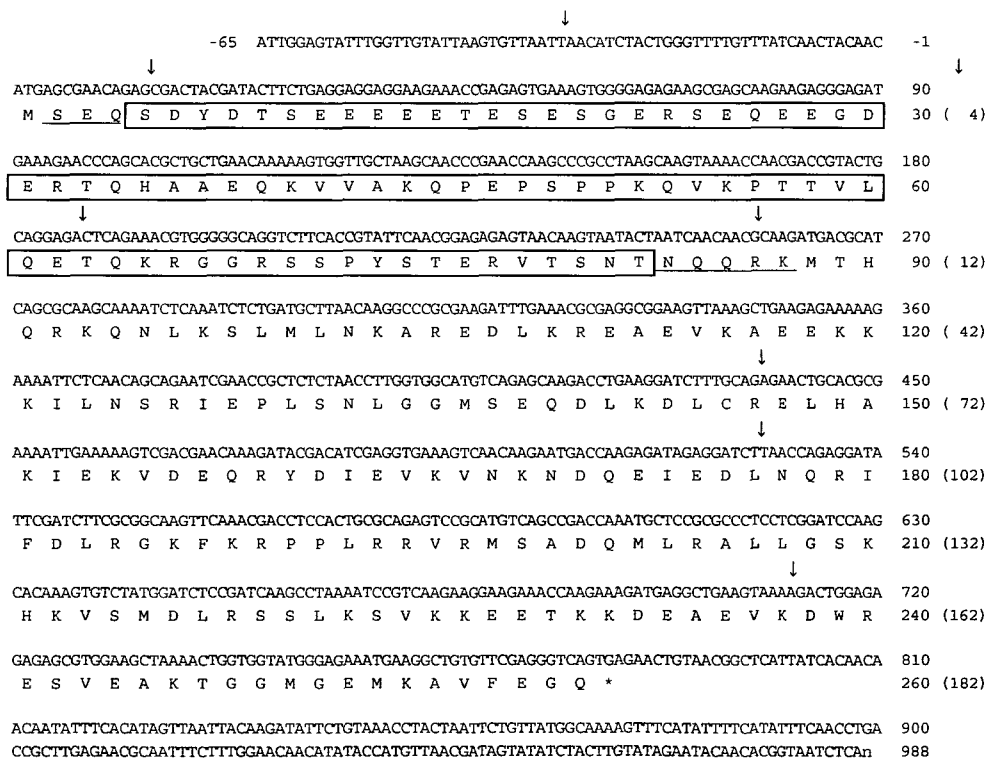

Fig. 1. **cDNA and deduced amino acid sequences of *Halocynthia roretzi* adult TnIs (bwTnI/cTnI).** The downward arrows (↓) indicate the positions of introns in the *Halocynthia* adult *TnI* gene. The amino acid residues of the cTnI specific region are boxed. The underlined residues (SEQNQQRK) were determined directly with an automated protein sequencer. The amino acid numbers in parenthesis are those of bwTnI. The determined nucleotide sequences have been submitted to the DDBJ under accession numbers AB001685 (*H. roretzi bwTnI* cDNA; corrected) and AB077758 (*H. roretzi cTnI* cDNA).

gion. This cDNA encodes 182 amino acid residues including the N-terminal nine residues which were missing in a previous study (*4*). Furthermore, with the carefully prepared TnI, we succeeded in directly detecting the N-terminal animo acids of Ser-Glu-Gln-Asn-Gln-Gln-Arg-Lys (residues 2 to 9) with a protein sequencer. We previously reported that the cDNA of *Halocynthia bwTnI* was constructed from 750 nucleotides, and encoded a protein of 173 amino acid residues (*4*). However, in this study, it is clear that *Halocynthia bwTnI* cDNA is constructed from 819 nucleotides, and encodes a protein of 182 amino acid residues. This is the same number of residues as in another ascidian species, *Ciona intestinalis bwTnI* (*5*). The mistake was unfortunately due to following two problems: (i) TnI was easily cleaved following the Arg-Lys sequence (residues 8 and 9) by internal proteases, this position being just before Met-10. (ii) The cDNA isolated before was truncated at nucleotide position 5, and so we mistook Met-10 as the initial Met.

As for the *Halocynthia* larval TnIs, we did not directly detect the N-terminal residues, but according to the alignment with other ascidian TnIs, the correct initial Met may be seven residues upstream from Met (positioned at 12 in Fig. 5a), which we previously reported as the initial Met (Ref. *4*, the sequence data, which have been submitted to DDBJ under accession numbers AB001686 and AB001687, were corrected).

The Cardiac TnI Isoform—In the previous study (*4*), we performed PCR, showing the same TnI isoform was expressed in the body wall and cardiac muscles. The primers employed were 5′-GCGAACAGAATCAACAACGC-3′ (corresponding to nucleotide positions 5 to 12 and 247 to 258 in Fig. 1) and R1, because we misjudged the former primer was within the 5′-untranslated region. When PCR is performed with primers F1 and R1, the former primer lies in the "real" 5′-noncoding region, and it is clear that the larger TnI isoform is expressed in the cardiac muscle (Fig. 2a). The *Halocynthia cTnI* cDNA comprises 1,053 nucleotides and encodes 260 amino acids, identical with *bwTnI* except that *cTnI* contains a 234 nucleotide insertion (encoding 78 amino acid residues, boxed sequences in Fig. 1) following the CAG codon of Gln-4.

The bwTnIs (namely bwTnI/cTnI common region) of *Halocynthia* and *Ciona* are composed of the same number of residues, and the identity between them is more than 75%. However, their cardiac-specific regions are different in length; that of *Ciona* is encoded by two exons, whereas the *Halocynthia* counterpart is encoded by three, and they show rather low homology except that their N-terminal parts are Glu-rich (Fig. 2b). The Pro-rich/hydrophobic/basic and AxExH motifs indicated by MacLean *et al.* (*5*) within the *Ciona* cardiac-specific region are not observed in the *Halocynthia* sequence. It is known that the vertebrate cTnI is phosporylated by protein kinase A (PKA), but there may be no PKA phosporylation site within ascidian cardiac-specific regions. As for ascidian cTnIs, only the somewhat extended and rather acidic N-terminus may be important.

Genomic Organization of the Adult TnI Gene and mRNA 5′-Leader trans-Splicing—We tried to amplify the *Halocynthia adTnI* gene by PCR with primers F1 and R1, but no product was amplified. Then we screened the genomic library with DIG-labeled *bwTnI* cDNA, and isolated some clones containing the *adTnI* gene. One of them contained the whole *adTnI* gene except the first 24 nucleotides of cDNAs (*bwTnI/cTnI* cDNAs), and then it was sequenced. The exon/intron organization of the *Halocynthia adTnI* gene is shown in Fig. 3a. Within the coding region, the nucleotide sequences of exons are exactly identical with cDNA, although two nucleotide substitutions are observed within the 3′-untranslated region. These are presumably caused by individual differences or allelic polymorphism. The *Halocynthia adTnI* gene is divided into eight exons by seven introns, and all introns conform to the GT-AG rule. Exons II, III, and IV must be cardiac TnI-specific ones. Genomic Southern analysis of *Eco*RI- or *Sal*I-digested DNA gave a single band (about 14 and 12 kbp, respectively; Fig. 3b). This suggests that the *Halocynthia adTnI* gene is a single-copy one, thus two isoforms (bwTnI and cTnI) must be expressed from the single gene through alternative splicing, like in *Ciona*.

To search for the first 24 nucleotides of the cDNA, we isolated several genomic clones and sequenced more than 10 kbp of genomic DNA upstream of the initiation ATG. How-

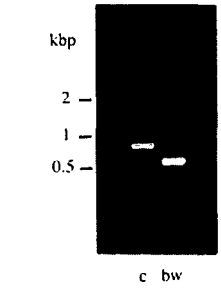

Fig. 2. **The longer TnI isoform (cTnI) is expressed in cardiac muscle.** (a) RT-PCR of two adTnI isoforms. The cDNAs prepared from cardiac (lane c) and body wall (lane bw) muscles were used as the template, respectively. The primers used for amplification were F1 and R1. The product derived from bwTnI cDNA is expected to be 845 bp-long, and cTnI is 611 bp. Left, size markers in kbp based on molecular markers. (b) Alignment of *Halocynthia* (Hr) and *Ciona* (Ci) cTnI specific regions. The downward arrows indicate the positions of introns in each gene. The identical residues are shown by asterisks (*), and gaps are inserted for maximal similarity, as shown by dashes (–).

b.

```
Hr   SDYDTSEEEEETESESGERSEQE-EGDERTQHAAEQKVVAKQPEPSPPKQVKPTTVLQETQKRGGRSSPYSTERVTSNT
          *  *    * *    **** *              *  ** *      *     *
Ci        ESSESEISTSEDESEQEDE--IEVPPVKKEPIVKEQPKP----AVESHNVEEK
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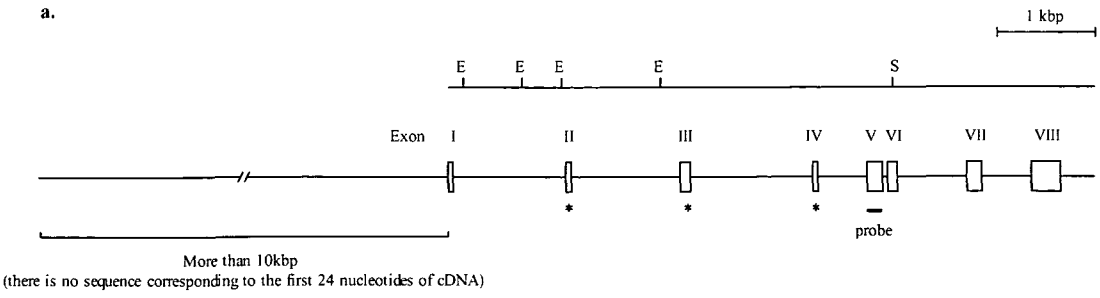

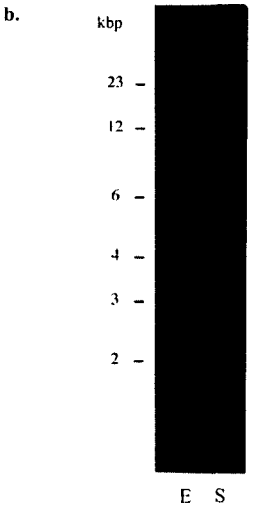

c.

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Hr TnI      ATTGGAGTATTTGGTTGTATTAAGTGTTAATTAACATCT
Ci TnI      ...C---.....A-----A....CAACCGGTATCAATT
Sp Ldh      G.......................AATTTGATAATTAAA
Hr 29k      ...........A..........ATTAAGCATTAAGTC
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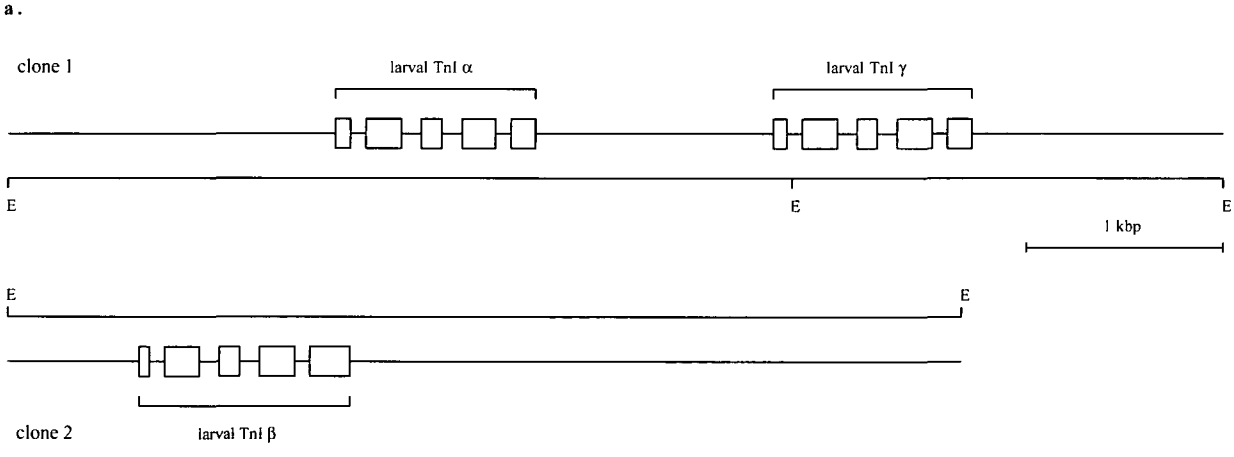
Fig. 3. ***H. roretzi* adult TnI gene.** (a) Structure of the Halocynthia adult TnI gene. Exons are shown as boxes and introns as bars. The *Eco*RI cleavage sites (E) and a single *Sal*I site (S) are indicated. The determined nucleotide sequences have been submitted to the DDBJ under accession number AB077763 (*H. roretzi* adult *TnI* gene). (b) Southern blot analysis of the *Halocynthia* adult *TnI* gene. Genomic DNA prepared from a single specimen of *H. roretzi* was digested with a restriction enzyme, *Eco*RI (lane E) or *Sal*I (lane S), and then hybridized to the DIG-labeled partial ascidian *bwTnI* cDNA (corresponding to exon V). Left, size markers in kbp based on molecular markers. (c) Alignment of the spliced leader (SL) sequences of ascidians. Identical nucleotides to those in *H. roretzi* adult TnI SL sequences are indicated by dots (.), and gaps are inserted for maximal similarity, as shown by dashes (–). Hr TnI, *Halocynthia roretzi* adult TnI; Ci TnI, *Ciona intestinalis* TnI; Sp Ldh, *Styela plicata* L-lactate dehydrogenase; Hr 29k, *Halocynthia roretzi* HR-29 protein.



b.

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                -182
HrMA4a      atggtca tcgcacttc tcattacgaattgcgc gtgataacaactg tcttcatttt
                -185
TnI-alpha   aatctgt ..t....cg ctagcataccag---- .........tt.. cggaaactgt
                -173
TnI-beta    aatctgt ..t....cg caagcatgccag---- ..a......tt.. caaaaactat
                -203
TnI-gamma   agtctgt ..t....cg caagtatgccag---- .........t... ctaaaagcat

                    B-region                  M-region
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Fig. 4. ***H. roretzi* larval TnI genes.** (a) Structure of *Halocynthia* larval *TnI* genes. Exons are shown as boxes and introns as bars. The *Eco*RI cleavage sites (E) are indicated. The determined nucleotide sequences have been submitted to the DDBJ under accession numbers AB077761 (*H. roretzi laTnIα* and *laTnIγ* genes) and AB077762 (*H. roretzi laTnIβ* gene). (b) Alignment of the 5′-untranslated regions of *H. roretzi laTnIs* and actin (HrMA4a). The B- and M-regions are boxed. Identical nucleotides to those in HrMA4a are indicated by dots (.), and gaps are inserted for maximal similarity, as shown by dashes (–). The nucleotides are numbered based on the initiation codon, ATG, the A of ATG being +1.

ever, we could not find that sequence. Does the *Halocynthia adTnI* gene possess a very large first intron within its 5′-untranslated region? Recently, Vandenberghe *et al.* provided an elegant answer to this question (*11*). They showed that the first 16 nucleotides of *Ciona TnI* cDNA is a spliced leader (SL) sequence, which is added through *trans*-splicing. According to their results, at least seven genes including *TnI* and the *Ciona* homologue of HR-29 of *H. roretzi* (*12*) undergo *trans*-splicing, namely their mRNAs possess an identical or very similar sequence at the 5′-end. Alignment of the *Ciona* SL-sequence and the *Halocynthia* "missing" sequence is shown in Fig. 3c. The *Halocynthia* sequence is slightly longer than the *Ciona* SL-sequence, but they show significant homology. Furthermore, a homology search revealed that the first 24 nucleotides of *Halocynthia adTnI* cDNAs were very similar to the 5′-end sequences of

a.

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                    10        20        30        40        50        60        70        80        90       100       110
Hr bwTnI            MSEQ--NQQRKMTHQRKQNLKSLMLNKAREDLKREAEVKAEEKKKILNSRIEPLSNLGGMSEQDLKDLCRELHAKIEKVDEQRYDIEVKVNKNDQEIEDLNQRIFDLRGK
Hr laTnI-alpha      MTE----STRKMTHQRKLQLKSLLLNGAREDLKREAEQKAEEKKKILNNRIESLGDLSSMSQQELMELCRELHAKTDKVDDERFDIELKVKKNDQEIEELNQKIFELRGK
Hr laTnI-beta       MAE----STRKMTHQRKLQLKSLLLSRAREDLKREEEQKAEEKKKILSNRIESLGDLSSMSQQELMELCRELHAKTDKVDDERFDIELKVKKNDQEIEELNQKIFELRGK
Hr laTnI-gamma      MADE---APRKMTHQRNRQLKSLMLKKAREDLKREAEQKAEEKKKILGNRIEALGDLSSMSQQELMDLCRELHARTDKVDDQRYDIQVKVKKNDAEIEELNQKIFELRGK
Ci bwTnI            MSEE--SGVRKMTHQRKMMLKSLMLNKAREDLKREMEQKAEAKKAELSQRLEPLSGLNSMSSQELMDLCRELHGKIDKVDEQRFDIEARVKKNDTEIEELNQKIFDLRGK
Pm TnI                 (+)QGQRKMTEQRKMNLKSLMLNKAREDLKVEAEHKAEEKKRVLNSRIEPLGNLSSMSESDLKDLARELNAKIDKIDEERYDLEVKVKKNDQEIEDLNQRIFDLRGK
Cs TnI              MADEDQTGARKMTHQRKMMLKSLMLNKAREDLKRETEQKAEEKKAALAKRIEPIGDIRSMSSQDLMDLCRELHAKIDQVDETRFDIEVRVKKNDQDIEDLNQKIFDLRGK
                           **** **   **** *  ****** * * *** **  *  * *       **   *  * ***       *  * *    * ***  ** *** ** ****
                           ↑                                                                                                ↑

                   120       130       140       150       160       170       180       190       200   207
Hr bwTnI            FKRPPLRRVRMSADQMLRALLGSKHKVSMDLRSSLKSVKKEETKKDE--AEVKDWRESVEAKTGGMGEMKAVFEGQ
Hr laTnI-alpha      FKRPPLRRVRMSADQMLRALLGSKHKVTMDLRSNLKTVK--ETKK
Hr laTnI-beta       FKRPPLRRVRMSADQMLRALLGSKHKVTMDLRSNLKTVK--EAKK
Hr laTnI-gamma      FKRPPLRRVRMSADQMLRALLGSKHKVTMDLRSNLKTVK--EPKK
Ci bwTnI            FKRPPLRRVRMSADQMLRALLGSKHKVSMDLRSNLKSVKKGGEKKED--AEVKDWRDNIEAKQG-MGGKKAVFEGAQ
Pm TnI              FKRPPLRRVRMSAGQMLRALLGSKHKVSMDLRSNLKSVKKEDTKKE----ETKDWRENVDAKQG-MSGMKAVFEHSQEEQSNHAIFSTGIWTINPML
Cs TnI              FKRPPLRRVRMSADQMLRALLGSKHKVSMDLRSNLKSVKKGDAKKDD--AEVKDWRDNVEAKAG-MGGKMAMFEGAKE
                    ************* ************* ***** ** **    **
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b.

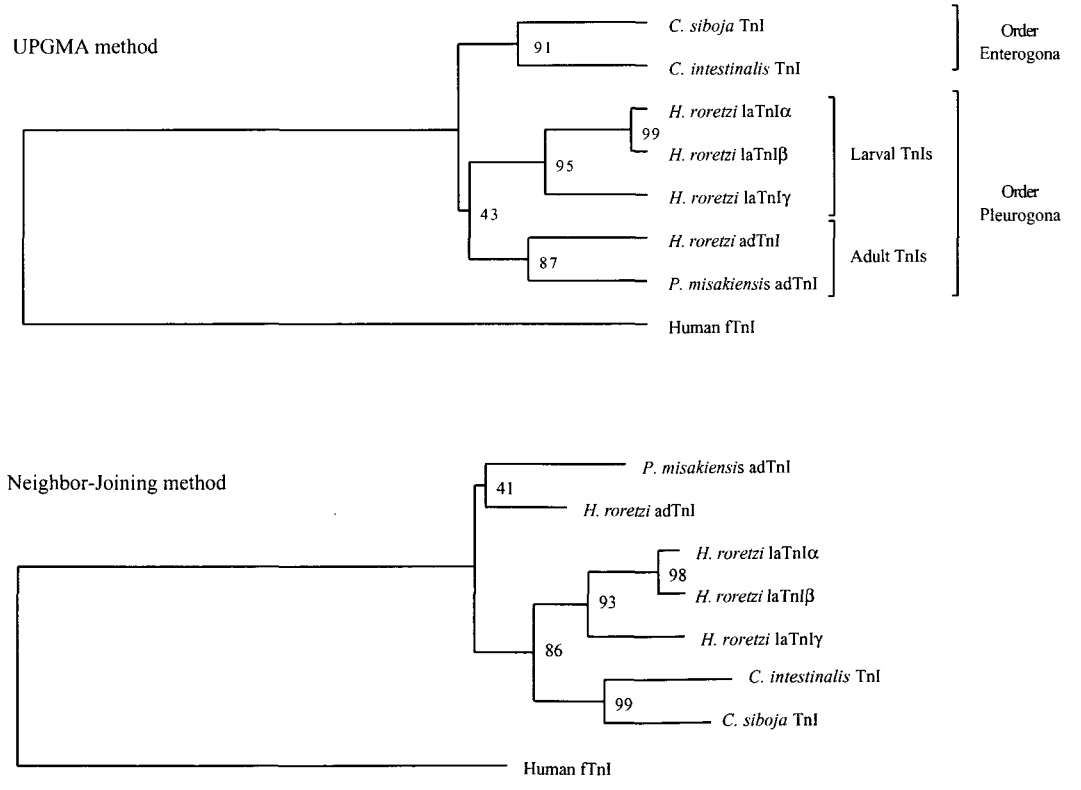

Fig. 5. **Evolution of ascidian TnIs.** (a) Alignment of the amino acid sequences of all known ascidian TnIs, with the CLUSTALW 1.7 program (*17*). The residues conserved in all chains are indicated by asterisks (*) and gaps are inserted for maximal similarity, as shown by dashes (–). Hr, *Halocynthia roretzi*; Ci, *Ciona intestinalis*; Pm, *Polyandrocarpa misakiensis*; Cs, *Chelyosoma siboja*. The N-terminus of *P. misakiensis TnI* is incomplete (shown by +), because the clone we isolated is truncated in the 5′-upstream region. (b) Phylogenetic trees were produced based on the above alignment (between the two arrows, corresponding to exons V and VI of *H. roretzi adTnI*) with the PHYLIP package (*18*), the UPGMA method (upper) and Neighbor-Joining method (lower) being used. Human fast skeletal TnI is used as an out group. The numbers at the forks indicate the percentage of 100 bootstrap resamplings that support these topological elements.

two other ascidian mRNAs (Fig. 3c): L-lactate dehydrogenase (Ldh) of *Styela plicata* (*13*) and HR-29. These results strongly suggest that the *Halocynthia adTnI* mRNAs also undergo trans-splicing of the 5′-leader sequences.

Structures of Larval TnI Genes—Using the *Halocynthia laTnIα* cDNA as a probe, we isolated some clones containing *laTnI* genes from the genomic library, two of which were sequenced. One of them (named clone 1) contained the full length *laTnIα* gene and the other (clone 2) contained the whole *laTnIβ* gene. On comparison with the previously reported cDNA sequences (*4*), some substitutions were observed within coding regions. *laTnIα* gene: Gly-23 (GGA) changed to Arg (AGA) in the genomic clone. *laTnIβ* gene: Ala-54 (GCT) changed to Ser (TCT), AAA encoding Lys-29 changed to AAG, and GAG encoding Glu-63 changed to GAA in the genome. These changes might be due to individual differences or allelic polymorphism. In addition, each gene has the complete sequence of the 5′-untranslated region of cDNAs, thus *Halocynthia laTnIs* mRNAs do not undergo *trans*-splicing. The exon/intron structure of the *Halocynthia laTnIs* genes is shown in Fig. 4a, both are divided into five exons by four introns, and all introns conform to the GT-AG rule.

One of the well studied genes of *Halocynthia* is actin (for a review see Ref. *14*). *Halocynthia* larval muscle actins are encoded by multi-copy genes, and at least five of them are clustered. Within the 5′-upstream region of HrMA4a, one of the larval actin genes, two *cis*-regulatory elements were defined (*15*). They are called the B-region and M-region, and are essential for larval muscle-specific expression. They consist of 9 and 13 nucleotide sequences respectively, and are separated by a 16 nucleotide spacer. Sequences well resembling these *cis*-regulatory elements are also observed within *Halocynthia laTnI* genes (Fig. 4b). In the case of *laTnIs*, the spacer between the two regions is rather short (12 nucleotides), but the 9 and 13 nucleotide sequences of the counterparts are well conserved. Although we did not detect the transcription start site of *laTnIs* correctly, but it is located at about 170–200 bp upstream from the initiation codon of *laTnIs*. This is a similar position to in the case of the B- and M-regions of HrMA4a (182 bp upstream from the initiation codon). Thus, the expression of *laTnI* genes may be regulated by the B- and M-regions, in the same manner as larval actins.

In addition, about 1.3 kbp downstream of *laTnIα*, we found another *laTnI* gene, named *laTnIγ* (Fig. 4a). The exon/intron organization of *laTnIγ* is identical with those of *laTnIα* and *laTnIβ*, and no stop codon nor frame-shift was observed within its coding region, and besides, *laTnIγ* also has B- and M-regions within its 5′-upstream region (Fig. 4b). These findings suggest that *laTnIγ* is a functional gene not a pseudo one, although we have not detected actual expression of it yet. Thus *Halocynthia* possesses at least three laTnI isoforms, and two of the genes are clustered in tandem, like larval actin genes. As far as we sequenced, there is no other *laTnI* gene, however, it is possible that *Halocynthia* has more *laTnI* genes, and the differences observed between cDNA and the genome may due to that.

Evolution of Ascidian TnIs—We determined the *TnI* cDNAs from two more ascidian species, *P. misakiensis* and *C. siboja*, and all known ascidian TnIs containing them are aligned in Fig. 5a. They show high homology, especially within the C-terminal half, which contains the actin/TnC binding site. Then we constructed phylogenetic trees from the N-terminal half of that alignment (Fig. 5b). The tree constructed by the UPGMA method (upper) is rather reasonable; TnIs can be roughly separated into two groups, the orders Enterogona and Pleurogona. The categorization of ascidians into these two orders was also supported by the results of analysis of 18S rDNAs (*16*). The tree suggests that the larval TnIs might have only been generated within the Pleurogona lineage after Enterogona/Pleurogona divergence. This scenario well agrees with the fact that *Ciona* does not possess larval TnI isoforms. However, the bootstrap value of the Pleurogona cluster is rather low (43%), and the other tree produced by the Neighbor-Joining method (lower) shows a different topology. To completely elucidate ascidian *TnI* evolution, it is necessary to determine the organization of *TnIs* from some other ascidian species, belonging to both the Enterogona and Pleurogona.

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