Ascidian Larva Reveals Ancient Origin of Vertebrate-Skeletal-Muscle Troponin I Characteristics in Chordate Locomotory Muscle

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Ascidians are protochordates related to vertebrate ancestors. The ascidian larval tail, with its notochord, dorsal nerve cord, and flanking rows of sarcomeric muscle cells, exhibits the basic chordate body plan. Molecular characterization of ascidian larval tail muscle may provide insight into molecular aspects of vertebrate skeletal muscle evolution. We report studies of the *Ci-TnI* gene of the ascidian *Ciona intestinalis*, which encodes the muscle contractile regulatory protein troponin I (TnI). Previous studies of a distantly related ascidian, *Halocynthia roretzi*, showed that different TnI genes were expressed in larval and adult muscles, the larval TnI isoforms having an unusual C-terminal truncation not seen in any vertebrate TnI. Here we show that, in contrast with *Halocynthia*, *Ciona* does not have a specialized larval TnI; the same TnI gene that is expressed in the heart and body-wall muscle of the sessile adult is also expressed in embryonic/larval tail muscle cells. Moreover the TnI isoform produced in embryonic/larval muscle is identical to that produced in adult body-wall muscle, i.e., a 182-residue protein with the characteristic chain length and overall structure of vertebrate skeletal muscle TnI isoforms. Phylogenetic analyses indicate that the unique features of *Halocynthia* larval TnI likely represent derived features, and hence that the vertebrate-skeletal-muscle -like TnI of *Ciona* is a closer reflection of the ancestral ascidian larval TnI. Our results indicate that characteristics of vertebrate skeletal muscle TnI emerged early in the evolution of chordate locomotory muscle, before the ascidian/vertebrate divergence. These features could be related to a basal chordate locomotory innovation—e.g., swimming by oscillation of an internal notochord skeleton—or they may be of even greater antiquity within the deuterostomes.

Introduction

The protochordates, including the major tunicate (urochordate) group, the ascidians, provide opportunities to study the molecular and developmental biology of organisms that are related to, and may resemble, early vertebrate ancestors (Berrill 1955; Wada 1998; Cameron, Garey, and Swalla 2000; Swalla et al. 2000). The ascidian larval tail shows hallmark chordate structures including a notochord, a dorsal nerve cord, and flanking rows of sarcomeric muscle cells that power the larval swimming activity (Katz 1983; Di Gregorio, and Levine 1998). Given its location, locomotory function, and ultrastructure, ascidian larval tail muscle is clearly homologous to vertebrate skeletal muscle (Meedel 1998), and its molecular characterization might be expected to provide insight into vertebrate skeletal muscle evolution. The larval tail and its muscle are resorbed during metamorphosis, and the sessile adult ascidian contains only nonlocomotory muscle tissues—the mantle or body-wall muscle and the heart—that are not obviously homologous to vertebrate skeletal muscle (Meedel 1998).

An integral aspect of muscle function is the contractile regulatory mechanism. One regulatory mechanism, based on the Ca^{2+}-sensitive troponin complex in cooperation with tropomyosin, is associated with sarcomeric thin (actin) filaments throughout the metazoa (Farah and Reinach 1995; Gergely 1998; Squire and Morris 1998; Gordon, Homsher, and Regnier 2000). Troponin/tropomyosin regulation also occurs in several unusual nonsarcomeric muscles (Toyota, Obinata, and Terakado 1979; Endo and Obinata 1981; Myers et al. 1996), including adult ascidian body-wall muscle (Toyota, Obinata, and Terakado 1979; Endo and Obinata 1981). The troponin complex consists of three subunits: (1) troponin C (TnC), a Ca^{2+}-binding subunit, (2) troponin T (TnT), a tropomyosin-binding subunit, and (3) troponin I (TnI), a subunit that interacts with actin, and with TnC and TnT, and that is the primary contractile regulatory subunit (Farah and Reinach 1995; Perry 1999).

Vertebrates have three TnI isoforms, sharing ~60% amino acid sequence identity, each encoded by a distinct gene (Dhoot and Perry 1979; Hastings 1997). Several short amino acid sequence motifs have been evolutionarily conserved across the metazoa (Kobayashi et al. 1989; Barbas et al. 1991), including a functionally critical 12-amino-acid segment near the middle of the molecule that binds actin and TnC and inhibits the actin-myosin contractile interaction (Talbot and Hodges 1981). Vertebrate skeletal muscle TnI isoforms (TnIfast and TnIslow) are characteristically shorter than most TnIs, ~180 amino acids long (Wilkinson and Grand 1978). In contrast, the vertebrate heart–specific TnIcardiac isoform is ~210–240 amino acids in length, similar in size to most protostome invertebrate TnIs (200–250 amino acids). Although they are relatively short, vertebrate skeletal muscle TnIs are not C-terminally truncated, as are several short TnI isoforms found in the protostome invertebrates—e.g., in the arthropod *Drosophila* (Barbas et al. 1991; Beall and Fyrberg 1991). Rather, the unique shorter chain length of vertebrate skeletal muscle TnIs reflects differences near the N-terminus (Wilkinson and Grand 1978), a region that interacts with TnC (Farah and Reinach 1995; Perry 1999).

Key words: contractile regulatory mechanism, muscle, chordate evolution, *Ciona intestinalis*, gene family evolution.

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Mol. Biol. Evol. 20(12):2113–2122. 2003
DOI: 10.1093/molbev/msg227
Molecular Biology and Evolution, Vol. 20, No. 12,

The evolutionary origins of vertebrate-characteristic features of skeletal muscle, such as short but C-terminally complete TnI, are not well understood. Studies of ascidian larval tail muscle may provide evolutionary insight, but to date they have led to enigmatic findings. Yuasa et al. (1997, 2002) found that embryonic/larval muscle of the ascidian *Halocynthia roretzi* expressed several closely related TnIs that were markedly truncated at the C-terminus; a distinct gene encoding a C-terminally complete TnI was expressed in adult muscles. In their C-terminal truncation, the larval muscle TnIs of *Halocynthia* do not resemble any vertebrate TnI, but they are reminiscent of some protostome invertebrate TnIs. Did vertebrate skeletal muscle evolve from an ancestral chordate muscle that, like *Halocynthia* larval tail muscle, expressed a C-terminally truncated TnI? Or is vertebrate skeletal-muscle–type TnI (short but with complete C-terminus) an ancient feature of chordate locomotory muscle, with the *Halocynthia* larval TnI C-terminal truncation perhaps representing a lineage-specific innovation (Yuasa et al. 2002)?

Here we report studies of the TnI expressed in larval tail muscle of *Ciona intestinalis*, an ascidian of the Order Enterogona/Suborder Phlebobranchiata, distantly related to *Halocynthia* (Order Pleurogona/Suborder Stolidobranchiata, or Order Stolidobranchia; see Wada (1998) and Swalla et al. (2000) for recent discussion of tunicate classification). We have previously characterized a vertebrate skeletal-muscle–like 182-residue C-terminally complete TnI expressed in Ciona adult body-wall muscle (MacLean, Meedel, and Hastings 1997). We now show that, in contrast to the mutually exclusive expression of specialized larval and adult TnI genes in *Halocynthia*, the same *Ciona* TnI isoform and gene that is expressed in adult muscle is also expressed in embryonic/larval tail muscle cells. Thus, unlike *Halocynthia*, *Ciona* expresses a TnI in larval tail muscle cells that has the typical features of vertebrate skeletal muscle TnI. Comparisons of amino acid sequences and genetic mechanisms of C-terminal truncation clearly indicate that the C-terminally truncated larval TnIs of *Halocynthia* represent a derived, rather than an ancestral, feature. Our findings indicate that vertebrate skeletal-muscle–characteristic features of TnI were developed in the locomotory muscle of chordates before the tunicate/vertebrate divergence.

Materials and Methods

Animals

Adult *Ciona intestinalis* were collected from the Sandwich Marina in the Cape Cod Canal, Massachusetts USA (except where indicated otherwise) and were maintained under constant illumination in a recirculating marine aquarium at 14°C. Collection of gametes and fertilization was as described elsewhere (Meedel, Farmer, and Lee 1997). Embryonic development was at 18°C.

RNA Isolation and Analysis

RNA was prepared by phenol extraction/salt precipitation from adult tissues or embryos as described (Meedel and Whittaker 1978; Meedel and Hastings 1993). Northern blot analysis was as described (Meedel and Hastings 1993) using a radiolabeled *Ci-TnI* antisense riboprobe derived from the cDNA clone pCTp2 (MacLean, Meedel, and Hastings 1997) and RNA size markers from Invitrogen Life Technologies. TnI mRNA 5′-RACE was performed using the Clontech AmpliFinder 5′-RACE kit as described (Vandenberghe, Meedel, and Hastings 2001). Reverse transcription polymerase chain reaction (RT-PCR) amplification of TnI mRNAs was based on primers GCTTAGCAACGCAACAAAA and GCAACATGCCAAAGAAAAATAC in the 5′- and 3′-untranslated regions, respectively. These primers amplify a 733-bp product from adult body-wall muscle RNA, a 874-bp product from heart RNA, and a 2.1-kb product from genomic DNA (MacLean, Meedel, and Hastings 1997). Prior digestion of RNA samples with DNase I (to remove traces of genomic DNA) and conditions for reverse transcription and PCR amplification were as described (Meedel, Lee, and Whittaker 2002).

Gene Structure Analysis

We determined the complete sequence (GenBank U94694) of a previously cloned 2.1-kb genomic DNA PCR product (MacLean, Meedel, and Hastings 1997) and located exons by comparison to body-wall muscle cDNA (GenBank U55261) and heart mRNA RT-PCR product (MacLean, Meedel, and Hastings 1997) sequences. Additional 5′-flanking sequence (GenBank AF237978) was cloned by an inverse polymerase chain reaction (IPCR) procedure (Ochman, Gerber, and Hartl 1988) using TGCGGTAATAAGTGAGGTC and AGCGAGAAATGGAACAAA as primers on circularized *Bst*YI fragments of *Ciona* genomic DNA (Vandenberghe, Meedel, and Hastings 2001). The resulting 2.8-kb IPCR product contained 2,067 bp of TnI DNA upstream of the ATG initiation codon; in 354 bp of overlapping sequence data, there were no differences between the IPCR product and the cloned 2.1 kb genomic PCR product.

In addition to the above-mentioned sequences, which were derived from Atlantic coast (Cape Cod) *Ciona* material, we also isolated TnI genomic DNA clones by hybridization screening of a lambda ZAP Express (Stratagene) phage library of partial *Sau*3A-cut *Ciona intestinalis* DNA (kindly provided by Dr. Robert Zeller) based on Pacific coast (Southern California) material, using the *Bst*YI IPCR product as probe. Two overlapping phage clones, one of which included 5,429 bp of DNA upstream of the ATG codon, were sequenced, including all exons (GenBank AF237979). Our Pacific TnI gene sequence is >99% identical to that present in the recently released *Ciona* genome sequence, which was also derived from a Southern California source (Dehal et al. 2002). In a 2.6-kb segment, including all exons and introns and 127 bp upstream of the ATG initiation codon, there were only 17 single-base differences, only one of which was in a protein-coding sequence (a synonymous substitution in alanine codon 176 (body-wall muscle TnI numbering). In the ''Pacific'' alleles the number, length, and distribution of exons corresponded exactly to those of the sequenced ''Atlantic'' allele, and they were ~96% identical in

sequence. There were six amino acid differences, one in exon 4 (the Pacific alleles encode EAKKAEL where the Atlantic sequence has EAEKAEL), and the rest in heart-specific exons 2 and 3. Introns were of similar lengths, although markedly divergent in sequence (<80% identity), and with 24 small (1–13 bp) indels and one large one (203 bp), in the first intron.

Expressed Sequence Tag Analysis

Expressed sequence tag (EST) analysis of tailbud embryo and larval mRNA populations was as previously described (Satou et al. 2001; Kusakabe et al. 2002; Satou et al. 2002) based on *Ciona* material collected in Japan. The EST sequence database can be accessed at http://ghost.zool.kyoto-u.ac.jp./indexr1.html. The National Center for Biotechnology Information accession number for EST cluster 00173 is UniGene Cluster Cin.13140.

TnI/β-gal Reporter Construct

The 1.5-kb TnI/β-gal reporter construct contained *Ci-TnI* genomic DNA derived from the *Bst*YI IPCR product (see above) (via a 1.8-kb *Kpn*I fragment, −1454 to +327, subcloned into pBluescriptIISK+ (Clontech)) extending from the *Kpn*I site at −1454 to a *Bpu1102*I site at −26 (nucleotides numbered with respect to the TnI ATG start codon.) The blunted *Bpu1102*I site was ligated to the *Sma*I end of a *Sma*I-*Bgl*II fragment of pSP72-1.27β-gal (Corbo, Levine, and Zeller 1997) carrying the nuclear β-gal reporter gene. The *Bgl*II end of the nuclear β-gal DNA fragment was ligated to the *Bam*HI site of the vector, pBluescriptIISK+. Electroporation of plasmid DNA constructs into one-celled embryos was as described by Corbo, Levine, and Zeller (1997). After 12 h, normally developed embryos were sorted and analyzed by β-gal histochemistry. Embryos were fixed for 30 min in seawater containing 1.5% paraformaldehyde, 0.1% Tween 80; they were then washed in 0.1% Tween 80 in phosphate buffered saline and transferred to staining solution (0.04% X-Gal, 2 mM $MgCl_2$, 0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide) for 1 h at room temperature.

Phylogenetic Analysis

TnI amino acid sequences encoded by DNA sequences corresponding to ancestral core exons II-V were aligned using Clustal W (Thompson, Higgins, and Gibson 1994) within the BioEdit 5.0.9 environment (Hall 1999). Phylogenetic analysis was carried out with MEGA software version 2.1 (Kumar et al. 2001) employing 100 bootstrap resamplings. Distance matrix methods (unweighted pair group method with arithmetic mean (UPGMA) and Neighbor Joining (NJ)) used pairwise gap deletion and Poisson correction. The NJ results were similar to the UPGMA results shown, except that *Ciona* TnI grouped more consistently with *Halocynthia* larval TnIs on bootstrap analysis. Maximum parsimony analysis used the close-neighbor interchange search method; PAUP 4.0 (Swofford 2000) generated the same consensus tree as did MEGA.

Results

TnI Gene Structure and Evolution

In *Ciona intestinalis*, the same TnI gene is expressed in adult body-wall muscle and heart, where it undergoes tissue-specific alternative splicing to generate 182- and 229-residue TnI isoforms, respectively (MacLean, Meedel, and Hastings 1997). We previously reported characterization of part of this gene including the heart-specific exons 2 and 3 (MacLean, Meedel, and Hastings 1997). Through further analysis of a PCR-cloned 2.1-kb segment of genomic DNA extending from the 5′ to the 3′-untranslated sequences (MacLean, Meedel, and Hastings 1997), and an inverse-PCR genomic DNA product extending 2,067 bp upstream of the ATG initiation codon (Vandenberghe, Meedel, and Hastings 2001), and comparison with body-wall muscle cDNA clone pCTp2 and RT-PCR products representing the heart-specific mRNA splice form (MacLean, Meedel, and Hastings 1997), we have determined the complete DNA sequence and intron-exon structure of the gene, here termed *Ci-TnI* to harmonize with *Ciona* genome project nomenclature (Chiba et al. 2003). Intron/exon structure was readily interpreted, notwithstanding a small number of presumably allelic single-base differences between the genomic DNA and mRNA-based sequences (the mRNA-based sequences both encode EAKKAEL where the genomic DNA encodes EAEKAEL in exon 4, and the heart mRNA-based sequence encodes K and V where the genomic DNA encodes E and I at heart TnI residues 40 and 48 [in exon 3]). In addition to this genomic DNA- and mRNA-based analysis of material collected from the Atlantic coast of North America, we also cloned and sequenced the *Ci-TnI* gene from Pacific coast material (see *Materials and Methods*) and found an identical exon organization.

The *Ci-TnI* gene comprises seven exons (fig. 1). There is a great similarity of exon organization and sequence to vertebrate TnI genes, particularly in the region encoding the C-terminal half of the protein (fig. 2). *Ci-TnI* exons 5–7 correspond precisely or approximately in length to exons 6–8 of the vertebrate TnI genes and share ~50%–80% amino acid sequence identity with them. The N-terminal half of the protein is less highly conserved both in amino acid sequence (<50% identity) and in exon organization; e.g., *Ci-TnI* exons 1 and 4 are each represented in vertebrate TnI genes by two separate exons. *Ci-TnI* gene organization resembles that of the recently reported *Halocynthia* adult TnI gene (Yuasa et al. 2002), although the latter has three rather than two serial heart-specific exons, making a total of eight exons.

Comparison of ascidian, vertebrate, and protostome invertebrate TnI gene structures suggests the overall evolutionary scenario depicted in figure 3. The ancestral metazoan TnI gene likely had five core exons, numbered I–V, of characteristic lengths and marked by short, conserved amino-acid sequence motifs, e.g., the actin/TnC-binding segment in exon IV, and other motifs denoted by boldface in figure 2 and described in the legend to figure 3. In addition, one or more facultative alternatively spliced near-N-terminal exons may have been present (surrounded by square brackets in figure 2), as is the case for extant *Ci-TnI*

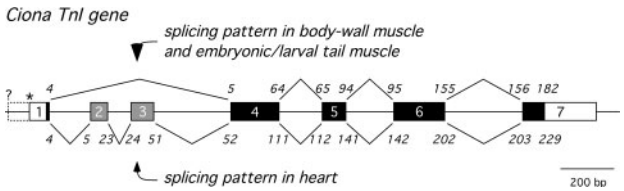

FIG. 1.—Structure of the *Ciona intestinalis* TnI gene, *Ci-TnI*. The gene comprises seven exons (numbered boxes). Protein-coding segments are black, except for the heart-specific alternatively spliced exons 2 and 3 (gray). Amino acid numbering for the body-wall muscle and embryo/larval TnI isoform is given above the gene schematic; that for the heart TnI isoform is below. All introns are located between codons. The question mark and dotted outline of the upstream end of exon 1 indicate that the precise transcription start site is not known. The asterisk marks the acceptor site to which a 16-nt leader RNA is trans-spliced. Distinct alleles of the gene from Atlantic and Pacific coast *Ciona* sources have the same structure (see *Materials and Methods*).

(MacLean, Meedel, and Hastings 1997) and *Drosophila* TnI genes (Barbas et al. 1991; Beall and Fyrberg 1991). After the protostome/chordate (deuterostome) divergence, several lineage-specific differences emerged, including (1) differing lengths of core exon II (42 codons in protostomes versus 15 codons in chordates), (2) the presence of independent within-codon (protostomes) and between-codon (chordates) introns at nearby but distinct sites in ancestral core exon III, and (3) the presence in protostomes but not in chordates of an intron within ancestral exon IV. This evolutionary schema strongly suggests that the ascidian/vertebrate differences in exons 1 and 4 (*Ci-TnI* numbering) are based on (1) insertion of a new intron into the protein-coding segment of ancestral exon I in the vertebrate lineage (apparently before the gene duplication events that established the TnIfast/TnIslow/TnIcardiac gene family) and (2) loss of the intron between ancestral exons II and III in the ascidian lineage.

Expression of the *Ci-TnI* Gene in Embryonic/Larval Tail Muscle Cells

Our initial studies had characterized *Ci-TnI* gene transcripts expressed in adult body-wall muscle and heart (MacLean, Meedel, and Hastings 1997) but did not assess whether the *Ci-TnI* gene was also expressed in embryos/larvae. Subsequent in situ hybridization studies detected RNAs hybridizing with *Ci-TnI* probes in larval tail muscle cells (Meedel, Lee, and Whittaker 2002; Satou et al. 2002), but these TnI-related RNAs were not characterized in molecular terms. To clarify the nature of the TnI mRNAs in *Ciona* embryo/larval tail muscle cells, we employed global EST analysis. In conjunction with the international *Ciona* genome consortium, the mRNA populations of several developmental stages of *Ciona* were characterized as ESTs by sequencing large numbers (>10,000) of randomly picked cDNA clones from each stage (Satou et al. 2002). Identical or near-identical overlapping EST cDNA sequences were grouped in clusters corresponding to individual mRNAs/genes. Cluster 00173 consisted of sequences related to *Ci-TnI*; a total of 55 ESTs (average length 675 bp) belonging to cluster 00173 were obtained from libraries derived from tailbud embryos and larvae, where they represented about 0.1% of the mRNA population (Chiba et al. 2003). Our further analysis of these EST cDNAs showed them to be ~99% identical to each other in overlapping regions, with ~1% of residues showing single-nucleotide heterogeneity (and, in untranslated regions, small indels), apparently reflecting allelic polymorphism. An assembled consensus sequence for cluster 00173 contained a 546-bp open reading frame that encoded a 182-residue TnI identical to the *Ci-TnI* product expressed in adult body-wall muscle. At the DNA sequence level, this open reading frame corresponded to, and was >99% identical to, *Ci-TnI* (Pacific allele AF237979) exons 1, 4, 5, 6, and 7. There were only two single-base differences (in the third positions of codons 46 and 174), both of which corresponded to allelic variation in the EST cluster in which the minority EST nucleotide corresponded to the one present in the genomic DNA sequence. The cluster 00173 consensus sequence included a total of 188 nt of 5′- and 3′-untranslated mRNA sequence, and these were ~99% identical to the corresponding *Ci-TnI* sequences. Thus cluster 00173 ESTs undoubtedly represent mRNAs transcribed from the *Ci-TnI* gene.

In 43 tailbud embryo/larval *Ci-TnI* ESTs that extended far enough in the 5′ direction to include the exon 1 junction, exon 1 was found in every case to be joined to exon 4 (body-wall-muscle splicing pattern) not exon 2 (heart splicing pattern). In 54 ESTs that extended far enough in the 3' direction to include the C-terminal protein-coding region, the complete C-terminal structure encoded by exon 7 was present in every case; there were no novel splicing products encoding C-terminally truncated TnIs similar to the *Halocynthia* larval TnIs. Thus the *Ci-TnI* gene is expressed in embryos/larvae, and the splicing pathway followed, and the TnI protein produced, correspond exactly to that used in adult body-wall muscle.

Additional studies independently supported this conclusion and gave further information. Developmental Northern blot studies, using *Ci-TnI* body-wall muscle cDNA clone pCTp2 (MacLean, Meedel, and Hastings 1997) as the hybridization probe, showed no hybridization with RNA from unfertilized oocytes or from embryos at 1.5, 3, or 4 h post-fertilization; a faint band of hybridization at 6 h (late gastrula); and strong and increasing hybridization at 9 h (neurula/early tailbud), 11 h (mid- tailbud), and 16 h (pre-hatch larva) of development (fig. 4*A*). By comparison with adult tissue RNA samples (fig. 4*B*), the embryonic/larval TnI mRNA corresponded in length to the body-wall muscle RNA (900 nt) rather than the longer, alternatively spliced heart RNA (1,050 nt) (MacLean, Meedel, and Hastings 1997). Reverse transcriptase PCR using primers based on *Ci-TnI* 5′- and 3′-untranslated mRNA sequences amplified a product from 16 h embryo RNA that co-migrated with the 733-bp product amplified from adult body-wall muscle RNA (data not shown); heart TnI mRNA generates a 874-bp product with these primers (MacLean, Meedel, and Hastings 1997). Developmental gene expression analysis by RT-PCR using the same primers confirmed the presence of *Ci-TnI* mRNA in 6 h embryos, as detected by Northern blot, and showed that very small amounts could be detected as early as 4 h postfertilization (64-cell stage) (data not shown). Similar

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Ciona Ci-TnI      exon 1      MSEE
Mus TnIcardiac    exons 1&2   *AD*SSDA
Homo TnIslow      exons 2&3   *P*VE
Coturnix TnIfast  exons 2&3   **DEE

Ciona Ci-TnI      exon 2      ESSESEISTSEDESEQEDE
Mus TnIcardiac                ------------------
Homo TnIslow                  ------------------
Coturnix TnIfast              ------------------

Ciona Ci-TnI      exon 3      IEVP-----PVKKEPIVKEQPEPAVESHNIEEK
Mus TnIcardiac    exon 3      AGE*QPAPA**RRR-SSANYRAY*T*P*AK---
Homo TnIslow                  --------------------------------
Coturnix TnIfast              --------------------------------

Ciona Ci-TnI      exon 4      SGVRKMTHQRKMMLK-SLMLNKAREDLKREMEQKAEAEKAELSQRLE-PLSGLNSMSSQELM
Mus TnIcardiac    exons 4/5   -KKS*ISAS**LQ**/T***QI*KQEME**A*ERRGEKGRV*RT*CQ-**-E*DGLGFE**Q
Homo TnIslow      exons 4/5   -RKP*I*AS**LL**/****A**K*CWEQ*H*ER-****VRYLAERI-*TLQTRGL*LSA*Q
Coturnix TnIfast  exons 4/5   -KK*RAATA*RQH**/*A**QL*VTEIEK*AAA*EVEKQN-YLAEHCP***LPG**--***Q

Ciona Ci-TnI      exon 5      DLCRELHGKIDKVDEQRFDIEARVKKNDTE
Mus TnIcardiac    exon 6      ****Q**ARV*****E*Y*V**K*T**I**
Homo TnIslow      exon 6      *******A*VEV***E*Y****KCLH*TR*
Coturnix TnIfast  exon 6      E**KK**A***S***E*Y*T*VKLQ*TNK*

Ciona Ci-TnI      exon 6      IEELNQKIFDLRGKFKRPPLRRVRMSADQMLRALLGSKHKVSMDLRSNLKSVKKGGEKKED
Mus TnIcardiac    exon 7      *AD*T***Y*********T*****I***A*MQ****TRA*E*L***AH**Q***EDIE*--
Homo TnIslow      exon 7      *KD*KL*VM***************V***A***************A*******EDTE*--
Coturnix          exon 7      L*D*S**L*********************A***********N****A***Q***EDTE*--

Ciona Ci-TnI      exon 7      ----AEVKDWRDNIEAKQGMGGKKAVFEGAQ
Mus TnIcardiac    exon 8      E--NR**G***K**D*LS**E*R*KK***
Homo TnIslow      exon 8      ER-PV**G***K*V**MS**E*R*KM*DA*KSPTSQ
Coturnix TnIfast  exon 8      EKDLRD*G***K***E*S**E*R*KM**AGES
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FIG. 2.—Comparison of amino acid sequences and exon organizations of the *Ciona Ci-TnI* gene and vertebrate TnIfast, TnIslow, and TnIcardiac genes. The *Ci-TnI*-encoded protein sequence is presented in seven blocks corresponding to individual exons, and the vertebrate sequences are also organized according to their known intron/exon structure. Asterisks mark residues identical to the corresponding Ci-TnI residue, and dashes show gaps introduced to maximize alignment. The Ci-TnI amino acid sequence is derived from genomic DNA sequence data (''Atlantic'' allele GenBank U94694 [gene], AAD09270.1 [protein]). Mouse (*Mus*) TnIcardiac data are from Ausoni et al (1994) (Z22784 [gene], CAA80459.1 [protein]). Human (*Homo*) TnIslow data are from Corin et al (1994) (L21905–11 [gene], AAC14461.1 [protein]). Quail (*Coturnix*) TnIfast data are from Baldwin, Kittler, and Emerson (1985) (M12132 [gene], AAB00122.1 [protein]). The number, lengths, and encoded protein sequences of *Ci-TnI* exons are similar to those of the vertebrate TnI genes, with three exceptions: (1) none of the vertebrate genes have an exon corresponding to *Ci-TnI* exon 2, (2) *Ci-TnI* exon 4 corresponds to a fusion of two exons present in each of the vertebrate genes, and (3) each of the vertebrate genes, but not the *Ci-TnI* gene, contains a codon-interrupting intron in the third or fourth codon (underlined amino acid in the top block of sequences), between the second and third codon bases. All other vertebrate introns, and all *Ci-TnI* introns, lie between codons. *Ci-TnI* exons 2 and 3 are used in heart muscle, but not in body-wall or embryo/larval tail muscle. The highly conserved inhibitory actin/TnC-binding segment is shown in boldface in *Ci-TnI* exon 6. Also in boldface are sequences that serve as signature motifs in identifying protostome/deuterostome versions of hypothetical ancestral core TnI exons (see figure 3 legend).

temporal expression profiles have been reported for other muscle genes whose expression is developmentally activated in the larval tail muscle cell lineages (Meedel and Whittaker 1983; Makabe et al. 1990; Araki et al. 1994; Kusakabe, Hikosaka, and Satoh 1995; Meedel, Farmer, and Lee 1997).

A 5′-RACE procedure was used to characterize the 5′-end of the larval *Ci-TnI* mRNA. 5'-RACE (rapid amplification of cDNA ends) products of identical size (~300 bp) were generated from larval RNA and from adult body-wall muscle RNA (data not shown). Leftward sequencing of both products from sites within exon 4 showed exactly the same 80-nt 5′-untranslated sequence, including a 16-nt trans-spliced leader (we have previously reported the body-wall muscle TnI mRNA 5′-untranslated sequence (MacLean, Meedel, and Hastings 1997; Vandenberghe, Meedel, and Hastings 2001)). Moreover in both 5′-RACE products the exon 4 sequence was spliced to exon 1 (body-wall muscle splicing pattern) and not to exon 3 (heart splicing pattern).

The foregoing evidence of Ci-TnI gene expression in tailbud embryos/larvae implies the presence of cis-regulatory elements able to drive transcription in these developmental stages. This was confirmed by transfection of a *Ci-TnI* reporter transgene construct into *Ciona* embryos by zygote electroporation. We have previously shown that a cytoplasmic β-gal reporter gene driven by 1.5 kb of DNA upstream of the *Ci-TnI* ATG start codon was expressed in embryonic tail, apparently in muscle cells (Vandenberghe, Meedel, and Hastings 2001). To further clarify the cellular basis of *Ci-TnI* transgene expression, we prepared a nuclear-targeted β-gal reporter driven by the same *Ci-TnI* upstream DNA. In transfected embryos stained at 12 h of development, nuclear staining of muscle cells was evident in almost all embryos (fig. 5). In some embryos all muscle cells showed strong nuclear β-gal expression. In the tail, there was no staining of peripheral cells (epidermis) or of central cells (notochord, nerve cord), and there was little staining in the trunk. These transgene studies indicate that the *Ci-TnI* gene contains

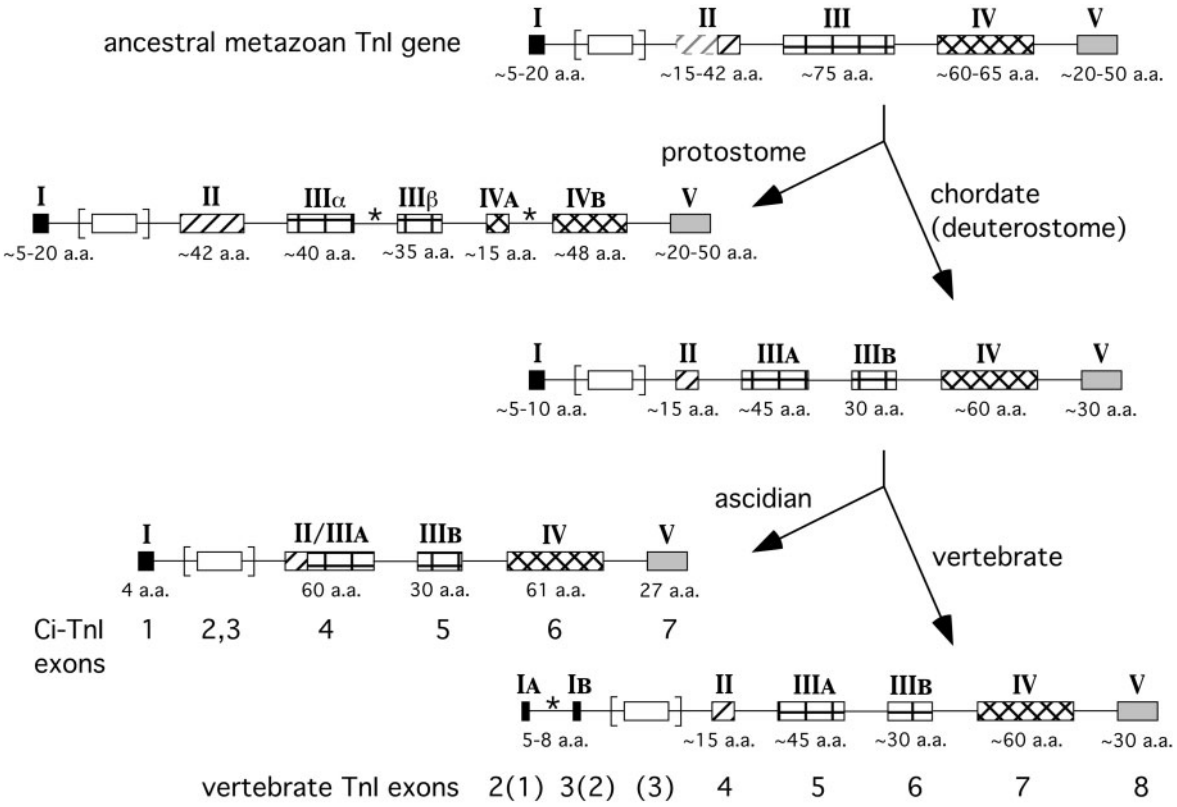

FIG. 3.—Evolution of TnI protein-coding exon organization. The top gene schematic shows the five proposed ''core'' exons (I–V) of the ancestral metazoan TnI gene, each marked with a different pattern. The remaining gene schematics outline the fates of the ancestral core exons during the evolution of protostome, deuterostome, ascidian, and vertebrate TnI genes. All codon-interrupting introns are marked with asterisks; unmarked introns lie between codons. The protostome TnI gene structure is a consensus based on the *Drosophila* TnI gene (Barbas et al. 1991; Beall and Fyrberg 1991) and the four *Caenorhabditis* genes (*tni-1, tni-3, tni-4,* and *unc-27:* GenBank NM_077505 [tni-1 mRNA], NP_509906 [tni-1 protein], WormBase F42E11.4 [*tni-1* gene]; CAB04737 [tni-3 protein], Z81593 [*tni-3* gene]; AAB94188 [tni-4 protein], AF039041 [*tni-4* gene]; NM_077087 [unc-27 mRNA], NP_509488 [unc-27 protein], WormBase ZK721.2 [*unc27* gene]). In the *Drosophila* gene, and in all the *Caenorhabditis* genes except *tni-3*, the DNA sequence encoding the actin/TnC-binding segment is split by an intron between the second and third bases of the conserved phenylalanine codon (GKFVP). The ancestral metazoan gene structure gives the most parsimonious derivation of its descendant genes. The only arbitrary feature is that we elected to show exon IV in the uninterrupted deuterostome form rather than in the interrupted protostome form; both are equally likely. The relationship between the generic ascidian and vertebrate TnI genes shown here and the specific genes compared in figure 2 is shown by exon numbering (Arabic numerals) below the gene schematics (numbers in brackets below the vertebrate TnI gene refer to the TnIcardiac gene). Apart from exon I, which encodes a short N-terminal segment, each of the five ancestral core exons contains a signature amino acid sequence motif that is conserved in both protostome (*Caenorhabditis tni-1*, and *Drosophila*) and deuterostome (*Ci-TnI*, and *Coturnix* TnIfast) genes. These are as follows: in exon II the last six residues are K/RK/RXXLK/R where X is any residue; exon III contains AXXD/EI/L six residues from the upstream end and K/RW/F/YD 11 residues from the downstream end; exon IV contains the actin/TnC binding sequence GKFV/KK/RPXLR/KR/KVR/S; exon V contains D/EW 3–8 residues from the upstream end. These motifs are in boldface in figure 2.

promoter/enhancer elements capable of driving tissue-specific expression in embryonic muscle cells.

Taken together, our EST, Northern blot, RT-PCR, 5′-RACE, and transgene expression data clearly show that the Ci-TnI gene is expressed in embryonic/larval muscle, and that a 182-residue C-terminally complete TnI is produced that is identical to that produced in adult body-wall muscle.

Discussion

The studies of Yuasa et al (1997, 2002) showed that the ascidian *Halocynthia* has distinct larval and adult TnI genes, the former encoding very short (149-residue) C-terminally truncated TnI isoforms quite different from vertebrate skeletal muscle TnIs. Ascidian larval tail muscle is homologous to vertebrate skeletal muscle, and it may reveal ancestral skeletal muscle features; this finding raises the question whether vertebrate skeletal muscle may have arisen from an ancestral chordate muscle that expressed a C-terminally-truncated TnI. However, our results show that in another ascidian, *Ciona*, the same TnI gene that is expressed in adult muscle is also expressed in larval tail muscle, where it produces a vertebrate skeletal-muscle–like 182-residue TnI that is not C-terminally truncated. Moreover, the available *Ciona* genome sequence data, which are thought to be near-complete, suggest that *Ci-TnI* is the only TnI gene in *Ciona* (Dehal et al. 2002); there is no evidence for an additional gene, either closely or distantly related, that might encode a C-terminally truncated isoform similar to the *Halocynthia* larva-specific TnI genes.

Our findings in *Ciona* raise the possibility that the C-terminally truncated larval TnIs of *Halocynthia* could

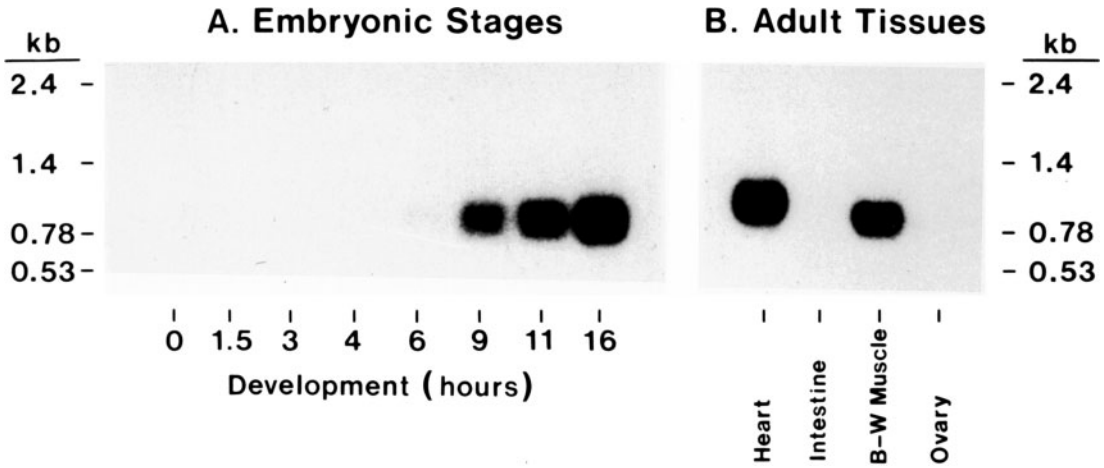

FIG. 4.—Northern blot analysis of *Ci-TnI* gene expression. Equal amounts of RNA from the indicated embryonic stages (panel *A*; 0 indicates unfertilized oocytes) or adult tissues (panel *B*) were subjected to Northern blotting followed by hybridization with an antisense riboprobe derived from cDNA clone pCTp2 and autradiography. All bands shown are images from a single gel and film and are directly comparable. The migration positions of a set of known RNA size standards are indicated at the left and right edges.

represent a derived, rather than an ancestral chordate, character, and this idea is strongly supported by amino acid sequence comparisons. As shown in figure 6, molecular phylogenetic analysis indicates that the gene duplication that gave rise to adult and larval TnI gene classes occurred in the tunicate TnI lineage after the divergence of the tunicate and vertebrate TnI lineages (see also Yuasa et al. [2002]). This relationship is also supported by gene structure data; all the *Halocynthia* TnI genes, adult and larval, share with *Ci-TnI* the exon 1 and exon 4 features that distinguish *Ci-TnI* from all the vertebrate TnI genes (see fig. 3). The level of sequence divergence between the *Halocynthia* adult and larval TnIs is very similar to their sequence divergence from *Ciona* TnI, suggesting that the initial adult/larval gene duplication occurred early in tunicate evolution. This is reflected graphically in the trifurcation of ascidian TnIs in the consensus phylogenetic trees shown in figure 6*B* and *C*. The simplest overall interpretation is that the ancestral chordate, like its descendants the vertebrates and the Enterogona ascidian *Ciona*, produced only C-terminally complete TnI, but that during tunicate evolution a gene duplication occurred in the *Halocynthia* lineage or its antecedents that permitted the evolution of larva-specific genes encoding C-terminally truncated TnIs. Consistent with its origin as an independent evolutionary innovation, the C-terminal truncation of *Halocynthia* larval TnIs has a different molecular genetic basis from those found in protostome invertebrates. In *Halocynthia* the stop codon that defines the C-terminal truncation is not created by a splicing event as in the alternatively spliced *Drosophila* TnI gene (Barbas et al. 1991; Beall and Fyrberg 1991), nor does it occur within ancestral core exon IV, as is the case for the *tni-4* gene of the nematode *Caenorhabditis* (K.E.M.H., unpublished analysis), but in a downstream exon that may or may not be derived from ancestral core exon V (see Yuasa et al. 1997, 2002). All of the evolutionary analyses indicate that the C-terminally complete larval TnI of *Ciona* more closely reflects the ancestral ascidian larval TnI than do the larval TnIs of *Halocynthia*.

Recent phylogenetic analysis suggests that the tunicate/vertebrate common ancestor—i.e., the stem chordate—may have been a motile organism with a tadpole-like body plan throughout life (Wada 1998). Its locomotory muscle would be homologous to both vertebrate skeletal muscle and ascidian larval tail muscle and would be expected to share features shared by the latter two muscles, including, as we show here, C-terminally complete TnI. Ascidian (*Ciona*) larval tail muscle TnI also

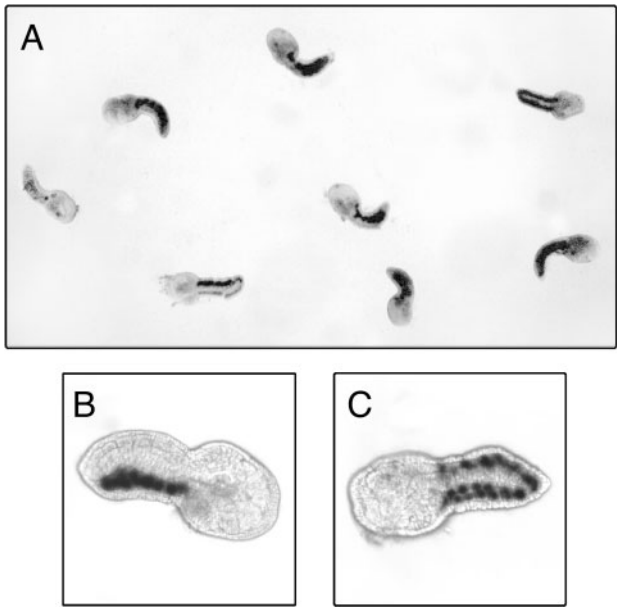

FIG. 5.—Expression of 1.5 kb TnI/β-gal construct in electroporated embryos stained at 12 h of development (tailbud stage). *A*. A low-magnification view of numerous embryos. Essentially 100% of normally developing embryos showed some expression. High-level expression was limited to tail muscle cells. Dorsal views of two embryos are shown in panels *B* and *C*. Expression was usually bilateral, in muscle cells on both sides of the notochord/nerve cord, but in some cases (panel *B*) expression was unilateral.

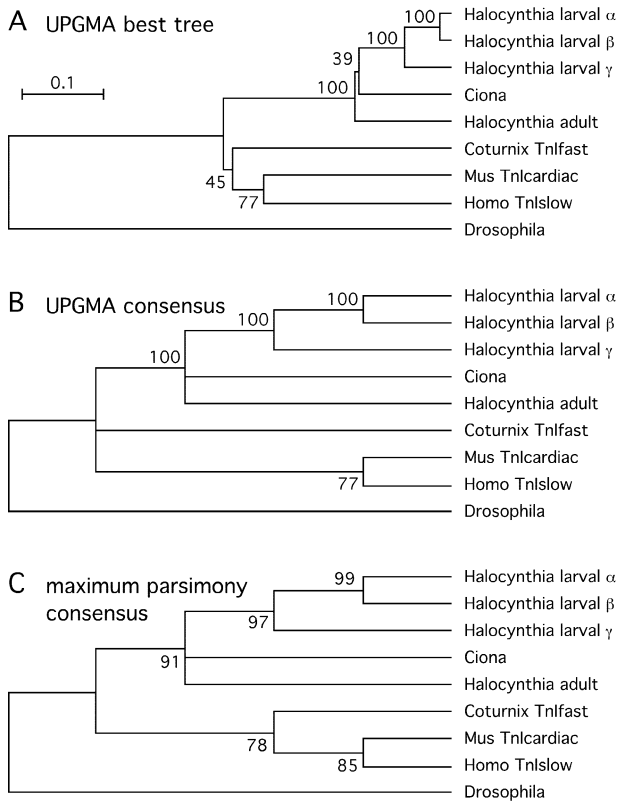

FIG. 6.—Phylogenetic trees showing relationships among ascidian and vertebrate TnI amino acid sequences. (*A*) Best UPGMA tree (branch lengths proportional to sequence divergence, scale indicated on bar). (*B*) UPGMA bootstrap consensus tree. (*C*) Maximum parsimony bootstrap consensus tree. In *B* and *C* branch lengths are not proportional to sequence divergence. Numbers show the number of times the sequence grouping represented by the corresponding branch occurred in 100 bootstrap resamplings. The aligned sequences, chosen with regard to the known gene structures, correspond to ancestral core exons II–IV (exon I is too short and variable to add information, and exon V is entirely—or almost entirely—lacking from the *Halocynthia* larval TnIs). The *Ciona* and vertebrate sequence sources were as in figure 2. The *Halocynthia* sequences were from the following GenBank accessions: larval alpha BAB83807.1, larval beta BAB83809.1, larval gamma BAB83808.1, and adult body-wall muscle BAB83810.1. The *Drosophila* sequence, used as outgroup, is from P36188.

shares with vertebrate skeletal muscle TnIs an unusually short chain length (~180 residues) in comparison with other C-terminally complete metazoan TnIs (>200 residues). This short chain length reflects two features of TnI gene organization that affect the N-terminal part of the protein. First, *Ciona* larval TnI and vertebrate TnIfast and TnIslow are ''core'' TnIs constructed solely of the five ancestral metazoan TnI core exons (exons I–V; fig 3) and are thus shorter than N-terminally extended TnIs produced by inclusion of additional near-N-terminal exons, as in the TnIs expressed in the vertebrate and ascidian hearts (Wilkinson and Grand 1978; Ausoni et al. 1994; MacLean, Meedel, and Hastings 1997). Second, these chordate core TnIs are shorter than the core TnIs of protostome invertebrates because the protein segment corresponding to ancestral core exon II is shorter (~15 codons versus ~42 codons; see fig. 3). *Halocynthia* larval TnIs (Yuasa et al. 2002), though differing markedly at the C-terminus, nonetheless share these chordate TnI near-N-terminal features. The sharing of these features by ascidian larval muscle and vertebrate skeletal muscle TnI indicates that they evolved early in chordate/deuterostome evolution and were already in place in the locomotory muscle of the ancestral chordate. The TnI N-terminal region is implicated in TnC binding (Farah and Reinach 1995; Ferrieres et al. 2000), so it is possible that these features reflect aspects of TnI:TnC interaction unique to chordate locomotory muscle. It appears that ''vertebrate'' muscle-type actin was also present in the ancestral chordate (Vandekerckhove and Weber 1984; Kusakabe et al. 1997), pointing to an early evolution of chordate locomotory muscle thin-filament characteristics. These features could be related to a basal chordate locomotory innovation, e.g. swimming by oscillation of an internal notochord skeleton, or they may be of even greater antiquity within the deuterostomes. Studies of TnI genes in other deuterostome groups, e.g, echinoderms, would shed further light on the origin and antiquity of chordate locomotory (vertebrate skeletal muscle)-type TnI.

Concerning modern ascidians, the biological implications of C-terminally truncated larval TnI in *Halocynthia*, but not in *Ciona*, are not clear. Current knowledge of TnI structure/function suggests that C-terminal truncation is likely to have a functional effect (Ramos 1999; Ferrieres et al. 2000; Murphy et al. 2000; Digel et al. 2001; Jin et al. 2001); however, the precise implications for contractile regulation in vivo are not clear. Indeed, there seem to be no obvious differences in larval tail muscle usage between these ascidian species. It is of interest to note that, parallel to observations on TnI, *Halocynthia* has separate genes encoding larval and adult isoforms of TnT (Endo et al. 1996), whereas the *Ciona* genome appears to contain a single TnT gene (Dehal et al. 2002; Chiba et al. 2003). It is plausible that the *Halocynthia* larval TnT gene could be specialized for function in a troponin complex containing a C-terminally truncated larval-specific TnI, a specialization that would not be relevant to *Ciona*, which uses the same TnI in larval and in adult (body-wall) muscle. Comparative functional and biochemical/biophysical studies of *Halocynthia* and *Ciona* larval tail muscle, although technically difficult because of small size, would be worthwhile and may lead to unique insight into the troponin contractile regulatory mechanism.

Because the *Ciona Ci-TnI* gene is expressed both in larval tail muscle and in adult body-wall muscle and heart, it presents opportunities for molecular genetic analysis of myogenic gene regulatory mechanisms operating in multiple muscle cell types and during both early embryonic development and subsequent metamorphosis/adult development. Moreover, a broader comparative analysis including the specialized adult and larval TnI genes of *Halocynthia* may provide insight into the evolutionary changes in gene regulatory elements that underlie the diversification of transcriptional specificities after gene duplication. Thus further studies of ascidian TnI genes are likely to lead to better understanding of the development and evolution of chordate muscle types and of the gene regulatory dynamics of gene family evolution.

Acknowledgments

This work was supported by a research grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada and the Faculty Research Committee of Rhode Island College. D.W.M. held an NSERC Postgraduate Scholarship.

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William Jeffery, Associate Editor

Accepted July 21, 2003