Tissue expression of four troponin I genes and their molecular interactions with two troponin C isoforms in *Caenorhabditis elegans*

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Gene duplication is a major genetic event that can produce multiple protein isoforms. Comparative sequence and functional analysis of related gene products can provide insights into protein family evolution. To characterize the *Caenorhabditis elegans* troponin I family, we analyzed gene structures, tissue expression patterns and RNAi phenotypes of four troponin I isoforms. Tissue expression patterns were determined using *lacZ/gfp/rfp* reporter gene assays. The *tni-1*, *tni-2/unc-27* and *tni-3* genes, each encoding a troponin I isoform, are uniquely expressed in body wall, vulval and anal muscles but at different levels; *tni-4* was expressed solely in the pharynx. Expressing *tni-1* and *-2* gene RNAi caused motility defects similar to *unc-27* (*e155*) mutant, a *tni-2* null allele. The *tni-3* RNAi expression produced egg laying defects while the *tni-4* RNAi caused arrest at gastrulation. Overlay analyses were used to assay interactions between the troponin I and two troponin C isoforms. The three body wall troponin I isoforms interacted with body wall and pharyngeal troponin C isoforms; TNI-4 interacted only with pharyngeal troponin C. Our results suggest the body wall genes have evolved following duplication of the pharynx gene and provide important data about gene duplication and functional differentiation of nematode troponin I isoforms.

Introduction

Muscle contraction is the result of a series of protein–protein interactions. In striated muscle, the thin filament complex of troponin (Tn) and tropomyosin (Tm) regulates contraction. The troponin complex components include the calcium binding protein, troponin C (TnC), the tropomyosin (Tm)-binding protein, troponin T (TnT), and troponin I (TnI), which is involved in inhibition of the actomyosin ATPase activity (Ohtsuki *et al.* 1986; Gordon *et al.* 2000). Recent studies of crystals of the core Tn complex suggest that in the absence of calcium, TnI binding to actin holds the Tm-Tn complex in a 'closed' state, thereby preventing myosin from binding to actin (Vassylyev *et al.* 1998; Takeda *et al.* 2003). Calcium released into the muscles following neural stimulation binds to TnC, which undergoes a conformational change that alters its relationship with TnI, resulting in the release of TnI binding to actin. Biochemical and physicochemical studies of muscle contraction have been carried out using proteins from different tissues of vertebrates.

Multiple forms of Tn subunits have been identified in fast skeletal (Sheng *et al.* 1992), slow skeletal (Grand & Wilkinson 1977) and cardiac muscles (Grand & Wilkinson 1976). Specific isoforms probably play important roles in determining the distinctive functional properties of the various muscle types. TnI isoforms are expressed in two patterns: a tissue-specific and a developmentally regulated pattern. An approach to understanding the role of TnI in the regulation of contraction is to study functional differences between various TnI isoforms. The most important molecular genetic mechanisms for generating protein isoform variants are alternative splicing from a single gene or transcription from different genes in a multigene family (Baldwin *et al.* 1985; Kobayashi *et al.* 1989; Barbas *et al.* 1993). TnI isoforms in *Drosophila* are produced from a single gene (Barbas *et al.* 1993) but those in vertebrates are produced from a multigene

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family (Hastings 1997). Interestingly, the two TnI isoforms of the protochordate ascidian, *Ciona intestinalis*, are encoded by a single gene (MacLean *et al.* 1997). It is likely that the alternative splicing mechanism is the ancestral type in the TnI genes of invertebrates, and that during vertebrate evolution, this mechanism has been abandoned in favor of transcriptional regulatory mechanisms (Hastings 1997). However, contrary to the alternative splicing mechanism of TnI genes in other invertebrates, there are four TnI genes encoding four different isoforms in *C. elegans* (WormBase).

After a new gene appears by gene duplication, an additional functional variant of the product can be produced by mutation and selective pressure (Gilbert 1978). Gene duplication is generally considered the primary engine of isoform diversity in the evolution of multigene families (MacLean *et al.* 1997). Differences in exon numbers, splicing sites and expression profiles are helpful in solving the evolutionary history of closely related genes (Baldwin *et al.* 1985). In globular proteins, it is known that modular structural units are often separated by different exons (Go 1983). Examination of the splicing pattern often reveals the relationships derived by the gene duplication process.

It is also of interest to know how molecular interactions within the Tn complex affect animal behavior. A genetic approach to the study of Tn functions is a powerful means by which to understand muscle function and animal behavior. Mutations in the *Drosophila* TnI and TnT genes produce muscle hypercontraction and have flying, walking, crawling and jumping defects (Fyrberg *et al.* 1990; Nongthomba *et al.* 2003, 2004).

The nematode *C. elegans* has two primary muscle types: body wall muscle for locomotion and pharyngeal muscle for feeding (Waterston 1988; Moerman & Fire 1997). These correspond to skeletal and cardiac muscles in vertebrates, respectively. In the worm pharyngeal muscle, development starts at the comma stage and body wall muscle elongation follows depending on progression after the twofold stage. Deficiencies of body wall TnC or Tm function in *C. elegans* causes the Pat (paralyzed arrest at embryonic twofold stage) phenotype (Williams & Waterston 1994) and in TnT, it causes Mup (muscle position abnormal) phenotype (Myers *et al.* 1996). The *mup-2/tnt-1* TnT gene is abundantly expressed in body wall muscle but at least four TnT genes are expressed in the worm (WormBase). Two different genes: *pat-10/tnc-1* and *tnc-2* encode the two isoforms of TNC that are expressed in body wall and pharyngeal muscles, respectively (Terami *et al.* 1999; our unpublished observation).

Nothing is known of the functional differences between the TnI isoforms in *C. elegans* or their evolutionary relationships. Investigation of these isoforms will provide important insights into the evolutionary divergence of these proteins but also contribute to solving problems regarding mechanisms of muscle filament assembly and functional interactions within the Tn complex. What was the evolutionary history of the original TnI gene that produced four genes? What is the evidence that this has led to functional diversity as detected by different patterns of gene expression and changes in *in vivo* and biochemical function? We can study the CeTnI genes as a model to investigate these questions in detail for a single protein family in a genetically amenable organism.

In this study, we cloned cDNA and genomic fragments of the *tni-2/unc-27* gene and completed the genome structures of three additional genes, *tni-1*, *tni-3* and *tni-4*, based on the *C. elegans* genome sequence database, WormBase. Tissue-specific expression patterns of these genes were studied by injecting promoter::*lacZ/gfp/rfp* gene fusion constructs. We found that three of four CeTNI isoforms are expressed in the body wall muscle tissues at different levels and that *tni-4* is expressed specifically in the pharynx. We have used RNAi to study the functions of the four TnI isoforms during muscle development. Finally, we performed *in vitro* protein overlay assays between four CeTNI and two CeTNC isoforms using anti-CeTNI and anti-CeTNC antibodies. Functional differences and evolutionary relationships among the four TnI isoforms in the worm are discussed. This is the first stage of characterizing all the isoforms of a troponin subunit in one animal.

Results

Structure: Cloning and mapping of four cDNAs and genomic clones of the TnI gene

Three positive clones were isolated by immunoscreening of a λZAPII cDNA library using antibody against *Ascaris* TnI (Nakae & Obinata 1993). One clone contained a 0.8 kb fragment, which showed a high homology to the *tni-2* gene and had the same sequence as the cDNA clones, cm15e4 and cm20c1 (Waterston *et al.* 1992) corresponding to the ZK721.2 ORF at the position of the *unc-27* gene. The other two clones were the partial transcripts missing the 5′end of the same gene. These results indicate that cDNA clones of the gene, *tni-2/unc-27* are abundant in the library. The *unc-27* (*e155*) mutation results in a stop at codon Gln^{10} (CAG→TAG; Q→Stop, arrowhead in Fig. 2). Together with the *tni*-2 gene, the 5′ ends of another three genes were defined by RT-PCR experiments (see Experimental procedures). The complete maps and genome organization of the four TnI genes are shown in Fig. 1. All four TnI gene transcripts

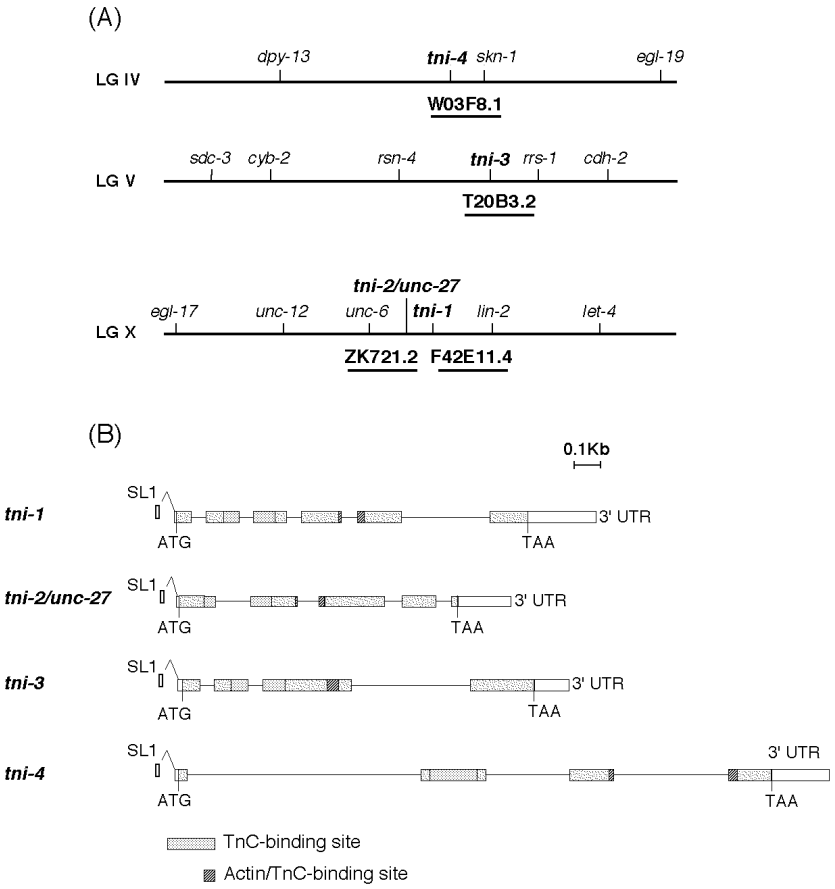


Figure 1 Genetic maps and gene organizations of *tni-1*, *-2*, *-3* and *-4*. (A) Genetic maps of four TnI genes and their cosmid clones. Genomic maps of linkage groups IV and V in relation to WO3F8.1 for *tni-4* and T20B3.2 for *tni-3*. Genetic map of linkage group X in relation to ZK721.2 for *tni-2/unc-27* and F42E11.4 for *tni-1*. (B) Gene organizations of four TnI genes: *tni-1*, *-2*, *-3* and *-4*. Lines indicate introns and filled boxes indicate exons. Untranslated regions are shown as open boxes. ATG and TAA indicate start and stop sites of translation, respectively. TnC-binding and actin/TnC-binding sites are shown in dotted and striped boxes, respectively. Accession numbers of the nucleotide sequences, genome organizations and cDNA sequences of *tni-1*, *-2*, *-3* and *-4* are available from the DDBJ/EMBL/GenBank under the accession numbers AB107358, AB107357, AB107359 and AB107583, respectively.

are *trans*-spliced by the SL1 spliced leader (Fig. 1B). The N-terminal amino acid sequences were deduced from the sequences obtained following RT-PCR (Fig. 2).

Sequence homology between TnI isoforms of *C. elegans* and other animals

The deduced amino acid sequences of the four TNI isoforms of *C. elegans* were aligned with those of *Drosophila* (Barbas *et al*. 1993), crayfish (Kobayashi *et al*. 1989), rabbit cardiac (Grand & Wilkinson 1976), slow (Grand & Wilkinson 1977) and fast muscles (Sheng *et al*. 1992). N-terminal extensions are common in the TnIs of invertebrates, those from the heart of *Ciona* and the cardiac TnI isoforms of vertebrates (Figs 2 and 3A). This region may have functional homology and indicate an evolutionary relationship of these muscles (Grand & Wilkinson 1976). Comparison of the TnC-binding site sequence of the TnIs showed more than 60% homology between TNI-1, -2, -3 and -4 isoforms of the *C. elegans*, *Drosophila* and crayfish but only about 20% homology with body wall and heart muscle isoforms of *Ciona*, cardiac, and the slow and fast muscles of rabbits (Figs 2 and 3A). Detailed inspection showed that the actin / TnC-binding sites CeTNI-1 and CeTNI-2 were identical and shared more than 82% homology with *C. elegans*, *Drosophila* and crayfish, but only around 50% homology to the isoforms of the body wall and heart muscles of *Ciona* and the cardiac, slow and fast muscles of rabbits (Fig. 3A). C-terminal extensions consisting of repeated glutamate residues are present in CeTNI-1, CeTNI-2 and CeTNI-3, but absent in CeTNI-4 (Fig. 3A). The presence of the C-terminal extension in the three isoforms was of particular interest because of their expression in body wall muscles. A phylogenetic tree was constructed from the alignment of TNIs from *C. elegans*, *Drosophila*, crayfish, *Ciona* body wall and heart, rabbit cardiac, slow and fast muscles (Fig. 3B). Gene structures, functional differences and evolutionary relationships of these TnIs are discussed below.

Localization: Tissue expression patterns of four TnI genes

The expression patterns of *tni-1/-2/-3/-4::lacZ*, *tni-2::gfp* and *tni-1/-3::rfp* constructs were observed (Fig. 4) and the results are summarized in Fig. 4A and Table 1.

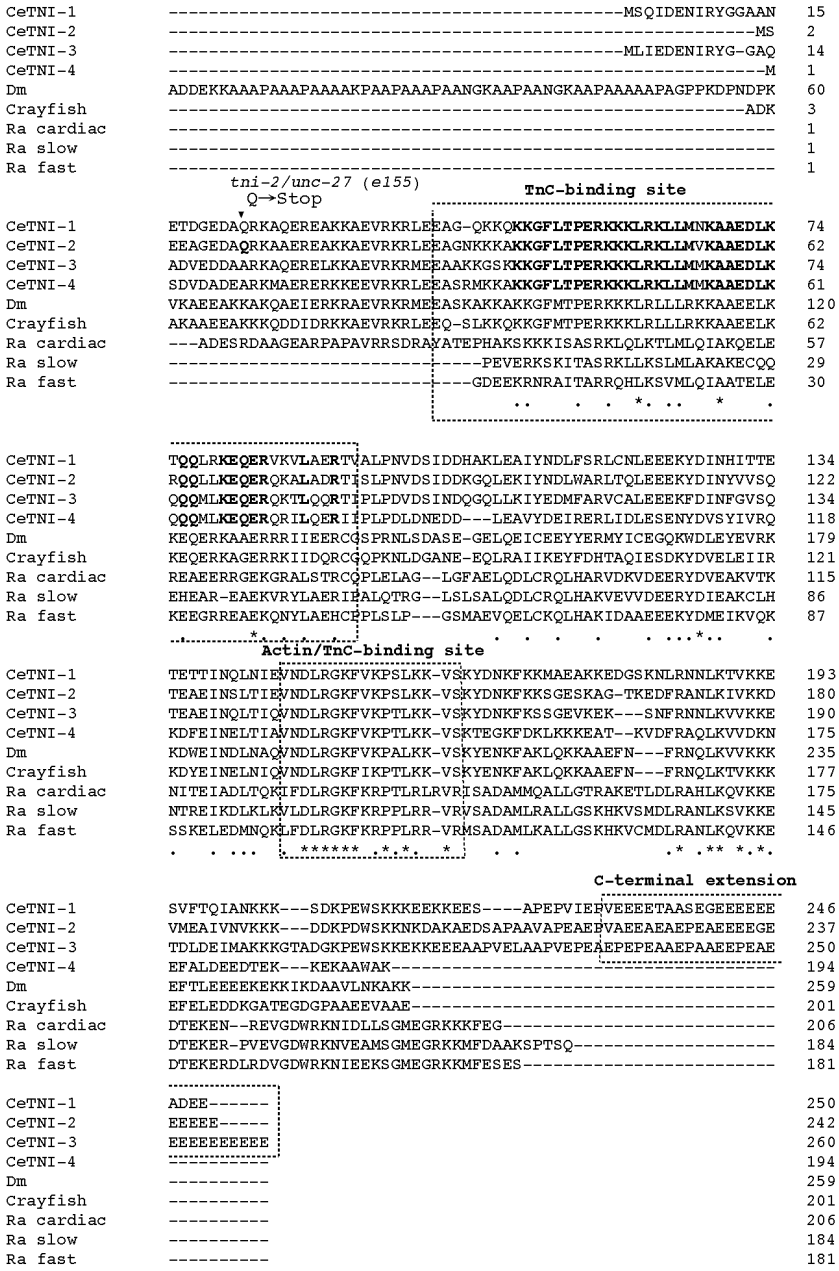


Figure 2 Sequence comparison of four CeTNIs of the worm and those of *Drosophila*, crayfish, rabbit cardiac, slow and fast muscles. Comparisons of the amino acid sequences of TNI-1, TNI-2, TNI-3 and TNI-4 of *C. elegans*, TNIs of *D. melanogaster* (Dm, accession number: P36188), *A. leptodactylus* (Crayfish, accession number: A31484), Rabbit cardiac (Ra cardiac, accession number: P02646), Rabbit slow (Ra slow, accession number: P02645) and Rabbit fast (Ra fast, accession number: P02643). The TnC-binding site, actin/TnC-binding domains were established by peptide-affinity binding studies (Wilkinson & Grand 1978). Locations of the TnC-binding site, actin/TnC-binding site and C-terminal extension are highlighted in boxes. Identity and similarity of amino acid residues are indicated by (.) and (*), respectively. Among *C. elegans* TNIs, identical amino acids are indicated in bold. Mutation site of *tni-2/unc-27* (*e155*) is indicated by an arrowhead: Gln^{10} stop for *e155*.

Expression of *tni-1/-2/-3::lacZ* began at the comma stage (Fig. 4B, panel e, f, g) and continued from the L1 to adult stages in body wall muscles (Fig. 4B, panel a, b, c). The *tni-1::lacZ* animals showed only weak expression in the body wall muscles but the *tni-2::lacZ* construct gave strong expression in all the body wall muscles (Fig. 4B, panel a, b). The detailed localization of the expression of both genes was confirmed by co-expression experiments using *tni-1::rfp* and *tni-2::gfp* (Fig. 4C, see later). The *tni-3::lacZ* construct was expressed in all the body wall muscles from the L1 to adult stages (Fig. 4B, panel c). In the adult stage, *tni-3::lacZ* expression in body wall muscles became weaker with time although strong expression persisted in the head and vulval muscles.

More details of body wall expression of the *tni-1*, *tni-2* and *tni-3* genes were observed in transgenic animals co-expressing *tni-1::rfp* and *tni-2::gfp* or *tni-2::gfp* and *tni-3::rfp* constructs. Interestingly, the *tni-1* gene was weakly expressed in the same cells as *tni-2* (Fig. 4C, panel a, b, c). The *tni-2* and *tni-3* genes were also expressed in the same cells of body

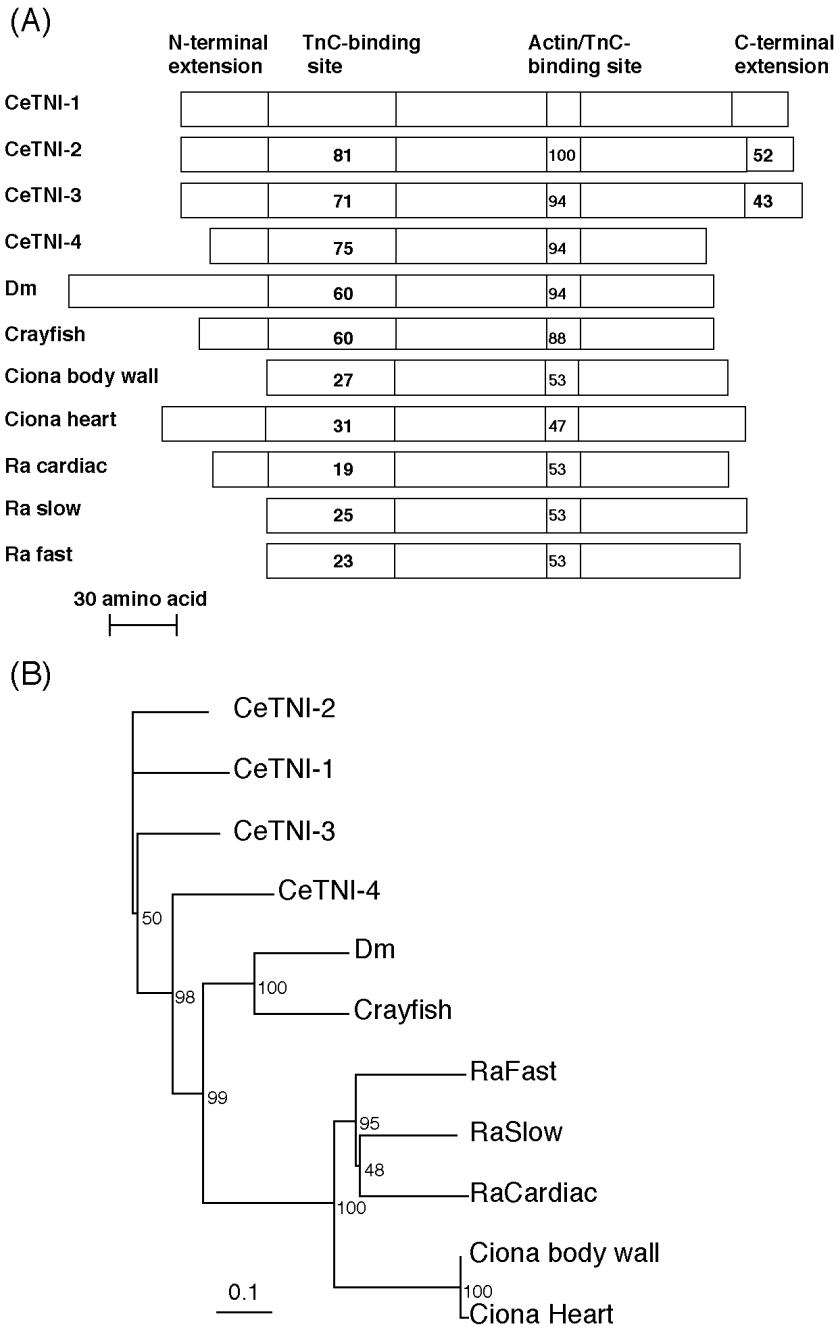


Figure 3 Structural homology and phylogenic alignment of TNI isoforms of invertebrates and vertebrates. (A) The two regulatory regions, TnC-binding and actin/TnC-binding sites showed conserved homology among invertebrates. Numbers represent percentage homology. TNI-1, TNI-2 and TNI-3 of *C. elegans* had unique C-terminal extensions. (B) Phylogenic alignment of four TNI isoforms of *C. elegans*; CeTNI-1, CeTNI-2, CeTNI-3 and CeTNI-4 and TNIs of *Drosophila*, crayfish, *Ciona* body wall, *Ciona* heart, rabbit cardiac (Ra cardiac), rabbit slow (Ra slow) and rabbit fast (Ra fast). The tree was derived using CLUSTALW and TreeView software. The numbers shown at the branch points represent the number of times the cluster denoted appeared during 100 bootstrap resamplings of the sequence data. Branch length sums indicate observed sequence differences. Scale bar shows 10% sequence difference. The amino acid sequences are the same as in Figure 2 and that of *Ciona* as in MacLean *et al.* (1997).

wall, vulval and anal muscles (Fig. 4C, panel d–l), but while *tni-2* was uniformly expressed in body wall muscles (Fig. 4C, panel b, d, g, j), the *tni-3* gene was strongly expressed in the head, vulval and anal muscles (Fig. 4C, panel e, h, k). Expression of *tni-2::gfp* was observed in the vulval and anal muscles (Fig. 4C, panel g, j) in addition to those muscles detected with *tni-2::lacZ* (Fig. 4B, panel b). This was the result of the inclusion of a longer 5′ upstream region of *tni-2* that includes the NdE-box enhancer for vulval expression (Fig. 5A, see later).

Because the *tni-1*, *tni-2* and *tni-3* genes were expressed in body wall muscles, we searched for the distribution of E-box (CANNTG) and the 1275, 1330 enhancer sequences which are the binding sites for the product of the CeMyoD gene, *hlh-1*, that is responsible for body wall muscle expression (Krause *et al.* 1994). E-box and

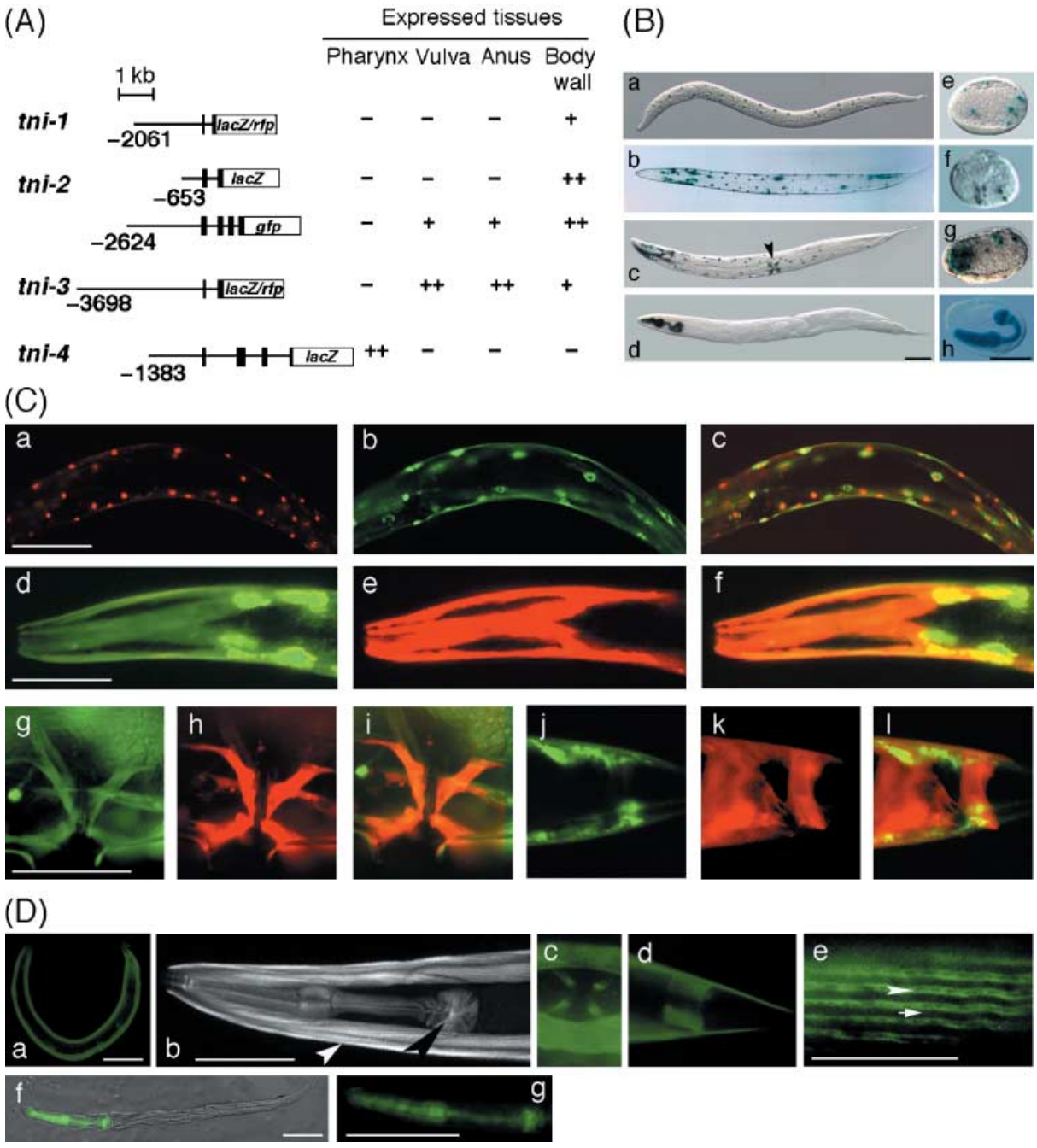


Figure 4 Expression patterns of four CeTNI isoforms and co-expression of three body wall isoforms. (A) Constructs of four *tni::lacZ/gfp/rfp* fusion genes and summary of expression profiles of these constructs. The relative levels of expression; ++, + and – are based solely on the intensity of the staining. Numbers represent the length of upstream regions and exons of each gene are indicated by black boxes. (B) Expression patterns of CeTNI isoforms in young adult and embryonic stages of *tni::lacZ* animals. a, Weak β-galactosidase expression of *tni-1::lacZ* was induced in body wall muscle. b, Strong expression of *tni-2::lacZ* was induced in body wall muscle. c, Strong β-galactosidase expression of *tni-3::lacZ* was induced in anterior part of the body and vulva muscle cells. The arrowhead indicates vulval expression. d, Strong β-galactosidase expression of *tni-4::lacZ* was induced in pharynx. β-galactosidase expression was restricted to the nuclei because the *lacZ* expression vector contained the SV40 nuclear localization sequence. e and f, Expressions of *tni-1* and *tni-2* were weakly observed in body wall at embryonic stage, respectively. g, *tni-3* was expressed in anterior body wall muscle from early embryonic stage. h, Strong expression of *tni-4* was in pharyngeal muscles at two-fold stage. In all panels, embryos are positioned with head pointing

Table 1 Summary of the results obtained in this study on four TnI genes

Structure (Figures 1–3)			**Localization** (Figure 4)	**Function** (Figure 6)	**Interaction** (Figure 7)	
Iso form/ Gene	ORF in cosmid (LG)★	cDNA (bp)/ Protein	Expressed tissues (a.a. residues)†	Phenotype of RNAi‡	CeTNC-1	CeTNC-2
CeTNI-1/*tni-1*	F42E11.4 (X)	1036/250	Body wall	Unc, poor backward movement	+	+
CeTNI-2/ *tni-2/unc-27*	ZK721.2 (X)	936/242	Body wall, vulva, anus	Unc, movement poor backward movement	+	+
CeTNI-3/*tni-3*	T20B3.2 (V)	935/260	Body wall, vulva, anus	Abnormal body morphology, Con, Egl, Pvl	+	+
CeTNI-4/*tni-4*	VO3F8.1 (IV)	775/194	Pharynx	Embryonic lethal, absence of pharynx	–	+

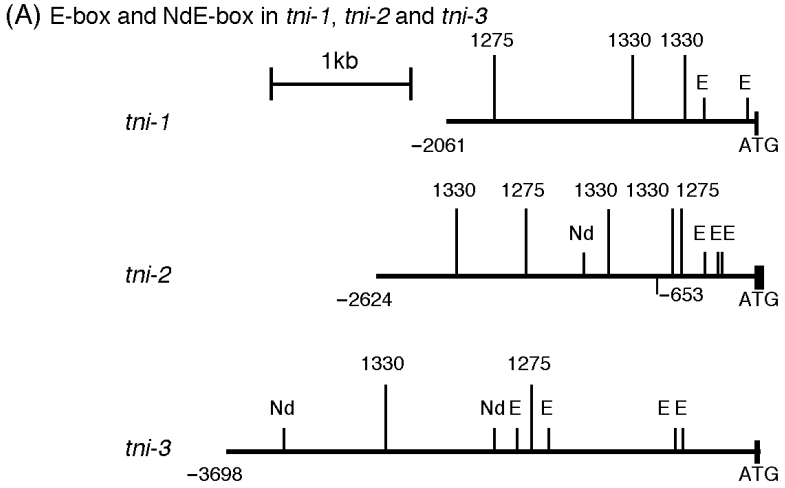
★*C. elegans* genome sequence database, WormBase (URL of this site is: http://www.wormbase.org/).
†The 5′ ends and the ATG positions of four TnIs were confirmed by RT-PCR in this study.
‡Differences of RNAi results (Simmer *et al.* 2004; Kamath *et al.* 2003; Maeda *et al.* 2001) reported in WormBase have been described in Discussion.

1275, 1330 enhancer sequences occur in the 5′ upstream regions of the *tni-1*, *tni-2* and *tni-3* genes responsible for their expression patterns (Fig. 5A). We also searched the CeTwist-binding site (CATATG) known as the NdE-box that is responsible for vulval expression (Harfe & Fire 1998) and found a single NdE-box in *tni-2* gene, located at position 1621 upstream of *tni-2::gfp* and two others in the *tni-3* gene, present at positions 1758 and 3587 in the upstream region of *tni-3::lacZ/rfp* (Fig. 5A). This is consistent with the observed vulval expression of the *tni-3::lacZ*, *tni-3::rfp* and *tni-2::gfp* gene constructs (Fig. 4B, panel c and Fig. 4C, panel g, h). Overall these results suggest that body wall expression of *tni-1*, *-2* and *-3* are regulated by the CeMyoD and *hlh-1*-enhancers and that vulval expression of *tni-2* and *tni-3* are regulated by the NdE-box-binding protein.

tni-4::lacZ was expressed only in the pharynx from the twofold to adult stages (Fig. 4B, panel d, h). We searched the *B* and *C* subelements in the upstream region of *tni-4* gene and *tni-4::lacZ* construct that are responsible for the pharyngeal expression of the myosin heavy chain gene, *myo-2* (Okkema & Fire 1994). Among the 31 nucleotide sequences of *B* subelement, the CEH-22 binding site (AAGTG) was conserved in *tni-4*. A second unknown binding site (AAAATG) in the *B* subelement was also found in the upstream region of the *tni-4* genes of *C. elegans* and the related nematode *C. briggsae* (Fig. 5B). Nucleotide alterations in the AAAATG region (*Bmut4*) reduce transcriptional activity of pharyngeal expression of *myo-2* (Okkema & Fire 1994). CEH-22, together with a second unknown factor could control the pharyngeal expression of *tni-4* in *C. elegans*. PHA-4 and PEB-1 are two binding proteins of the *C* subelement (Kalb *et al.* 2002). The 5′ upstream regions of the *tni-4* genes of both *C. elegans* and *C. briggsae* have a partial homology to the PHA-4 binding site of *myo-2* (Fig. 5C) suggesting that PHA-4 could also regulate *tni-4* pharyngeal expression.

TNI localizations were also detected by indirect immunofluorescence staining of wild-type animals using anti-

to the left. Bar represents 50 μm. (C) Co-expression of three body wall isoforms in transgenic animals. a, Weak expression of *tni-1::rfp* in body wall muscles. b and d, Strong expression of *tni-2::gfp* in body wall muscles and head region, respectively. c, Merged image of *tni-1::rfp* and *tni-2::gfp*. e, Strong expression of *tni-3::rfp* in the head region. f, Merged image of *tni-2::gfp* and *tni-3::rfp*. g, Lateral view of the vulva of *tni-2::gfp*. h, Lateral view of the vulva of *tni-3::rfp*. i, Merged image of the vulva of *tni-2::gfp* and *tni-3::rfp*. j, Lateral view of the anal part of *tni-2::gfp*. k, Lateral view of the anal part of *tni-3::rfp*. l, Merged image of the anal part of *tni-2::gfp* and *tni-3::rfp*. Bar represents 50 μm. (D) Immunostaining of wild-type animals with the anti-CeTNI-2 antibody (a–e) and anti-CeTNI-4 antibody (f–g). a, Staining of the body wall muscles. b–d, Each panel shows the following high magnification of head region; arrowhead indicates the staining of body wall and pharynx (b), Vulval muscles (c), Anal muscles (d), High magnification fluorescence micrograph of body wall musculature (e). Note that the region stained with the anti-CeTNI-2 antibody is located in the I-band of the thin filament region. Arrow indicates A bands, arrowhead indicates I-bands. f, Staining of the pharyngeal muscles. g, High magnification of the pharyngeal muscles. Bar represents 50 μm.



(B) *B* subelement in *tni-4*

B207	5′ CEH-22	Second factor binding site	3′
Ce myo-2	−519 **AAGTG**GTTGTGTGGATAAGAGTAGC**AAAATG**		−489
Ce tni-4	−504 **AAGTG**agaaaaa--ATtA---TAtt**AAAATG**		−479
Cb tni-4	−275 **AAGTG**tgT----cttTgtG---gtag**AAATG**		−252

(C) *C* subelement in *tni-4*

C183	5′ PHA-4 PEB-1		3′
Ce myo-2	−434 TCTC**GTTGTTTGC**CGTCGGATGTCTGCC		−407
Ce tni-4	−822 gCTt**GTTGTT**gt**C**CtgatagaaTtTaaa		−794
Cb tni-4	−251 aaaa**GT**gaa**TTGC**CtTCGGgaGcCccgt		−224

Figure 5 Regulatory sequences of the body wall and pharynx expressions of the TnI genes. (A) Distribution of E-box, enhancers of *hlh-1* and NdE-box in the 5′ upstream of the *tni-1*, *-2* and *-3*. E: E-box, 1275, 1330: the enhancer sequences of *hlh-1* (Krause *et al*. 1994), Nd: NdE-box. (B) Comparison of the *B207* sequence of *myo-2* and the upstream regions of *tni-4* in *C. elegans* and *C. briggsae*. (C) Comparison of *C183* sequence and the 5′ upstream regions of *tni-4* in *C. elegans* and *C. briggsae*. Conserved nucleotides are shown in boldface uppercase. CEH-22, PHA-4 and PEB-1 binding sites are underlined.

CeTNI-2 and anti-CeTNI-4 antibodies (Fig. 4D). The anti-CeTNI-2 antibody strongly stained the body wall muscle (Fig. 4D panel a), and faintly the pharyngeal muscle (Fig. 4D, panel b, resulting from the cross reactivity of anti-CeTNI-2 antibody, shown in Fig. 7A, left panel), vulval muscles (Fig. 4D, panel c) and anal muscles (Fig. 4D, panel d). At high magnification the anti-CeTNI-2 antibody stained the thin filaments in the I-band regions that also contain actin (Fig. 4D, panel e). The anti-CeTNI-4 antibody stained only the pharynx muscles (Fig. 4D, panel f, g). These results confirm the expression patterns determined using the reporter gene constructs.

Function: RNAi analysis of four TnI genes

Single or multiple losses of TnI function were assessed by RNAi experiments. Because muscles are required for most activities of *C. elegans*, observations were made of body morphology, locomotion, egg laying and viability of eggs. Seventy percent of *tni-1* (*RNAi*) (n = 28) animals at L1 stage could not elongate their bodies and were unable to move, especially their posterior parts. Animal motility is more clearly detectable in liquid medium at the adult stage. Wild-type (n = 20) and *tni-1* (*RNAi*) animals showed 120 (± 4.5 s.d.) and 32.4 (± 3.6 s.d.) waves/min, respectively. *tni-2* (*RNAi*) (n = 25) animals showed only 1.5 (± 0.5 s.d.) waves/min and 80% of these animals were without sinusoidal trails on NGM (Nematode Growth Medium) plate (Fig. 6B). The null *unc-27* (*e155*) (n = 20) animals showed 24.8 (± 2.7 s.d.) waves/min, and *tni-2* (*RNAi*) towards *unc-27* (*e155*) (n = 20) animals showed 9.4 (± 2.8 s.d.) waves/min. *tni-2* (*RNAi*) animals exhibited a more severe uncoordinated motility (Fig. 6B) than that of the *unc-27* (*e155*) animals. The TNI-band intensity of *unc-27* (*e155*) animals was detected as 10% of the wild-type in Western analysis (not shown). This result

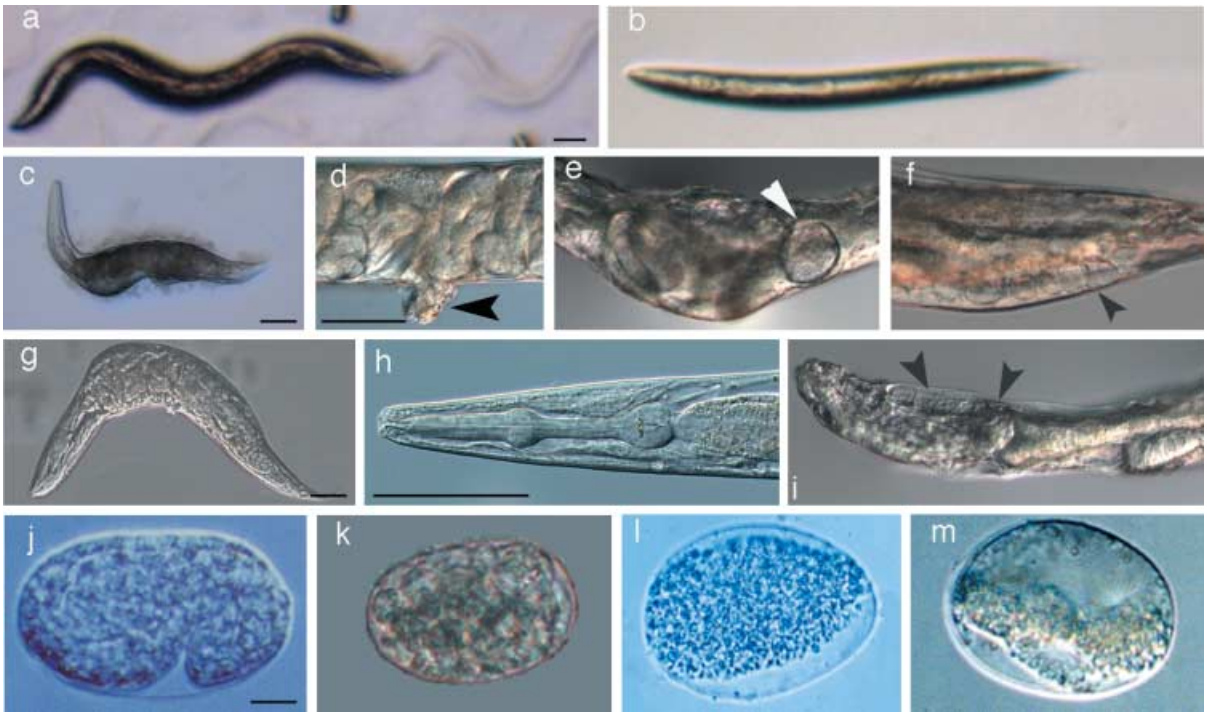
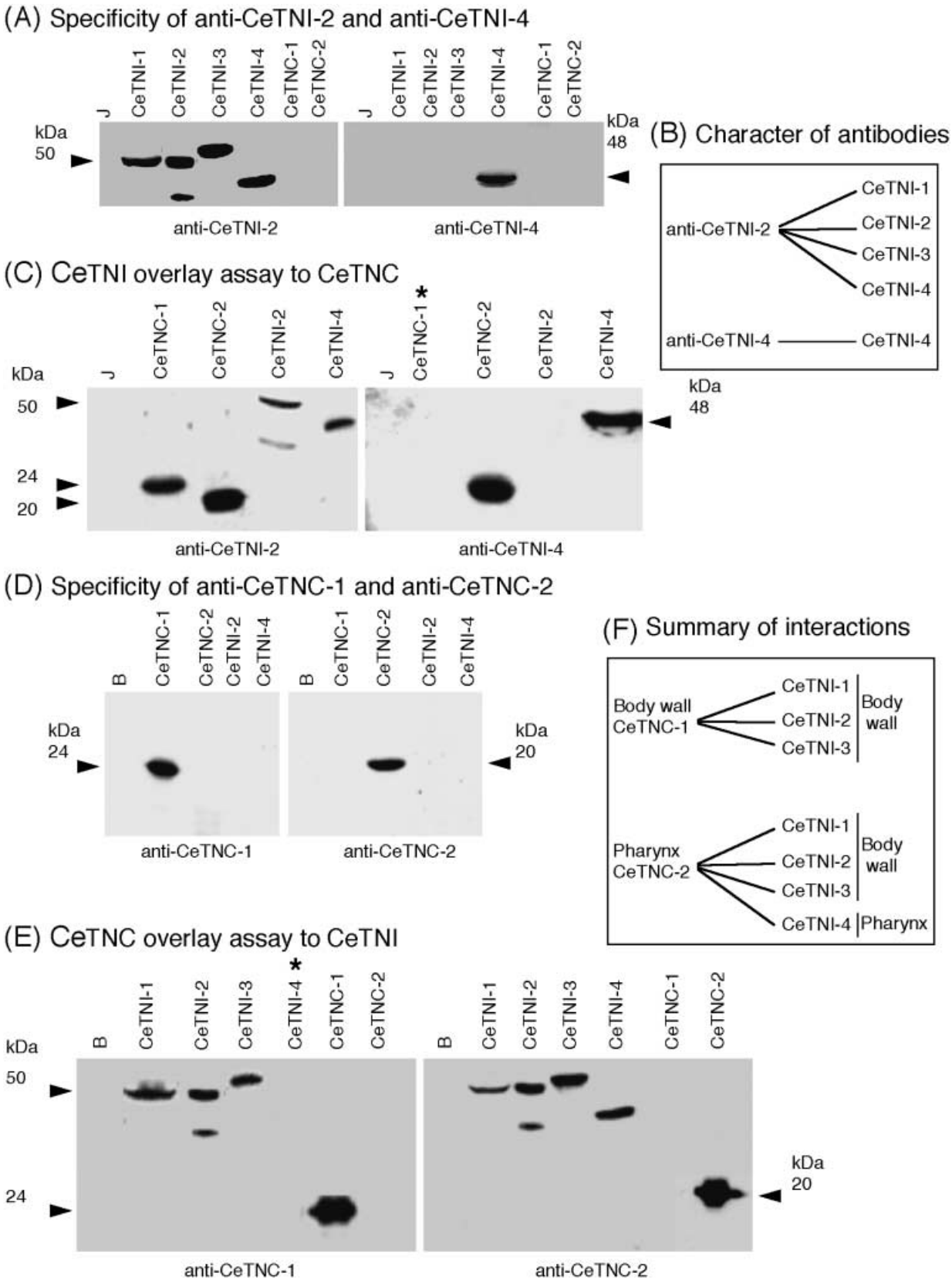


Figure 6 RNA interference of four CeTNI isoforms. (A) Wild-type animal and (B) *tni-2* (*RNAi*) animal on NGM plate. (C–F) *tni-3* (*RNAi*) animal phenotypes in young adult stage; (C) Overall morphology of young adult stage, Arrowheads indicate high magnification of (D) protruding vulva, (E) vacuole-like structures near the vulva, and (F) vacuole-like structures near the intestines and anus, respectively. (G) *tni-1/-2/-3* (*RNAi*) animal phenotypes in L2 stage. (H) Normal head region of wild-type at high magnification. (I) Head region of *tni-1/-2/-3* (*RNAi*) animal at high magnification. Arrowheads indicate the abnormal head muscle cells and deformed pharynx. (J) Wild-type embryo at twofold stage at 500 min. (K–M) *tni-4* (*RNAi*) embryos; (K) At 400 min, development of egg ceased at gastrulation stage, which would have attained at least the twofold stage in wild-type embryo. (L) At 36 h, cells had shrunk and reduced in number. (M) At 72 h, cells at the midsection towards the upper part were alive while those of the lower part were not viable and could not reach 'pretzel'-stage. Bars represent 50 μm.

is consistent with the deformed muscle morphology of the *unc-27* (*e155*) mutant animals (Burkeen *et al.* 2004). These authors also reported that the null allele *unc-27* (*e155*) does not produce the most severe defects compared to the weak alleles. This means that mis-sense mutations (especially those classifiable as dominant negative mutants) or RNAi treatments (perhaps resulting from incomplete isoform specificity) can have more serious effects on filament assembly and muscle formation than null mutations. We cannot rule out the possibility that a severe functional defect of *tni-2* (*RNAi*) animals on motility may arise from some interference of other isoforms as a result of nonspecific binding by related RNAi species.

Seventy percent of *tni-3* (*RNAi*) ($n = 25$) animals showed abnormal body morphology, egg laying defects (Egl) and protruding vulva (Pvl) from L4 to adult stages (Fig. 6C–F). These animals were severely constipated (Con) and their intestines were filled with *E. coli* (Fig. 6C). There were no vulval contractions and the animals were unable to lay eggs (Fig. 6D). Vacuole-like structures were found near the vulva (Fig. 6E) and in the region of the anus (Fig. 6F).

More than 90% of animals injected with the *tni-1/-2/-3* (*RNAi*) mixture ($n = 27$) exhibited from the L1 stage abnormal body morphology, prominent swelling near the vulva and intestines with similar phenotypes to those of *tni-3* (*RNAi*) animals observed at the L4 stage (Fig. 6G). *tni-1/-2/-3* (*RNAi*) animals with many vacuoles near the vulva, intestine and anus, and were severely constipated, moved uncoordinatedly and exhibited the Egl phenotype at the adult stage (not shown) similar to *tni-3* (*RNAi*) animals. The cuticles of young adults with the Egl phenotype had burst and were actually punctured. Compared to *tni-2* (*RNAi*) and *tni-3* (*RNAi*) animals, the head region of *tni-1/-2/-3* (*RNAi*) animals had abnormal muscle cells with deformed pharynxes (Fig. 6I). Phenotypic effects in these RNAi animals appeared mainly restricted to muscles expressing these genes (Fig. 4B,C).



tni-4 (*RNAi*)-treated (n = 20) animals produced 70% dead embryos before the comma stage (Fig. 6K–M). This is in contrast to wild-type control worms injected only with buffer (n = 21) which showed 3% dead embryos. Embryonic cells totally degraded between 400 and 500 min and by approximately early 400 min, embryos had shown developmental arrest at gastrulation, the 28-cell stage (Fig. 6K). Initially, arrested embryos exhibited a severe shrinkage as a result of loss of water (Fig. 6L) but by 500 min further degradation decreased cell size with more cell loss (Fig. 6M). Fifteen percent of surviving *tni-4* (*RNAi*) animals showed no pharynx pumping but grew normally. Generally, at about 350 min, cell proliferation largely ceases and the completely formed internal cylinder of the pharynx starts to pump at about 760 min. *tni-4* (*RNAi*)-treated embryos arrested development before the pharynx pumping was initiated. Despite the presence of transcription factors such as *pha-4* (Kalb *et al*. 2002), *peb-1* (Kalb *et al*. 2002), *ceh-22* (Okkema & Fire 1994) and other muscle genes known to be expressed in the pharynx, the embryonic arrest indicates that completion of normal pharynx formation requires *tni-4* function. This is consistent with previous RNAi experiments with two pharynx Tm isoforms, CeTMIII and CeTMIV, which showed that developmental effects appeared at a much earlier stage than body wall muscle development (Anyanful *et al*. 2001).

Using the RNAi feeding (Kamath *et al*. 2003; Simmer *et al*. 2004) and soaking methods (Maeda *et al*. 2001), *tni-1* (*RNAi*) animals showed dumpy, Egl and Unc phenotypes. *tni-2* and *tni-3* (*RNAi*) animals appeared wild type and *tni-4* (*RNAi*) animals grew slowly. Our RNAi study (by microinjection) gave much more severe phenotypes and was consistent with the tissue expression patterns (Table 1, Figs 4 and 6), suggesting that this method more accurately reflects the results of other approaches to determine gene function.

Interaction: *In vitro* molecular interaction between four CeTNI and two CeTNC isoforms

Anti-CeTNI-2 antibody cross-reacted with four CeTNIs but did not cross-react with the CeTNCs (Fig. 7A, left panel, Fig. 7B). The smaller band of CeTNI-2 may have come from limited proteolysis of CeTNI-2 in bacteria or from a second initiation product of the construct. Anti-CeTNI-4 antibody specifically reacted only with CeTNI-4 (Fig. 7A, right panel, Fig. 7B). CeTNI-2 and CeTNI-4 were overlaid on to two CeTNCs and were detected using anti-CeTNI-2 and anti-CeTNI-4 antibodies, respectively. The overlay indicated that CeTNI-2 bound to both CeTNC-1 and CeTNC-2 (Fig. 7C, left panel), but that CeTNI-4 specifically bound to CeTNC-2 (Fig. 7C, right panel).

After determining the specificity of anti-CeTNC-1 and anti-CeTNC-2 antibodies by Western analysis (Fig. 7D), we performed overlay assays of each CeTNC on the four CeTNIs. CeTNC-1, the body wall type isoform, bound specifically to all three body wall type CeTNIs (CeTNI-1, -2, -3) (Fig. 7E, left panel, Fig. 7F) but that of pharynx type CeTNC-2 bound to all four CeTNIs (Fig. 7E, right panel, Fig. 7F).

Figure 7 Western analysis and protein overlay assay of CeTNI and CeTNC isoforms. (A) Western analysis of four CeTNIs with anti-CeTNI-2, anti-CeTNI-4 antibodies. Arrowheads indicate the molecular sizes of kDa. (B) Character of anti-CeTNI-2 and anti-CeTNI-4 antibodies. (C) Protein overlay assay of CeTNI-2 and CeTNI-4 isoforms on CeTNC-1 and CeTNC-2. Overlaid CeTNI-2 and CeTNI-4 were detected by anti-CeTNI-2 antibody (left panel) and by anti-CeTNI-4 antibody (right panel). Arrowheads indicate 24 kDa and 20 kDa were the positions of CeTNC-1 and CeTNC-2, respectively. (D) Western analysis of two CeTNC isoforms with anti-CeTNC-1 and anti-CeTNC-2. (E) Protein overlay assay of CeTNC-1 and CeTNC-2 isoforms. Overlaid CeTNC-1 and CeTNC-2 were detected by anti-CeTNC-1 antibody (left panel) and by anti-CeTNC-2 antibody (right panel), respectively. Lane J: Total proteins of *E. coli* JM109; Lane B: Total proteins of *E. coli* BL21 (DE3); other lanes: Total protein of *E. coli* haboring each of expression construct, which is producing CeTNI-1, CeTNI-2, CeTNI-3, CeTNI-4, CeTNC-1 and CeTNC-2, respectively. Arrowheads indicate 50 kDa CeTNI-1, CeTNI-2 and CeTNI-3 and 48 kDa CeTNI-4. Fifteen percent and 12% acrylamide gels were used for CeTNI and CeTNC analysis, respectively. It was noted that CeTNC-1 and CeTNI-4 did not interact with anti-CeTNI-4 and anti-CeTNC-1 antibodies, respectively, as shown by asterisks ((C) right and (E) left panel). (F) Summary of interaction between isoforms of CeTNI and CeTNC. This was obtained from the results of (C) and (E). More details are described in the text.

Discussion

We have obtained various forms of evidence to determine the evolution of the *C. elegans* family of TnI isoforms. The experiments focused on examining gene and protein sequences, gene structure, determining their expression patterns, using RNAi to cause isoform-specific *in vivo* interference and assessing interactions between the four TnI and two TnC isoforms using binding assays.

Structural differences among four TnI genes and TNI isoforms of *C. elegans*

We have fully characterized the gene structure of the four TnI genes in *C. elegans* and the amino acid sequences of

the TNI isoforms they produce. Results obtained on TnI gene structures (Fig. 1) and phylogenetic analysis from the sequence comparisons (Figs 2 and 3) allowed us to determine the evolutionary relationships of the four TnI genes. In particular, we found that the TnC-binding sites of three of the genes, *tni-1*, *-2* and *-3* are split by an intron while that of *tni-4* is not. The actin/TnC-binding sites are split differently in the *tni-3* and *tni-4* genes, but the amino acid sequences are identical between them. Although the presence of N-terminal extensions of the four CeTNI are a common feature found in other invertebrates, the C-terminal extensions of CeTNI-1, -2 and -3 are unique. From these results we speculate that the *tni-4* gene could be closer to the ancestral gene for the four TnI genes in *C. elegans* (Fig. 3A,B). This assumption was strongly supported by the following expression and functional analyses.

Functional differences in four CeTNI isoforms

Differences in expression patterns between the four TnI genes

Following gene duplication, it is proposed that new isoforms can evolve so that the organism can achieve new capabilities (MacLean *et al.* 1997), in this case by the evolution of different muscle types. A comparison of the tissue and stage-specific expression patterns should therefore be revealing about the evolution of this gene family. The TnI expression patterns obtained using the *tni-1::rfp* and *tni-2::gfp* or *tni-2::gfp* and *tni-3::rfp* transgenic animals were important in identifying major differences in expression of the three body wall muscles TnI isoforms (Fig. 4C). The three genes, *tni-1*, *tni-2* and *tni-3*, were co-expressed in all the body wall muscles, but at different levels (Fig. 4C, panel a–l) under the control of the 5′ upstream regulatory sequences (Fig. 5A).

The *tni-4* gene is expressed solely in the pharyngeal muscles (Fig. 4B, panel d) which are formed at much earlier stages of the nematode development than the body wall muscles. The *tni-3* gene could have duplicated from the original and formed a body wall isoform. Compared to the rhythmic contractions of the pharynx, the body wall muscle contractions are stimuli-dependent and calcium-regulated.

Phenotypic differences by RNAi treatment in four CeTNI isoforms

The effects of interfering with isoform function using isoform-specific RNAi constructs shows that the CeTnI genes have clearly evolved to achieve differences in functional importance. While RNAi for the *tni-1* and *-2* genes produce abnormal locomotion, only RNAi treatment interfering with *tni-3* function led to abnormal muscle morphology, egg laying defects, and constipation, the latter two as a result of an absence of contractions of the vulva and anus, and associated vacuolization. This suggests that within the body wall-expressed genes, the *tni-3* gene may be especially important. Inhibition of *tni-4* gene expression produced large numbers of dead embryos from injected individuals, with survivors showing no pharyngeal pumping; RNAi treated embryos arrested at gastrulation long before pumping was initiated. Clearly, the multiple TnI genes have evolved to encode functionally different isoforms with different expression patterns.

Nongthomba *et al.* (2003, 2004) report on muscle hypercontraction phenotypes in *Drosophila* caused by the mutant alleles of the TnI genes. We observed uncoordinated phenotypes in *tni-1* (*RNAi*) and *tni-2* (*RNAi*) animals that showed weak backward movements. This suggests that defects in Ca^{2+} regulation can produce hypercontraction or Unc phenotypes in *C. elegans* as observed in *Drosophila*. Currently, no mutant has been isolated in the *tni-3* gene, but the phenotypes seen with the *tni-3* (*RNAi*) animals (Fig. 6C–F), from egg laying defect (Egl), protruding vulva (Pvl) and constipation (Con), it is possible to isolate some *tni-3* mutants suggest *tni-3* mutants might be recoverable by selecting for these phenotypes. Isolating mutants in the *tni-3* gene would allow us to determine the sites responsible for the functional differences in this isoform compared to the others.

Interaction patterns between four CeTNI and two CeTNC isoforms

The interaction between TnI and TnC is a pivotal one in the function of the Tm-Tn complex. We have therefore explored TnC-TnI *in vitro* binding to determine whether there are isoform-specific differences that may indicate that the isoforms have functionally diverged during evolution. The results of the protein overlay assay (Fig. 7F) imply that the body wall CeTNI-2 bound to the body wall and pharynx types of CeTNC (Fig. 7C, left panel) whereas pharynx CeTNI-4 only bound to pharynx CeTNC-2 (Fig. 7C, right panel). In contrast, the body wall CeTNC-1 bound to three body wall CeTNI isoforms (Fig. 7D, left panel) but pharynx CeTNC-2 bound to both body wall and pharynx types of CeTNI isoforms (Fig. 7E, right panel).

Recently, the crystal structure of the core Tn complex has been determined. The authors stated that the interaction sites between Tn and Tm/actin could be unique in each molecule of the complex in different tissues and animals (Takeda *et al.* 2003). Using our system, a reverse genetic approach in which a designed gene can be introduced in the animal should be possible and would allow

investigation of which parts of the molecule, especially the isoform-specific parts, function to produce muscle-specific changes in function that subsequently contribute to animal behavior.

It is known that vertebrate cardiac TnC and TnI interact with each other more weakly than the corresponding components of skeletal muscle (Liao *et al.* 1994). At present, neither the site of interaction between CeTNI and CeTNC, nor the location of antibody detection sites of either antibodies are known. Molecular dissection techniques with epitope mapping of CeTNI will help to determine which part of the molecule interacts with other molecules (Hamada *et al.* 2002). If a difference can be found in the binding site the results will suggest coevolution between the CeTNIs and CeTNCs.

Functional relations to other animal TnIs

The C-terminal region of TnI is essential for Tm–actin and TnC interaction in the case of rabbit and chicken (Van Eyk & Hodges 1988). It is not known why the C-terminal extensions, which are extremely rich in glutamate, are needed for the function of the three body wall TnI isoforms in *C. elegans* (Figs 2 and 3A) and also in *C. briggsae* (not shown). In fact, there is, as yet, no direct evidence that these extensions are important for TnI functions. However, the C-termini of TnTs in *Drosophila* and crayfish *Astacus* show conserved glutamate-rich extensions (Fyrberg *et al.* 1990; Benoist *et al.* 1998; Domingo *et al.* 1998) that it has been proposed might enhance cooperation of Tn-Tm complexes within thin filaments (White *et al.* 1987). The CeTNI may have a homologous function. Glutamate has a negative charge, which may contribute to protein–protein interactions under different Ca^{2+} concentrations. The C-terminal hydrophilic regions of the three body wall type CeTNIs could be important for interactions with CeTNC-1 in body wall muscles.

The evolutionary relationships of the nematode TnI genes

The pattern that emerges from our studies of gene structure, protein sequence, expression patterns and biochemical function (CeTNI-CeTNC binding) is that the CeTnI genes fall into two distinct groups: the body wall-expressed genes (*tni-1*, *-2* and *-3*) and the pharyngeal expressed gene, *tni-4*. Of these, the *tni-4* gene appears most likely to be close to the ancestral gene from which this protein family evolved.

The data suggest that *tni-3* may be the original body wall-expressed gene. It is expressed widely in the body wall and the RNAi studies show that it is required and has a greater effect on a number of body wall muscle functions — movement, vulval and anal contractions, while *tni-1* and *tni-2* gene functions seem restricted to locomotion. We propose that the *tni-2* gene, that encoded CeTNI-2 for locomotion, arose from *tni-3*, and that *tni-1* and *tni-2* are close to each other in three points, represent the most recent gene duplication. This is supported by our observation that they are (i) located on the same chromosome (Fig. 1A), (ii) show a high homology of amino acid sequences, especially at the actin/TnC-binding site (Figs 2 and 3A), and (iii) are expressed in the same cells (Fig. 4C, panel c). Although these genes are functionally related to each other, *tni-1* expression was weaker (Fig. 4) and occurred in fewer body wall muscles. We propose that *tni-1* is a 'spare' gene; that is, it is the one of a duplicated pair of genes that has acquired the potential to function in a newly appeared tissue-specific manner in evolution. The relation between *tni-1* and *tni-2* readily fulfills these criteria. Currently, the *C. elegans tni-2* gene encodes a body wall muscle isoform necessary for locomotion as seen from the RNAi treatments. *tni-1* can be 'a spare gene' if it is sufficiently required for new function in a new functional tissue or in a different pattern of tissues. As more complete genome sequence data accumulate, this kind of gene, 'a spare gene', should be reported from other organisms. The relation between gene duplication and functional divergence to produce new or modified function tissues, changes in morphology and development processes is one of the key subjects in evolution. To know how a spare gene can get a function in newly appeared tissues is an interesting subject for the future.

Why has gene duplication been important in the evolution of protein families? Our studies have demonstrated with the TnI family in *C. elegans* not only that these genes likely share a common genetic origin but that they have acquired functional differences. Some of the amino acid differences between CeTNI isoforms must be important, suggesting that one TNI isoform could not readily substitute for another. To test for functional differences, it is necessary to introduce amino acid substitutions in different isoforms in those regions that interact with other members of troponin complex. As the actin/TnC-binding site between CeTNI-1 and CeTNI-2 or CeTNI-3 and CeTNI-4 are identical, this approach is hard to design. Introducing substitution into TnC-binding sites in four TnI genes could be possible but more efficient approaches could be done by injecting the chimera construct of the promoter/coding regions into the mutant animals.

It is well established that different protein classes undergo molecular evolution at different rates, presumably reflecting

different functional constraints. However, it is also the case that different isoforms of the 'same' protein, encoded by a multigene family, may evolve at different rates. Excitation-contraction coupling in body wall for locomotion, vulva for egg laying and anal for defecation show functionally different patterns and these are likely to be reflected at molecular level. This may explain why three CeTNI isoforms function within the body wall muscles although we cannot identify each of the three functions. Alternatively, the presence of more than one isoform may be necessary to modulate muscle function. As we have established TnI studies at the molecular, tissue and whole animal levels in the nematode, we can use this system to investigate in more detail the functional differences of CeTNI isoforms at the molecular and physiological levels. It is also possible to develop the worm model to study TnI mutations in human myopathies, including cardiomyopathy.

Experimental procedures

Nematode strain and culture

The *C. elegans* strains; wild-type Bristol N2, mutant *unc-27* (*e155*), microinjected with the reporter genes and RNAi treated animals used in this study were grown under standard conditions (Brenner 1974).

DNA handling and sequencing

Conventional methods were used for all DNA manipulations except where otherwise stated. The purified pBluescript SK (–) plasmids with the cloned RACE fragments were sequenced by Dye Terminator cycle sequencing kits with a 373 A DNA sequencer (Perkin Elmer, Applied Biosystems Division). DNASIS™ and GENETYX-MAC were used for sequence data analysis.

Cloning strategy and detection of mutation

A polyclonal antibody against *Ascaris* TnI (Nakae & Obinata 1993) used for immunoscreening of λZAPII cDNA library was provided by Dr Barstead (Oklahoma Medical Research Foundation, Oklahoma City, OK). The pCTnI-2 plasmid contained the cDNA insert at the *Eco*RI site of pBluescript SK (–). The 6.5 kb *Eco*RI fragment of *tni-2* and 5 kb *Hin*dIII genomic fragments of *tni-1* were obtained from a genomic DNA library constructed with size-fractionated genomic fragments cloned into a processed plasmid (pUC118). All positive colonies were identified by ECL System (Amersham Corp).

Double-stranded DNA was purified using a single worm PCR (polymerase chain reaction) method (Barstead & Waterston 1991). The *Taq* polymerase used for PCR was from TaKaRa Biochem. Amplified product was sequenced for localization of mutation site of *unc-27* (*e155*).

RNA isolation and 5′ RACE

Total RNA was isolated from wild-type nematodes using the TRIzol LS reagent (Gibco BRL), following the manufacturer's instructions. The isolated RNA was stored in three times its volume at 99% (v/v) at –80 °C.

Reverse transcription (RT)-PCR and 5′ RACE reactions were performed using RACE system (Gibco BRL), following the manufacturer's instructions. Primers used were TNI1–2a (5′-CGAAGCTTCTTCTTACGCTC-3′) for *tni-1*, TNI2–2a (5′-CCGAGCTCAGCAGCCTTGTTC-3′) for *tni-2*, Ti3Ex3A2 (5′-AAGTCGACATTTCATCGTAGACTGCCTCG-3′) for *tni-3* and Ti4Ex3A2 (5′-AAACTAGTATAGTACGCTGCTGAAG-GGTCT-3′) for *tni-4*. These cDNA products were tail-tagged with terminal deoxynucleotidyl transferase (TdT, Gibco BRL) and used to tag the cDNA products and PCR amplification for second strand DNA syntheses that were performed with SL1 primer as sense and TNI1–1a (5′-TCTTGGCCTTCTTCTTGTTTCCGG-3′) for *tni-1*, TNI2–1a (5′-CTTCTTTCTTTCTGGAGTC-3′) for *tni-2*, Ti3Ex3A1 (5′-GACGAACGATGTAGCTGA-3′) for *tni-3* and Ti4Ex3A1 (5′-GGGATAGTACGCTGCTGAAGGG-3′) for *tni-4* as anti-sense. Southern hybridization was used to confirm the presence and size of fragments of RACE products which were then cloned into pBluescript SK(–) for sequencing.

Construction of reporter gene fusions

We prepared *tni-1::lacZ*, *tni-2::lacZ*, *tni-3::lacZ*, *tni-4::lacZ*, *tni-1::rfp*, *tni-2::gfp* and *tni-3::rfp* fusions that accurately reflected the wild-type expression patterns. All constructs summarized in Fig. 4A are available upon request. Several transgenic *C. elegans* lines bearing extra chromosomal arrays containing the promoter::repoter fusion plasmids were obtained by microinjection together with the plasmid pRF4 as a marker. Strains with the extra chromosomal arrays were stained to detect β-galactosidase activity (Fire 1992). All constructs were assayed in more than two independent transgenic lines.

RNA mediated interference

The four TnI genes were amplified separately from their cDNAs by PCR using M13 (–21) and M13RV primers (Gibco). The cDNAs corresponding to their full coding regions were inserted in the pBluescript SK(–) vector. RNA was synthesized *in vitro* from the DNA template with Ambion MAXIscript™ using T3 and T7 polymerases. DNA removal, RNA extraction, suspension and annealing were performed as described (Montgomery *et al.* 1998). The dsRNA was injected into the gonads of wild-type worms. For triple RNAi, dsRNAs of three genes were mixed together and injected into wild-type worms. After recovery, worms were transferred to fresh NGM plates and further transferred to another fresh culture plates every 12 h. The injected worms are referred to as P0, and subsequent transfers as P1, P2, etc. The embryos for observation were washed from the plate by the hypochlorite method as described (Lewis & Fleming 1995) at 300 and 500 min; 12, 18, 24, 36, 60 and 72 h and kept for 5 h at 20 °C in M9 buffer for any developmental change that might occur (Anyanful *et al.* 2001). The approximate timing of *tni-4* (*RNAi*)

eggs were determined so as to allow comparison to control, buffer injected embryos. A Nomarski interference microscope (Zeiss Axioplan 2) at 400× was used for observing embryos and worms.

Troponin C and troponin I expression vectors

A 650 bp *Eco*RI fragment of cDNA clone, pCTNC1 (Terami *et al*. 1999), was inserted into the *Eco*RI site of pET28 (+) (Novagen). A 650 bp *Kpn*I-*Eco*RI fragment of *tnc-2* was cloned into the *Kpn*I and *Eco*RI sites of pUC119 (STRATAGENE) from yk366f10 cDNA clone. A *Sal*I-*Eco*RI fragment from this plasmid was recloned into the *Sal*I and *Eco*RI sites of pET28b (+).

A 1 kb *Eco*RI to *Kpn*I fragment of *tni-1* was cloned into the *Eco*RI and *Sma*I sites of pGEX-4T2 from yk103h4 cDNA clone. A 1 kb *Eco*RI fragment of *tni-2* was cloned into the *Eco*RI site of pGEX-4T3 (Pharmacia Biotech.) from cDNA clone, cm15e4 of *tni-2* ligated into pBluescript SK(–). The 1 kb *Eco*RI to *Kpn*I fragment of *tni-3* was cloned into the *Eco*RI and *Sma*I sites of pGEX-4T1 from yk147e9 cDNA clone. PCR product of *tni-4* encoding the *tni-4* cDNA without its own stop codon was generated and subcloned in frame at the *Bam*HI and *Sma*I sites of pGEX-4T2 from yk328a10 cDNA clone.

Antibody preparation, immunostaining, Western analysis and protein overlay assay

Antisera used in this study were prepared by immunizing rabbits with gel homogenates containing 1 mg of the His-tag fusion peptide which was isolated by preparative SDS-PAGE and the corresponding band was excised from the Coomassie Blue stained gel (Hamada *et al*. 2002). Briefly, the immunization procedure was as follows: antigens were injected three times into rabbits at 2-week intervals. Antiserum was collected 1 week after the last injection. Immunostaining employed β-mercaptoethanol-collagenase treatment (Williams & Waterston 1994) for worms. Antibody was affinity-purified from the antiserum by adsorption on to a membrane to which the band of the corresponding fusion peptide had been transferred. The antibody concentration was estimated from the density of the IgG band using BSA as a standard. The amount of protein loaded in each lane was approximately 0.002 μg. Western analysis was performed using the ECL detection system (Amersham International Plc.). GST-troponin I fusion proteins were over-expressed in the host strain JM109 and BL21 (DE3) and reacted with bacterial protein including TNC. Using bacterially expressed proteins, we characterized the interactions between CeTNI and CeTNC by overlay assay (Terami *et al*. 1999).

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