Aberrant Splicing of an Alternative Exon in the *Drosophila* Troponin-T Gene Affects Flight Muscle Development

Upendra Nongthomba,^{*,†,1} Maqsood Ansari,^{*,1,2} Divesh Thimmaiya,[†] Meg Stark*
and John Sparrow^{*,3}

^{*}Department of Biology, University of York, York, YO10 5DD, United Kingdom and [†]Molecular Reproduction Development and Genetics, Indian Institute of Science, Bangalore, 560 012 India

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ABSTRACT

During myofibrillogenesis, many muscle structural proteins assemble to form the highly ordered contractile sarcomere. Mutations in these proteins can lead to dysfunctional muscle and various myopathies. We have analyzed the *Drosophila melanogaster* troponin T (TnT) *up^l* mutant that specifically affects the indirect flight muscles (IFM) to explore troponin function during myofibrillogenesis. The *up^l* muscles lack normal sarcomeres and contain “zebra bodies,” a phenotypic feature of human nemaline myopathies. We show that the *up^l* mutation causes defective splicing of a newly identified alternative TnT exon (10a) that encodes part of the TnT C terminus. This exon is used to generate a TnT isoform specific to the IFM and jump muscles, which during IFM development replaces the exon 10b isoform. Functional differences between the 10a and 10b TnT isoforms may be due to different potential phosphorylation sites, none of which correspond to known phosphorylation sites in human cardiac TnT. The absence of TnT mRNA in *up^l* IFM reduces mRNA levels of an IFM-specific troponin I (TnI) isoform, but not actin, tropomyosin, or troponin C, suggesting a mechanism controlling expression of TnT and TnI genes may exist that must be examined in the context of human myopathies caused by mutations of these thin filament proteins.

TROPONIN T (TnT), troponin I (TnI), and troponin C (TnC) form the troponin complex, which with tropomyosin (Tm) act as a regulatory switch for striated muscle contraction (reviewed in GORDON *et al.* 2000). Striated muscle contraction, including that of *Drosophila* indirect flight muscles (IFM) and the tergal depressor of the trochanter (TDT) or jump muscle (PECKHAM *et al.* 1990), is activated by Ca²⁺, although the IFM also require an applied strain. At low intracellular Ca²⁺ concentrations, the TnI subunit inhibits actomyosin activity. When intracellular Ca²⁺ concentration is increased, Ca²⁺ ions bind to TnC, leading to conformational changes in the Tn–Tm complex, removing the TnI inhibition, and activating actomyosin crossbridge activity. Biochemical studies have shown that TnT is required for both complete inhibition and activation of actomyosin activity in the absence or presence of Ca²⁺ (POTTER *et al.* 1995; OLIVEIRA *et al.* 2000).

The importance of TnT is underlined by the discovery of mutations that cause human familial cardio- and nemaline myopathies (PERRY 1998; JOHNSTON *et al.*

2000; ROBERTS and SIGWART 2001; MORIMOTO *et al.* 2002; TOWBIN and BOWLES 2002).

Mutations of TnT genes in other model genetic organisms can result in severe muscle phenotypes and may also affect the expression of other thin filament protein genes. In *Caenorhabditis elegans* TnT (*CeTnT-I*), gene mutations can cause detachment of body wall muscles. It has been argued that this results from prolonged force development caused by an inability of the muscles to relax once Ca²⁺-dependent muscular activation has occurred (MYERS *et al.* 1996; MCARDLE *et al.* 1998). Studies of *silent heart* mutants in the zebrafish *TnT2* gene indicate that the muscle phenotype arises by reduced expression and accumulation of TnT2 message and protein combined with reductions of other thin filament proteins (SEHNERT *et al.* 2002). Recent studies of *D. melanogaster* TnI mutations, *heldup-2*, *hdp²* (where muscle detachment occurs once muscle contraction is activated), and *hdp³* (an IFM–TDT-specific TnI null), suggest that hypercontracted muscle phenotypes can be associated with coordinated reductions in message and protein levels of other thin filament proteins (NONGTHOMBA *et al.* 2003, 2004). To understand how TnT mutants cause defective muscle development requires a more complete understanding of how they can affect the expression and assembly of other muscle proteins.

The IFM are dispensable for survival under laboratory conditions and there are IFM-specific isoforms of many

¹These authors contributed equally to this work.

²Present address: Department of Genetics, University of Karachi, Karachi 75270, Pakistan.

³Corresponding author: Department of Biology, University of York, York YO10 5DD, United Kingdom. E-mail: jcs1@york.ac.uk

muscle structural proteins, making *Drosophila* IFM an effective genetic model for muscle studies (VIGOREAUX 2001; NONGTHOMBA *et al.* 2004). Moreover, myofibrillogenesis, from myotube formation to development of functional muscle fibers, can be easily followed *in vivo* (NONGTHOMBA *et al.* 2004). *Drosophila* muscle proteins show substantial sequence homology to their vertebrate counterparts and thin filament structure is similar to that of vertebrates (CAMMARATO *et al.* 2004). Vertebrate TnT binding domains are conserved in *Drosophila* (BENOIST *et al.* 1998), implying common roles during muscle contraction. *Drosophila* TnT isoforms differ from all vertebrate isoforms in having a polyglutamate C-terminal extension of unknown function (BENOIST *et al.* 1998; DOMINGO *et al.* 1998).

A single gene, *upheld* (*up*) encodes all *Drosophila* TnT isoforms (FYRBERG *et al.* 1990; BENOIST *et al.* 1998). It contains 11 exons of which exons 3, 4, and 5 are alternatively spliced in different muscles (BENOIST *et al.* 1998; MAS *et al.* 2004). The IFM- and TDT-specific TnT isoform, the smallest, is encoded by an mRNA made only from constitutive exons (FYRBERG *et al.* 1990; BENOIST *et al.* 1998). All *up* mutants were recovered by their effects on the IFM (absence of flight or wing position), but most, such as *up*¹⁰¹ (FYRBERG *et al.* 1990) are due to mutations in constitutive exons and show behavioral defects associated with myopathy in other muscle groups (NAIMI *et al.* 2001). However, the effects of the *up*² and *up*³ mutations are restricted to the flight and jump muscles. This is difficult to explain if the IFM–TDT-specific isoform is encoded solely by constitutive exons. These mutants accumulate little or no TnT protein in the IFM and only a few Z-bands and thin filaments are seen. The various *up* mutations show varying degrees of IFM disorganization (DEAK *et al.* 1982; HOMYK and EMERSON 1988; FYRBERG *et al.* 1990; BARTHMAIER and FYRBERG 1995).

The *up*¹ and *up*¹⁰¹ are the only extant *upheld* alleles. The phenotypic effects of *up*¹ are almost identical to those of *up*² and *up*³ (FAHMY and FAHMY 1958; DEAK *et al.* 1982), but a detailed molecular characterization is lacking. From an analysis of *up*¹, we confirm recent findings (HERRANZ *et al.* 2005) for an additional alternative *Drosophila* TnT exon 10, known as exon10a. We show that it is alternatively spliced to produce an IFM–TDT-specific isoform and that the *up*¹ mutation affects splicing of this exon, causing an absence of TnT message and protein in adult IFM. We discuss these findings and the effects of the *up*¹ mutation on muscle development and human nemaline muscle disease.

MATERIALS AND METHODS

Fly strains: Flies were maintained on a yeast-agar medium at 25°. For *Drosophila* genetic notation, see FlyBase (<http://www.flybase.org>). An *up*¹ stock, *ccw*¹ *sma*¹ *up*¹ *mal*^{F1}/FM7c, was procured from the Bloomington Stock Center. Canton-S and *Texas* were used as wild-type controls. Other genotypes are described in NONGTHOMBA *et al.* (2004).

Behavioral experiments: Walking and larval crawling experiments were performed as described by NAIMI *et al.* (2001). For jump tests, flies, with their wings removed, were placed on a 2-mm ruled grid; jumps were induced by lightly touching the dorsal thorax with a paintbrush and 8–10 jumps observed for each fly. The number of grid lines jumped was scored. Flight tests were carried out as described by DRUMMOND *et al.* (1991).

Microscopy: Polarized light microscopy was performed as described previously (NONGTHOMBA and RAMACHANDRA 1999) and recorded using a Nikon Diaphot microscope with polarizing optics. Thoraces were prepared for TEM as described by KRONERT *et al.* (1995). Sections were viewed and photographed using a Tecnai 12 BioTwin electron microscope.

Pupal/adult sample preparation: Pupae were aged following FERNANDES *et al.* (1991) and the puparia removed. Heads, thoraces, and abdomens were separated using a sharp blade and transferred immediately to ice-cold 70% ethanol (for RNA experiments) or (for the preparation of protein samples) to either 50% ethanol or York modified glycerol (YMG; 20 mM KPi buffer, pH 7.0, 1 mM NaN₃, 1 mM DTT, 2 mM MgCl₂, 50% glycerol, 1% Triton X-100) if demembration was required. For the dissection of pupal and adult IFM and TDT muscles, thoraces were cut along the midline and the hemi-thoraces separated. The muscles were detached, isolated, and transferred to either fresh 50% ethanol or YMG and left overnight at 4° or –20°.

Genomic DNA isolation, RNA extraction, and synthesis of the first-strand cDNA: Genomic DNA was isolated from flies as described in the Berkeley *Drosophila* Genome Project (<http://www.fruitfly.org/about/methods/inverse.pcr.html>). For RNA extraction, staged whole organisms (embryos, larvae, pupae dissected from their puparia, and newly eclosed adult flies) were collected and fixed in ice-cold 70% ethanol; if required, dissections of IFM and TDT muscles occurred at this stage. The samples were then transferred to either TRI Reagent (Sigma) or RLT lysis buffer (QIAGEN RNeasy kit) on ice with the addition of 2-mecaptoethanol as indicated by the manufacturer. Total RNA was extracted using the TRI reagent method or a QIAGEN RNeasy mini kit and the mRNA reverse transcribed using first-strand RT–PCR kits from Stratagene by following the manufacturer's instructions.

PCR amplification: Semiquantitative analysis of the IFM mRNA for various thin filament proteins was performed as described previously (NONGTHOMBA *et al.* 2003). PCR amplifications of TnT genomic DNA and cDNA were done with the following primers: P1 (exon 1 sense primer) 5'-AACCGCAG CATTCGCTCCTA-3'; P2 (exon 11 anti-sense primer) 5'-CTC GAGAATAGCAAGTTGTTAACTAC-3'; P3 (exon 7 sense primer) 5'-ATCGAAGGATTCGGCGAGGCTA-3'; P4 (5' UTR forward primer) 5'-GAACCGCAGAATTCGCTCCTAC-3'; P5 (exon 10a anti-sense primer) 5'-TGCGCTGAGTGAATCTTTCCTG-3'; P6 (exon 10b anti-sense primer) 5'-GGTGTATTGCTCCTTCTTC TCG-3'; P7 exon 10a-specific anti-sense primer) 5'-TTGTGC GCTGAGTGAATC-3'; and P8 (exon 10b-specific anti-sense primer) 5'-CGGTGTATTGCTCCTTCT-3'. Primers for the *ribosomal protein-49* (*rp49*) gene were used as an internal RNA standard for extraction, loading, and amplification against which the expression levels of the muscle genes were normalized.

Between 20 and 30 cycles were used for all the measurements of gene expression. PCR reaction products were assessed by 1.2% agarose gel electrophoresis. Gel images were captured using the JHBIO gel documentation system and gel quantification was done using the SpotDenso tool of the AlphaEaseFC software package from Alpha Innotech. The data were processed using MS Excel.

Gel purification, cloning, and sequencing of PCR products: PCR products were purified using QIAGEN gel purification kits, ligated to pGEM-T Easy vector (Promega),

FIGURE 1.—IFM abnormalities in 3- to 5-day-old *up*¹ flies. (A) Polarized light micrograph of wild-type IFM. (B) *up*¹ thorax showing thinned and broken fibers (arrow) as well as muscle fibers that have detached from the posterior apodemes (bottom left). (C) *up*¹/+ hemithorax showing normal myofibers. A–C: star, DLM; I, DVM. Anterior to top right. Bar, 0.22 mm. (D) EM of TS of wild-type IFM. (E) EM (TS) of *up*¹ IFM shows almost complete absence of myofibrillar organization, scattered thick and thin filaments and occasional hexagonal filament lattice patches (arrowhead). Serial repeated electron-dense structures, zebra bodies, are visible (arrow). (F) EM (TS) of *up*¹/+ heterozygote IFM shows myofibrils (Myo) with normal thick-thin filament lattice at their center but surrounded by irregular, loosely packed filaments (arrows). (G) EM of LS of wild-type IFM myofibrils showing regular sarcomeres. (H) EM (LS) of *up*¹ IFM lack sarcomeres, except for short sarcomere-like structures (arrowhead), irregularly streamed M- and Z-bands, and zebra bodies (arrow). (I) EM (LS) of *up*¹/+ IFM show more myofibrillar organization than *up*¹ hemizygotes, although Z-bands and M-lines appear streamed and thick and thin filaments can cross from one myofibril to the next (arrows). Bar for D–F (as in D) and for G–I (as in G), 1.6 μ m.

and transformed into *Escherichia coli* DH5 α cells. QIAGEN Miniprep kit was used for plasmid preparations. DNA sequencing employed T7 sequencing primers or primers used to generate the fragments and was done by either the Sequencing Facility, Department of Biochemistry, University of Oxford, or Macrogen. A minimum of three clones was recovered from each PCR and every clone sequenced three times; output was analyzed using Lasergene software (DNASTAR).

Protein techniques: One-dimensional gel electrophoresis and Western blotting of muscle proteins were conducted as outlined in NONGTHOMBA *et al.* (2001) with antibodies described in SAIDE *et al.* (1989) or NONGTHOMBA *et al.* (2004). For 2-D gel electrophoresis, the IFM from 30 flies were dissected in 50% ethanol and transferred to 250 μ l of rehydration buffer [8 M urea, 2% CHAPS in dH₂O, 0.018 M DTT, and 5 μ l immobilized pH gradients (IPG) buffer, pH 4–7]. Homogenized samples were centrifuged to remove particulate matter and 125 μ l of each supernatant adsorbed onto 7-cm pH 4–7 IPG strips, covered with 2–3 ml of Plusone Dry Strip cover fluid (Pharmacia Biotech) and left overnight at room temperature. Strips were electrophoresed at 200–2000 V for 1.5 hr (by increasing voltage by 200 V every 15 min), held at 2000 V for 10 min, and finally at 2500 V for 3 hr. Sample strips were then incubated in SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, and 2% SDS in dH₂O with 460 mM DTT) for 15 min and a second dimension electrophoresis performed using a 13% SDS-PAGE gel.

RESULTS

***upheld*¹ mutant affects both flight and jumping but not walking or larval crawling:** The *up*¹ mutation was generated by chemical mutagenesis (FAHMY and FAHMY 1958). Originally the wings-up trait was 100% penetrant in flies raised at 29° but only 60% at 18°; the wings-up phenotype

is recessive, but heterozygotes and homozygotes cannot fly, so flightlessness is dominant (DEAK 1977; DEAK *et al.* 1982). In the viable homozygous *up*¹ line, generated by recombination from the *ccw*¹ *sma*¹ *up*¹ *mal*^{F1} chromosome for this study, the penetrance of the wings-“up” phenotype in hemizygous males and homozygous females is 89–92% irrespective of temperature (18–29°).

The jumping ability of *up*¹ flies is poor from eclosion onwards and further deteriorates with age, suggesting a progressive TDT myopathy. Young flies, 1–2 days old, jump 1.4 ± 0.7 mm ($n = 18$) compared to 22.8 ± 3.3 mm ($n = 22$) for Canton-S. By day 6, few *up*¹ flies could jump and by day 10 none could. *up*¹/+ heterozygous flies jumped 7.6 ± 3.2 mm ($n = 16$), better than *up*¹ flies but not as well as wild type. Jumping ability of *up*¹/+ heterozygotes remained constant for at least 15 days after eclosion.

*up*¹ has no effects on walking ability, unlike the *up*¹⁰¹ allele (NAIMI *et al.* 2001). All flies, including Canton-S and *up*¹/+ heterozygotes, could climb the 10.5-cm height of the measuring system in 6–14 sec; larval crawling was not affected (data not shown). Overall, the behavioral data suggest that the *up*¹ phenotype is restricted to the IFM and TDT.

***up*¹ IFM and TDT muscles are disorganized:** In polarized light, *up*¹ thoraces showed partially hypercontracted IFM with some detachment of fibers (Figure 1B). IFM from *up*¹/+ heterozygous females looked normal (Figure 1C), suggesting that *up*¹ hypercontraction is recessive. The severity of the IFM phenotype does not correlate with wing position, consistent with our observations

FIGURE 2.—Age-dependent myopathy of *up'* TDT. Polarized light micrographs of TDT (star) and the first set of DVM (I) from wild type (A) and TDT (B) of 6-day-old *up'* flies. Reduced birefringence in B indicates disorganized TDT (arrow), DVM (arrowhead) myofibrils. IFM appear more severely affected than TDT. Bar, 0.15 μ m. EM (TS) of wild-type TDT (C) show the characteristic pattern of myofibrils (Myo), 6-day-old *up'* TDT (D) with disorganized myofibrils and streamed lattice (arrows). EM of (LS) of wild-type TDT (E) show well-organized sarcomeres and Z-bands in registration between neighboring myofibrils (Z) and 6-day-old *up'* TDT (F) with severely disorganized sarcomeres (arrow) and streamed Z-bands. Bar for C–F, 2 μ m.

on other muscle mutants that this character is not a reliable indicator of IFM structure or function (NAIMI *et al.* 2001; NONGTHOMBA *et al.* 2003).

The transverse section (TS) electron micrographs (EMs) of homozygous *up'* IFM show a lack of normal myofibrillar organization (Figure 1E). Myofibrillar structure is severely disrupted (compare to wild type, Figure 1D) and the hexagonal lattice of thick and thin filaments is absent, except in a few places. Fuzzy, electron-dense structures that appear to be incompletely formed Z-lines are seen in most areas. Longitudinal sections (LS) of *up'* IFM confirm the almost complete absence of organized myofibrils; most areas contain disorganized skeins of thick filaments (Figure 1H), although occasional myofibril-like structures are seen with some semblance of sarcomeres. These are narrower and shorter (1 μ m) than normal adult sarcomeres (3.3 μ m); the Z-lines are less densely stained than wild type and exhibit streaming. These myofibrils are reminiscent of nascent myofibrils from the earliest stages of myofibrillogenesis (compare to Figure 3C). Thick and skewed Z-lines arranged in short serial arrays that we have previously called “tiger-tails” (NONGTHOMBA *et al.* 2004) are seen, which are similar to zebra bodies, structures described in some human nemaline myopathies (LAKE and WILSON 1975). We will use this term for these *Drosophila* structures.

The IFM of *up'*/+ heterozygotes show a more regular filament lattice (Figure 1F) than that seen in *up'* homozygotes and myofibrils are easily identified. LS show a continuous sarcomeric arrangement, but the Z-lines are wavy (Figure 1I); this may be a mild form of streaming. Frequently, the micrographs show filament bundles that extend from one myofibril to a neighboring one. Myofibrils show a nonuniform cross-section and peripheral

filaments are incorporated into the central lattice (Figure 1F). Similar IFM disruption phenotypes are seen in the TnI IFM–TDT-specific splice mutant *hdp³* (NONGTHOMBA *et al.* 2004) and are common features of many fly muscle protein mutants (VIGOREAUX 2001).

Polarized light and EM confirm the progressive myopathy of the *up'* jump muscles. One day after eclosion, the *up'* TDT myofibrils are well organized with a uniform birefringence in polarized light indistinguishable from the wild-type controls (Figure 2A). However, within 3 days, gaps appear in the fibers (Figure 2B) that worsen with age. This suggests a decreased structural order with time. EMs of *up'* TDT from 1 to 2-day-old flies (not shown) are indistinguishable from wild type, but in 5-day-old *up'* flies, a disorganized TDT myofibrillar lattice (Figure 2D) is seen (compare to wild type, Figure 2C). The individuation of the myofibrils and the intracellular membranes are lost. Compared to wild type (Figure 2E), the *up'* TDT (Figure 2F) have disorganized myofibrils with short sarcomeres, in which streamed Z-lines are no longer in register with those of neighboring myofibrils. In extensive regions, Z-lines are broken and sarcomeric structures are absent.

Developing *up'* IFM show late pupal disorganization:

As all IFM fibers, both dorsolongitudinal muscle (DLM) and dorsoventral muscle (DVM), are fully formed and mostly attached to the cuticle, we conclude that the mutation causes no major defects of myoblast fusion, myotube formation, and fiber formation during *up'* IFM development. Examination of early fiber development by light microscopy showed no obvious differences from wild type. The first mutant effect appears to be a separation of the fibers, usually from anterior attachment sites, starting at 80 hr after puparium formation (APF), similar to that seen in adults (Figure 1B). EMs of *up'* IFM

FIGURE 3.—EM of developing myofibrils. (A) TS of wild-type IFM myofibrils at 44 hr APF show filament lattice assembling inside a ring of microtubules (arrow). (B) TS of 44 hr APF *up'* myofibrils; the microtubular arrangement is like wild type, but fewer thick and thin filaments are seen. (C) LS of 46 hr APF wild-type myofibrils with well-defined sarcomeres (arrowheads show Z-bands). (D) LS of *up'* mutant myofibril at a similar age. Myofibrils (Myo) are normal, except the Z-bands are diffuse (bulbous) and form short zebra bodies (arrows). Bar, 0.2 μ m for A and B and 0.5 μ m for C and D.

during early myofibril assembly (42–44 hr APF) show (Figure 3B) that myofibrils begin to assemble normally, like those of the controls (Figure 3A), within rings of microtubules. LS of wild-type controls (Figure 3C) show nascent Z-bands as a single line demarcating each sarcomere, while the *up'* Z-bands (Figure 3D) are broad and bulbous. These may be early assemblies of multiple Z-bands, as seen later as zebra bodies. However, at slightly earlier stages of insect flight muscle development the Z-bands are broader (AUBER 1969), so possibly at this stage of development some aspects of the early *up'* phenotype are due to normal IFM development that is slightly delayed.

Exon 10 of the *up* gene is alternatively spliced: The restriction of the *up'* phenotypes to the IFM and TDT suggested that it might affect an unidentified IFM–TDT-specific TnT isoform, missed in previous studies (FYRBERG *et al.* 1990; BENOIST *et al.* 1998; ADAMS *et al.* 2000). An extensive PCR amplification was performed of first-strand cDNA synthesized from RNA obtained from carefully dissected wild-type and *up'* IFM of 1-day-old adults. Sequencing of the wild-type amplified products revealed the presence of exon 10a (GenBank accession no. AY665838). This recently discovered exon (HERRANZ *et al.* 2005) lies 5' to the previously identified old exon 10, now designated 10b. Exon-specific primers and RT–PCR revealed that the mRNA including exon 10a is expressed specifically in IFM and TDT and that this expression starts during midpupal stages (Figure 4A). Exon 10a is apparently not used in other tissues or body parts, perhaps explaining its designation as part of an intron in previous studies (FYRBERG *et al.* 1990; TALERICO and BERGET 1994). Exon 10b is expressed in all the muscles and developmental stages, except in the very early pupal stage (Figure 4A); these pupae express neither TnT isoform but are only 8–10 hr APF, a stage at which the larval muscles are still degenerating and the pupal/adult muscle cells are yet to initiate sarcomere for-

mation. Although both exons are found in IFM message, the expression level of the exon 10b message appears much less than 10a in adult IFM. Sequencing of adult IFM cDNA clones showed that 7/9 clones (78%) contained the new 10a exon and 2/9 (22%) contained exon 10b, confirming exon 10a as encoding the major IFM isoform. Exon 6 can form two splice variants with inclusion or exclusion of a GTG codon (that can also act as 5' splice donor site) and our sequencing confirmed that both isoforms are coexpressed as previously described (BENOIST *et al.* 1998). The gene structure of the *Drosophila* TnT gene *upheld* and its splice isoforms expressed in IFM are shown in Figure 4B.

Sequencing of the flanking introns shows that at the 3' splice site, the intron preceding exon 10b has the consensus CAG sequence of *Drosophila* introns (MOUNT *et al.* 1992), while that preceding 10a has TAG at this position (Figure 4C). The peptide sequence of the two exons shows considerable residue conservation, but the exon 10a-encoded polypeptide has the greater number of potentially phosphorylatable threonine residues (Figure 4C).

The *up'* phenotype is due to mis-splicing of TnT exon 10a: Semiquantitative PCR amplification of first-strand cDNA made from total RNA isolated from dissected wild-type and *up'* IFM, TDT, and whole thoraces was conducted (Figure 5A). Sample loading was adjusted to produce equivalent levels of the *rp49* internal control and 22 cycles of amplification were used; previous experiments with these samples determined that none of the PCR signals were at saturating levels. A strong product band for the exon 10a-containing RNA is seen in the wild-type IFM, thorax, and TDT samples but is totally absent in the *up'* samples and is replaced by a faint doublet (see below). As expected from the data in Figure 4, the wild-type TDT and thorax samples show a strong signal for the 10b exon and a much weaker one

FIGURE 4.—Expression pattern of TnT gene exons 10a and exon 10b. Primer P3 (sense exon 7) with either primer P2 (antisense exon 10a) or primer P6 (antisense exon 10b) detect isoform-specific splicing with products of 369 and 259 bp, respectively. (A) Exon 10a messages are expressed in thoraces of 60–90 hr APF pupae (MP, mid-pupae), adult IFM, and TDT. Unspliced 10a containing transcripts (top bands) are detected at all stages except early pupae (EP) 8–12 hr APF and in all adult body parts, but not in TDT and IFM. The exon 10b isoform is expressed at all developmental stages except EP and tissues, although at significantly lower levels in the IFM. MM, molecular marker. (B) Gene structure of the *upheld* gene. Solid boxes, constitutive exons; striped boxes, alternatively spliced exons. Exon 6 produces 2 alternative mRNA differing by 3 bp (1 codon) due to a 5'GTG acting as an alternative splice donor site (FYRBERG *et al.* 1990). Thin lines joining the exons show how the IFM and TDT isoforms are produced. (C) Exon 10 sequences. Top lines show the alignment of exon ends (exon in top case, intron in bottom case). In the intron preceding exon 10a, the usual splice acceptor site CAG is changed to TAG. Alignment of the translated exons (bottom lines) shows that the 2 isoforms share 50% conserved residues and that exon 10a has 7 potential phosphorylation (5 Thr, 2 Tyr) sites and 10b has 4 (2 Ser, 1 Thr, 1 Tyr). One Thr is conserved between the two and there is a substitution of a Thr (10a) for a Ser (10b) at one position and a Ser (10b) replaces a Tyr (10b) at another.

for the IFM sample. Visual comparison of the wild-type and *up^l* IFM and TDT samples suggests that while the mutant has no effect on exon 10b use in the IFM, the absence of the 10a-containing mRNA in TDT is compensated by increased (127%) use of exon 10b. Densitometric analysis of this gel and two other replicates (Figure 5B) confirms this (t -test, $P = 0.017$) for TDT but in the IFM shows evidence for a slight but significant (t -test, $P = 0.003$), increase in exon 10b usage (32%). However, despite this modest increase of exon 10b usage in the IFM, absolute levels remain low compared to that required to compensate for the missing 10A iso-

form (compare 10a and 10b signals in Figure 5A). In the TDT, the increase in exon 10b-containing mRNA looks likely to have a more significant compensatory effect.

The effect of the *up^l* mutation appears to prevent the IFM-specific alternative splicing of exon 10a. We amplified the genomic DNA from whole wild-type and *up^l* flies using primers for exon 7 (P3) and 10b (P6). Sequencing six clones from three different PCR reactions for each genotype produced only one nucleotide difference between the wild type and *up^l*. The 3' end of the intron preceding the wild-type exon 10a is TAG (Figure 4C). Whereas the *Drosophila* consensus for all introns is C (68%) A (100%) G (100%), a T at the first position occurs in 27% of *Drosophila* genes surveyed (MOUNT *et al.* 1992). In *up^l*, TAG is changed to TTG (see Figure 5C). Since the A at this position is invariant in *Drosophila* splice sites, this change almost certainly accounts for the failure of proper splicing and nonaccumulation of the exon 10a-containing isoform in *up^l* IFM.

Using the exon 10a-specific primers, two faint PCR bands of similar but clearly different sizes were seen in IFM and TDT from *up^l* flies (Figure 5A). These *up^l* IFM gel bands were excised and cloned; wild-type IFM 10a and 10b bands were also cloned. While the wild-type cloned inserts were the same length, the *up^l* inserts, as expected, were either larger or smaller than the wild-type controls. When sequenced, all the *up^l* clones showed that splicing had occurred at one of two cryptic splice sites (AG), one in intron 9 and the other within exon 10a (Figure 5C). The more 5' site generates an inserted sequence upstream of the normal splice site; this insert will not maintain the normal reading frame of TnT. The more 3' site generates an in-frame deletion of the N-terminal 12 amino acids of the sequence encoded by exon 10a.

Western blots showed (Figure 5D) a complete absence of TnT in adult *up^l* IFM, indicating that only the TnT-10a (isoform encoded by the exon 10a-containing mRNA) isoform normally accumulates in adult IFM and that neither of the misspliced mRNAs generates a protein that accumulates in the IFM. However, in early pupal IFM (42–50 hr APF), the wild type and *up^l* both show a strong signal, although visual inspection suggests it may be less in *up^l*, which we take to indicate that in early stages of sarcomere formation the exon 10b isoform is the major TnT isoform, with the exon 10a isoform being expressed at lower levels at this stage. We have not confirmed this quantitatively. As anticipated from the evidence for increased levels of the 10b mRNA in *up^l* TDT (Figure 5B), there is no apparent decrement in the levels of TnT detected in the *up^l* TDT muscles.

Since *up^l* early myofibrillogenesis is quite normal, this supports the conclusion that during early IFM myofibrillogenesis in wild-type pupae, the TnT-10b isoform is expressed and is the major isoform but is replaced at later stages by expression and accumulation of the TnT-10a isoform. It is not clear why there should be 10b/10a TnT isoform switching during IFM development; the

FIGURE 5.—Expression of TnT message and protein accumulation is affected by the *up¹* mutation. (A) Semiquantitative RT-PCR of wild-type (+/+) and *up¹* (-/-) IFM, thorax, and TDT mRNA using sense primer P4 and isoform-specific antisense primers P7 (10a) and P8 (10b). Exon 10a and exon 10b mRNA accumulates in wild-type IFM and TDT muscles (as these are the major thoracic muscles the thorax samples are expected to show their combined expression pattern). Exon 10a-containing mRNA is absent in *up¹* (-/-) IFM, thorax, and TDT RNA. The 10a-specific primer produces two weak bands, one smaller and one larger than the wild-type product. Exon 10b mRNA is at low levels in *up¹* IFM, similar to wild-type IFM. In *up¹* TDT, the mutation increases accumulation of exon 10b mRNA. The lower band is the rp49 control product.

uct. (B) Densitometric comparisons of exon 10b signals shown in A (three replicate muscle samples). The *up¹* exon 10b mRNA is significantly elevated (IFM, 1.32-fold; *t*-test, $P < 0.003$; TDT, 2.3-fold; *t*-test, $P < 0.017$) compared to wild type. (C) Genomic TnT sequence in vicinity of exon 10A shows altered splice site (TTG; boxed) in *up¹* flies, exon (capital letters), and the 2 cryptic splice sites (ag and AG) that in *up¹* produce a longer mRNA with inserted intron 9 sequence and a shorter mRNA with 36 nucleotides deleted from exon 10a, respectively. (D) Western blots with anti-TnT antibody of proteins from whole thoraces, adult IFM, adult TDT, and pupal (42–50 hr APF) IFM; anti- α -tubulin antibody was used to standardize loadings. Comparison of wild-type and *up¹* samples shows a reduction in TnT in whole *up¹* thorax, no difference in TDT, a reduction in *up¹* pupal IFM, and an absence of TnT in *up¹* adult IFM. The *up¹* results confirm that missplicing does not lead to accumulation of mutant TnT despite *up¹* adult IFM containing some exon 10b mRNA (A); absence of detectable TnT in adult *up¹* IFM argues for no significant synthesis of the 10b isoform during late pupal stages and removal of previously accumulated 10b TnT.

isoforms do not differ in polypeptide chain length, as exons 10a and 10b are of equal size (Figure 4C), but the polypeptides they encode differ in charge and potential phosphorylation sites.

Expression of messages and assembly of the other thin filament proteins are affected in *up¹* IFM: The IFM–TDT-specific TnI null *hdp³* mutation reduces mRNA levels and accumulation of other thin filament proteins (NONGTHOMBA *et al.* 2004). Semiquantitative RT-PCR of *up¹* IFM for actin, both IFM TnI isoforms, and TnC4 mRNA showed a large reduction only in the level of the late pupal-adult TnI isoform mRNA (Figure 6A), although a slight reduction in TnC4 mRNA may be indicated. Western blots of IFM proteins from different developmental stages showed that the small TnI isoform (without the exon 3-encoded N-terminal extension) was present at early stages of *up¹* myofibrillogenesis at wild-type levels. The larger TnI isoform, normally expressed at later stages of IFM development, did not accumulate in *up¹* (Figure 6B, a). TnC4 is the only other thin filament protein that showed significant reductions at the message level (Figure 6A). However, while TnC4 was detected (and identified by MALDI-ToF and MS/MS sequencing) in wild-type IFM, it was not found in *up¹* IFM (Figure 6B, b); since TnC4 mRNA was detected (see above), this may indicate that in the absence of TnT and the larger TnI isoform, the TnC4 polypeptide does not

accumulate. We did not detect by either MALDI-ToF or MS/MS peptide sequencing the TnC1 isoform in wild-type IFM despite a report that this isoform is also present in *Drosophila* IFM (QIU *et al.* 2003), but this may reflect their observation that TnC4 is the major isoform. Despite a lack of visible reductions in actin and Tm mRNA levels, the accumulation of the proteins was significantly reduced (Figure 6B, c). Arthrin, the IFM-specific, ubiquitinated actin (BALL *et al.* 1987) was absent in *up¹* IFM. There were no reductions in α -actinin, a Z-disc protein.

***up¹* muscle disorganization is partially suppressed by headless myosins:** Mutations causing IFM hypercontraction, such as *hdp²* and *up¹⁰¹*, can be suppressed by genetically manipulating the amount of functional myosin assembled into thick filaments and thereby the destructive unregulated muscle contractions (NONGTHOMBA *et al.* 2003). *Mhc¹⁰* is a myosin heavy chain null mutant that removes all the thick filaments from the IFM. The Y97 transgene expresses headless myosin and in wild-type background is co-incorporated into the sarcomeres with the myosin, producing relatively minor effects on sarcomere structure (CRIPPS *et al.* 1999). In strains homozygous for the IFM-specific myosin null, *Mhc¹⁰*, carrying two copies of the Y97 transgene produces recognizable sarcomeres, although with rather variable length (CRIPPS *et al.* 1999). Incorporating the Y97 construct and replacing one *Mhc⁺* gene copy with the *Mhc¹⁰* mutant in *up¹* (*up¹/Y; Mhc¹⁰/Mhc⁺*;

FIGURE 6.—*upl* effects on the expression and accumulation of IFM thin filament proteins. (A) RT-PCR amplifications from wild-type (+/+) and *upl* (-/-) IFM show a large decrease in mRNA levels for the larger TnI isoform in *upl* IFM but only a slight decrease in TnC4. No changes were apparent in other thin filament protein message levels, including the smaller TnI isoform. (B) Western blots of IFM proteins from wild-type and *upl* IFM wild type and *upl* IFM show normal accumulation of the smaller TnI isoform at early developmental stages (I, 40–48 hr APF; II, 60–68 hr APF), but in *upl* IFM, the larger isoform, which in wild-type IFM replaces the smaller isoform in the adult (III), fails to accumulate, although some of the smaller isoform persists in the *upl* IFM. (b) Coomassie blue-stained 2-D IFM protein gel show that the abundant TnC4 isoform wild-type IFM (arrow) identified by MALDI-ToF and MS/MS sequencing analysis (data not shown) of the excised spot is absent in *upl* IFM (arrowhead). The open arrow indicates the CG3544 protein product also identified by MALDI-ToF and MS/MS sequencing (data not shown). (c) *upl* IFM show reductions in actin and Tm accumulation although their message levels were quite normal. In *upl* IFM, arthrin is completely absent; levels of α -actinin, a Z-band protein, are unchanged from wild type.

Y97) produces flies with normal wing posture and normal IFM morphology under polarized light (Figure 7A). This suggests that the partial hypercontraction phenotype of *upl* flies (Figure 1B) involves inappropriate levels of actomyosin activity. Despite the normal fiber appearance, EMs reveal severe myofibrillar disruption (Figure 7, B–D). The *upl*/Y; *Mhc*⁺/*Mhc*⁺; Y97 IFM show a phenotype indistinguishable from that of *upl* (Figure 1, E

and H). The zebra bodies remain a prominent phenotypic feature (Figure 7B) but are not seen in the *upl*/Y; *Mhc*¹⁰/*Mhc*⁺; Y97 flies where the assumed decreased amounts of full-length myosin permit quite normal Z-bands to form. Thick and thin filaments assemble between them (Figure 7C), producing small regions of hexagonal lattice (Figure 7D); in most areas filaments remain disorganized. This is very similar to the *hdp*³ IFM

FIGURE 7.—Reduction of myosin content partially suppresses the *upl* phenotype. Polarized light micrograph of an *upl*; *Mhc*⁺/*Mhc*¹⁰; Y97 hemithorax (A) shows wild-type DLM birefringence (star) and muscle pattern. Bar, 0.15 mm. (B) EM (LS) of *upl*; *Mhc*⁺/*Mhc*¹⁰; Y97 show zebra bodies (arrowhead), small myofibrils (Myo), Z-discs (arrow), and dispersed thin and thick filaments, whereas in C, LS of *upl*; *Mhc*¹⁰/+, Y97/Y97, IFM show sarcomere-like assemblies with Z-bands (Z) (arrows), although of variable size, orientation, and shape. (D) TS of *upl*; *Mhc*¹⁰/+, Y97/Y97 myofibrillar assemblies (Myo) and filament lattice (arrow). Bar, 1 μ m for B and C and 0.5 μ m for D.

phenotype that is also not suppressed completely by reducing myosin head concentration in the same way (NONGTHOMBA *et al.* 2004), whereas in the case of *hdp²* (a TnI missense mutation) *hdp²/Y; Mhc¹⁰/Mhc⁺; Y97* flies, the IFM ultrastructure is completely restored (NONGTHOMBA *et al.* 2003). We have argued that this TnI null mutation has its primary molecular effects on the regulatory mechanism that controls the amount of thin filament messages expressed during early IFM development. Similar interpretations can be made for *up¹*.

DISCUSSION

Developmental and tissue-specific expression of the *upheld* gene: The IFM are oscillatory muscles specialized to produce wing beat frequencies of >200 Hz during flight (VIGOREAUX 2001). Stretch as well as Ca^{2+} is required to activate IFM muscle contraction (PECKHAM *et al.* 1990; AGIANIAN *et al.* 2004). IFM contain homologs of most major thin filament proteins, but the IFM express different isoforms, probably to cope with their different functional demands (FYRBERG *et al.* 1983; KARLIK and FYRBERG 1986; VIGOREAUX 2001; QIU *et al.* 2003; HERRANZ *et al.* 2004; NONGTHOMBA *et al.* 2004). These isoforms are generated through expression of different structural genes or intragenically by use of alternative promoters or alternative transcript splicing (for review, see BERNSTEIN *et al.* 1993).

The single *upheld* (*up*) gene is the only TnT gene present in the Drosophila genome and produces different TnT isoforms by alternative splicing. Previously, it was believed that the IFM–TDT-specific isoform was generated from a message containing only constitutively expressed exons (FYRBERG *et al.* 1990; BENOIST *et al.* 1998). As a result, mutations, such as *up²* and *up³*, whose phenotypic effects were restricted to the IFM and TDT, were difficult to explain (FYRBERG *et al.* 1990). Our studies with the *up¹* mutation revealed a similar conundrum that has now been resolved by the identification of a new TnT exon, 10a (HERRANZ *et al.* 2005), which we have shown is expressed only in the IFM and TDT (Figure 4A) and whose splicing is affected by the *up¹* mutation in the TnT gene. Exon 10a and its alternative exon 10b are mutually exclusively spliced. Homologs to these two alternative TnT gene exons in *D. melanogaster* occur in the TnT genes of other insects (HERRANZ *et al.* 2005). Exon 10b-containing message is present in all stages where muscles are developing and is probably expressed in all muscles; exon 10a usage is only found in the IFM and TDT or late pupae that will contain these muscles (Figure 4A). Our data show that exon 10b-containing mRNA is a minor component in adult IFM and does not produce a significant amount of TnT, since in the absence of exon 10a-containing message, due to the *up¹* mutation, no TnT is detectable in Western blots (Figure 5D). However, a significant TnT signal is seen in IFM samples from early *up¹* pupae. As this cannot be trans-

lated from exon 10a-containing message, we presume that it is the 10b isoform, which at this stage must be the major TnT isoform. This isoform is clearly removed from the IFM during later stages. This is the first indication of TnT isoform switching in a Drosophila muscle. We have previously shown a TnI isoform switch during Drosophila IFM development (NONGTHOMBA *et al.* 2004; Figure 6). The switching of these TnI and TnT isoform pairs may occur at the same time; these experiments remain to be done. However, as binding partners, this may reflect a significant change in the properties of the developing troponin complex. Since this switch occurs in the pupae long before the IFM are called upon to power adults in flight, it is unclear what purpose this switching achieves. Developmental and muscle-specific isoform switching is widespread in vertebrate striated muscles and changes due to alternative transcript splicing have been reported for cardiac TnT (cTnT) in vertebrates (COOPER 1998; CHARLET *et al.* 2002).

***up¹* IFM phenotype and the roles of TnT during myofibrillogenesis:** The *up¹* mutation replaces the AG splice acceptor site flanking exon 10a with TG and that prevents the production of mature mRNA containing exon 10a. The altered splice site does not reduce the production of exon 10b-containing mRNA and protein in the IFM (Figure 5, A and D). In the TDT, where both isoforms are normally expressed, even in young adults, the lack of 10a message in the *up¹* mutants is at least partially compensated for by a significantly increased accumulation of exon 10b-containing message (Figure 5, A and B). The observation that jumping of the mutant flies is poor argues either that this compensation fails to provide sufficient functional TnT or that there is a need in these muscles for both isoforms.

The *up¹* mutation affects the accumulation of a number of other thin filament proteins (Figure 6, A and B), including the 40-kDa TnI isoform, TnC, the Tm2 isoform, actin, and arthrin (mono-ubiquitinated actin). The obvious explanation is that in the absence of the TnT, the troponin complex fails to assemble, affecting thin filament assembly or stability, leading to increased turnover of unincorporated proteins. Short term *in vivo* radiolabeling of flies homozygous for the null *KM88* mutant of the IFM-specific *Act88F* actin gene showed that in the absence of F-actin, troponin subunits and Tm are synthesized but do not accumulate (J. SPARROW, unpublished data). Increased protein turnover cannot be the whole solution, especially for mutants of the more peripherally associated thin filament proteins such as the troponin complex.

The *up¹* IFM also show reduced message levels of the IFM-specific TnI-exon 3 isoform and TnC4 (Figure 6A) and nonaccumulation of these proteins. These are specific effects, as other thin filament protein messages are not affected, including the mRNA for the small TnI isoform, actin, and Tm2. Despite this, all the mature IFM thin filament proteins are either absent (TnC4, the

large TnI isoform, and arthrin) or reduced, as for actin and Tm 2 (Figure 6B). This phenomenon whereby mutation in one protein reduces the amounts of other thin filament proteins has been reported previously. In the IFM of the *up²* and *up³* mutants, an absence of TnT was correlated with reductions of Tm and actin (FYRBERG *et al.* 1990). We recently showed (NONGTHOMBA *et al.* 2004) that an IFM-specific TnI null mutant reduced mRNA levels of TnC, TnT, Tm, and actin but not of myosin heavy chain or α -actinin. In zebrafish mutations in the *Tnnt2* gene (cardiac TnT), “*silent heart*” (*sih*) reduce TnT expression accompanied by reductions in Tm and TnI mRNA levels (SEHNERT *et al.* 2002). The mechanism(s) for such a coordinated reduction in the amount of thin filament transcripts and proteins are still obscure. In early stages of *up¹* IFM development, the 10b TnT isoform is present, as is the small TnI isoform, but at later stages when the TnT-10a isoform is not made, the late pupal-specific larger TnI fails to accumulate. This suggests that part of the mechanism for coordinated reduction in message levels involves a monitoring of whether the wild-type proteins can assemble into the myofibrils or not.

Unlike the *hdp³* mutation where IFM elongation does not occur due to unregulated actomyosin interactions during myofibril assembly (NONGTHOMBA *et al.* 2004), *up¹* IFM elongate and complete fiber morphogenesis (Figure 1B). Initial myofibril assembly occurs normally (Figure 3, B and D), due probably to the presence of the TnT-10b isoform during early IFM development (Figure 5D). This should allow the normal assembly of the smaller TnI (30 kDa) isoform into the thin filaments (Figure 7B), inhibiting actomyosin interactions and allowing the developing fibers to elongate (NONGTHOMBA *et al.* 2004). However, later in normal development, this TnI is replaced by the larger (40 kDa) TnI isoform (NONGTHOMBA *et al.* 2004). TnT is a central component of the troponin complex serving to anchor the other components, TnI and TnC, to Tm (FARAH and REINACH 1995; GORDON *et al.* 2000). Failure to incorporate the TnT-10a isoform into *up¹* IFM leads to nonaccumulation of both 40-kDa TnI and TnC4 protein (Figure 7B). As a result, during the later stages of IFM development, the thick and thin filaments (mostly lacking troponin complexes) will interact with each other in an unregulated manner, tearing the fibers (Figure 1, B, E, and H) to produce disassembly of myofibrils and sarcomeres.

The lost *up³* allele, reported with similar phenotypes to *up¹*, can now be explained by the newly defined TnT gene structure. The *up³* mutation was a 265-bp deletion in the intron between exons 9 and 10b (exons 7 and 8 of FYRBERG *et al.* 1990), which included part or all of exon 10a. It was argued (FYRBERG *et al.* 1990) that *up²* had a nucleotide change from GTAAGT to GTAAAT in a splice donor site immediately after exon 7 (exon 9 of present study, a constitutive exon). However, this splice donor site change was probably polymorphic, as it is

unlikely to have affected exon 7 splicing. Since neither mutation is now available, we cannot confirm their genetic lesions. The most plausible explanation is that both led to missplicing of exon 10a.

Possible functional effects of the TnT exon10 alternative splice variants in IFM and TDT: Our results show that alternative splicing of exons 10a and 10b occurs in both the IFM and TDT muscles, but whereas the major TnT isoform in the IFM contains the exon 10a encoded sequence, both the exons in the TDT are used to produce two different TDT isoforms in similar amounts. This suggests that activation of oscillatory flight muscle (IFM) by calcium and stretch and of the tubular TDT muscle, which is activated by calcium alone (PECKHAM *et al.* 1990), requires different ratios of these two TnT isoforms. In dragonflies, intraspecific TnT splice variants differentially affect Ca^{2+} sensitivity and force production (MARDEN *et al.* 2001). Whether this is the function of the *Drosophila* switch from the TnT-10b isoform to the TnT-10a isoform (Figure 5D) in mature IFM is unclear. During early IFM development, there is minimal muscle function. In the TDT of wild-type flies, both isoforms are present, but in *up¹* flies where increased TnT-10b expression compensates for the absence of TnT-10a (Figure 5, B and C), the muscle structure is normal at eclosion, but jumping is poor and deteriorates with age. Either this is due to a reduction in total TnT present in the *up¹* TDT or TnT-10b can compensate structurally for TnT-10a but cannot do so functionally.

Alignment of the *Drosophila* TnT amino acid sequences with their cardiac vertebrate counterparts (data not shown) reveals that the exon 10-encoded polypeptides correspond to residues 236–262 in the human cTnT sequences, within the C-terminal part of the so-called TnT T2 region (residues 183–288). Partial atomic structures of the human cardiac troponin-Tm complex (TAKEDA *et al.* 2003) show that this region of TnT makes major interactions with TnC, TnI, and Tm. The *Drosophila* exon 10-encoded polypeptides show little homology with the corresponding cardiac sequences they align with (in whole protein alignments). Of the many conserved cardiac sequence residues in this region, only one, E255, is totally conserved when exons 10a and 10b are included. In this situation, it is impossible to speculate on the functional differences that might arise from the multiple amino acid changes resulting from exon 10 alternative splicing. TnT in *Drosophila* flight muscles is phosphorylated (DOMINGO *et al.* 1998), as it is in vertebrate striated muscles where it can affect muscle performance (reviewed by METZGER and WESTFALL, 2004). The sites of *Drosophila* TnT phosphorylation are not known. Exon 10a contains seven potential phosphorylation sites (Figure 4C); replacing this exon with exon 10b reduces these sites by three. However, sequence homology provides no clues as to whether any of these are likely to be phosphorylated, as the residues do not coincide with known phosphorylation sites in the T2 region of

vertebrate cTnT (METZGER and WESTFALL 2004). In the IFM and TDT, different TnI isoforms are expressed (NONGTHOMBA *et al.* 2004), so there appear to be two TnI/TnT isoform pairs: the small (30 kDa) adult TnI isoform with TnT-10b and the large (40 kDa) IFM-TDT-specific TnI with TnT-10a. Alternative exon 10 usage may affect the interaction with the different TnI isoforms with which they are coexpressed to modulate muscle activation/inhibition.

TnT and human myopathies: Human familial nemaline myopathies are characterized by nemaline rods and muscle weakness (NORTH *et al.* 1997; SPARROW *et al.* 2003). Most are caused by mutations in nebulin (PELIN *et al.* 1999) or the *ACTA1* α -actin gene (NOWAK *et al.* 1999; SPARROW *et al.* 2003), although two are reported in the slow skeletal Tm, *TPM3* (LAING *et al.* 1995; TAN *et al.* 1999), and TnT, *TNNT1*, genes (JOHNSTON *et al.* 2000). Nemaline rods are modified Z-bands. The *up¹* IFM contain electron-dense bodies that appear to be structural homologs of nemaline rods and structures referred to as zebra bodies in human myopathy. Similar structures were reported for the *up²* and *up³* (FYRBERG *et al.* 1990) and *hdp³* TnI mutations (BEALL and FYRBERG 1991; NONGTHOMBA *et al.* 2004). Perhaps, as in humans, the development of the nemaline rod structures and/or zebra bodies may be a characteristic feature of many thin filament protein mutations.

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