The complete genomic organization of the *Drosophila* troponin T (TnT) gene shows many interesting features, including the presence of a microexon of only 3 nucleotides conserved among *Drosophila*idae. It is the smallest *bona fide* exon so far described, placing a new lower limit on the nucleotide number required for correct splicing. Four muscle-type specific transcripts are generated by developmentally regulated alternative splicing. Exons 3, 4, and 5 are absent in the transcript present in jump and flight muscles. A total of 11 exons are present in the adult hypodermic muscles transcript, whereas the microexon is absent in the larval hypodermic musculature. The two isoforms differ in a lysine residue. Post-translational regulation of the flight muscles/tergal depressor of the trochanter-specific isoform is involved in flight and/or jump function. The interaction domains of TnT in the tropomyosin-troponin complex are strongly conserved in the known vertebrate and invertebrate TnT sequences, whereas the terminal regions show an important variability. The COOH-terminal region shows important phylogenetic variations, whereas the NH2-terminal domain is associated with specific muscle types in a particular organism, a finding that discloses a selective value for these domains in the functionality of distinct muscles in different organisms.

Much of our understanding of the regulation of muscle contraction derives from studies of vertebrate skeletal muscle. The process is controlled by the reversible binding of Ca2+ to the thin filament protein troponin (1, 2). Although there are differences in the known details of this process in different muscles, the basic process is similar (1–3). The detailed structure and protein domains involved in the formation of the tropomyosin-troponin complex, and hence the details of how it functions, are not known (3).

The role of the highly asymmetric TnT1 protein in the regulatory mechanism of muscle contraction is still unclear, but previous reports in vertebrate muscles indicate that it is a key component in linking together and regulating all the components of the complex, i.e. tropomyosin, troponin I, and troponin C (4–6). In addition, it increases the cooperativity of actin-tropomyosin and provides Ca2+ sensitivity to the thin filament (7). TnT is one of the proteins known to present a very complex isoform pattern both at the transcript level through differential splicing and at the protein level through post-translational modification (8). Furthermore, mutations in cardiac TnT are responsible for a large proportion of familial hypertrophic cardiomyopathy cases (9) and changes in cardiac TnT isoform expression have been correlated with heart disease (10). In *Drosophila*, mutant analysis revealed phenotypes with muscle abnormalities, indicating a possible effect of this protein on myofibrillar assembly and/or stability (11). More recently, the analysis of *Caenorhabditis elegans* mutants in TnT have demonstrated that they are defective in embryonic body wall muscle cell contraction, sarcomere organization, and cell positioning (12). This information justifies the increasing interest in this molecule, recognizing a more important role for TnT in muscle contraction than previously thought.

An organism such as *Drosophila* offers important advantages for biochemical studies, as they can be complemented with genetic investigations (see Refs. 13 and 14). *Drosophila* develops two distinct sets of muscles during its life cycle, embryonic/larval and adult musculature. Larval muscles, including the body wall musculature, the gut and the dorsal heart muscles, are superfibrillar nonfibrillar muscles. In the adult, the hypodermic musculature and the tergal depressor of the trochanter (TDT) are tubular and contract synchronously. TDT is involved in jumping, a preparatory step in the initiation of the flight response. In the thorax, however, the majority of the musculature is made up of the large indirect flight muscles (IFM), which are responsible for flight. As in the larva, the adult gut and all the visceral muscles, including the dorsal heart vessel, are tubular supercontracting muscles (15). *Drosophila* flight muscles have been one of the historically used models in the studies of striated muscle (16, 17). Unlike vertebrate muscles, calcium activation results in low tension and low ATPase activity and they show the property of stretch activation in the presence of Ca2+; a stretch of just 1–3% in muscle length triggers a large amplitude delayed rise in tension. This is the underlying mechanism that allows wing beat frequencies of up to 300 Hz in *Drosophila* even higher in...
other insects. Although this phenomenon is present in all muscles, only in IFM and mammalian heart muscles are its effects large enough to be functionally significant (18). IFM are also classified as asynchronous, since the frequency of wing beats does not correspond to the frequency of nerve impulses.

Drosophila fibers contain homologues of the proteins found in vertebrate muscles: actin, myosin heavy chains, myosin light chains, α-actinin, tropomyosin, troponin C, I, and T (15). In fact, all these contractile proteins are members of isoform families usually encoded in several divergent genes, a molecular diversity that reflects the functional diversity of the different muscles. The type- or stage-isoform specificity in muscle fibers has been maintained in many cases across evolution, suggesting specific roles for each isoform (8, 15, 19). However, the functional significance of expressing specific isoforms in different muscles remains a critical unsolved issue (8). In Drosophila, in contrast, contractile protein isoforms arise mainly by alternative splicing of one or a few genes (20). Often this diversity is increased by post-translational modifications (13, 21, 22). Recently, the importance of phosphorylation in the regulation of flight muscle activity has been shown in three muscle proteins, the myosin regulatory light chain, flightin and miniparamysin (23–26). Interestingly, in the case of Drosophila as in other invertebrate prostates, TnT is considerably larger than in chordate counterparts carrying a carboxyl-terminal extension (11, 12). In addition, some insect muscles contain peculiar myofibrillar proteins such as arthrin, troponin H, flightin, and mp20 (15). The presence of specific protein components and/or specific isoforms in these muscle-types should explain, at least in part, their special mechanical properties (27–33).

The evident complexity of TnT regulation makes the study of TnT mRNA expression in Drosophila of particular interest. Several specialized muscles, for example IFM or TDT, are relatively homogeneous, facilitating biochemical studies correlating isoform-specific mRNA and protein expression with fiber type. A comparative analysis in Drosophila and other fiber types could be important to the understanding of regulation and function of TnT gene splicing.

In this article, we report the complete characterization of the gene encoding the Drosophila TnT (11). The gene has several interesting structural features, including a first untranslated exon of 80 nucleotides, a microexon of just 3 nucleotides in interesting structural features, including a first untranslated exon of 80 nucleotides, a microexon of just 3 nucleotides in several divergent genes, a molecular diversity that reflects the functional diversity of the different muscles. The type- or stage-isoform specificity in muscle fibers has been maintained in many cases across evolution, suggesting specific roles for each isoform (8, 15, 19). However, the functional significance of expressing specific isoforms in different muscles remains a critical unsolved issue (8). In Drosophila, in contrast, contractile protein isoforms arise mainly by alternative splicing of one or a few genes (20). Often this diversity is increased by post-translational modifications (13, 21, 22). Recently, the importance of phosphorylation in the regulation of flight muscle activity has been shown in three muscle proteins, the myosin regulatory light chain, flightin and miniparamysin (23–26). Interestingly, in the case of Drosophila as in other invertebrate prostates, TnT is considerably larger than in chordate counterparts carrying a carboxyl-terminal extension (11, 12). In addition, some insect muscles contain peculiar myofibrillar proteins such as arthrin, troponin H, flightin, and mp20 (15). The presence of specific protein components and/or specific isoforms in these muscle-types should explain, at least in part, their special mechanical properties (27–33).

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In this article, we report the complete characterization of the gene encoding the Drosophila TnT (11). The gene has several interesting structural features, including a first untranslated exon of 80 nucleotides, a microexon of just 3 nucleotides in length and two polyadenylation sites that are used somewhat differentially during development. A single promoter is utilized for the expression of the four TnT mRNA products generated by developmentally regulated alternative splicing. The transcripts present muscle-type specificity. The results strongly indicate the existence of post-translational regulation for the smallest IFM/TDT specific isoform, together with a direct or indirect role of these modifications in flight and/or jump function.

### EXPERIMENTAL PROCEDURES

#### Isolation of the Genomic Clones—Two genomic DNA libraries (EMBL3 from Drosophila melanogaster and Drosophila viridis) were screened following a standard protocol (13). Two TnT cDNA probes were used: probe P2 (395-bp fragment) covering exon 2 to exon 6 and probe H2 (510-bp fragment) covering exon 8 to exon 11. The exon numbering used here (see Fig. 1) modifies that previously described (11). Positive clones and subclones were purified and cloned in pUC18 and pBlueScript and mapped for positions of restriction endonuclease cleavage and by Southern analysis with TnT cDNA probes and oligonucleotides derived from the TnT cDNA sequences (11). Four D. melanogaster clones were obtained containing overlapping inserts of 11, 11.5, 14.5, and 15 kb, covering a region of 25 kb (Fig. 1). One D. virilis clone was obtained containing an insert of 11.3 kb. The 15-kb D. melanogaster clone covers almost the complete gene, including 5 kb upstream from the transcriptional initiation site. The D. virilis clone covers the complete gene (in this case, approximately 9.2 kb) as well as close to 2 kb upstream of the transcriptional start. Several oligonucleotides covering distinct regions of the DNAs, were synthesized and used as primers for the sequencing of the clones (35).

#### Mapping of 5' Termini Primed Extension—Primed extension assays were conducted basically as described (36). The primers used were: E2R (5′-CTCCTTCATCGTCGAGCATT-3′) and E3R (5′-CTCCTCGG GGTTCCTCCT-3′) complementary to nucleotides 26–43 and 71–88, respectively, of the TnT cDNA sequence (11). Oligonucleotides were end-labeled with 50 μCi γ-32P-ATP and annealed at 65 °C/20 min with 25 μg total RNA from different developmental stages. The annealed primers were extended with 25 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) at 42 °C/120 min. The primers were used on cloned genomic DNA templates to generateideoxy sequencing ladders that were compared with the primer extension products to determine the distance in nucleotides from the designated primers to the TnT transcription initiation sites. The primer extensions were electrophoresed on a 6% polyacrylamide-urea sequencing gel.

#### Reverse Transcriptase-Polymerase Chain Reactions—RT-PCR using RNA from distinct developmental stages, dissected muscles as well as body parts of flies, were performed according to standard protocols (34). Oligonucleotides covering different exons were used as primers. For exon 1 (5′-TGGTAAGCGCGAGCATC-3′) and exon 6 (5′-ACCCCGTGGCTGCTT-3′) and (5′-GGAGAGGGCGATCCAGAG-3′); for exon 11 (5′-CTCCGCTCCCTGATCTT-3′) and (5′-TGAAATGCAAGTGTATTTAAA-3′); for exon 5 (5′-TGCTGCGTGTTGAG-3′) and (5′-GGCGCCTGCATCTCGACGATGAAGTACAC-3′). Products were analyzed in 2% agarose gels. Bands were excised, cloned in pGEMT, and sequenced using T3 and T7 primers as described (35).

To determine the presence of two functional polyadenylation signals, assays were performed as follows: A first step was carried out using oligo(dT) as a primer to transcribe exclusively polyadenylated RNAs from different stages of development, namely, embryos, larvae, late pupae, and adults. Next, a PCR amplification was carried out using, in this case, an oligonucleotide covering exon 11 of the TnT gene as a direct primer and oligo(dT) as an indirect primer. Two different oligo(dT) probes were used as probes for Southern blot analysis as products; the first, hybridizing a sequence just after the first polyadenylation signal and the second, after the second polyadenylation signal. PCR fragments were amplified through 30 cycles (94 °C/30 sec, 55 °C/30 sec, 72 °C/30 sec) using Taq polymerase (Boehringer Mannheim).

In Situ Hybridization and Riboprobe—Two complementary oligonucleotides were synthesized with the 66-bp sequence corresponding to exons 3 and 5. For cloning in pGEMT, ApaI and NsiI sequence were added as adapters on each end. The pGEMT polylinker was eliminated to avoid nonspecificity in the riboprobe. A PvuII/NsiI fragment (275 bp) from exon 6 was also cloned in the same vector. Riboprobes were labeled with digoxigenin using the DIG RNA labeling kit (T7) (Boehringer Mannheim). For in situ hybridizations, specimens were embedded in Tissue-Tek OCT compound (Miles) and frozen at −80 °C. 20-μm sections were mounted on slides and hybridized at 55 °C overnight. After washing, sections were incubated overnight with an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody at 4 °C.

### RESULTS

#### The Structure of the Tropomin T Gene: Identification of the Transcriptional Initiation Sites and the 5′ Upstream Regions—After screening of two EMBL3 genomic libraries from two Drosophila species using TnT cDNA probes from the 5′ and 3′ regions of the gene, four different genomic clones from D. melanogaster and one from D. viridis were isolated (see “Experimental Procedures”). Clones covering the TnT genes and 5′ upstream regions were subcloned and the restriction mapping completed (Fig. 1). Primer extension analysis (Fig. 2) confirmed that the published TnT cDNAs were incomplete at the 5′ end. Different approaches were used in the identification of the 5′-untranslated exon(s). Comparison of D. melanogaster and D.
virilis genomic sequences allowed the identification of a 97% conserved region of 80 nucleotides, 2.3 kb away from the 5′ end of the published cDNAs (11). The size of this sequence, the putative first exon, matched very well with the results previously obtained by primer extension. The existence of this exon was finally demonstrated by RT-PCR using RNAs from different stages of development. The sequence of the first exon of the D. melanogaster TnT gene is presented in Fig. 2B. The primer extension assays also indicated the existence of several transcriptional initiation sites. Five different initiation sites were distributed throughout a 19 nucleotide sequence and were used with almost the same intensity throughout Drosophila development (Fig. 2A).

In addition, during our analysis of the differential muscle-type RNA expression of the TnT gene (see below), the presence was demonstrated of another unidentified exon (exon 4), which is only 3 nucleotides in length. When RNA from late pupae were cloned by RT-PCR and sequenced, comparison revealed the existence of a GAA trinucleotide in one cDNA absent in the already published TnT cDNA clones (11). The following experiments were done to confirm the existence of a possible trinucleotide exon. First, we found that the trinucleotide was conserved in the sequenced D. virilis adult cDNA (Fig. 3A) even though D. melanogaster and D. virilis diverged more than 50 million years ago. This conservation through evolution indicates the importance of this trinucleotide for insect TnT function. Second, the introns in which the putative exons should appear were sequenced in both species, revealing that a GAA trinucleotide was, indeed, present in both genomic sequences (Fig. 3B). The microexon was flanked by acceptor and donor splice consensus sequences, namely the AG at 3′ splice junction and GT at the 5′ splice junction of the gene (38, 39).

Fig. 1. Genomic structure and organization of the D. melanogaster TnT gene. In the upper part, a schematic representation of the troponin T gene is shown with the numbered exons in boxes. P2 and H2 correspond to the cDNA probes used in the screening. The complete restriction mapping of the DNA region is presented. In the lower part, the four genomic clones analyzed are indicated with the name of the isolated clones in boxes.

Fig. 2. Identification of the transcription initiation sites of the D. melanogaster TnT mRNAs at distinct developmental stages. A, primer extension analysis of the TnT transcription units. Total RNA from different developmental stages were annealed with oligonucleotides complementary to troponin T and extended with reverse transcriptase. Embryos of 3 h and 16 h (E3 and E16), 1st and 2nd instar larvae (LI and LII), early and late pupae (Pe and Pl), imagoes (Ad), and control (C). E2R and E3R oligonucleotides were the primers used in the assays. The same primers were used with the appropriate genomic clones to generate the dideoxy sequencing ladders shown in A. The transcription start sites are indicated by arrowheads. Numbering starts at nucleotide +1, corresponding to the main initiation start of the TnT as detected in these assays. B, the inferred 5′ end sequence of the TnT transcript is shown, incorporating the sequence of the identified exon 1. Arrowheads indicate the location of the initiation sites. The boxed sequence, TCAGT, indicates the initiator element.
Fig. 3. A 3-base pair microexon, exon 4, occurs in the D. melanogaster and D. virilis TnT genes. RT-PCR was performed with purified RNA from late pupae. Oligonucleotides covering exon 1 and exon 6 of D. melanogaster and exon 1 and 5 of D. virilis TnT genes were synthesized, used as primers, and the products analyzed as described under "Experimental Procedures." In panel A, the agarose gels with the RT-PCR products and a diagram indicating the exon organization of the products are shown; a, b, and c correspond to the D. melanogaster products and d to the D. virilis product. Numbers in the diagram label the exons present in each cloned fragment. In the lower part of panel A, partial sequences of the cloned products are shown with the GAA boxed, used as primers, and the products analyzed as described under "Experimental Procedures." In panel B, a comparison is presented of the upstream and downstream genomic sequences of microexon 4 in D. melanogaster and D. virilis. The trinucleotide and the AG-containing 3′ splice junction appear in three contiguous boxes. At −6 nucleotides from the 3′ splice junction, pyrimidine-rich regions are identified. Hypothetical branch points, A(s) adjacent to the pyrimidine-rich regions, are also marked with a filled circle. Additional conserved sequences located upstream of the 3′ splice sites are underlined.

exons of the Drosophila TnT gene have been sequenced and compared. All donor and acceptor splice sites correlate with the splice junction consensus for Drosophila (40). In particular, GTAAGT was the donor splice sequence in 8 of the 11 exons, including the microexon (data not shown).

Comparison of the genomic clones of both species at the 3′ end of the genes also revealed the existence of another conserved polyadenylation site, AATAAA, in the D. melanogaster gene at 96 bp of the one previously published (11). To determine whether the newly identified polyadenylation signal was functional, PCR assays were carried out (see "Experimental Procedures"). The analysis indicated that both signals were used all through development. The first signal appears to be used preferentially in embryos, whereas both are found in the transcripts in the adult musculature (data not shown).

Regions extending 250–400 bp upstream of the initiation starts in both Drosophilidae were sequenced. The alignment of both sequences allowed the identification of putative functional motifs in the proximal 5′ regions of both genes. Analysis of these two regions confirmed that they contained regulatory elements of the TnT genes (Fig. 4). The pentanucleotide TCAGT, a consensus sequence for the eukaryotic promoter capsite (41) and for the regulatory initiator element (42), is present in the sequenced TnT transcriptional initiation sites and is highly conserved in the two Drosophilidae. Furthermore, TnT proximal sequences in both species show AT-rich regions, between −35 and −40 from the initiation site. A region extending to around 90–100 nucleotides upstream of the initiation starts was more than 85% conserved, indicating that these regions may correspond to the TnT minimal promoters. A conserved CCAAT element was also detected at approximately the canonical distance, between −101 and −106 from the initiation start. In addition, two conserved E boxes (the binding site for nautilus, the Drosophila MyoD), found to be critical in numerous vertebrate muscle promoters (43), were also detected at −170 and −245 in the D. melanogaster 5′ region of the TnT gene. On the other hand, the CG elements, also important in eukaryotic promoters, have not been clearly detected in the proximal promoter regions of TnT genes.

In summary, it is possible to say that Drosophila TnT gene is organized into 11 exons using two polyadenylation sites separated 96 bp in different muscle types. Moreover, the gene appears to be controlled by a single promoter that regulates the expression of the different mRNAs.
products of the RT-PCR were isolated, subcloned in pGEMT and sequenced. In the analysis of the 5′ region of the mRNA, oligonucleotides covering the amplification of DNA fragments from exon 1 to exon 6 were used as primers (Fig. 5A). Detailed comparison of the cloned and sequenced RT-PCR products obtained from larvae and late pupae allowed the identification of four different transcripts that differ in the choice of exons 3, 4, and 5. In Fig. 5B, the exonic organization of the alternative spliced 5′ regions of the transcripts is indicated. The larger transcripts detected in larvae and pupae differ only in the presence of the microexon (exon 4). To explore whether the 3′ region was alternatively spliced and since exon 11 encodes for the sequence encoding the polyglutamic tail (Fig. 5C), the complete analysis of the RT-PCR sequenced products revealed that TnT transcripts showed only alternative splicing in the 5′ region sharing the last 8 exons.

The muscle specificity of these transcripts was examined in greater detail. RT-PCR assays using RNA of dissected muscles were performed with TDT, IFM, visceral muscles from larvae, adult, and larval hypodermic muscles (Fig. 6). Since alternative splicing had been detected only in the 5′ end of TnT, the study was focused on this region. Analysis and comparison of the sequenced RT-PCR products revealed that transcripts were differentially expressed in a tissue-specific manner during development (Fig. 6). The transcript present exclusively in TDT and flight muscles thus does not include exons 3, 4, and 5 and is first detected in late pupae (Fig. 5A). Hypodermic muscles show two distinct transcripts. In the adult hypodermic muscles, the 11 exons of the gene were present, whereas in the larval hypodermic musculature, the microexon was absent. The hypodermic transcripts thus differ only in the microexon. In visceral muscles from larvae, the same transcript is found as in larval hypodermic muscles; that is, the microexon is absent. Finally, the fourth transcript does not include exons 4 and 5 in its sequence. This transcript is present in larval preparations (37), two different TnT isoforms have been identified. To confirm the muscle specificity of some of these transcripts, two distinct riboprobes were prepared (see “Experimental Procedures”) for in situ hybridization experiments (Fig. 7). As expected, the riboprobe covering exon 6 (275 bp size), a constitutive exon, hybridized to both embryonic/larval and adult musculature, and was present in all muscles (Fig. 7, A and B). The riboprobe covering exons 3 and 5 (66 bp size) also hybridized to both the embryonic/larval and adult musculature but was absent in the IFM and TDT muscles (Fig. 7, C and D). The transcript that excludes exons 4 and 5 using the same approach was difficult to ascribe, since these exons are 3 and 24 bp in length, respectively. In conclusion, the present results revealed that the TnT gene encodes four transcripts specific to different muscle types.

Drosophila TnT Protein Isoforms and Muscle Function—Late embryos, second and third instar larvae, early and late pupae, newly emerged nonflying imagos, and adults of 19 h were analyzed by Western blot using a Drosophila polyclonal antibody against TnT (Fig. 8A). As described in embryonic and larval preparations (37), two different TnT isoforms have been visualized (Fig. 8A, arrows on the left). These isoforms should
correspond to the two distinct transcripts present throughout embryonic and larval musculature development. In the pupal stages, clear isoform mobility modifications are appreciated due to the important changes occurring at that stage, first the histolysis of the larval musculature (Fig. 8A, Pe), followed by the onset of adult musculature formation. In the adult stages, four protein bands are clearly appreciated. The two bands with higher molecular weights have the same mobility as those present in embryos and larvae. The bands with lower molecular weight (Fig. 8A, arrows on the right) are specific to adult stages and are present exclusively in thorax preparations (37). They appear first in late pupae preparations with a slightly lower mobility of the higher mobility isoform(s) specific to TDT and IFM muscles. Two-dimensional gel separations of mixed preparations of adults with larvae and adults with pupae were carried out to better identify the distinct isoforms (data not shown). The results confirm that some changes seen in the protein pattern arise not only because distinct transcripts are expressed but also from post-translational modifications of the synthesized proteins. The analysis of the stages covering adult muscle formation from late pupae to adults indicates that important changes occur. The theoretical pi of the TnT is 4.5 (pl/MW Program, University of Geneva) and two-dimensional analysis reveals a pi range from 5.5 to 6.5. The two high molecular weight bands showing the same apparent mobility as those present in embryos and larvae first appear in late pupae and seem to undergo no important modifications throughout adult muscle formation (white arrows). In late pupae, some remnants of the embryos and larvae TnT proteins are still detectable in adult samples. In summary, important changes appear associated mainly with the flight and jump isoform. Some or all of these changes may be due to phosphorylation. In vivo
phosphorylation experiments revealed that TnT isoforms are phosphorylated (37). The changes in TnT isoform phosphorylation during development also suggest a role for phosphorylation in TnT function.

**DISCUSSION**

The *Drosophila* TnT gene covers 10.5 kb and is composed of 11 exons. There are several previously unreported features in the exonic organization of the *Drosophila* TnT gene. Intron 1, downstream of the first untranslated exon, is unusually large (2.3 kb in length) when compared with the average size of the introns in *Drosophila*. Large first introns in *Drosophila* muscle genes have been related to the presence in these introns of regulatory motifs involved in the regulation of their expression (44). Also new is exon 4, a GAA trinucleotide, detected in some of the TnT mRNAs of *D. melanogaster* and *D. virilis*, two species separated by more than 50 million years of evolutionary time. The micro exon has been found conserved in the genomic sequences of the two species, flanked by proper splicing signals (38, 39). To our knowledge, it is the smallest *bona fide* exon so far documented at this level of detail. The finding of this exon reduces the limit of the minimum size of exonic nucleotide sequence required for correct splicing. Analysis of the conserved intron sequences upstream and downstream of the micro exon in both *Drosophilidae* indicates that there are several conserved motifs quite different from the previously suggested splicing signals for short exons in vertebrates (45–49). In addition to the pyrimidine-rich region, two sequence elements (underlined in Fig. 3) appear in the upstream region adjacent to the 3′ splice side: a 35 nucleotide element between 105 and 140 nucleotides and a poly(A) sequence (11 A) between 151 and 161 nucleotides in *D. melanogaster*. These conserved sequences differ from those previously reported (48). No conserved sequences have been found downstream of the micro exon, and no G-rich repeat elements appear upstream or downstream of the micro exon (48). These results question the evolutionary generality of this type of signal, suggesting that splicing signals for short exons in vertebrates may differ from those of invertebrates. Alternatively, the enhancer elements required to splice short exons could vary among different microexons. More microexons need to be studied to clarify the mechanism(s) involved in micro exon splicing.

Splicing variability of the TnT genes in vertebrates and invertebrates resides mainly in the 5′ exons. Interestingly, exon 3 in the *Drosophila* TnT gene encodes a glutamic acid-rich domain homologous to a variable spliced exon in vertebrate embryonic cardiac TnT absent in the adult isoform (50, 51). This acidic domain has also been described in an isoform present in chicken fast leg muscles (52). Exon 3 is also alternatively spliced in *Drosophila* TnT. It is absent in the flight and jump muscle isoform but present in all other isoforms. The analysis of the mature transcripts present in *D. melanogaster* dissected muscles show that micro exon 4 is alternatively spliced and is present exclusively in the adult hypodermic and visceral muscles. These transcripts contain the 11 exons of the TnT gene in its entirety. The larval hypodermic muscle transcript does not incorporate micro exon 4, however. Since the incorporation of micro exon 4 in the mature RNA introduces an additional lysine in the amino acid sequence of the protein, this amino acid is the only difference between the larval and adult hypodermic muscle TnT isoforms. Comparison of the vertebrate and invertebrate TnT genes reveals that this additional lysine is present neither in chordate TnT genes (50, 52-54) nor in the report nematode and mollusc TnT genes (12, 55), whereas its presence in the *D. virilis* TnT gene confirms its significance for dipteran. In this respect, one should recall that *Drosophila* develops two distinct sets of muscles during its life cycle: embryonic/larval and adult musculatures with different functional properties. Larval muscles are supercontractile nonfibrillar muscles responsible for larval movements. The hypodermic musculature in the adult, in which micro exon 4 is incorporated, contracts synchronously and cooperates with the IFM and TDT muscles in wing and leg movements (15, 26). Finally, exon 5, also a very short exon coding for only 8 amino acids, gives specificity to other yet unidentified *Drosophila* muscle types.

It may be of interest to point out that there is an important size variability in the amino-terminal region of the TnTs, which is longer in vertebrates especially the cardiac isoforms. The amino-terminal regions of urochordate (ascidian) TnTs are slightly shorter and similar to *Drosophila* and mollusc TnTs. On the other hand, the nematode amino-terminal domain is similar in size to the vertebrate skeletal muscle TnT. Related to the splicing variability in the 5′ region, a repeating metal-binding sequence present exclusively in certain avian orders is not detected in any of the invertebrate TnTs so far studied (56). When metal ion is bound to this variable domain, an overall reconfiguration of the TnT is detected through structural relationships between this variable region and other domains of the intact TnT molecule (57).

The situation is slightly different in the case of the COOH-terminal region of TnT. This region also presents important variability but is not created by an alternative splicing mechanism. The only reported exception occurs in vertebrate fast skeletal muscle TnT, in which two alternatively and mutually exclusive miniexons have been described in this region. Interestingly, when the currently known TnT protein sequences are compared (8, 11, 12, 50, 54, 55), there is an even clearer size increase trend in the carboxy-terminal domains (Fig. 9); in urochordates such as ascidians, they are slightly longer than in vertebrates with an extension of approximately 10 amino acid residues. Molluscs, such as *Chlamys nipponensis* (55) and *Patinopecten yessoensis* (56) present extensions in these regions of 55 amino acid residues. *C. elegans* TnT has a carboxy-terminal extension of approximately 100 residues, whereas insects (*D. melanogaster* and *D. virilis*) have even larger extensions including an additional polyglutamic tail of 30 residues. The extensions in invertebrates have poor sequence similarity, but are rich in polar amino acids, particularly in glutamic acid residues. *Drosophila* is the extreme in this trend with its polyglutamic tail. This highly acidic tail is possibly also conserved in the crayfish *Astacus* (data not shown). This domain is expressed in all *Drosophila* TnT isoforms indicating the importance of this domain for arthropod muscle function. Results from our laboratory showing that *Drosophila* TnT binds Cu²⁺ in *vitro* may be related to this domain (37). The phenotype of a *C. elegans* mutation, *map-2* (e2346ts), which partially truncates this extension (12), demonstrates that at the 3-fold stage, embryos show a muscle displacement that affects muscle contraction coordination. Muscle cells in this mutant express TnT. In most of them, thin and thick filaments seem to assemble normally, but in some, they do not appear to become stably assembled, either because assembly is defective or because it is unstable (12). A TnT mutant in *Drosophila*, *upheld* (11), generates a truncated polypeptide lacking the COOH-terminal extension (11). Homozygous *upheld* flies have no TnT in their flight muscles and virtually no thin filaments, presumably because the truncated molecules are rapidly degraded. It would seem that the role of TnT in the invertebrate troponin complex regulating muscle contraction is slightly different and possibly more complex than in vertebrates.

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Differential RNA Expression of Drosophila Troponin T Gene

By in vitro analysis, the TnT interaction domains with tropomyosin or with TnI and TnC have been identified in two different regions in vertebrates, located in the middle and close to the carboxyl-terminal end of the vertebrate protein (4–6, 59, 60). By alignment of the known vertebrate and invertebrate TnT sequences, an appreciable similarity has been found in these two regions (11, 12, 55) (Fig. 9). In summary, these findings support the idea that the interaction domains of TnT allow the whole tropomyosin-troponin complex to be bound to the thin filament are fully conserved during evolution, whereas in the other domains there is important variation. No splicing has been detected in these domains. Although these findings document the essential role of these interaction regions in the contractile apparatus, they also indicate the importance of the amino- and carboxyl-terminal regions in the modulation of TnT function. These regions present important variability whose analysis reveals that although some of these domains are conserved among species, orders, or even phyla, comparison within the same organism as in our studies in Drosophila shows their importance in determining specific stage or type isoforms. Distinct isoforms are ascribed to specific muscle fibers, giving muscle type specificity. We suggest that these TnT regions modulate muscle function and are in some way responsible for differences in the contraction in addition to their evolutionary peculiarities, such as the 10 residues specific to the nematode and the polyglutamatic tail specific for diptera/arthropods.

The modulation of TnT function appears to be slightly different from another key muscle protein, myosin. Myosin heavy chain modulation of the activity in different muscles occurs by alternative splicing in the functional domains of the protein (15, 61). In TnT, the binding domains are conserved and the changes in the NH2- and COOH-terminal regions of the protein modulate its function, possibly through the reconfiguration of the protein.

A distinct form of modulation could reside at the level of post-translational modifications. The complexity of the TnT isoforms deduced from the transcript pattern of the Drosophila TnT gene increases with the appearance of important changes produced by post-translational modifications of the isoforms. Our studies reveal that some of these changes appear exactly at the moment that flies acquire the flight and jump capability (see Fig. 8) as has been seen in the honey bee (37). It is worth pointing out that the theoretical pI of the highest molecular weight Drosophila TnT isoform is 4.5 (62), but the pI determined in two-dimensional analysis ranges from 5.5 to 6.5. Recent in vitro studies demonstrated that TnT could be a substrate for transglutamination, a reaction strictly dependent on the presence of calcium ions (63). Previous in vitro studies have demonstrated that vertebrate TnT isoforms are phosphorylated (64); furthermore, in vivo phosphorylation experiments in our laboratory (37) revealed that Drosophila TnT isoforms are also phosphorylated and change during development, suggesting a role for phosphorylation in TnT function. Two conserved phosphorylation sites for casein kinase II (58) have been found in all the invertebrate carboxyl-terminal extensions known (underlined in Fig. 9).

Our findings strongly suggest that post-translational modifications occur during TnT protein synthesis, giving rise to a more basic pI than the theoretical one. Phosphorylation could be a more sophisticated level of regulation implicated in the acquisition of more specific muscle capability in a temporary manner, as is the case of the flight or jump acquisition of the thorax muscles after hatching. The occurrence of some of these modifications has been documented in vertebrate TnTs, but their functional significance is still unclear. Drosophila with its molecular genetic approaches is a very suitable model system to test the functionality of this huge isofrom variability.

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FIG. 9. Amino acid sequence comparison of the carboxyl-terminal regions of the known vertebrate and invertebrate TnTs. The sequences corresponding to the interaction domains located near the carboxyl-terminal region of the currently known TnTs have been aligned. The comparison within the same organism as in our studies in D. mel, D. melanogaster, C. elegans, Patino, Patinopecten, Acipenser, Chicken, and Rabbit is shown. The amino acid sequence comparison of the carboxyl-terminal regions of the known vertebrate and invertebrate TnTs. The sequences corresponding to the interaction domains located near the carboxyl-terminal region of the currently known TnTs have been aligned. The comparison within the same organism as in our studies in D. mel, D. melanogaster, C. elegans, Patino, Patinopecten, Acipenser, Chicken, and Rabbit is shown.
Differential Muscle-type Expression of the *Drosophila* Troponin T Gene: A 3-BASE PAIR MICROEXON IS INVOLVED IN VISCERAL AND ADULT HYPODERMIC MUSCLE SPECIFICATION

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