MOLECULAR AND CELLULAR BIOLOGY, Mar. 1993, p. 1433–1439
0270-7306/93/031433-07$02.00/0

Vol. 13, No. 3

Abnormal Muscle Development in the *heldup*^{3} Mutant of *Drosophila melanogaster* Is Caused by a Splicing Defect Affecting Selected Troponin I Isoforms

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Received 2 September 1992/Returned for modification 2 November 1992/Accepted 9 December 1992

The troponin I (TnI) gene of *Drosophila melanogaster* encodes a family of 10 isoforms resulting from the differential splicing of 13 exons. Four of these exons (6a1, 6a2, 6b1, and 6b2) are mutually exclusive and very similar in sequence. TnI isoforms show qualitative specificity whereby each muscle expresses a selected repertoire of them. In addition, TnI isoforms show quantitative specificity whereby each muscle expresses characteristic amounts of each isoform. In the mutant *heldup*^{3}, the development of the thoracic muscles DLM, DVM, and TDT is aborted. The mutation consists of a one-nucleotide displacement of the 3′ AG splice site at the intron preceding exon 6b1, resulting in the failure to produce all exon 6b1-containing TnI isoforms. These molecular changes in a constituent of the thin filaments cause the selective failure to develop the DLM, DVM, and TDT muscles while having no visible effect on other muscles wherein exon 6b1 expression is minor.

We have reported that the haplolethal (HL) region of the *Shaker* gene complex (*ShC*) of *Drosophila melanogaster* (13) harbors the structural gene for troponin I (TnI) (2) and ascribed the *heldup* (*hdp*) mutations, originally described by Deak (10), to the TnI gene on the basis of genetic complementation tests. Later, Beall and Fyrberg (4) showed that some *hdp* mutants lack several TnI isoforms. However, since the mutant phenotype consists in the severe depletion of selected muscles, it is not clear whether the lack of specific TnI isoforms is the cause or the consequence of the muscle defect.

We find that the allele mutation *hdp*^{3} consists of a single nucleotide change at the A(G/G) 3′ border of the intron preceding exon 6b1 of TnI, which is converted to A(A/G). The splicing complex in the mutant does not seem to recognize the new splice site and consequently does not express exon 6b1-containing RNA isoforms in any muscle, including those that do not show the mutant phenotype. The muscles not affected by the absence of exon 6b1 are those in which the expression of this exon would have been quantitatively minor.

TpI is a constituent of the thin filaments and plays a regulatory role in muscle contraction (33). It binds to actin, blocking the actin-myosin interaction in the resting state. Upon Ca^{2+} entry, TnI shifts to interact with troponin C, leaving actin free and allowing the relative displacement of thin and thick filaments. Muscle physiology in insects shows peculiar features which sustain their extraordinary performance during wing beat (25). In the insect fibrillar flight muscles (DLM and DVM), calcium alone is not enough to produce full activation. The Ca^{2+}-activated muscles are further activated in both tension and ATPase activity by small strains (1 to 3%) of their fibers. This property, first shown in the giant waterbug *Lethocerus* sp. (32), is also seen in *D. melanogaster* (23) and enables the wings to beat at high frequency. It is expected that the diversity of mechanical properties of insect muscles will be based on a wide repertoire of regulatory elements such as TnI (reviewed in reference 6).

After genomic sequencing of the entire TnI transcription unit and extensive polymerase chain reaction (PCR) assays, we have identified a large repertoire of TnI isoforms. Assays of hybridization to tissue sections and stage-specific Northern (RNA) blots with exon-specific probes indicate that these isoforms are qualitatively as well as quantitatively regulated in their expression. Finally, gene dosage experiments suggest that the TnI family might interact functionally with other products encoded within *ShC* that are now being studied.

MATERIALS AND METHODS

PCR of RNA and genomic DNA. Total RNA was extracted by the thiocyanate-phenol method (7); 5 μg was transcribed to cDNA by using the First-Strand cDNA Synthesis Kit (Pharmacia), with the degenerate hexanucleotide as a primer. cDNA was further amplified with *Taq* DNA polymerase (Boehringer) under standard conditions (18). For total TnI mRNA amplification, we used the sense 5′-CCGTGGATCGTCGGACCGTTC-3′ (within exon 1) and the antisense 5′-CTGATCCAAATCCATTGTGGAC-3′ (within exon 10) primers. After this amplification, two bands are observable in agarose electrophoresis gels stained with ethidium bromide. The largest of these bands corresponds to exon 3-containing isoforms, as revealed by Southern transfer and specific hybridization.

Amplification of genomic DNA was carried out in six overlapping fragments. The following primers were used for each fragment: 5′-CAGTTCCTTGAGTTCACCTC-3′ (within the intron upstream of exon 2) and 5′-ACCTCCTCG GCCTTCACTTT-3′ (within exon 3) for a 948-nucleotide (nt) fragment; 5′-CAGAAAAAGGCAGCAGCTCC-3′ (within exon 3) and 5′-GTACCTCAACATCCTTGCGT-3′ (within exon 6a1) for a 1,363-nt fragment; 5′-AGACACACT CAAATCTCTGA-3′ (within exon 6a1) and 5′-TTGGGCCT TGACTTTCTGCA-3′ (within exon 6b2) for a 2,157-nt frag-

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ment; 5′-TACCGCAGCCGAACTGCAAA-3′ (within exon 6b2) and 5′-CTCCCAGTCTTTTTTCCTGA-3′ (within exon 6b1) for a 1,141-nt fragment; 5′-GCGAATTGCAAGAGATT TGCG-3′ (within exon 6b1) and 5′-ATTTAGCACAGCG GCATCTT-3′ (within exon 9) for a 3,045-nt fragment; and 5′-TTCGCCAAGCTGCAGAAGAA-3′ (within exon 8) and 5′-CTGATCAAATCCATTGTGGAC-3′ (within exon 10) for a 1,003-nt fragment. All of these fragments were subcloned for further sequence analysis. Nucleotide sequences were determined by the dideoxy chain-terminating method (29).

Northern blots and in situ hybridizations. For developmental analysis of new exons, we used poly(A)$^+$ RNA as described previously (2). For mutant analysis, we used total RNA extracted as described above. Specific probes were labeled with [α-^{32}P]dATP by the random primer method (12) after amplification and subcloning of the corresponding exon. In situ hybridizations were carried out in 10-μm sections from paraffin-embedded flies, using ^{35}S-labeled antisense RNA probes as described previously (24).

Antisera and Western immunoblots. Antibodies against TnI were raised against a β-galactosidase fusion protein. For this construction, we inserted a *Bam*HI-*Hin*dIII fragment from cDNA A16 (2) in the polylinker of vector pUR 290 (27). In this manner, a 205-amino-acid segment of the TnI polypeptide encoded in cDNA A16 is fused in frame with *Escherichia coli* β-galactosidase. The fusion protein was purified from *E. coli* DIH 101 extracts after electrophoresis and electroelution from the polyacrylamide gel strip containing the band. Rabbits were immunized and sera were collected by standard procedures (14).

Total proteins were extracted at different developmental stages by homogenization in 20 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$–5% 2-mercaptoethanol–2% sodium dodecyl sulfate (SDS) and boiling for 10 min. Protein analysis was performed by SDS-polyacrylamide gel electrophoresis (12% initial acrylamide concentration) and Western transfer to nitrocellulose membranes. For the results reported here, a crude antiserum (J2) was used at a 1:1,000 dilution.

Histology and evaluation of functional phenotypes. Histological analysis of mutant phenotypes was carried out on 10-μm sections of paraffin-embedded flies stained with toluidine blue. Flight and jumping performance was evaluated in a manner similar to that described by Homyk and Emerson (16). Briefly, flight performance was evaluated by pushing the flies over the edge of a 1-m-high platform. Flies were scored as nonfliers (− in Table 1) if they fell to the bottom and as fliers (+ in Table 1) if they did not. Jumping performance was tested by gently blowing a puff of air on each fly and scoring the length of the jump. The arbitrary limit of 1 cm was used to separate jumpers from nonjumpers. The loss of ground support and the blow of air are chosen as stimuli because it is known that they trigger the scape pathway through the cervical giant interneuron. Jumping performance was tested only in flightless strains because flight initiation requires initial jump, and consequently, fliers should be jumpers.

Nucleotide sequence accession number. The EMBL/GenBank data base nucleotide sequence accession number for the TnI transcription unit is X58188 DMTROPONI.

RESULTS

Differential splicing of 13 exons yields 10 TnI isoforms which are expressed in specific muscles. In a previous report (2), we described the HLI transcription unit of the HL region of *ShC*. The sequence of the 11 exons known at that time indicated that HLI encodes *Drosophila* TnI, as determined from sequence comparison with TnIs from other organisms. Also, the *hdp* mutants were ascribed to this transcription unit on the basis of tests of allelomorphism with all known mutations in the HL region of ShC. We have now sequenced the 12 kb of genomic DNA that encompass the transcription unit, and the region has been scanned for areas with high values of *Drosophila* codon usage. As a result, the total number of exons has been increased to 13 (Fig. 1). Also, extensive PCR assays in the wild type and mutants as well as Northern analyses (see below) have been used. Together, the results of these studies indicate that the complete repertoire of TnI isoforms has been identified.

Among the 13 exons identified, 4 deserve special mention. We refer to them as the exon 6 family, in view of their sequence similarities (Fig. 1). Also, they are mutually exclusive, for no mRNA has been found with more than one exon 6, or without any, after PCR experiments in the wild type and the *hdp* mutants. Furthermore, any combination with more than one exon 6 would break the reading frame.

Within the exon 6 family, sequence similarities permit grouping into two subfamilies, 6a and 6b, each composed of two members, 6a1-6a2 and 6b1-6b2. Amino acid sequence identity is 32% between the two subfamilies and 47% between members of each subfamily. The amino acid similarities among exons 6 are concentrated in one half of the sequence and represent the TnI attribute. The acidic residues at positions 168, 173, 175, and 184 (Fig. 1) are conserved in all *Drosophila* exons 6 and at the homologous positions in all known TnIs. In contrast, the other half of exon 6 sequences contains the characteristic that differs among isoforms. The acidic residue at position 158 of exon 6b1 is a peculiar feature of this exon since it is occupied, instead, by a basic amino acid in the other exons 6 and at the homologous positions of other organisms. Another differential aspect of this region is the number of cysteines. There is one cysteine encoded in exon 5, a constitutive exon, none in exon 6a1, one in exons 6a2 and 6b2, and two in exon 6b1 (Fig. 1). The one at position 157 is a constant in all known TnIs except for the exon 6a1-containing isoforms of *D. melanogaster* and the crayfish. Most likely, different isoforms would give rise to variable domains, depending on the particular exon 6 present. A further indication of the functional significance of exons 6 comes from their pattern of expression and the location of TnI mutations.

Exons 6a2 and 6b2 are newly described here. They are scarcely expressed in the wild type, which explains the failure to detect them in the screening of cDNA libraries. Their detection is now possible as a result of sequencing of the genomic DNA and the subsequent PCR amplification assays. Northern blots hybridized with exon 6a2-specific probes show some weak signal in the embryo and larval stages, and the blots must be overexposed in order to find a trace of signal in the adult stages. In situ hybridizations to tissue sections indicate that all embryonic and larval somatic muscles express this exon. By contrast, exon 6b2 shows a relatively high signal in the adult stage and a very low level in the embryo and larva (data not shown).

Exons 6a1 and 6b1 are more abundant and were previously reported (2) as 6a and 6b. 6a1 is expressed at all developmental stages in all muscles. The highest expression in the adult is found in the somatic muscles of the abdomen (Fig. 2A). Exon 6b1 is weakly expressed at larval stages, but coincident with muscle histogenesis during metamorphosis, its level increases and reaches maximal values in the thoracic muscles (Fig. 2B). Among them, the expression of

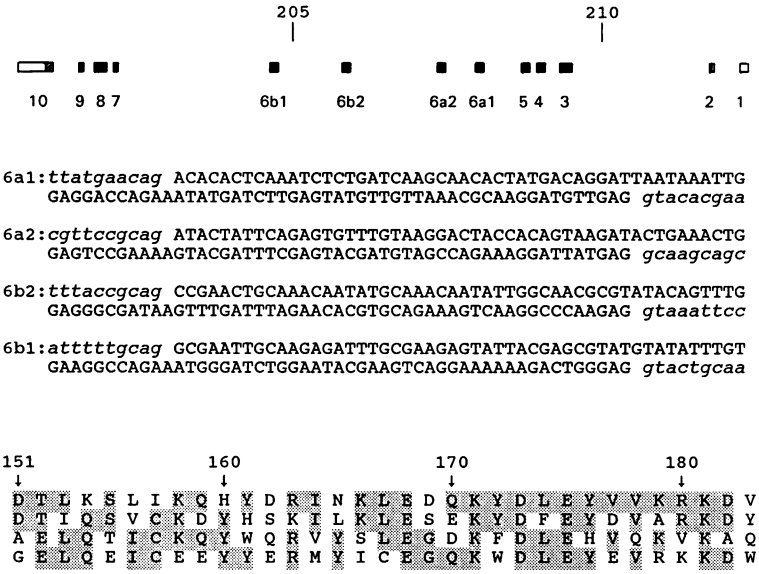

FIG. 1. TpI gene organization. The transcription unit extends over 12 kb of genomic DNA and includes 13 exons. The 3′-5′ orientation reflects the proximal-distal transcription of TnI RNA on the X chromosome. Coordinates at the top are from Baumann et al. (3). Coding segments are indicated by filled boxes. The cross-hatched box indicates a fragment of exon 10 between two alternative stop codons. The sequences of exons 6 are indicated by capitals, and the intron borders are indicated by lowercase letters. Note the unusual GC dinucleotide at the 5′ of intron following exon 6a2. Amino acid sequences of exons 6 are shown in one-letter code, with the identities shaded. Residues are numbered according to Barbas et al. (2).

exon 6b1 shows quantitative differences. TDT is the muscle with the highest level, followed by DVM, DLM, and leg muscles. 6b1 expression is very low in the abdomen and not detectable in the proboscis.

The full repertoire of native TnI isoforms has been elucidated after mRNA analysis by systematic Northern blots and PCR assays. Amplifications with an antisense primer from exon 9 and sense primers from each of the alternative exons 6 demonstrated that all four combinations are possible in the wild type, during both larval and adult stages. Similarly, amplifications with an antisense primer from exon 10 have shown that every exon 6 can combine with exon 10, skipping exon 9. By contrast, when a sense primer from exon 3 was assayed with antisense oligonucleotides for each exon 6, only the combination 3-6b1 gave the amplified product (data not shown). It should be noted that the joint expression of exons 3 and 6b1 occurs only in DLM and DVM muscles and corresponds to the cDNA A16 previously described (see Fig. 3 in reference 2). The complete set of TnI isoforms found after this study is shown in Fig. 3. Eight of them are expressed during the embryo and larval instars, and two additional ones are expressed at the adult stage (see the legend to Fig. 3).

To analyze TnI expression at the protein level, we raised antibodies against a β-galactosidase–TnI fusion protein. Western blots of adult total proteins incubated with the

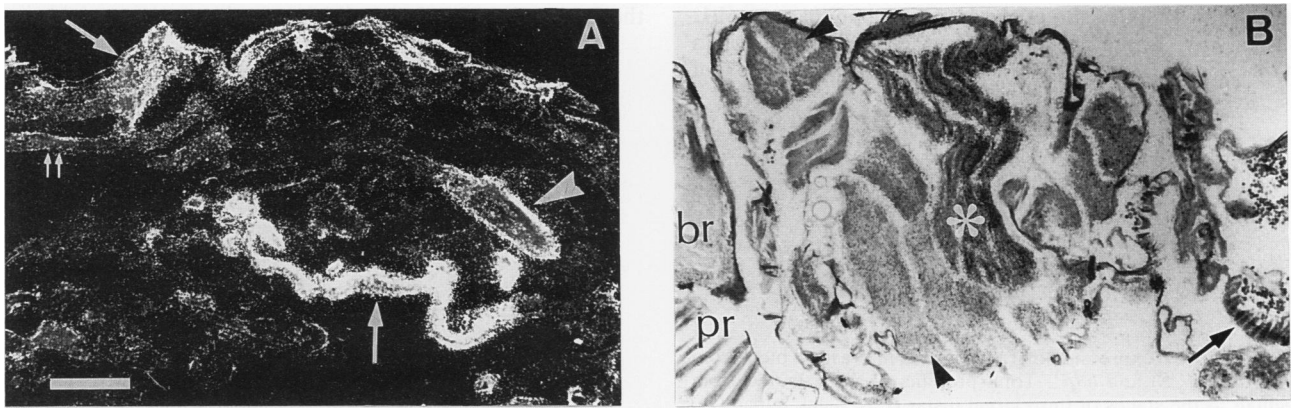

FIG. 2. In situ expression of major exons 6. Shown are sagittal sections of wild-type late pupae hybridized with exon 6a1 (A)- and 6b1 (B)-specific probes. The 6a1 signal is very strong in abdominal muscles (single arrows) and in the splacnic muscles of the gut (large arrowhead). DLM fibrillar muscles show a weaker hybridization signal (small arrows). The 6b1 signal is maximal in the tubular thoracic muscle TDT (asterisk), while fibrillar muscles (arrowheads) and tubular muscles of legs show a lower level of hybridization. The signal is very weak in abdominal muscles (long arrow) and seems to be absent from the proboscis (pr) muscles. Anterior is to the left. Sections are counterstained with toluidine blue. br, brain. Bar = 200 μm in both panels.

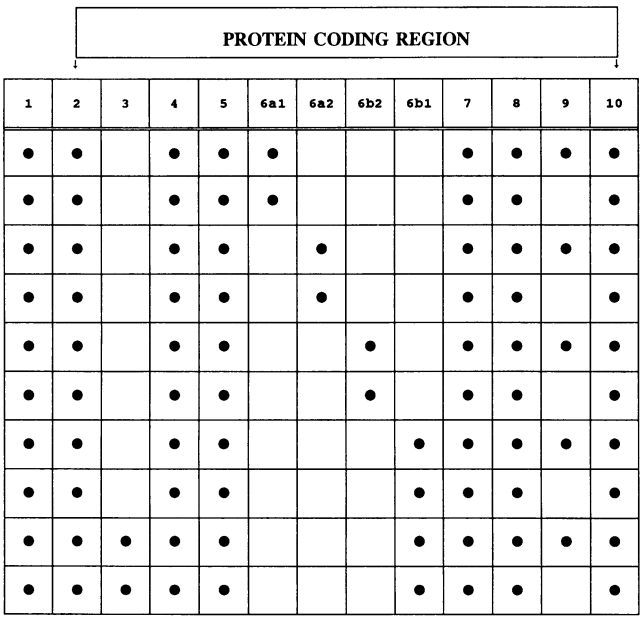

FIG. 3. The family of *Drosophila* TnI isoforms. Ten combinations from 13 exons are found after Northern hybridizations with specific probes and extensive PCR analysis of mRNA. All possible combinations of alternative exons were assayed with the corresponding primers (see text). Only the 10 isoforms presented here resulted amplified. Larvae express only isoforms without exon 3, while the adult shows the whole repertoire.

anti-TnI antibodies identify a band of 30 kDa (apparent molecular size) and a doublet of 40 kDa (Fig. 4). In the larval material, only the 30-kDa band is detected. Since exon 3 is specific for the adult stage, we interpret these results as indicating that the 40-kDa doublet corresponds to the protein isoforms containing this exon. At the onset of metamorphosis, all signs of TnI disappear coincident with muscle tissue histolysis. At the pupal stage, coincident with myogenesis, TnI reappears but the 40-kDa doublet initiates as a single band instead of the doublet found in the adult. It is plausible that some TnI protein isoforms undergo some type of maturation process after eclosion.

The *hdp*^{3} mutant phenotype is caused by a single nucleotide change at the 3′ splice site of the intron preceding exon 6b1. The allele *hdp*^{3} is one of the most extreme *hdp* mutations in terms of muscle structure (2, 10). Serial observations of

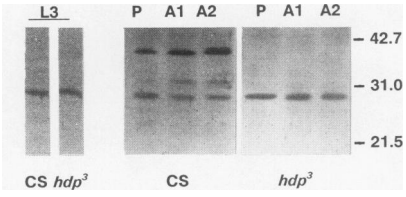

FIG. 4. Protein expression of TnI isoforms in Western blots from the wild type (CS) and *hdp*^{3}. Total proteins were extracted from individuals at the stages of third larval instar (L3), black pupae (P), younger than 12-h adults (A1), and older than 2-day adults (A2). Reference molecular sizes are indicated at the right in kilodaltons. At L3, only the 30-kDa isoforms are expressed in CS as well as in *hdp*^{3}. Note the conversion, after eclosion, of the upper (40-kDa) band into a doublet. This band(s) corresponds to exon 3-containing isoforms, and it is completely absent in the mutant.

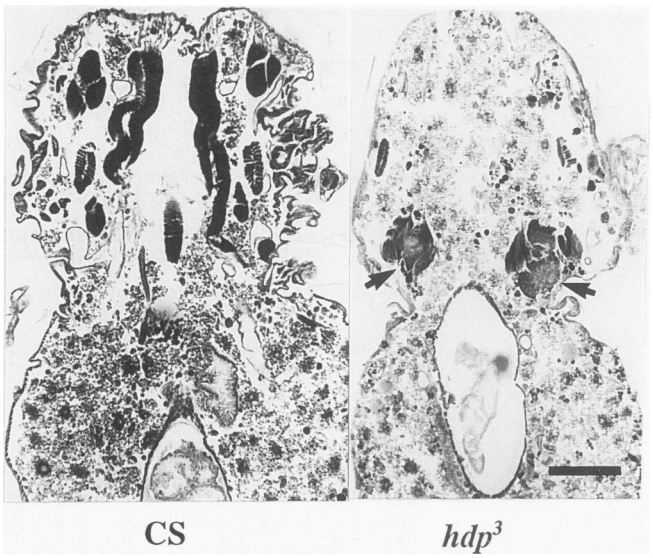

FIG. 5. The *hdp*^{3} mutant phenotype. Shown are horizontal sections of late pupae of CS and *hdp*^{3} females. Note the failure of DLM, DVM, and TDT muscle development in the mutant. Only small remnants (arrows) can be found occasionally. Bar = 250 μm.

myogenesis during metamorphosis at the optical microscope level show that DLM, DVM, and TDT muscles initiate assembly but collapse soon afterwards (Fig. 5). Newly eclosed adults show occasional remnants of muscle tissue without any trace of sarcomeric organization. All other somatic and visceral muscles appear normal in structure. Previous observations at the electron microscope level had shown some thin filaments within the sarcoplasm (4).

To locate the sequence defect in this allele, we amplified total RNA from *hdp*^{3} flies. The only exons absent from these PCR amplifications are 3 and 6b1. The sequences of the remaining 11 exons in the mutant are normal. Furthermore, we amplified genomic mutant DNA in six overlapping fragments (see Materials and Methods). After sequencing all exons and extensive regions of the intron borders, we found that the only significant change with respect to the wild type is in the 3′ border of the intron preceding exon 6b1, where the universal G residue is substituted by an A (Fig. 6). Since the first nucleotide of exon 6b1 is a G, the mutation yields, in

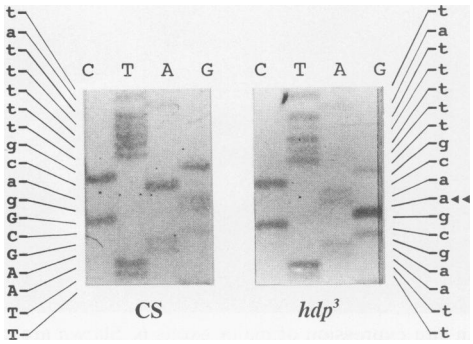

FIG. 6. Sequence change in the TnI gene of *hdp*^{3}. The sequence gel corresponds to the border between exon 6b1 and the upstream intron. Amplified genomic DNA from CS and *hdp*^{3} flies was used. Nucleotides of the wild-type exon 6b1 are in capitals. The nucleotide change in the mutant is indicated by two arrowheads.

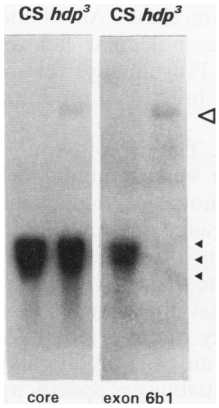

FIG. 7. Splicing defect in *hdp*^{3}. Shown are exon-specific hybridizations to Northern blots from wild-type and mutant adult total RNA. The probe used for each hybridization is indicated below. The same blot was used throughout. As a core probe, we used a fragment containing the constitutive exons 7, 8, and 10. Three main bands of TnI (full arrowheads) can be obtained. Note the lack of exon 6b1-containing isoforms in the mutant and the consequent appearance of a very large form (open arrowhead) interpreted as a precursor.

effect, a one-nucleotide relative displacement of the AG splice signal.

The transcriptional consequences of the mutation were studied in a Northern blot hybridized with exon-specific probes (Fig. 7). Hybridization with an exon 6b1-specific probe showed that exon 6b1-containing isoforms are absent in the mutant. Also, a much larger RNA is detected in the mutant. We reasoned that this new RNA could represent transcripts aborted at the moment of exon 6b1 processing. This putative precursor hybridized also with probes specific for exons 3, 6b2, and 7, 8, and 10, and it was not detected in poly(A)$^+$ RNA blots (data not shown). The same total RNA Northern blot was hybridized with probes specific for the remaining exons 6 and revealed no alteration in the mature TnI mRNAs with respect to the normal pattern. At the protein level, the *hdp*^{3} mutant shows no alteration in the larval material, while in the adult, only the 30-kDa band is detected in Western blots (Fig. 4). This observation is compatible with the absence of exon 3-containing mRNAs.

These molecular changes are the foundation of the mutant phenotype: failure to develop DVM, DLM (fibrillar), and TDT (tubular) muscles. It should be noted that other tubular muscles in the adult and the larvae, wherein exon 6b1 should have been expressed, do not show the mutant phenotype. This finding is an indication of the differential quantitative requirements of each TnI isoform in each muscle, a concept that we refer as quantitative specificity (11).

Dosage effect on the mutant expression of *hdp*^{3}. Since the only defective TnI isoforms are those containing exon 6b1, the mutant phenotype can be ascribed solely to them. It is generally recognized that proper muscle function requires a precise stoichiometry of all its constituents. Gene dosage experiments allow in vivo exploration of these requirements. Table 1 shows the structural and functional consequences of modifying the relative amounts of TnI isoforms and the proteins encoded in the adjacent genes. It is observed that a 50% reduction in exon 6b1-containing isoforms (♀ *hdp*^{3}/+), while normal levels of all remaining isoforms, are maintained, leads to abnormal muscle structure and function. The muscle phenotype is restored to wild type if a normal dosage for exon 6b1 TnI isoforms is reached [♂ *hdp*^{3}; *Dp(1;3)JC153*/+]. However, a further increment of dosage [♂ *hdp*^{3}; *Dp(1;3)JC153/Dp*(1;3)*JC153*] again causes a functional defect, since these flies are unable to fly or jump. Also, this genotype shows a reduced viability with respect to the control. The control [genotype ♂ +; *Dp(1;3)JC153/Dp(1;3)JC153*], in which the same dosage is present on a nonmutant background, does not yield any obvious functional defect. These observations are compatible with the interpretation that other proteins encoded in some of the genes duplicated within *Dp(1;3)JC153* must keep a stoichiometric relationship with all or some TnI isoforms in order to yield normal muscle activity. This relationship should affect the physiology of the muscles rather than their structure, since ♂ *hdp*^{3}; *Dp/Dp* flies show normal muscle histology and wing position even though they are unable to jump or fly (Table 1).

DISCUSSION

Structural features of TnI. We have shown that TnI of *D. melanogaster* is produced as a family of 10 isoforms which have significant structural differences with respect to the alternative exons 3 and 9 as well as the mutually exclusive exons 6. The number of cysteines in each isoform is determined by the exon 6 present. This feature is likely to contribute to the formation of a variable number of protein domains containing S-S bonds. In vitro experiments with vertebrate TnI have shown that cysteines may need to be reduced in order to be functional (17), allowing for a regulatory mechanism based on the oxidation state of the mole-

TABLE 1. Structural and functional phenotypes of TnI gene dosage effects[a]

Genotype	Muscular phenotype			Functional phenotype		
	DLM	DVM	TDT	Wing position	Jumping	Flight
♂ +; *Dp/Dp*	+	+	+	+	+	+
♂ *hdp*^{3}	−	3	3	Up	−	−
♀ *hdp*^{3}/+	1	+	2	+[b]	−	−
♂ *hdp*^{3}; *Dp*/+	+	+	+	+	+	+
♂ *hdp*^{3}; *Dp/Dp*	+	+	+	+	−	−

[a] Structural phenotypes are based on the histological evaluation of at least five serially sectioned 2-day-old flies. They were obtained, whenever possible, from the same sib. Symbols: +, normal; 1 to 3, increasing structural defects in myofibril package, attachment to the hypoderm, order within the sarcomere, etc.; −, muscle practically absent. *Dp* is *Dp(1;3)JC153*. This rearrangement contains all known genes within *ShC* (13), and they are transcribed at a rate according to the sex of the genome in which the rearrangement is located. Functional phenotypes were assayed as described in Materials and Methods. Three- to five-day-old flies (n = 10) of each genotype were tested for flight and jumping performance.

[b] Aged ♀ *hdp*^{3}/+ flies tend to develop a wings-up phenotype.

cule. In this context, the *Drosophila* TnI isoforms that contain only one cysteine would not be subject to this regulation. Sequence comparison of *Drosophila* exons 6 with the equivalents in other organisms shows that certain residues (e.g., K158 in Fig. 1) are highly conserved. The existence of isoforms with a significant variation at these positions (e.g., exon 6a1 isoforms) suggests that TnIs play rather diverse functions. Other structural features, possibly involved in steric interactions with other proteins, might be the hydrophobic sequence of exon 3, which is very similar to that of some tropomyosin isoforms (19). Also, TnI has a modified leucine zipper motif in common with the myosin heavy chain (MHC) (2), which could provide the structural basis for another interaction.

Since TnI is known to play a very strategic role in the regulation of muscle physiology, it is not surprising that this type of protein will interact with thin as well as thick filaments. In fact, TnI interactions with MHC have been proposed (6) on the basis of double-mutant suppression. Genetic studies are under way to detect functionally significant interactions of TnI. In this context, the gene dosage experiments described in this report suggest that some proteins encoded within *ShC* might interact with TnI and contribute to proper muscle physiology. This suggestion is based on the observation that hdp^3 male mutants in which the TnI and neighboring genes are in excess of dosage can neither fly nor jump (Table 1). More precisely, the progressive increment of dosage of the region on a mutant background first restores the structural and functional abnormalities [genotype ♂ hdp^3; *Dp(1;3)JC153/+*] and later causes functional impairement [genotype ♂ hdp^3; *Dp(1;3)JC153/Dp(1;3)JC153*]. It is plausible that some of the proteins encoded in the vicinity of TnI are functionally related to it and need to maintain a certain stoichiometry among them. Studies on the region are under way to clarify this issue.

TnI in muscle structure and function. Actin and myosin are the major components of thin and thick filaments, respectively. Null mutations at the corresponding structural loci yield muscles devoid of thin or thick filaments (5), indicating a relative independence in the self-assembly of filaments. TnI is a minor constituent of thin filaments and plays a functional role within the complex of three troponins and tropomyosin (33). Thus, it is surprising to find that a mutation lacking some but not all TnI isoforms yields such dramatic effects on muscle development.

At present, we can only speculate on the mechanism that links partial TnI depletion with failure to develop specific muscles. It is plausible that the stoichiometric changes caused by the elimination of some TnI isoforms are more deleterious than the dearth of isoforms per se. The results from the gene dosage constructions (Table 1) seem to indicate that other proteins encoded within *ShC* might be required in specific ratios with respect to TnIs. Homyk and Emerson had proposed that HL regions might represent clusters of genes encoding muscle-related proteins (16). Perhaps what we refer to today as simply stoichiometric relationships will turn out to be more elaborated regulatory relationships among the HL components. The finding that TnI is present in 10 different isoforms invites one to reconsider the scope of functional roles of this protein. It is important to realize that any given muscle can have several TnI isoforms (e.g., DLM expresses at least two mutually exclusive exons 6, 6a1 and 6b1) whose specific roles in the control of muscle contraction are still unknown. It will be important to determine whether TnI isoforms within a single muscle have different localizations along the myofibrils and/or play diverse functions. All of these considerations serve to illustrate the molecular heterogeneity that each muscle cell conceals. Presumably, this molecular diversity sustains an equivalent functional repertoire that is yet to be explored.

TnI gene expression. The use of exon 3 is a distinguishing feature of the fibrillar versus tubular developmental pathways. In turn, the choice of this exon limits the choice repertoire of exons 6, since only exon 6b1 is compatible with exon 3. This hierarchy of sequential decisions represents constitutive steps of the respective myogenic programs. It is generally admitted that these types of selective features result from the activity of cell-specific *trans* factors whose mechanisms of action are still largely unknown. In addition, beyond this type of qualitative specificity, it seems appropriate to introduce the term quantitative specificity. A case in point is that of exon 6b1-containing isoforms which are expressed in practically all muscles observed, albeit with different intensities (Fig. 2B). The total absence of these isoforms causes a visible phenotype only in the DLM, DVM, and TDT muscles, while the remaining muscles seem to operate normally. It seems reasonable to propose that in addition to the *trans* factors that select which exon will be expressed, another set of *trans* factors must specify the relative amount to be expressed and that this amount is a biologically significant parameter. We envision that at least some of the *trans* factors act on the splicing complex, yielding cell-specific spliceosomes for each isoform to be expressed.

Splicing control. The mutation hdp^3 consists in the substitution of the universal G at the 3′ splice site of the intron preceding exon 6b1 by an A generating, in effect, a one-nucleotide displacement of the AG signal. In turn, the mutation modifies the sequence context at the 3′ end from cagG to caaG. These changes result in the accumulation of a precursor RNA, possibly from interruption of splicing (Fig. 7).

It could be argued that, as has been found in many cases, splicing in the mutant does indeed take place, generating aberrant RNAs in which exon 6b1 has been skipped (1, 8, 15, 22, 28), or, if the new AG signal is used, a stop codon appears in the frameshifted sequence of the new exon 6b1. Both types of RNAs will possibly be unstable and rapidly degraded (21). However, splicing of the mutant RNA beyond exon 6b1 appears unlikely because PCR amplifications in hdp^3 have never yielded an abnormal mRNA, and in any event, the appearance of the precursor RNA would not be justified if splicing of exon 6b1 took place. It is somewhat surprising that the one-nucleotide displacement of the AG signal interrupts splicing. In vitro studies have shown that this type of change results, most frequently, in exon skipping or, seldom, in a sequence frameshift (31). Also, in vitro experiments have shown that an AAG triplet can substitute for the more common signal CAG (30). In addition to the possible divergence between in vitro and in vivo conditions, an important difference between those cases and hdp^3 is that in the latter case, 6b1 is an alternative exon, while most of the available information on signals and mechanisms of splicing concerns constitutive exons.

Two other cases of *Drosophila* mutations at the 3′ splice site of alternative exons are known with some detail. We reported previously that Sh^{E62} is an identical type of mutation in the alternative exon 20 of the V transcription unit of the *ShC* (20). In that case, neither exon skipping nor splicing interruption occurs. Instead, the intron preceding exon 20 is maintained in the final mRNA, which is polyadenylated and

translated (at least in a heterologous system) into an aberrant protein. It should be realized, however, that exon 20 is the penultimate exon in this transcription unit, and once the choice of exon 19 is made, exon 20 becomes obligatory (i.e., not truly alternative) (20). The second case is that of Mhc^{10}, which consists in the same type of change at the 3′ border of the intron preceding alternative exon 15a of the MHC locus (9). In that case, splicing is severely reduced but not eliminated. It might be safe to conclude that in addition to the AG signal, nucleotide context is more important for splicing of the 3′ end of alternative exons than for splicing in constitutive exons. This difference might be particularly important when, as is the case here, there is no polypyrimidine tract to facilitate branch point formation (26). Interestingly, Mhc^{10}, in which there is a basal splicing activity (9), does contain a suitable polypyrimidine tract in the corresponding intron. A final consideration for potential *cis* factors affecting alternative splicing can be found upon inspection of the genomic DNA sequence. Several long (up to 25-nucleotide) inverted repeats can be found in most introns, especially among those separating exons 6. The matching of selected repeats could sequester or expose different sets of exons.

ACKNOWLEDGMENTS

We are grateful to M. A. Arevalo for oligonucleotide synthesis.

We thank the Fondation pour l'Etude du System Nerveux Central et Périphérique (Généve) for a fellowship to J.A.B. This work was supported by grants from the DGICYT (PB87-0416) and EEC (SC1000124.C; SCIENCE Programme).

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