Muscle Abnormalities in *Drosophila melanogaster heldup* Mutants Are Caused by Missing or Aberrant Troponin-I Isoforms

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Abstract. We have investigated the molecular bases of muscle abnormalities in four *Drosophila melanogaster* heldup mutants. We find that the heldup gene encodes troponin-I, one of the principal regulatory proteins associated with skeletal muscle thin filaments. heldup, heldup, and heldup mutants, all of which have grossly abnormal flight muscle myofibrils, lack mRNAs encoding one or more troponin-I isoforms. In contrast, heldup, an especially interesting mutant wherein flight muscles are atrophic, synthesizes the complete mRNA complement. By sequencing mutant troponin-I cDNAs we demonstrate that the molecular basis for muscle degeneration in heldup is conversion of an invariant alanine residue to valine. We finally show that degeneration of heldup thin filament/Z-disc networks can be prevented by eliminating thick filaments from flight muscles using a null allele of the sarcomeric myosin heavy chain gene. This latter observation suggests that actomyosin interactions exacerbate the structural or functional defect resulting from the troponin-I mutation.

Rapid and precisely controlled movements of skeletal muscles are critically dependent upon orderly assembly, maintenance, and functioning of myofibrils. These contractile organelles have been extensively studied for several decades, and we have an accordingly sophisticated understanding of their structure and protein composition, as well as working hypotheses to explain how actomyosin interactions generate force in a regulated manner. Despite these impressive advances in our knowledge, many aspects of myofibril assembly and functioning remain controversial (for review refer to Epstein and Fischman, 1991). It is expected that these issues will be resolved only by continuing to investigate the roles played by particular contractile proteins.

Analyses of muscle and cytoskeletal mutants can relate defects of contractile proteins to the syndromes of abnormalities that they engender, and thus foster a deeper understanding of the roles played by each. A convenient system in which to conduct such studies is the indirect flight musculature of *Drosophila melanogaster* (reviewed by Fyrberg and Beall, 1990). Accordingly, we are investigating the molecular bases of several mutants wherein myofibrils do not assemble or function properly.

We recently turned our attention to the allelic series of *Drosophila heldup* mutations. The locus was originally defined by a single mutant, wings-up A, reported by Hotta and Benzer (1972). Flies having the mutation, subsequently renamed heldup by Deak (1977), cannot fly because flight muscles degenerate during late pupation and early adulthood. In the ensuing years three additional alleles, heldup, heldup, and heldup were isolated and partially characterized by Deak et al. (1982). Barbas et al. (1991) recently found that heldup mutations fail to complement certain lethal alleles of the *Drosophila* troponin-I gene, suggesting that troponin-I was the affected protein. However, these observations could be due to the failure of mutations within two closely linked genes to complement (a phenomenon referred to as intergenic noncomplementation, see Deak et al., 1982; Homyk and Emerson, 1988). Accordingly, we sought to establish directly whether or not troponin-I is perturbed in heldup mutants, and thus to further elucidate how the molecular defects engender the muscle abnormalities.

We report here that the heldup locus encodes troponin-I, a principal component of the thin filament–linked system of regulatory proteins. We demonstrate that three heldup mutants having profoundly disrupted myofibrils, heldup, heldup, and heldup, fail to synthesize one or more isoforms of troponin-I. We additionally show that heldup, an especially interesting mutation wherein myofibrils of affected flies form normally but degenerate during the pupal/adult transition (Hotta and Benzer, 1972; Deak et al., 1982), is due to the conversion of an invariant alanine residue to valine. Finally, we used electron microscopy to demonstrate that myofibril degeneration in heldup flight muscles can be prevented by eliminating thick filaments, implying that the troponin-I mutation increases cross-bridge tension beyond the limits that can be supported by the myofilament lattice, or that it impairs the ability of myofibrils to maintain their highly ordered filament lattices even when generating only normal levels of force and tension.

Materials and Methods

Drosophila Strains

heldup, heldup, heldup, and heldup strains were obtained from Dr. Ted

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Homyk (University of Virginia). Strains bearing T(Y)W32 and T(Y)ZP translocations were obtained from the Bowling Green stock center. Flies used for all experimental work were reared at 25°C. The Canton-S strain was taken to be wild-type in every instance.

Electron Microscopy of Indirect Flight Muscles

Our protocol is based upon that of Reedy and Reedy (1985). Whole thoraces were fixed in 3% (vol/vol) glutaraldehyde, 0.2% (wt/vol) tannic acid in buffered Ringer's (110 mM NaCl, 2 mM KCl, 3 mM MgCl₂, 20 mM KPi [pH 6.8]) at 4°C overnight. Muscle fibers were dissected from thoraces in buffered Ringer's and rinsed once with the same solution, then twice with 0.1 M NaPO₄ (pH 6.0). Fibers were postfixed in 1% OsO₄, 0.1 M NaPO₄, 10 mM MgCl₂ (pH 6.0) for 1 h on ice, rinsed in cold water three times, and dehydrated through 50, 70, 95, and 100% ethanol series. The fibers were infiltrated with Epon812/dodecenylsuccinianhydride/ araldite (1:7:2 by vol) and the resin polymerized. Silver-gold sections were cut, stained with uranyl acetate and lead citrate, and observed in the electron microscope.

Isolation of Troponin-I Genomic and cDNA Clones

Falkenthal et al. (1984) extensively screened the Drosophila genomic library of Maniatis et al. (1978) with 32P-labeled RNA of developing Drosophila indirect flight muscles. One clone isolated in the course of this work, lambda dmpt61, was localized by Falkenthal et al. (1984) to subdivision 17A of polytene chromosomes, very close to the location wherein heldup mutations were mapped by Homyk and Emerson (1988). We obtained a small amount of lambda dmpt61 DNA from S. Falkenthal, and packaged it into viable phage particles using a commercially available extract (Amersham Chemical Co., Arlington Heights, IL). Infection of E. coli cells, phage growth, and large scale preparation of phage DNA was according to standard protocols. The mRNA complementary 5.2 kb EcoRI fragment of lambda dmpt61 was subcloned in plasmid pUC19 and used for further experiments.

To isolate lambda dmpt61 cDNAs we screened the Q4 pupal-stage Drosophila cDNA library of Poole et al. (1985) with the mRNA-complementary 5.2-kb EcoRI fragment of the same phage. A 771-nucleotide clone, TnI cDNA1, encoding a complete 208-codon open reading frame and 5'-and 3'-untranslated regions, was thus isolated. The conceptually translated protein sequence was similar to sequences of vertebrate and invertebrate troponin-I isoforms. This clone and its derivative sequences were used for experiments described herein.

Hybridization to Southern-blotted DNA

Hybridizations were performed at 42°C in 50% vol/vol formamide, 5× SSC (SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 5× Denhardt's solution, 50 mM NaPO₄ (pH 6.8) containing 40 µg of denatured calf thymus DNA/mL. Filters were washed twice at room temperature and three times at 68°C in 2× SSC, 0.1% (wt/vol) NaPO₄, 0.1% (wt/vol) sodium pyrophosphate, 0.1% (v/v) SDS. Hybridizing fragments were visualized by exposure to X-ray film.

Colocalization of the Troponin-I Gene and heldup Mutations

Homyk and Emerson (1988) previously demonstrated that heldup mutants could not be complemented by a translocated portion of the X chromosome that included the region between the 16F3-6 subdivision and the centromere, but not one that included the region between 16F5-8 and the centromere. To simultaneously refine our chromosomal localization of the troponin-I gene and establish whether heldup mutants were likely to be due to perturbations of it, we Southern blotted DNA of males having these same translocations (named T(Y)W32 and T(Y)ZP, respectively) and hybridized the filter to 32P-labeled lambda dmpt61 DNA. T(Y)W32/FM7 or T(Y)ZP/FM7 females were mated to yw/Y males. Male offspring having a normal yw chromosome and a T(Y)ZP translocation chromosome were recognized by the y", non-Bar phenotype (Homyk and Emerson, 1988). DNA was prepared using standard methods, digested with EcoRI nuclease, and transferred to nitrocellulose. After hybridization and exposure to X-ray films, lanes were scanned using a densitometer. This analysis revealed that yw(T(Y)W32) males had two copies of lambda dmpt61 DNA, while yw(T(Y)ZP) males had only one. These results established that lambda dmpt61 DNA was within the 16F subdivision, and not 17A as reported by Falkenthal et al. (1984). Together with the mapping result of Homyk and Emerson (1988), our data are consistent with the hypothesis that heldup mutants have defective troponin-I genes.

RNA Preparation, Electrophoresis, and Blotting

RNA was extracted from synchronously developing Drosophila cultures or (in the case of mutants) late pupae by the SDS-phenol technique (Spradling and Mahowald, 1979). RNAs to be separated were denatured by heating for 15 min at 65°C in a buffer containing 50% formamide and 17% formaldehyde. Electrophoresis buffer contained 20 mM sodium acetate, 1 mM EDTA. For details of RNA transfer and hybridization see Fyrberg et al. (1983).

Characterization of heldup Alleles

Troponin-I cDNAs were synthesized from late pupal RNA of particular mutants using the polymerase chain reaction (PCR) method. 2 µg of poly(A)⁺ RNA, or 50 µg of total RNA, was denatured by heating to 70°C, and annealed to 10 µg/ml of oligo (dT) or an appropriate antisense oligonucleotide primer, then transcribed using highly purified reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 90 min at 37°C. The cDNA product was deproteinized using phenol-chloroform, precipitated with salt and ethanol, and resuspended in 10 mM Tris, 1 mM EDTA (pH 8.0). PCR amplifications were carried out in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM nucleotide triphosphates, and 0.01% gelatin. Sense and antisense primers were added to a final concentration of 100 pM (~1 µg each in a final volume of 100 µl), and the mixture was incubated for 25 cycles of denaturation, renaturation, and extension (94°/1 min, 45°/1 min, and 72°/2 min, respectively) in the presence of highly purified Taq polymerase. The following primers were used: 5'-translated region (encoding BamHI cloning site) GGATCCAAAAACACAAATCTAA, 3'-translated region (antisense, and incorporating EcoRI cloning site) GGAATTCATCAGTGAGCATGTT-GGA, exon 4 (antisense, and incorporating a BamHI cloning site) GGATCCCTGTCGGGTCAAG, exon 4 CTGAACAGGAGATGCCATAGA. To recover PCR products containing exon 3 (using the 5'-translated region and exon 4 antisense primers) it was necessary to add 10% DMSO to PCR reactions, presumably to adequately denature DNA so as to avoid terminating replication by Taq polymerase. cDNA products recovered from each mutant were cloned in pGEMBL or pUC plasmid vectors using restriction enzyme sites incorporated within the primers, and nucleotide sequences determined using the dideoxy terminator method (Sanger et al., 1977). In the case of heldup², the identity of the single mutated nucleotide was confirmed by sequencing both sense and antisense DNA chains of several independently derived clones.

Results

We began our investigation by reexamining the indirect flight muscles of four heldup mutants using electron microscopy, our aim being to deduct or at least delimit the molecular basis of muscle abnormalities. Preliminary analyses of heldup² and heldup³ mutants were reported by Deak et al. (1982). Fig. 1 illustrates flight muscles of recently eclosed heldup², heldup¹, heldup⁴, and heldup¹ adults. In heldup² flight muscles (A) myofibrils are recognizable, but disruptions of sarcomeric organization are readily apparent when

Figure 1. Electron micrographs of heldup² flight muscles. A illustrates a longitudinal section of heldup² flight muscles (for comparison to wild-type myofibrils, refer to the inset of A, Fig. 5 A of Fyrberg et al., 1990, and Fig. 1 of Fyrberg and Beall, 1990). Myofibrils of newly eclosed heldup² mutants are recognizable, but the order and periodicity of many sarcomeres is perturbed. B-D illustrate flight muscles of heldup¹, heldup³, and heldup⁴ mutants, respectively. Thick and thin filaments are readily apparent, but sarcomeres are never seen. Z-discs are absent, although electron-dense material resembling Z-discs is frequently seen. Bars: (A, inset) 2 µm; (B-D) 1 µm.
Figure 2. Electron micrographs of flight muscles of heldup; Ifm(2)2 double mutants. A illustrates a longitudinal section of flight muscles having the Ifm(2)2 myosin heavy chain null mutation. Extensive pseudomyofibrillar networks of thin filaments and Z-discs (I-Z-I brushes) fill the sarcoplasm. B shows comparable sections of heldup2; Ifm(2)2 double mutants. The phenotype is identical, demonstrating that the heldup2 mutants synthesize and accumulate the full complement of thin filaments. C illustrates flight muscles of heldup3; Ifm(2)2 double mutants. Both heldup3 and heldup5 (not shown) have a severe deficit of thin filaments, and consequently I-Z-I arrays are not continuous. D illustrates muscles of heldup4. As in heldup3 and heldup5, there is a paucity of actin filaments, although it is not as severe as in heldup3 and heldup5. I-Z-I networks of heldup4 are nevertheless discontinuous. Bar, 1 μm.
Figure 3. Degeneration of heldup2 myofibrils during the pupal-adult transition. A and B illustrate longitudinal and cross-sections of myofibrils in late pupal stages of heldup2 (for comparison, refer to the inset of Fig. 1 A, Fyrberg et al., 1990, and Fig. 1 of Fyrberg and Beall, 1990). Sarcomeric organization is nearly normal, although occasional minor perturbations of Z-disc structure are apparent. In cross-sections, note the numerous discontinuities in myofibrillar lattices, the irregular diameters of myofibrils, and the frequent occurrence of very small filament bundles surrounding myofibrils. Such features are never seen during the assembly of wild-type myofibrils. C and D illustrate heldup2 myofibrils in adults 1 and 2 d after eclosion. Note the progressive loss of lateral filament register and the concomitant breakdown of Z-disc structure. Bar, 1 μm.
compared to wild-type myofibrils (refer to inset of A). In flight muscles of heldup	extsuperscript{+}, heldup	extsuperscript{2}, and heldup	extsuperscript{3} (B–D, respectively) myofibrils are absent from newly eclosed adults. Both thick and thin filaments having apparently normal morphology can be seen within the sarcoplasm. However, we never found normal Z-discs in flight muscles of these three mutants, although diffuse electron-dense material that appeared similar to Z-discs could be seen. Our collective observations therefore demonstrated that all four heldup mutations profoundly disrupt myofibrillar organization, thus confirming and extending prior observations of Deak et al. (1982).

To better resolve the structure of thin filament/Z-disc networks we examined flight muscles of heldup; Ifm(2)2 double mutants wherein all myosin heavy chains and thick filaments of flight muscles were eliminated by the Ifm(2)2 myosin heavy chain null mutation (Chun and Falkenthal, 1988; Beall et al., 1989). flies having only the myosin heavy chain defect have pseudomyofibrillar arrays of thin filaments and Z-discs running continuously throughout the sarcoplasm (refer to Fig. 2 A; Chun and Falkenthal, 1988; Beall et al., 1989). Muscles of 5-d-old heldup; Ifm(2)2 double mutants appeared identical (B), demonstrating that they have the full complement of thin filaments and Z-disc proteins. However, the remaining three double mutants all have reductions of thin filament/Z-disc networks. C illustrates a typical section of heldup; Ifm(2)2 flight muscles. Networks of thin filaments and Z-discs are discontinuous, and it is clear that there is a profound paucity of thin filaments. D illustrates a comparable section of heldup; Ifm(2)2 flight muscles. Again, I–Z–1 networks are discontinuous, either due to the scarcity of long actin filaments or to disruptions of the continuity and/or organization of thin filaments and Z-discs. Results for heldup	extsuperscript{3} were essentially identical to those for heldup	extsuperscript{+} (data not shown). Finally, we noted that in heldup	extsuperscript{+}, heldup	extsuperscript{2}, and heldup	extsuperscript{3} the absence of myosin apparently improved the morphology of Z-discs (compare Figs. 1 and 2), suggesting that actomyosin interactions exacerbate the myofibrillar disorganization caused by these heldup alleles. We emphasize, however, that in all three mutants thin filament reductions were seen even in the absence of myosin.

By analyzing flight muscles during several stages of their development in heldup	extsuperscript{+} mutants we confirmed that the associated syndrome of myofibrillar abnormalities is less severe than those seen in heldup	extsuperscript{+}, heldup	extsuperscript{2}, and heldup	extsuperscript{3} flight muscles (refer also to Hotta and Benzer, 1972; Deak et al., 1982). heldup	extsuperscript{+} flight muscle myofibrils form nearly normally until they are approximately two-thirds of their final diameter. This is illustrated in Fig. 3, A and B. Viewed in longitudinal sections (A), the only apparent abnormality was a slight perturbation of Z-disc morphology. Viewed in cross-sections, myofilament lattices are fairly normal, although internal inconsistencies in packing arrangement were frequently apparent, as were wide variations in average myofibril diameter (refer to B). During the final day of adult muscle development heldup	extsuperscript{+} myofibrils progressively degenerate. By the time the adult ecloses (C), thick and thin filaments have moved out of lateral register, causing skewing of M-lines and Z-discs. Only one day later (D), crossstriations have nearly disappeared as Z-discs and other transverse structures continue to fragment. Note by reference to Fig. 2 B, that thin filament/Z-disc networks of heldup	extsuperscript{+} flight muscles lacking myosin appear normal, and furthermore, do not degenerate. Hence, actomyosin interactions seem to cause the myofibrillar degeneration in this mutant.

To understand the molecular basis for heldup mutations we wished to isolate the relevant gene and document the lesions associated with each allele. Falkenthal et al. (1984) previously reported the isolation of a candidate recombinant lambda clone, named dmpt6l, by probing the Drosophila genomic library of Maniatis et al. (1978) with labeled lambda dmpt6l DNA. During larval stages we find abundant levels of 1.1- and 1.2-kb mRNAs. Experiments of Barbas et al. (1991) and our unpublished results suggest that this length heterogeneity is attributable to alternative poly (A) addition sites. During early pupation, when Drosophila has virtually no intact muscle cuclature, no complementary RNA is detected. During later stages of pupation we find an abundant 1.3-kb RNA, as well as the continued accumulation of 1.1 and 1.2-kb species. The larger 1.3-kb mRNA is due to the inclusion of two adult exons (3 and 9) utilized exclusively in flight muscles (refer to Fig. 6 and Barbas et al., 1991).

Figure 4. Temporal accumulation of troponin-I mRNA during Drosophila development. 10 µg of poly(A)+ RNA from larval, pupal, and adult stages of development was electrophoresed on a denaturing agarose gel, transferred to nitrocellulose, and hybridized to 32P-labeled lambda dmpt6l DNA. During larval stages we find abundant levels of 1.1- and 1.2-kb mRNAs. Experiments of Barbas et al. (1991) and our unpublished results suggest that this length heterogeneity is attributable to alternative poly (A) addition sites. During early pupation, when Drosophila has virtually no intact muscle cuclature, no complementary RNA is detected. During later stages of pupation we find an abundant 1.3-kb RNA, as well as the continued accumulation of 1.1 and 1.2-kb species. The larger 1.3-kb mRNA is due to the inclusion of two adult exons (3 and 9) utilized exclusively in flight muscles (refer to Fig. 6 and Barbas et al., 1991).
To establish whether lambda dmpt6l was likely to encode a principal contractile protein we used a 5.2-kb EcoRI fragment of the phage DNA to probe a developmentally staged series of Drosophila RNAs using the RNA blot-hybridization technique. Results, shown in Fig. 4, demonstrated that two mRNA species (1.1 and 1.2 kb) were abundantly expressed throughout the larval stages, while an additional 1.3-kb mRNA is expressed during those pupal stages wherein adult muscles form. No mRNA accumulation is seen in early pupa, a stage when the metamorphosing animals are virtually devoid of muscles. The heterogeneity, abundance, and temporal accumulation pattern of these transcripts suggested that lambda dmpt6l encoded several isoforms of a principal contractile protein. These observations were consistent with the criterion by which the clone had been initially isolated by Falkenthal et al. (1984).

We next used the 5.2-kb mRNA-complementary EcoRI fragment of lambda dmpt6l to probe a cDNA library representing mRNA of late pupae, the stage during which indirect flight muscles of the adult thorax form. We isolated a 771-nucleotide EcoRI fragment and completely sequenced both strands. A 208-amino acid open reading frame, displayed in Fig. 5, was deduced. The encoded protein is similar to troponin-I isoforms of other organisms, and we thus named the sequence troponin-I cDNA. As expected from evolutionary relationships, the similarity is greatest between Drosophila and crayfish, and somewhat lower in comparison to vertebrate forms. In particular, two long stretches of the Drosophila sequence (residues 36-68 and 143-170) were identical to those of crayfish. The latter sequence overlaps with the portion of troponin-I (residues 127-151) thought to interact with both troponin-C and the \( \text{NH}_2 \) terminus of actin (Wilkinson and Grand, 1978; Van Eyk and Hodges, 1988). The function of the residue 36-68 stretch is not well documented, but may be a secondary site for troponin-C binding (Grand et al., 1982; Hitchcock-DeGregori, 1982) and is close to a region of troponin-I believed to interact with troponin-T (Chong and Hodges, 1982). Finally, note that our deduced sequence is identical to a composite sequence of several Drosophila troponin-I cDNAs recently published by Barbas et al. (1991), although they did not recover the cDNA shown here. In independent experiments we have sequenced three alternately spliced exons (exons 3, 6a, and 9) and correlation of the primary sequence to exons please refer to Barbas et al. (1991).

To directly establish whether heldup mutants have missing or defective isoforms of troponin-I we used the polymerase chain reaction (PCR) to amplify and sequence the encoded mRNAs. Oligo (dT) or antisense primers were annealed to 2 \( \mu \)g of late pupal poly(A)+ mRNA recovered from each mutant, and the mix was transcribed using reverse transcriptase. Oligonucleotide primers complementary to the 5'- and 3'-untranslated regions and/or various internal sites were annealed, and the intervening DNA amplified by 25 cycles of polymerase chain reaction. We separated the resultant products on 5% acrylamide or 1.5% agarose gels. Results are summarized in Fig. 6, A–C. In the first experiment we analyzed whether transcripts or each mutant contain exon three, which encodes a proline–alanine–rich sequence that accumulates only within flight muscles (Barbas et al., 1991; our unpublished data). The region of troponin-I mRNAs between the 5'-untranslated region and codons 38-43 of exon 4 was amplified using oligonucleotide primers (refer to Materials...
As can be seen in A, pupal RNA of wild type and heldup2 have mRNA containing exon 3, while it is absent from or markedly reduced in transcripts of heldup3, heldup4, and heldups. Larval RNA also lacks transcripts containing exon 3, confirming that it encodes a peptide that accumulates only in adults. Illustrates an experiment designed to assess the presence of alternate exon 6b, also found only in adult muscles. Two electrophoretically separable products having apparent sizes of 700 and 770 nucleotides are recovered (transcripts containing exons 3 and 9 were not recovered in this experiment unless DMSO or another denaturing reagent was included in the PCR reaction mixture). By completely sequencing each of these products we find that the more slowly migrating species is identical to our troponin-I cDNA1, while the more rapidly migrating one differs only in the identities of codons 90–124. This region corresponds precisely with those encoded by two alternate exons (denoted 6a and 6b) of the troponin-I gene (Barbas et al., 1991, our unpublished data). mRNAs encoding the more slowly migrating form, which contains alternative exon 6b, are abundant in adult muscles but absent from larvae, and also from heldup3 and heldup5 mutants. Thus both mutants lack mRNA encoding a major adult isoform of troponin-I, presumably because they are unable to splice all combinations of tropinin-I primary transcripts.

In a third experiment, we amplified mutant mRNA sequences using one primer encoding codons 139–144 of exon 8, and one encoding the 3′-untranslated region. Electrophoretic separation of the products yielded two species, and sequencing of these products revealed that the faster migrating band contains a 3′ exon present in both larvae and adults (exon 10 of Barbas et al., 1991), while the more slowly migrating protein contains a 3′ exon unique to adult troponin-I cDNAs (exon 9 of Barbas et al.), as well as exon 10. Inspection revealed that in heldup3, heldup4, and heldup5 the majority of mRNA encoding the adult muscle-specific exon is absent. Accumulation of heldup2 troponin-I
mRNA is again unaffected, demonstrating that this mutant differs from the other three in that it is capable of specifying the entire complement of troponin-I mRNAs. From these experiments it is clear that muscle abnormalities in three of the four heldup mutants are due to a failure to synthesize particular alternately spliced mRNAs that are present only in adult muscles, while no such defect is apparent in heldup'.

Since our extensive electrophoretic analysis of heldup' cDNAs failed to reveal any differences relative to wild type, we determined the complete nucleotide sequences of all three PCR-generated heldup' cDNAs. This work revealed that the identity of only a single codon is altered. Codon 55 encodes alanine (GCC), an invariant residue in all troponin-I isoforms, including those of vertebrates, sequenced to date (refer to Fig. 5). As shown in Fig. 7, in heldup' a single GC>AT transition has converted codon 55 from alanine (GCC) to valine (GTC). The codon is located within the constitutively expressed exon 4 (refer to Fig. 6), hence it is expected to affect all Drosophila muscles. All sequence data are available from EMBL/GenBank/ DDBJ under accession number 59376.

Discussion

Genetic methods offer a convenient means to further assess the role of particular contractile proteins in myofibril assembly and function, especially in organisms having expendable systems of muscle fibers. To conduct such an approach systematically it is essential to characterize contractile protein genes, determine their chromosomal locations, and relate mutant alleles to the syndromes of myofibrillar abnormalities that they engender. In this manuscript, we have documented such a study of the troponin-I gene in Drosophila melanogaster. We have shown that the heldup locus encodes troponin-I, a principal component of skeletal muscle myofibrils, documented that each of four heldup mutants has missing or aberrant troponin-I isoforms, and used electron microscopy to further characterize the syndromes of myofibrillar defects caused by each mutation. These results demonstrate that the full complement of troponin-I is essential for normal myofibrillar assembly, as heldup', heldup', and heldup' mutants, each lacking one or more troponin-I isoforms, have grossly abnormal myofibrils. Furthermore, our analyses of heldup' prove that a chemically conservative amino acid replacement within troponin-I can lead to myosin-dependent myofibrillar degeneration.

It is not yet possible to precisely describe why reductions of troponin-I isoforms disrupt myofibrils, but consideration of available data delimits the scope of tenable hypotheses. In this and previous work we have documented that reduction or elimination of tropomyosin, troponin-T, and troponin-I all diminish the number of thin filaments in Drosophila flight.
muscles, even in the absence of myosin heavy chain (Fyrberg et al., 1990; this article; our unpublished observations). These observations could be explained by either of two models. In the first, stoichiometric imbalances of troponin or tropomyosin would preclude thin filament assembly. In this model thin filaments would form only if all of the component proteins were present in the correct levels. Ultrastructural observations of troponin-T null allele heterozygotes (refer to Fyrberg et al., 1990) do not support this notion. In such heterozygotes the concomitant twofold reduction of troponin-T synthetic capacity does not eliminate thin filaments, rather, approximately one-half of the normal complement forms and is incorporated into myofibrillar lattices. In the second model the effect of troponin and tropomyosin deficits would be to reduce the amount of tropomyosin available for binding to actin filaments. Since tropomyosin is the protein most widely distributed on the actin filament surface it is well placed to stabilize F-actin against the activities of severing enzymes or proteases. Evidence that tropomyosin plays such a role has been documented both in vitro and in vivo (Wegner, 1982; Bonder and Moosiker, 1983; Liu and Bretscher, 1989). Levels of stable actin filaments thus may be directly determined by availability of tropomyosin or of the number of troponin molecules competent to stabilize tropomyosin binding. All of the extant flight muscle mutant data is in accord with this model, although mutagenesis of tropomyosin and tests of abilities of derivatives to bind actin filaments in vivo will be required to directly substantiate the hypothesis.

The phenotypes of heldup	extsuperscript{1}, heldup	extsuperscript{3}, and heldup	extsuperscript{7} mutants provide insights into troponin-I splicing patterns as well as the functions of the various isoforms, heldup	extsuperscript{1}, which lacks RNA containing exons 3 and 9, is unable to fly but can jump weakly (Homyk and Emerson, 1988; our unpublished data). It follows that mRNAs containing exons 3 and 9 must function mainly in flight muscles. This observation corroborates an mRNA localization experiment published by Barbas et al. (1991), who showed that an exon 3-specific riboprobe labeled only flight muscles. The fact that heldup	extsuperscript{1} lacks mRNAs containing both exons 3 and 9 further suggests that they are always spliced coordinately. heldup	extsuperscript{3} and heldup	extsuperscript{7} mutants lack mRNAs containing exons 3, 6a, and 9. We suspect that the common defect is inability to splice exon 6a, and we further hypothesize, based upon our PCR analyses of mutant mRNAs, that splicing of exon 6a is requisite, but not sufficient, for splicing of exons 3 and 9. In contrast to heldup	extsuperscript{1}, heldup	extsuperscript{3} and heldup	extsuperscript{7} can neither jump nor fly (Homyk and Emerson, 1988; our unpublished data), suggesting that exon 6a is important for jump muscle function. Finally, it is likely that troponin-I mRNAs that contain exon 6a, but not 3 or 9, are also utilized within flight muscles, as heldup	extsuperscript{7} flight muscles have a more normal content of thin filaments than do those of heldup	extsuperscript{3} and heldup	extsuperscript{1} which lack mRNAs containing exon 6a.

The heldup	extsuperscript{7} troponin-I missense mutation, like two previously characterized troponin-T missense mutations associated with indented thorax and upheld	extsuperscript{10} flightless mutants, is recessive (Deak, 1982; Homyk and Emerson, 1988) and minimally disrupts myofibril assembly in hemizygotes and homozygotes. Near the completion of adult muscle development, normal-appearing myofibrils are present in expected amounts, and Western blotting with antibodies recog-
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