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*^{3}, *heldup*^{4}, and *heldup*^{5} mutants, all of which have grossly abnormal flight muscle myofibrils, lack mRNAs encoding one or more troponin-I isoforms. In contrast, *heldup*^{2}, 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*^{2} is conversion of an invariant alanine residue to valine. We finally show that degeneration of *heldup*^{2} 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 intensively 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*^{2} by Deak (1977), cannot fly because flight muscles degenerate during late pupation and early adulthood. In the ensuing years three additional alleles, *heldup*^{3}, *heldup*^{4}, and *heldup*^{5} 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 a 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*^{3}, *heldup*^{4}, and *heldup*^{5}, fail to synthesize one or more isoforms of troponin-I. We additionally show that *heldup*^{2}, 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*^{2} 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^{2}, *heldup*^{3}, *heldup*^{4}, and *heldup*^{5} strains were obtained from Dr. Ted

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Homyk (University of Virginia). Strains bearing *T(1:Y)W32^P* and *T(1:Y)V7^P* 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 $MgCl_2$, 20 mM KMOPS [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 $NaPO_4$ (pH 6.0). Fibers were postfixed in 1% OsO_4, 0.1 M $NaPO_4$, 10 mM $MgCl_2$ (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/dodecenylsuccinic anhydride/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 ^{32}P-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 $NaPO_4$ (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) $NaPO_4$, 0.1% (wt/vol) sodium pyrophosphate, 0.1% (w/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 be complemented by a translocated portion of the X chromosome that included the region between the 16F3-6 subdivision and the centromere, but not by 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(1:Y)W32^P* and *T(1:Y)V7^P*, respectively) and hybridized the filter to ^{32}P labeled lambda dmpt61 DNA. *T(1:Y)W32^P/FM7a* or *T(1:Y)V7^P/FM7a* females were mated to *ywf/Y* males. Male offspring having a normal *ywf* chromosome and a *T(1:Y)* 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 *ywf/T(1:Y)W32^P* males had two copies of lambda dmpt61 DNA, while *ywf/T(1:Y)V7^P* 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).

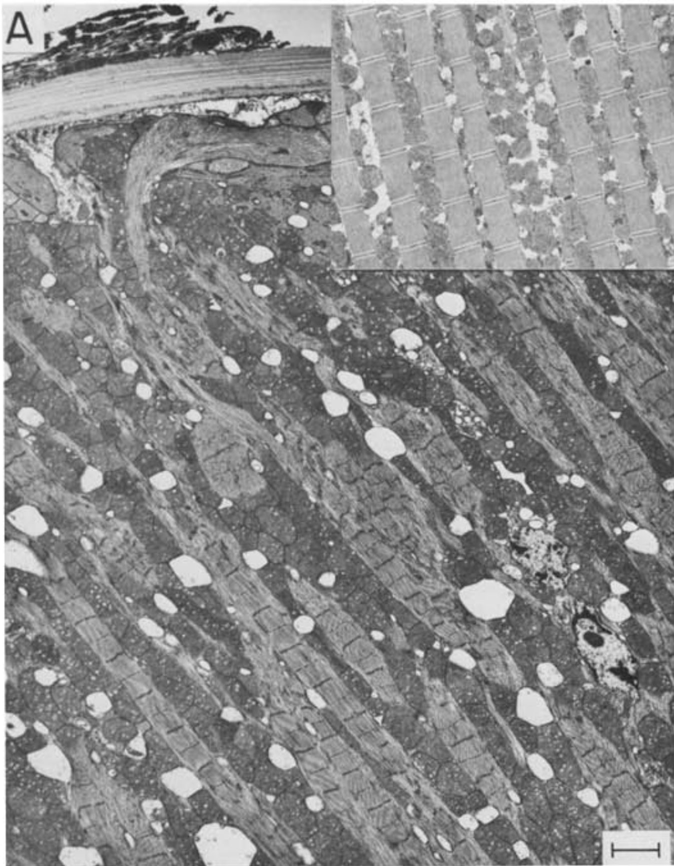
Characterization of heldup Alleles

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 $MgCl_2$, 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' untranslated region (incorporating BamHI cloning site) GGGATCCAAAAA-CACAAATCAAA, 3' untranslated region (antisense, and incorporating EcoRI cloning site) GGAATTCACTAGTGGACGTGT-GGA, exon 4 (antisense, and incorporating a BamHI cloning site) GGGATCCTCTCTGGG-GTCATGAA, exon 8 CGTCAAGCCAGCCCTGAAG. To recover PCR products containing exon 3 (using the 5'-untranslated 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 pEMBL 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 *heldup^2*, 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 deduce or at least delimit the molecular basis of muscle abnormalities. Preliminary analyses of *heldup^2* and *heldup^5* mutants were reported by Deak et al. (1982). Fig. 1 illustrates flight muscles of recently eclosed *heldup^2*, *heldup^3*, *heldup^4*, and *heldup^5* adults. In *heldup^2* flight muscles (*A*) myofibrils are recognizable, but disruptions of sarcomeric organization are readily apparent when

Figure 1. Electron micrographs of *heldup* flight muscles. *A* illustrates a longitudinal section of *heldup^2* 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 *heldup^2* mutants are recognizable, but the order and periodicity of many sarcomeres is perturbed. *B–D* illustrate flight muscles of *heldup^3*, *heldup^4*, and *heldup^5* 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.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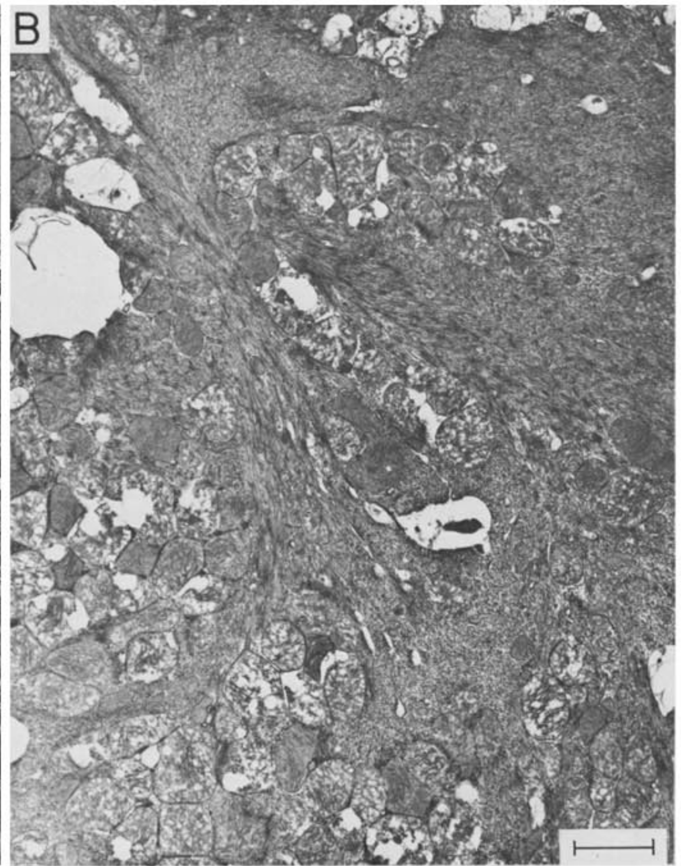
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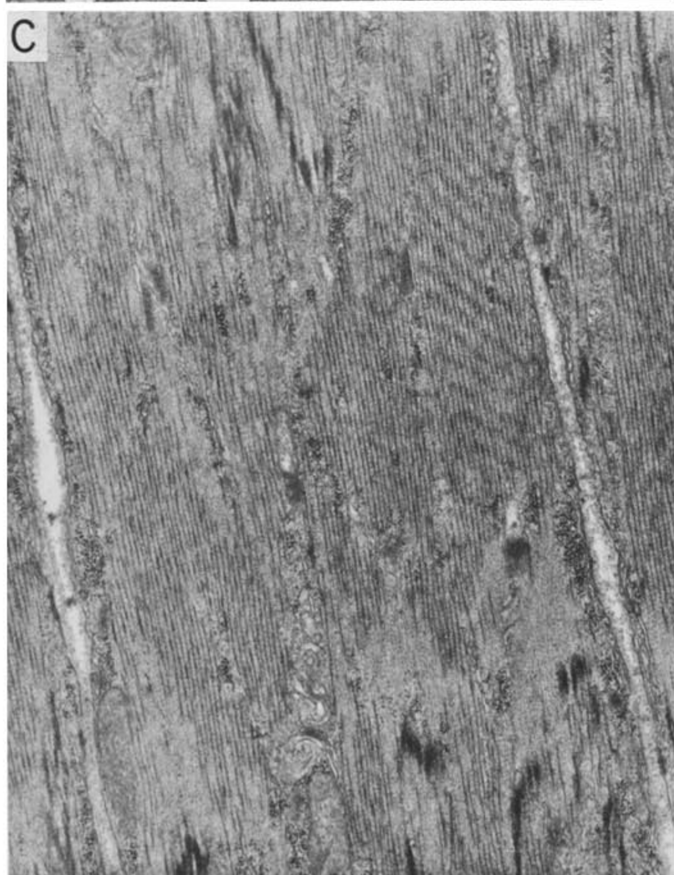
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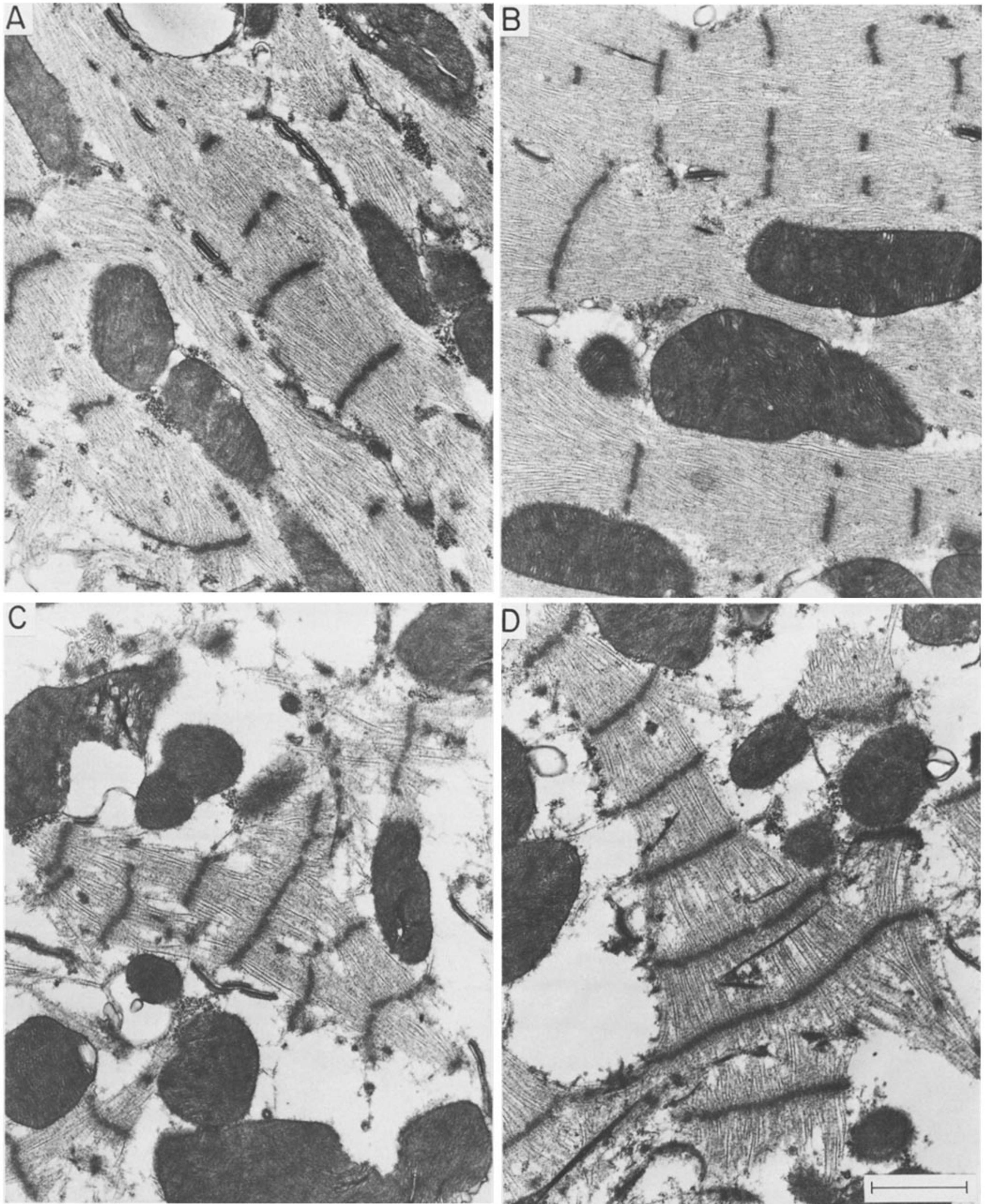

Figure 2. Electron micrographs of flight muscles of *heldup*; *Ifm(2)2* double mutants. *A* illustrates a longitudinal section of flight muscles having the *Ifm(2)2* myosin heavy chain null mutation. Extensive pseudomyofibrillar networks of thin filaments and Z-discs (I-Z-I brushes) fill the sarcoplasm. *B* shows comparable sections of *heldup*^{2}; *Ifm(2)2* double mutants. The phenotype is identical, demonstrating that the *heldup*^{2} mutants synthesize and accumulate the full complement of thin filaments. *C* illustrates flight muscles of *heldup*^{3}; *Ifm(2)2* double mutants. Both *heldup*^{3} and *heldup*^{5} (not shown) have a severe deficit of thin filaments, and consequently I-Z-I arrays are not continuous. *D* illustrates muscles of *heldup*^{4}. As in *heldup*^{3} and *heldup*^{5}, there is a paucity of actin filaments, although it is not as severe as in *heldup*^{3} and *heldup*^{5}. I-Z-I networks of *heldup*^{4} are nevertheless discontinuous. Bar, 1 μm.

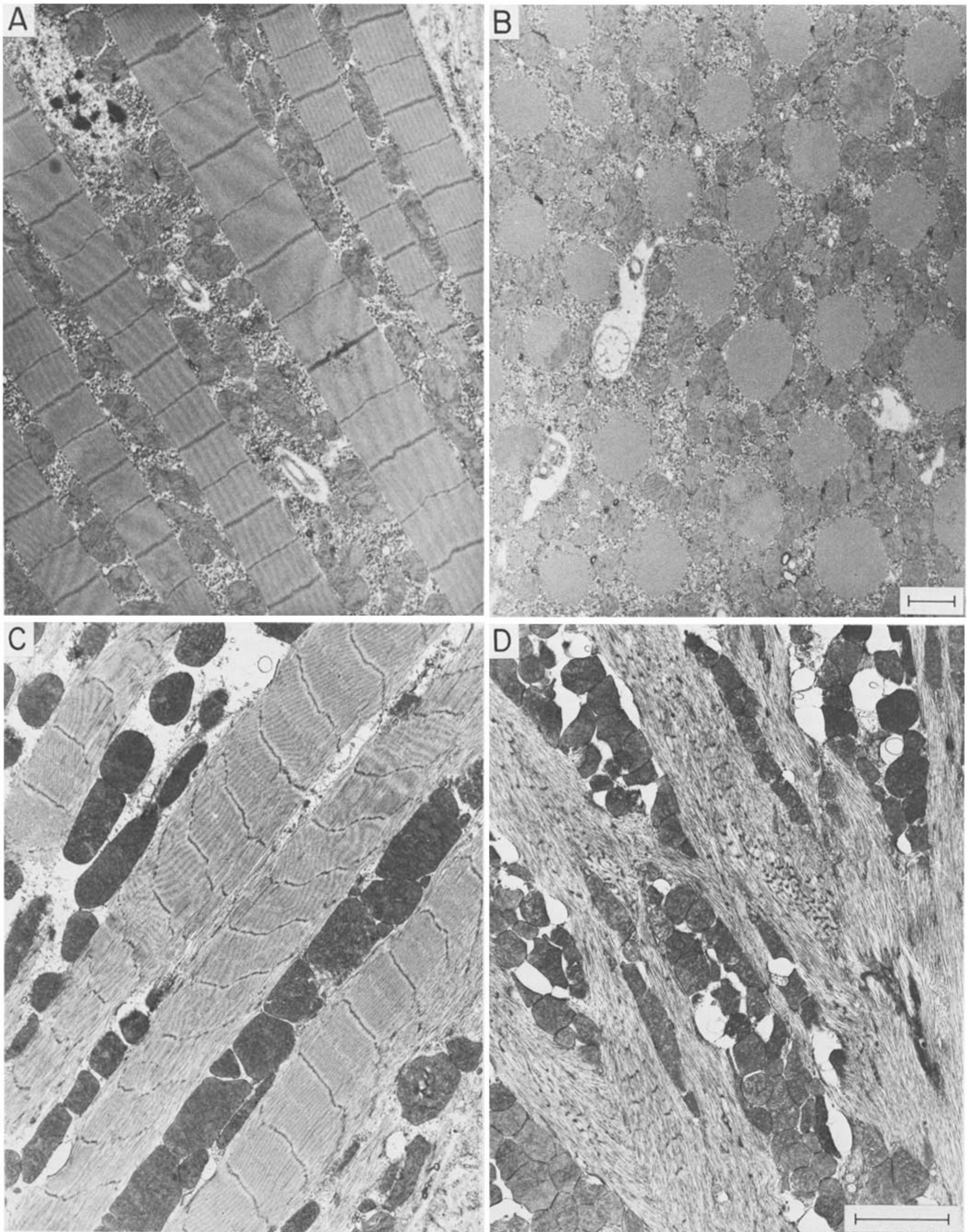

Figure 3. Degeneration of *heldup*[2] myofibrils during the pupal–adult transition. *A* and *B* illustrate longitudinal and cross-sections of myofibrils in late pupal stages of *heldup*[2] (for comparison, refer to the inset of Fig. 1 *A*, Fyrberg et al., 1990, and Fig. 1 of Fyrberg and Beall, 1990). 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 *heldup*[2] 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*[3], *heldup*[4], and *heldup*[5] (*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*[2]*;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*[3]*;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*[4]*;Ifm(2)2* flight muscles. Again, I-Z-I 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*[5] were essentially identical to those for *heldup*[3] (data not shown). Finally, we noted that in *heldup*[3], *heldup*[4], and *heldup*[5] 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*[2] mutants we confirmed that the associated syndrome of myofibrillar abnormalities is less severe than those seen in *heldup*[3], *heldup*[4], and *heldup*[5] flight muscles (refer also to Hotta and Benzer, 1972; Deak et al., 1982). *heldup*[2] 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*[2] 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*), cross-striations 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*[2]

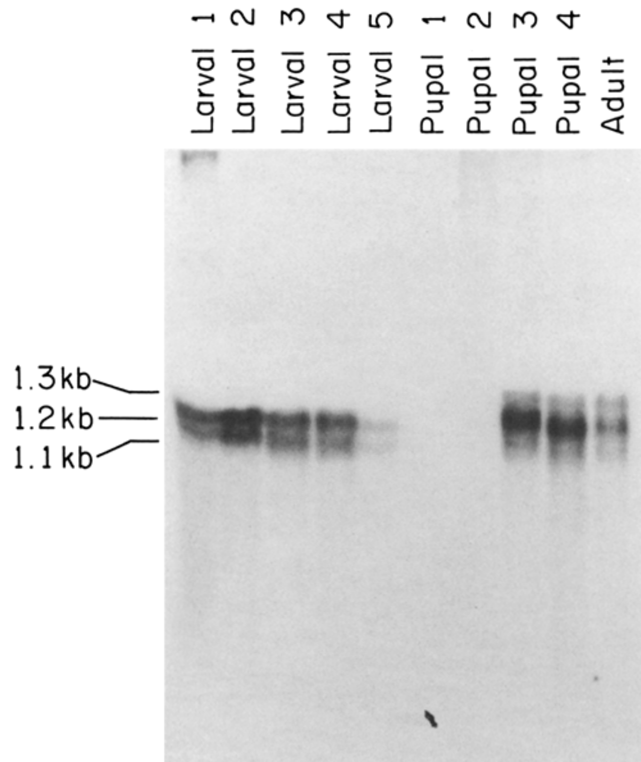

Figure 4. Temporal accumulation of troponin-I mRNA during *Drosophila* development. 10 μg of poly(A)$^+$ RNA from larval, pupal, and adult stages of development was electrophoresed on a denaturing agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled lambda dmpt61 DNA. During larval 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 musculature, 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).

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 dmpt61, by probing the *Drosophila* genomic library of Maniatis et al. (1978) with ^{32}P-labeled RNA of indirect flight muscles. They localized the insert of *Drosophila* DNA within lambda dmpt61 within the 17A subdivision of the X-chromosome, very close to the map position of *heldup* mutants (the 16F subdivision) determined by Homyk and Emerson (1988). We obtained a small quantity of dmpt61 DNA from S. Falkenthal, and demonstrated by quantitative Southern blotting that it resided between the same two X-chromosome translocation breakpoints (termed W32 and V7) where Homyk and Emerson (1988) localized the *heldup* locus (data not shown, refer to Materials and Methods for a description of these experiments).

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Drosoph. 1      MADDEAKKAKQAEIERKRAEVRKRMEEASKAKKAKKGFMTPERKKKLRLLLRKKAAEELKK
Crayfish 1 adkakaaEeaKkKQddIdRKkAEVRKRlEEqS-lKKqKKGFMTPERKKKLRLLLRKKAAEELKK
Bov.car. 1 adrsggstAgdtvpApppvrRrssanyrayAtephakKKskisasRKlqLktLmlqiAkqELer
Chicken skel.                              1  msdeeKKrraataRrqhLksamlqLAvtEieK

62 EQERKAAERRRIIEERCGSPRNLSDASEGELQEICEEYYERMYICEGQKWDLEYEVRKKDWEINDLNAQVND
64 EQERKAgERRkIIdqRCGQPkNLdgAnEeqLraIikEYfdhtaqiEsdKyDvElEiirKDyEINeLNiQVND
65 EaEerrgEkgRalstRC-QPleLaglgfaELQdlCrqlhaRvdkvdeeryDvEakVtKnitEIaDLNqkifD
33 EaaaKevEkqnylaEhC-pPlsLpg-SmqELQElCkklhakidsvdeeryDtEvklqKtnkEleDLsqklfD

134 LRGKFVKPALKKV--SKYENKFAKLQKKAAE-FNFRNQLKVVKKKEFTLEEEEKEKKPDWSKGKPGDAKV
136 LRGKFiKPtLKKV--SKYENKFAKLQKKAAE-FNFRNQLKtVKKKEFelEddk--------gateGDgpa
136 LRGKFkrPtLrrVriSadammqAlLgarAkEtldlRahLKqVKKedtekE---nrevgDWrKnidalsgm
103 LRGKFkrPpLrrVrmSadamlrAlLgsKhkvnmdlRanLKqVKKedtekEkd-lrdgvDWrKnieeksgm

201 KEEVEAEA
195 aEEVaAE

203 egrkkkfeg
172 egrkkmfeages
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Figure 5. Conceptual translation of the 771-nucleotide *Drosophila* troponin-I cDNA containing exon 6a but lacking exons 3 and 9, and comparison of the primary sequence to troponin-I isoforms of other species. The 208-residue *Drosophila* troponin-I primary sequence is compared to sequences of crayfish (Kobayashi et al., 1989), bovine cardiac (Leszyk et al., 1988), and chicken skeletal muscle (Wilkinson and Grand, 1978) troponin-Is. Note that the *Drosophila* sequence is most similar to that of crayfish, and, furthermore, that *Drosophila* residues 36–68 and 143–170 are identical to their crayfish counterparts. The latter region overlaps with the portion of troponin-I known to bind actin and troponin-C (residues 127–151). For the sequences of exons 3, 6b, and 9, and correlation of the primary sequence to exons please refer to Barbas et al. (1991).

To establish whether lambda dmpt61 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 pupation, a stage when the metamorphosing animals are virtually devoid of muscles. The heterogeneity, abundance, and temporal accumulation pattern of these transcripts suggested that lambda dmpt61 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 dmpt61 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 cDNA1. 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 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 of Barbas et al., 1991) not included in troponin-I cDNA1. The sequences of each also match those deduced by those authors (data not shown).

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 μg 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

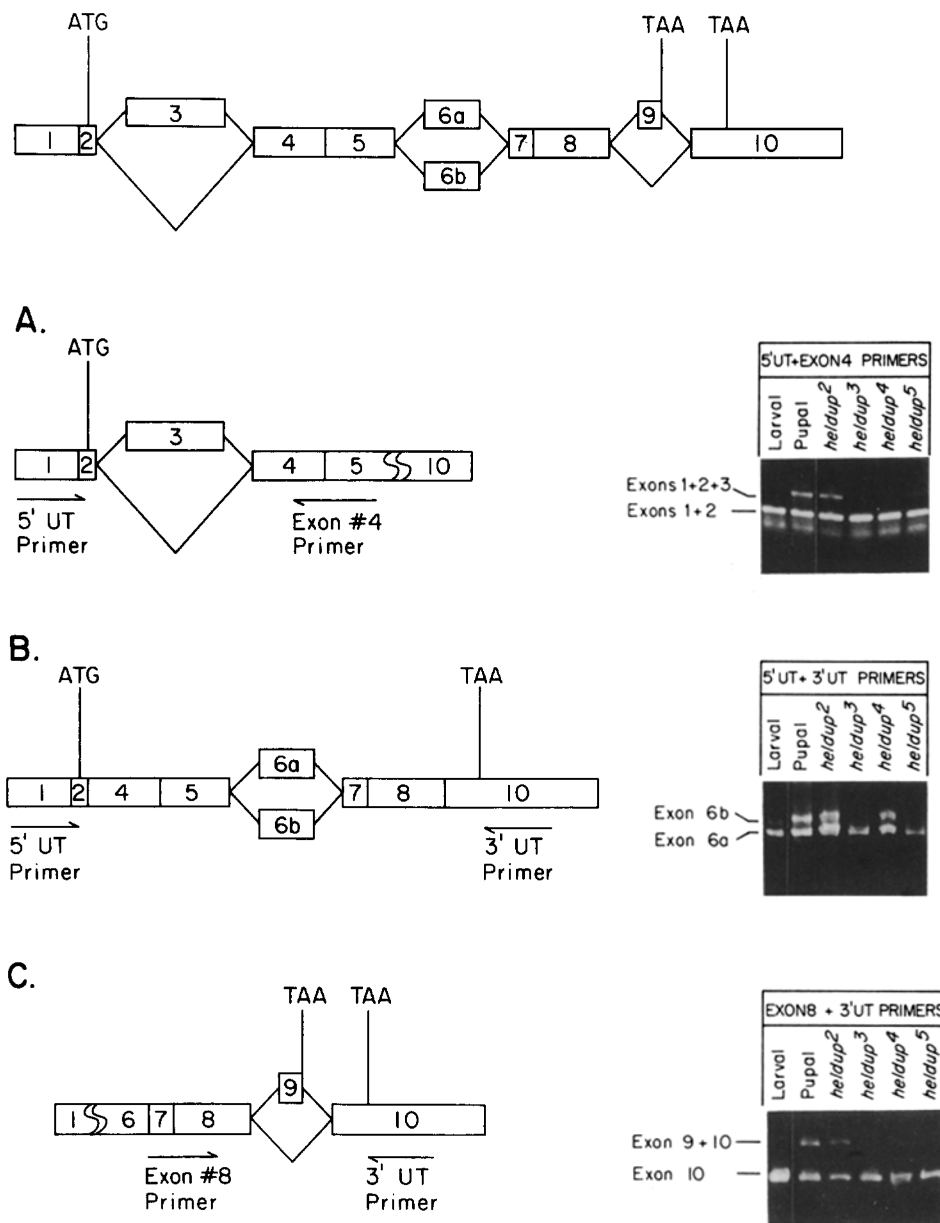

Figure 6. Electrophoretic separation of PCR-amplified sequences of particular *heldup* mRNAs. The upper portion of the figure is a schematic representation of *Drosophila* troponin-I mRNA and the locations of segments encoded by alternatively spliced exons. *A*, *B*, and *C* illustrate products recovered using PCR and defined oligonucleotide primers. In every case, mutant mRNA was derived from late stages of pupae. *A* illustrates products recovered using a pair of primers derived from the 5′ untranslated region and exon 4 (for the sequences of primers used in these experiments refer to Materials and Methods). The more slowly migrating band includes exon three, while the more rapidly migrating product does not. Note that exon 3 is utilized only in adults, and that mRNA including this exon is absent or underrepresented in *heldup³*, *heldup⁴*, and *heldup⁵* mutants. *B* illustrates a comparable experiment using primers derived from 5′ and 3′ untranslated regions. Two products, having apparent sizes of 700 and 770 nucleotides, can be separated on 5% acrylamide gels. *heldup³* and *heldup⁵* lack the upper band, which we have shown by nucleotide sequencing to include exon 6b. *C* illustrates a third experiment that utilized exon 8 and 3′-untranslated primers. The more slowly migrating band includes exon 9, and encodes a troponin-I isoform having a distinct carboxy-terminal sequence. *heldup³*, *heldup⁴*, and *heldup⁵* lack most of the mRNA that includes this exon.

and Methods for primer sequences and reaction conditions). 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 *heldup⁵*. Larval RNA also lacks transcripts containing exon 3, confirming that it encodes a peptide that accumulates only in adults. *B* 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 troponin-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 product 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

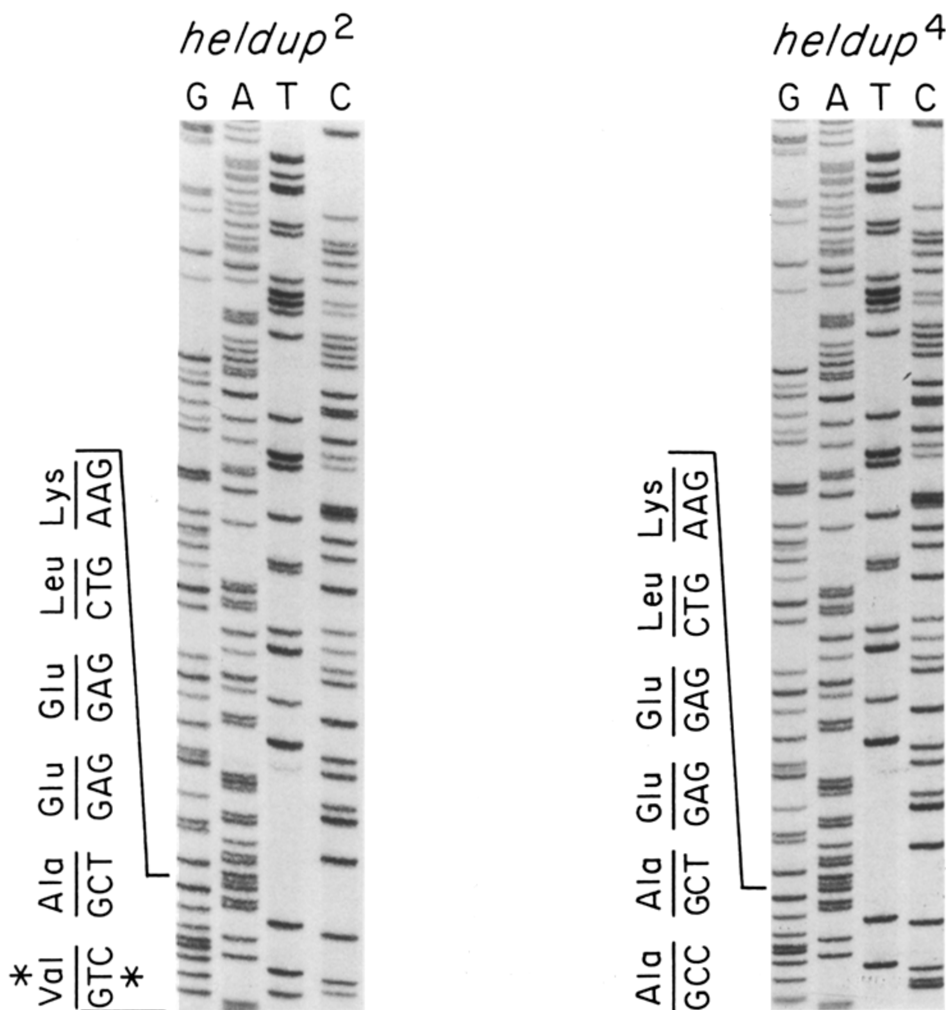

Figure 7. Nucleotide sequence of the *heldup*[2] mutation. Polymerase chain reaction generated *heldup*[2] cDNAs were subcloned into pUC19 plasmid vectors and sequenced using the method of Sanger et al. (1977). For comparison, we also show the comparable sequence of *heldup*[4] troponin-I cDNA. A single GC>AT transition converts codon 55 from alanine (GCC) to valine (GTC). The codon is located within the constitutively expressed exon 4 (refer to Fig. 6), hence is expected to affect all *Drosophila* muscles. All sequence data are available from EMBL/GenBank/DDBJ under accession number 59376.

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*[2].

Since our extensive electrophoretic analysis of *heldup*[2] cDNAs failed to reveal any differences relative to wild type, we determined the complete nucleotide sequences of all three PCR-generated *heldup*[2] 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*[2] a single GC>AT transition has converted codon 55 to valine. The single amino acid change must alter troponin-I structure and function and thus confer the observed syndrome of myofibril degeneration.

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*[3], *heldup*[4], and *heldup*[5] mutants, each lacking one or more troponin-I isoforms, have grossly abnormal myofibrils. Furthermore, our analyses of *heldup*[2] 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 Mooseker, 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***^{3}, ***heldup***^{4}, and ***heldup***^{5} mutants provide insights into troponin-I splicing patterns as well as the functions of the various isoforms. ***heldup***^{4}, 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***^{4} lacks mRNAs containing both exons 3 and 9 further suggests that they are always spliced coordinately. ***heldup***^{3} and ***heldup***^{5} 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***^{4}, ***heldup***^{3} and ***heldup***^{5} 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***^{4} flight muscles have a more normal content of thin filaments than do those of ***heldup***^{3} and ***heldup***^{5} which lack mRNAs containing exon 6a.

The ***heldup***^{2} troponin-I missense mutation, like two previously characterized troponin-T missense mutations associated with ***indented thorax***^{3} and ***upheld***^{101} 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 recognizing tropomyosin, actin, and troponin-T demonstrates that all are present in normal levels (Fyrberg et al., 1990; our unpublished data). However, these myofibrils begin degenerating as adult muscles mature, and within 1 or 2 d after adult emergence, extensive structural abnormalities can be seen. In all three cases, myofibrillar degeneration can be prevented by eliminating myosin heavy chain. One hypothesis to explain these observations is that the troponin mutations increase the level or duration of myosin cross-bridge tension, creating conditions wherein myofibrils can neither complete assembly nor maintain their near crystalline myofilament arrangement. If this is the case, then the muscle atrophy theoretically could be suppressed by myosin heavy chain mutations that impaired the ability of cross-bridges to generate force, or enhanced by myosin mutations that reduced the ability of crossbridge activity to be regulated. A second and seemingly equally plausible hypothesis is that the relatively mild troponin-T and -I point mutations slightly reduce the affinity of troponin–tropomyosin for the thin filament surface, and thus create conditions wherein thin filament arrangement can be maintained in the absence of myosin, but not while undergoing myosin interactions during crossbridge cycles. The lack of stability could be due to enhanced proteolysis of the thin filament/Z-disc networks, or inability of these networks to incorporate nascent proteins at the rate necessary for replacement of Z-disc or thin filament proteins lost due to turnover.

It remains possible that some of the myofibrillar defects observed in troponin-I and -T mutants lacking particular isoforms are due to myosin-induced disassembly of thin filament/Z-disk networks. The morphology of Z-discs in ***heldup***^{3}, ***heldup***^{4}, and ***heldup***^{5} mutants was markedly improved by elimination of myosin heavy chains in flight muscles. However, we emphasize that myosin effects could not be the primary cause, as even in its absence the levels of thin filaments are sharply reduced by the troponin deficiencies.

A final issue for discussion concerns why ***heldup*** mutations profoundly disrupt flight muscle formation and maintenance, yet only moderately or minimally affect the functioning of larval and adult tubular muscles. In the case of ***heldup***^{3}, ^{4}, and ^{5}, only adult isoforms of troponin-I are eliminated, hence larval muscles should be unaffected. Since these three mutants are probably due to splicing defects, maintenance of adult tubular muscle function may be due to the ability of these fibers to suppress the splicing defects and generate at least some normal transcripts. In the case of ***heldup***^{2}, all muscles are expected to have the mutant troponin-I, as the affected exon (exon 4) is used within all fibers. ***heldup***^{2} hemizygous males and homozygous females may survive because the isoforms of tropomyosin, troponin-T, and/or myosin expressed within larval and adult tubular muscles mitigate the troponin-I defect, or because the physiological demands placed upon these muscles in laboratory-reared flies can be met by myofibrils having defective troponin-I. In either case, it is worth noting that ***heldup***^{2} hemizygous males are poorly viable (Homyk and Emerson, 1988), strongly suggesting that the alanine to valine replacement indeed has a substantive deleterious effect in larval muscles.

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References

Barbas, J. A., J. Galceran, I. Krah-Jentgens, J.-L. de la Pompa, I. Canal, O. Pongs, and A. Ferrus. 1991. Troponin-I is encoded in the haplolethal region of the *Shaker* gene complex of *Drosophila*. *Genes & Dev.* 5:132-140.

Beall, C. J., M. A. Sepanski, and E. A. Fyrberg. 1989. Genetic dissection of *Drosophila* myofibril formation: Effects of actin and myosin heavy chain null alleles. *Genes & Dev.* 3:131-140.

Bonder, E. M., and M. S. Mooseker. 1983. Direct electron microscopic visualization of barbed end capping and filament cutting by intestinal microvillar 95-kdalton protein (villin): a new actin assembly assay using the *Limulus* acrosomal process. *J. Cell Biol.* 96:1097-1107.

Chong, P. C. S., and R. S. Hodges. 1982. Photochemical cross-linking between rabbit skeletal troponin subunits. *J. Biol. Chem.* 257:11667-11672.

Chun, M., and S. Falkenthal. 1988. *Ifm(2)2* is a myosin heavy chain allele that disrupts myofibrillar assembly only in indirect flight muscles of *Drosophila melanogaster*. *J. Cell Biol.* 107:2613-2621.

Deak, I. I. 1977. Mutations of *Drosophila melanogaster* that affect muscles. *J. Embryol. Exp. Morphol.* 40:35-63.

Deak, I. I., P. R. Bellamy, M. Bienz, Y. Dubuis, E. Fenner, M. Gollin, A. Rahmi, T. Ramp, C. A. Reinhardt, and B. Cotton. 1982. Mutations affecting indirect flight muscles of *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* 64:61-81.

Epstein, H. F., and D. A. Fischman. 1991. Molecular analysis of protein assembly in muscle development. *Science (Wash. DC)*. 251:1039-1044.

Falkenthal, S., V. P. Parker, W. W. Mattox, and N. Davidson. 1984. *Drosophila melanogaster* has only one myosin alkali light chain gene which encodes a protein with considerable amino acid sequence homology to chicken myosin alkali light chains. *Mol. Cell Biol.* 4:956-965.

Fyrberg, E., and C. Beall. 1990. Genetic approaches to myofibril form and function in *Drosophila*. *Trends Genet.* 6:126-131.

Fyrberg, E. A., J. W. Mahaffey, B. J. Bond, and N. Davidson. 1983. Transcripts of the six *Drosophila* actin genes accumulate in a stage- and tissue-specific manner. *Cell.* 33:115-123.

Fyrberg, E., C. C. Fyrberg, C. Beall, and D. Saville. 1990. *Drosophila melanogaster* troponin-T mutations engender three distinct syndromes of myofibrillar abnormalities. *J. Mol. Biol. 216:657*-675.

Grand, R. J. A., B. A. Levine, and S. V. Perry. 1982. Proton-magnetic-resonance studies on the interaction of rabbit skeletal muscle troponin-I with troponin-C and actin. *Biochem. J.* 203:61-68.

Hitchcock-Degregori, S. E. 1982. Study of the structure of troponin-I by measuring the relative reactivities of lysines with acetic anhydride. *J. Biol. Chem.* 257:7372-7380.

Homyk, T. H., Jr., and C. P. Emerson, Jr. 1988. Functional interactions between unlinked muscle genes within haplo-insufficient regions of the *Drosophila* genome. *Genetics.* 119:105-121.

Hotta, Y., and S. Benzer. 1972. Mapping of behaviour in *Drosophila* mosaics. *Nature (Lond.)*. 240:527-535.

Kobayashi, T., T. Takagi, K. Konishi, and J. A. Cox. 1989. Amino acid sequence of crayfish troponin-I. *J. Biol. Chem.* 264:1551-1557.

Leszyk, J., R. Dumaswala, J. D. Potter, and J. H. Collins. 1988. Amino acid sequence of bovine cardiac troponin-I. *Biochemistry.* 27:2821-2827.

Liu, H., and A. Bretscher. 1989. Disruption of the tropomyosin gene in yeast results in the disappearance of actin cables from the cytoskeleton. *Cell.* 57:233-242.

Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, D. Quon, G. K. Sim, and A. Efstrataidis. 1978. The isolation of structural genes from libraries of eucaryotic DNA. *Cell.* 15:687-701.

Poole, S., L. M. Kauvar, B. Drees, and T. Kornberg. 1985. The *engrailed* locus of *Drosophila*: structural analysis of an embryonic transcript. *Cell.* 40:37-43.

Reedy, M. K., and M. C. Reedy. 1985. Rigor crossbridge structure in tilted single filament layers and flared-X formations from insect flight muscle. *J. Mol. Biol.* 185:145-176.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463-5467.

Spradling, A. C., and A. P. Mahowald. 1979. Identification and genetic localization of mRNAs from ovarian follicle cells of *Drosophila* melanogaster. *Cell.* 16:589-598.

Van Eyk, J. E., and R. S. Hodges. 1988. The biological importance of each amino acid residue of the troponin-I inhibitory sequence 104-115 in the interaction with troponin C and tropomyosin-actin. *J. Biol. Chem.* 263:1726-1732.

Wegner, A. 1982. Kinetic analysis of actin assembly suggests that tropomyosin inhibits spontaneous fragmentation of actin filaments. *J. Mol. Biol.* 161: 217-227.

Wilkinson, J. M., and J. A. Grand. 1978. Comparison of amino acid sequences of troponin-I from different striated muscles. *Nature (Lond.)*. 2781:31-35.